

ISOTOPE AND RADIATION RESEARCH ON ANIMAL DISEASES AND THEIR VECTORS

PROCEEDINGS OF A SYMPOSIUM, VIENNA, 7-11 MAY 1979
JOINTLY ORGANIZED BY IAEA AND FAO



INTERNATIONAL ATOMIC ENERGY AGENCY, VIENNA, 1980

**ISOTOPE AND RADIATION RESEARCH
ON ANIMAL DISEASES AND THEIR VECTORS**

PROCEEDINGS SERIES

ISOTOPE AND RADIATION RESEARCH ON ANIMAL DISEASES AND THEIR VECTORS

**PROCEEDINGS OF THE INTERNATIONAL SYMPOSIUM
ON THE USE OF ISOTOPES
FOR RESEARCH AND CONTROL OF VECTORS
OF ANIMAL DISEASES,
HOST PATHOGEN RELATIONSHIPS AND THE
ENVIRONMENTAL IMPACT OF CONTROL PROCEDURES,
JOINTLY ORGANIZED BY THE
INTERNATIONAL ATOMIC ENERGY AGENCY
AND THE FOOD AND AGRICULTURE ORGANIZATION
OF THE UNITED NATIONS,
AND HELD IN VIENNA, 7-11 MAY 1979**

**INTERNATIONAL ATOMIC ENERGY AGENCY
VIENNA, 1980**

**ISOTOPE AND RADIATION RESEARCH
ON ANIMAL DISEASES AND THEIR VECTORS
IAEA, VIENNA, 1980
STI/PUB/525
ISBN 92-0-010080-5**

© IAEA, 1980

Permission to reproduce or translate the information contained in this publication may be obtained by writing to the International Atomic Energy Agency, Wagramerstrasse 5, P.O. Box 100, A-1400 Vienna, Austria.

Printed by the IAEA in Austria
February 1980

EDITORIAL NOTE

The papers and discussions have been edited by the editorial staff of the International Atomic Energy Agency to the extent considered necessary for the reader's assistance. The views expressed and the general style adopted remain, however, the responsibility of the named authors or participants. In addition, the views are not necessarily those of the governments of the nominating Member States or of the nominating organizations.

Where papers have been incorporated into these Proceedings without resetting by the Agency, this has been done with the knowledge of the authors and their government authorities, and their cooperation is gratefully acknowledged. The Proceedings have been printed by composition typing and photo-offset lithography. Within the limitations imposed by this method, every effort has been made to maintain a high editorial standard, in particular to achieve, wherever practicable, consistency of units and symbols and conformity to the standards recommended by competent international bodies.

The use in these Proceedings of particular designations of countries or territories does not imply any judgement by the publisher, the IAEA, as to the legal status of such countries or territories, of their authorities and institutions or of the delimitation of their boundaries.

The mention of specific companies or of their products or brand names does not imply any endorsement or recommendation on the part of the IAEA.

Authors are themselves responsible for obtaining the necessary permission to reproduce copyright material from other sources.

FOREWORD

To solve the world-wide problems of famine, malnutrition and environmental pollution it is imperative that all techniques and resources for the protection of animals and plants be mobilized. N'gana (animal trypanosomiasis) alone profoundly affects the socio-economic development of Africa. Its vector, the tsetse fly, is widespread and prevents agricultural development over much of this continent of 7 million square kilometres. The need to control this and other related animal diseases has been emphasized by a mandate from the 1974 World Food Conference of the United Nations. If n'gana alone could be eliminated, the cattle population could be increased by at least 120 million head with a resultant yearly increase of meat production of 1.5 million tons.

To discuss these problems the Symposium on the Use of Isotopes for Research and Control of Vectors of Animal Diseases, Host Pathogen Relationships and the Environmental Impact of Control Procedures was convened in Vienna by the International Atomic Energy Agency from 7 to 11 May 1979. It was an integral part of the IAEA and FAO's effort to promote a greater awareness of the actual and potential application of nuclear techniques in the resolution of problems in the control of arthropod vectors of animal diseases and of animal pathogens, and in pesticide management. It was the first of its kind to be convened by the sponsoring organizations and was intended as a forum for the exchange of information on the most recent developments in these fields.

A total of 58 participants from 19 countries attended, and 37 papers were presented, which covered a variety of topics, including the sterile insect technique as applied to tsetse flies. Several papers were presented covering its various aspects such as mass rearing, sterility induction, ecology, behaviour and computer modelling. Other topics emphasized were pathogenesis and immunology of vector-borne diseases such as trypanosomiasis, anaplasmosis, babesiosis and leishmaniasis. Also included were presentations of insect repellents and the biotransformation and degradation of labelled pesticides. The papers and the discussions resulting from these presentations are published in these Proceedings.

CONTENTS

TICKS; TSETSE FLIES; TRYPANOSOMES: GENERAL CONSIDERATIONS (Session 1)

Programme for the control of African animal trypanosomiasis and related development (IAEA-SM-240/35)	3
<i>P. Finelle</i>	
Discussion	14
Pathogenesis of trypanosome infections in cattle (IAEA-SM-240/19)	15
<i>Max Murray, W.I. Morrison, D.L. Emery, G.W.O. Akol, R.A. Masake, S.K. Moloo</i>	
Discussion	31
General discussion on tsetse flies	33

BABESIOSIS AND ANAPLASMOSIS (Session 2)

Bovine anaplasmosis with emphasis on immune responses and protection (IAEA-SM-240/22)	37
<i>M. Ristic</i>	
Discussion	54
Studies on the preference of <i>Babesia microti</i> and <i>Babesia hyalomysci</i> for erythrocytes of a particular age group and on their pathogenesis using iron-59 (IAEA-SM-240/7)	57
<i>H.S. Hussein</i>	
Discussion	65
Immune mechanisms in <i>Babesia</i> -infected animals (IAEA-SM-240/36)	67
<i>R.S. Phillips</i>	
Discussion	75
Bovine babesiosis: Protection of cattle by the inoculation of irradiated piroplasms (IAEA-SM-240/1)	77
<i>R.E. Purnell, D. Lewis, D.W. Brocklesby</i>	
Discussion	94
Non-specific immunization against babesiosis (IAEA-SM-240/14)	95
<i>F.E.G. Cox</i>	
Discussion	104

Uptake of ^3H purines as an in vitro test to measure viability of <i>Babesia</i> parasites following chemotherapy, irradiation and other treatments (IAEA-SM-240/21)	107
<i>A.D. Irvin, E.R. Young, R.E. Purnell</i>	
Discussion	116

TRYPANOSOMIASIS AND LEISHMANIASIS (Session 3)

Pathophysiology of trypanosomiasis in the bovine (IAEA-SM-240/28)	121
<i>J.D. Dargie</i>	
Discussion	129
Secondary immunodeficiencies associated with African trypanosomiasis (IAEA-SM-240/27)	133
<i>R.J. Terry, K.M. Hudson, M. Faghihi Shirazi, D. May</i>	
Discussion	146
Role of biologically active substances in the pathogenesis and immunology of trypanosomiasis (IAEA-SM-240/15)	149
<i>I.R. Tizard, A. Mellors, K. Nielsen</i>	
Discussion	158
Microbial stasis of <i>Leishmania enriettii</i> in activated guinea pig macrophages (IAEA-SM-240/3)	161
<i>C.M. Grocock, E.J.L. Soulsby</i>	
Discussion	177
Patterns of development of trypanosomes and related parasites in insect hosts (IAEA-SM-240/20)	179
<i>D.H. Molyneux</i>	
Discussion	189
Efficiency of wild <i>Glossina pallidipes</i> Austen as vectors of pathogenic African trypanosomes (IAEA-SM-240/38)	191
<i>L.H. Otieno, N. Darji, J. Olobo, P. Onyango</i>	
Discussion	197
Leishmaniasis vector behaviour in Kenya (IAEA-SM-240/39)	199
<i>M.J. Mutinga</i>	
Discussion	204

INSECT REPELLENTS; PESTICIDES (Session 4)

Long-term protection of animals from tsetse bites through controlled-release repellents (IAEA-SM-240/23)	207
<i>R. Galun, M.N. Ben-Eliahu, D. Ben-Tamar, J. Simkin</i>	
Discussion	217

Application of isotope-labelled compounds in the study of the chemical stability of pesticides (IAEA-SM-240/5)	219
<i>M. Rösseler, D. Luther, H.-C. Abendroth, H. Koch</i>	
Discussion	231
Biotransformation of lindane in mammals and its significance for safety assessment of the pesticide (IAEA-SM-240/6)	233
<i>U.V. Gopalaswamy, M.P. Phondke, A.S. Aiyar</i>	
Discussion	244

STERILE INSECT TECHNIQUE AND STERILITY INDUCTION (Session 5)

La lutte contre <i>Glossina palpalis gambiensis</i> Vanderplank par lâchers de mâles irradiés en Haute-Volta — Etude de paramètres opérationnels (IAEA-SM-240/2)	249
<i>D. Cuisance, H. Politzar, M. Clair, E. Sellin, Y. Taze, G. Bourdoiseau, J. Février</i>	
Discussion	264
Integration of sterile insects and pesticides for the control of the tsetse fly <i>Glossina morsitans morsitans</i> (IAEA-SM-240/4)	267
<i>D.A. Dame, D.L. Williamson, P.E. Cobb, D.B. Gates, P.V. Warner, A.G. Mtuya, H. Baumgartner</i>	
Discussion	278
General discussion on the FAO Animal Health Division field research on tsetse flies	281
Use of the sterile male technique as an adjunct to insecticidal and physical methods for stable fly control on the island of St. Croix, U.S.V.I. (IAEA-SM-240/16)	283
<i>R.S. Patterson, G.C. LaBrecque, D.F. Williams</i>	
Discussion	294

ISOTOPE APPLICATIONS; PHYSIOLOGY (Session 6)

Use of isotopes as model substances to elucidate the mode of transmission of pathogens to animals and plants by arthropod vectors (IAEA-SM-240/10)	299
<i>W.J. Kloft, H.L. Cromroy, E.S. Kloft</i>	
Midgut absorption of proteins by tsetse flies <i>Glossina morsitans morsitans</i> Westwood (Diptera: Glossinidae) (IAEA-SM-240/12)	313
<i>G. Nogge, M. Giannetti</i>	
Discussion	317

Use of gamma-irradiated blood for feeding tsetse flies (IAEA-SM-240/13) ...	319
<i>B. Bauer, K.H. Iwannek, H.J. Hamann, G. Adamsky</i>	
Discussion	325
Role of adult fat body and milk gland in larval nourishment of	
<i>Glossina morsitans</i> (IAEA-SM-240/9)	327
<i>P.A. Langley, E. Bursell</i>	
Discussion	334

ECOLOGY AND BEHAVIOUR OF TSETSE FLIES (Session 7)

Construction details of the Challier/Laveissière biconical trap	
(IAEA-SM-240/40)	339
<i>L. Ryan, D.H. Molyneux</i>	
Discussion	353
Tsetse movement in wind fields: Possible epidemiological and	
entomological implications for trypanosomiasis and its control	
(IAEA-SM-240/40A) (<i>Abstract only</i>)	355
<i>D.H. Molyneux, D.A.T. Baldry, C. Fairhurst</i>	
Discussion	356
Dynamics and behaviour of tsetse populations (IAEA-SM-240/30)	359
<i>D.J. Rogers</i>	
Discussion	367
Field studies with irradiated <i>Glossina morsitans morsitans</i> Westwood	
(Diptera: Glossinidae) from a colony maintained on a membrane	
feeding regimen (IAEA-SM-240/24) (<i>Abstract only</i>)	369
<i>M. Weiss, W. Takken</i>	
Discussion	369
Feeding and rearing behaviour in tsetse flies (IAEA-SM-240/31)	371
<i>L.H. Otieno, Y. Youdeowei</i>	
Discussion	379
Factors affecting the efficiency of the sterile insect release method for	
tsetse (IAEA-SM-240/25)	381
<i>C.F. Curtis</i>	
Discussion	394
La multiplicité des accouplements chez les glossines et la méthode des	
lâchers de mâles stériles (IAEA-SM-240/37)	397
<i>R.C. Pinhão</i>	
Discussion	408

MASS REARING OF TSETSE FLIES: COMPUTER MODELLING (Session 8)

Recent advances in the rearing of <i>Glossina pallidipes</i> Austen (IAEA-SM-240/32)	413
<i>M.E. Leegwater-Van der Linden</i>	
Discussion	422
Cross-breeding experiments with <i>Glossina morsitans morsitans</i> (Westwood) wild genotype and phenotype genetic marker <i>ocra</i> (IAEA-SM-240/33)	425
<i>A.M.V. Van der Vloedt</i>	
Discussion	437
Rearing of <i>Glossina palpalis palpalis</i> Robineau-Desvoidy on freeze- dried blood (IAEA-SM-240/34) (Abstract only)	439
<i>H. Wetzel</i>	
Discussion	439
Serum substitutes for feeding <i>Glossina palpalis palpalis</i> Robineau- Desvoidy (Diptera: Glossinidae): II. The influence of serum albumin on the fecundity and weight of the progeny (IAEA-SM-240/17) (Abstract only)	441
<i>W. Takken</i>	
Discussion	441
Growth of laboratory populations of tsetse flies: Some mathematical investigations (IAEA-SM-240/11)	443
<i>W. Timischl</i>	
Discussion	455
List of Chairmen of Sessions and Secretariat of the Symposium	457
List of Participants	459
Author index	467

**TICKS; TSETSE FLIES; TRYPANOSOMES:
GENERAL CONSIDERATIONS**

(Session 1)

PROGRAMME FOR THE CONTROL OF AFRICAN ANIMAL TRYPANOSOMIASIS AND RELATED DEVELOPMENT

P. FINELLE
Food and Agriculture Organization
of the United Nations,
Rome

Abstract

PROGRAMME FOR THE CONTROL OF AFRICAN ANIMAL TRYPANOSOMIASIS AND RELATED DEVELOPMENT.

After a statement on the problems raised by African animal trypanosomiasis and its control, and the development of currently affected areas, the Programme is outlined describing the methodology for tsetse and trypanosomiasis control operations and the basic approach for implementation of related development. The activities carried out during the five-year preliminary phase are summarized. The operational phase of the Programme is now being launched. A series of Preliminary Assistance Missions to Governments for the evaluation and preparation of control schemes, development strategies and projects is being mounted.

1. INTRODUCTION

Trypanosomiasis is probably the only disease that has profoundly affected the settlement and economic development of a major part of a continent. The magnitude of the problem accords trypanosomiasis control an obligatory and high priority for increasing agricultural production in support of general economic development in Africa, a view emphasized by the 1974 World Food Conference of the United Nations in Resolution XI whereby FAO was given the mandate 'to launch, as a matter of urgency, a long-term programme for the control of African animal trypanosomiasis as a project of high priority.'

2. THE TRYPANOSOMIASIS PROBLEM

African trypanosomiasis are parasitic diseases affecting both humans and animals and are transmitted by tsetse flies (*Glossina* spp.). They are prevalent in Africa between latitudes 15°N and 21°S representing a surface area of approximately

10 million km². The incidence and severity of the disease are dependent upon local conditions and, in consequence, result in

- (a) areas where virtually no livestock development can be attempted;
- (b) areas where some livestock breeds, tolerant to the disease, can be maintained;
- (c) areas where trypanosomiasis-susceptible livestock can be reared by using curative and prophylactic trypanocides despite the presence of tsetse flies.

In addition to direct losses from the disease and the financial requirements for control operations, animal trypanosomiasis also indirectly affects

- (a) *human health* through protein deficiencies caused by the shortage of meat and milk;
- (b) *livestock production*, since trypanosomiasis prevents the introduction of improved breeds and can also cause overstocking in tsetse-free areas consequently resulting in deterioration of rangelands;
- (c) *agricultural production*, through the lack of draught animals and manure;
- (d) *the rural economy* by preventing integrated agriculture and livestock production and contributing to transhumance since some pastures are seasonally rendered unsafe through fly advances;
- (e) *the national economy* since the deficit in animal production compels affected countries to import meat and dairy products.

Although animal trypanosomiasis imposes constraints in rural activities in vast areas, it must be recognized that it has contributed to preservation of natural resources, particularly in areas where *Glossina morsitans* is prevalent.

Some species of tsetse flies are vectors of *human trypanosomiasis* (sleeping sickness), a disease that poses a major problem for public health in Africa. It is estimated that about 35 million people reside in endemic trypanosomiasis areas. In most countries the disease has been brought under control through strict medical surveillance and treatment but, where surveillance has been relaxed, it continues to be a serious problem with the ever present threat of developing into epidemic proportions. This situation is further aggravated by improved communication systems and labour migrations, thus increasing the risk of spread between countries with common boundaries.

With present knowledge it is impossible to assess approximate losses caused by animal trypanosomiasis. However, an indication of the magnitude of the problem may be gained by an estimation of the theoretical potential for meat production, should the disease be controlled.

Area of the tsetse-infested zone that could be used for livestock	7 million km ²
Average potential carrying capacity	20 cattle per km ²

Total potential population of tsetse-infested zone	140 million cattle
Present population of tsetse-infested zone	20 million cattle
Possibility of increasing the cattle population in the areas infested at present	120 million head
Average cattle productivity in Africa ¹	12.5 kg/head annually
Additional meat production	1.5 million tonnes/year
Value of additional meat production (50 ¢ per kg)	US\$ 750 million

Although very approximate and theoretical and not taking into consideration substantial loss of productivity in other sectors of the economy, these estimates show how control could contribute to the development of a sound and balanced rural economy in 35 African countries affected by tsetse flies.

3. CONTROL METHODS

Trypanosomiasis and tsetse control methods have been greatly improved during the last 25 years, and several of these have been applied effectively and economically. These include

- (a) the use of trypanocidal drugs;
- (b) the rearing of trypanotolerant animals;
- (c) vector control by ground and aerial spraying of insecticides.

Research is at present being carried out on the release of sterilized insects and immunization of livestock.

3.1. Trypanosomiasis control

3.1.1. *Vaccination*

An immunization procedure (vaccine or acquired trypanotolerance) would be the method of choice for trypanosomiasis control. Research is being carried

¹ Total annual meat production/total population of all classes of cattle.

out in various institutes, particularly in the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya, which devotes the most of its activities to this problem; results of major importance have already been obtained. For various technical reasons, practical achievements will only be possible after long and in-depth research work has been carried out. In spite of the recent discovery of a method for the *in vitro* culture of a trypanosome species, immunization against animal trypanosomiasis is still only a research objective.

3.1.2. Trypanocidal drugs

Although the armoury of trypanocides is restricted and some are subject to fluctuation in availability, all are highly active against the various species of trypanosomes either as curatives or prophylactics, several of the latter group providing protection from infection for 2–4 month periods. Nevertheless, chemotherapy is bedevilled by development of resistance to drugs. The formation of chemoresistance is of great importance because it persists even after passage of trypanosomes through tsetse. Furthermore, trypanosome strains may be resistant to several drugs belonging to different chemical groups. In consequence, prophylactic drugs should only be used where strict adherence to the treatment regimen is possible. Chemoprophylaxis is therefore limited under conditions of traditional animal production. However, the use of chemoprophylaxis is justified in treatment of slaughter stock that is at risk when crossing infested zones to reach markets. In spite of constraints, trypanocides are being used on an expanding scale; the number of treatments carried out annually in recent years in Africa is estimated to be in excess of 25 million. Chemotherapy must, however, be regarded as a palliative measure only. It requires an efficient and well-equipped veterinary service to gain maximum benefits; administration costs, however, may be high because any regime requires repeated treatments.

3.1.3. Trypanotolerance

Many tsetse-infested areas have some livestock production because of the existence of trypanotolerant cattle, sheep and goats. This is particularly noticeable in West Africa where an important cattle population exists, composed of N'Dama and the West African short-horn breeds. Trypanotolerance is, however, only relative and can break down when the challenge is severe. Moreover, stress factors such as malnutrition, intercurrent infectious diseases, parasitism and excessive work decrease the degree of tolerance.

Trypanotolerance has apparently been produced by natural selection, which suggests a genetic basis. Recent research on changes in immunological responses and blood biochemical factors between trypanotolerant and non-trypanotolerant animals tends to confirm the possibility of breeding for tolerance, which should be an

effective means of increasing the number of tolerant animals and the degree of tolerance.

The development of the rearing of trypanotolerant animals has proved to be an effective method in areas where the disease risk is not too great and which are ecologically similar to the places of origin of these animals. It is, however, a costly method requiring considerable financial input with economic benefits ensuing slowly because of the slow biological cycle. One of the major limiting factors is the small number of breeding stock available.

Wild animals are also trypanotolerant and survive in tsetse-infested areas. The exploitation of some species of wild ruminants is an optional development tool, particularly in marginal areas where the trypanosome challenge is high and tsetse eradication is not technically and/or economically feasible.

3.2. Tsetse control

3.2.1. *Biological control*

Biological control of tsetse through predators, parasites and pathogens is only a remote possibility at present. Release of sterile males, genetical control (cytogenetical manipulations) may eventually serve to augment existing methods in integrated control campaigns. Applied research on the release of sterile males is receiving increasing attention. Although promising results have been obtained, effectiveness and economic implications are still to be determined. Some methods of ecological control that have been used extensively in the past, notably manipulation of the habitat, are still useful but labour costs and the risk of soil erosion preclude the application on a large scale. Removal of food sources by game elimination cannot be recommended, principally because of the increasing concern for environmental protection.

3.2.2. *Chemical control*

At present there are no practical alternatives to the use of insecticides for large-scale tsetse control. Control of tsetse by application of insecticides has already achieved considerable success especially in Nigeria, Botswana and elsewhere, where extensive areas have been rendered fly-free. Initially, ground-spraying techniques have been used, but in recent years encouraging results have been obtained with various methods of aerial application. More precise knowledge of the ecology of tsetse has made it possible to limit application of insecticides to vegetation representing the true habitat, resulting in substantial economies in control operations. Aerial spraying can eliminate tsetse completely in extensive isolated habitats, the costs of such operations are comparable with, if not lower than, that of ground spraying. Aerial spraying is at present the method of choice

for the implementation of large-scale control programmes because of efficacy, speed of operation and relatively small manpower requirement. It will have an increasingly extensive application in the foreseeable future. Although both application methods have been considerably improved, there is still a need for refinement in the interest of greater effectiveness and decreased environmental pollution. These can be effected by greater discrimination in application from the ground or more selectively from a helicopter to 10% or less of the vegetation or by sequential low-volume broadcast application by fixed-wing aircraft.

Chemical control involves the use of organochlorine insecticides which may have undesirable side-effects on non-target species. Future control operations will require compounds that are rapidly biodegradable to ensure minimum accumulation in the environment in order to avoid long-term deleterious effects on non-target species.

3.2.3. *Re-invasion*

Whichever chemical control technique is used, total elimination of tsetse is always difficult to achieve, the treated zone being at risk to re-invasion from adjacent infestations or by recrudescence of small foci that are difficult to locate. Isolation is at present obtained by establishing barriers that have been deforested; these are usually sufficiently wide to prevent voluntary crossing by tsetse. Barriers may also be maintained by repeated spraying of a persistent insecticide on the periphery of the treated zone. Restriction of movement of vehicles, livestock and possibly game across these barriers, however essential, is difficult to ensure, and all these are capable of carrying tsetse flies over considerable distances. It is envisaged that the release of sterile males in the zone surrounding an area rendered tsetse-free by chemical control would minimize the risk of re-invasion. However, the problem of re-invasion will largely be obviated if tsetse eradication is followed by planned settlement. It is therefore essential that control be followed by well-defined area development projects.

4. OUTLINE OF THE PROGRAMME

At the outset it must be emphasized that the aims of the Programme are not confined to the control of the disease or its vector, but include the planning and implementation of sound follow-up programmes for land use and rural development in the areas selected for the application of control measures.

4.1. Trypanosomiasis/tsetse control

Several effective control methods are available, but as a result of the number of tsetse species and their geographical distribution over a wide ecological range, they must be adapted to local conditions with due consideration of resources available. In general, several techniques should be used simultaneously.

In the absence of an immunization procedure which, in spite of recent progress, is still only a research objective, the control of tsetse fly remains the only available approach to *elimination of the disease*, provided that treatment campaigns are carried out to eliminate existing trypanosome infections to avoid mechanical transmission of the disease by blood-sucking insects other than the tsetse. For large-scale tsetse control operations there are at present no practical alternatives to the use of insecticides applied either from the ground or by aircraft. The release of sterile males could be useful to achieve tsetse elimination after reduction of the natural population has been achieved by application of non-persistent insecticides. Tsetse control by insecticide application is suited to savannah conditions but project areas must have sufficient development potential to justify the requisite capital expenditures and be susceptible to successful prevention of re-invasion from adjacent infestations.

Other control techniques are only palliative measures that may be used to great advantage in areas where tsetse control cannot be done for various reasons or where it has not as yet been attempted.

In spite of constraints, particularly the development of chemoresistance, chemotherapy, either curative or preventive, is being used on an expanding scale and remains the most commonly used control technique.

The rearing of trypanotolerant livestock is an alternative to tsetse or trypanosomiasis control, particularly in moist savannah and forest areas, for which effective and economic tsetse control methods have yet to be devised. Similarly, the exploitation of relatively resistant wild animals is an optional development tool, particularly in marginal areas where the trypanosome challenge is high and tsetse control not technically or economically feasible.

4.2. Tsetse area development

The task of rural development is even more complex in the presence of the tsetse. There can be no truly uniform methodology for tsetse area development. The approach must always be specifically designed according to available resources and to local development objectives, i.e. it must be area-specific. In some situations the main economic justification for a tsetse eradication attempt may be the savings that can be realized by eliminating the need for recurrent expenditure to control re-invasion of neighbouring areas. In other situations the economic considerations may be secondary to the social or humanitarian motives, for example, incorporating

nomad communities into the development process or relieving land shortage of overcrowded rural areas. In all situations, however, the process will involve removal or reduction of the environmental conservation afforded by the tsetse and therefore carefully planned and operated controls and support systems will be indispensable to minimize spontaneous or haphazard exploitation and associated risk for the environment.

Although development methodology must be area-specific, the overall strategy for each tsetse area must adhere to broad common guidelines which should incorporate the following sequential steps with each culminating in a decision as to whether and when to proceed to the next:

- (a) assessment of the need for development of the area; this involves determination of existing and predictable human and animal population pressures and food needs in relation to observable resource trends, and may take into account social and cultural aspirations of the relevant rural communities;
- (b) determination of the level of need for, and prospects for, securing an international/sub-regional approach to control where the tsetse distribution extends across national frontiers;
- (c) survey of the natural resource potential of the area and assessment of the technical, social and economic possibilities for area development;
- (d) investigation of the epizootiology of trypanosomiasis and the distribution and bionomics of tsetse in the area; this information will lead to provisional selection of a control strategy, assessment of logistical and infrastructural requirements, and formulation of field trials to test the technical and economic feasibility of alternative control techniques;
- (e) field trials: (i) to test the technical and economic feasibility of alternative tsetse and trypanosomiasis control/eradication techniques and methods in the area; (ii) to assess the feasibility of tsetse eradication. If not feasible, consideration of the comparative advantages of chemotherapeutic measures or the use of trypanotolerant cattle; (iii) to determine stock carrying capacities and technical, economic and social feasibility of alternative range improvement, and/or crop production systems, and/or wildlife management schemes; (iv) to determine the risk of tick-borne and other diseases; (v) training of key field staff must be given high priority and should be initiated very early in this phase of activities.

In the event of a decision being taken to proceed with full-scale or phased area development, the following institution-building activities should receive high priority:

- (a) initiation of appropriate training programmes to provide adequate local capability in planning, implementing and determining conservation levels; and in associated fields such as environmental-impact monitoring and defined support requirements;
- (b) appropriate establishment or strengthening of tsetse control units;
- (c) creation of co-ordination machinery to secure the requisite level of inter-disciplinary co-ordination at the national level;
- (d) creation, where necessary, of appropriate legislation to provide the requisite legal jurisdiction for controlling development of the area;
- (e) survey and demarcation of the development area;
- (f) formulation and implementation of public relations programmes designed to enlist understanding, confidence and active participation by communities to be involved in the area development.

The value of any land use development plan must be determined ultimately by the quality of implementation which follows its formulation. In this context experience has confirmed the necessity to avoid delay between successful application of tsetse and trypanosomiasis control measures, and the inception of area development and associated administrative controls. From this it follows that the formulation of appropriate land use development plans must be completed and developed into action programmes equipped with adequate resources for successful implementation before large-scale tsetse and trypanosomiasis control efforts are launched.

5. PRESENT STATUS OF THE PROGRAMME

The *five-year preparatory phase* for implementation of the Programme for the Control of African Animal Trypanosomiasis and Related Development involved training of personnel at all levels; applied research and field trials to improve control techniques; surveys, promotion of rearing of trypanotolerant livestock; assessment of the socio-economic implications of trypanosomiasis and its control

including land use after tsetse elimination; planning, management, co-ordination and funding of the main Programme.

Additional funds have been made available in the FAO Programme of Work and Budget for 1978-79 for increased activities which have included surveys and feasibility studies in 16 countries for the development of a data base concerning the problem of trypanosomiasis and the economic implications; to provide technical information and proposals for trypanosomiasis and tsetse control projects for consideration by interested assistance agencies; co-operation with and support to research institutions to stimulate applied research on trypanosomiasis and tsetse control; Expert Consultations on co-ordination and funding of research on trypanosomiasis; Joint WHO Expert Committee and FAO Expert Consultation on Trypanosomiasis; Expert Consultations to discuss the management structure and co-ordination of Programme activities; preparation of a training manual on tsetse control as well as several technical publications; compilation of a list of the various control, research, training and development activities currently carried out in countries concerned with tsetse and trypanosomiasis control including support provided by bilateral and multilateral assistance agencies.

Particular emphasis has been given to training at all levels in order to strengthen national structures and allow the establishment in each country concerned of specialized units comprising appropriate multidisciplinary technical staff, who will be involved in control activities, land use planning and subsequent development of the tsetse-free areas. These training activities have been carried out not only in co-operation with the African governments concerned but also with other interested institutions such as the Organization of African Unity, WHO, international research laboratories and multilateral or bilateral assistance agencies. Of special importance have been the three postgraduate training courses carried out in co-operation with France, the Federal Republic of Germany and the United Kingdom; the two FAO/OAU/WHO Training Seminars on Trypanosomiasis and Tsetse Control, implemented in co-operation with France and the United Kingdom and with the assistance of the International Laboratory for Research on Animal Diseases (ILRAD), the International Centre of Insect Physiology and Ecology (ICIPE), the Organisation de Coordination et de Coopération pour la Lutte contre les grandes Endémies (OCCGE), the Ecole de Lutte anti-tsé-tsé (ELAT) and the Institut d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (IEMVT); the Tsetse and Trypanosomiasis Information and News Service jointly sponsored by FAO/OAU/WHO, the Centre for Overseas Pest Research (COPR) of the Ministry of Overseas Development, United Kingdom, and the Institut d'Elevage et de Médecine Vétérinaire des Pays Tropicaux, France. The close co-operation that has been established among these institutions has permitted the implementation of major activities which would have been impossible for one agency to accomplish alone.

The Programme for the Control of African Animal Trypanosomiasis and Related Development is now entering its operational phase with the implementation of multidisciplinary Preparatory Assistance Missions to governments.

6. PROPOSED ACTION

It is clearly recognized that governments must determine the level of emphasis to be accorded to tsetse/trypanosomiasis control activities in relation to other national commitments, and that the pattern of all area development programmes, being dependent upon socio-political philosophies and priorities, will vary substantially from country to country. However, it is assumed that for the time being the main approach to the problem will be in the form of localized tsetse control operations, confined to certain well-defined areas in certain countries. However, with the increase in human population there will be a time in the not-too-distant future when overall national control schemes will have to be considered. Nearly all of these will require a co-ordinated subregional approach between neighbouring countries as fly belts usually extend across national borders.

The FAO Programme for the Control of African Animal Trypanosomiasis and Related Development is being developed to provide or channel support for national and sub-regional programmes for tsetse area development wherever needed. This will particularly concern the following:

At the national level

- (a) *Preparatory Assistance Missions*, if required by governments, to plan the development of tsetse-infested areas for agricultural or livestock production. This could encompass advice on the method of control to be applied, the economics of the operation itself, land-use planning and determination of cost-benefit relationships.
- (b) *Establishment and implementation of Technical Assistance Projects*
 - (i) Establishment of specialized services needed for the purpose of tsetse and trypanosomiasis control.
 - (ii) Training of staff required for the units under (i).
 - (iii) Assistance in the formulation of control policies and implementation of projects.
 - (iv) Assistance in the establishment and implementation of area development schemes.

At the regional level

- (a) Training at scientific and technical levels to create a specialist cadre for tsetse/trypanosomiasis control, and to cope with research needs (seminars, postgraduate training courses, manuals, film material).

Strengthening of training facilities for middle-level control personnel.

- (b) Sponsor applied research to secure control methodologies for specific ecological conditions by studies of tsetse ecology, improvement of insecticide application equipment, trials with new insecticides, evaluation of various control methods and effect on non-target organisms in the environment.
- (c) Promote the rearing of trypanotolerant livestock through:
 - (i) the assessment of trypanotolerant breeds
 - (ii) the establishment of selection and multiplication centres
 - (iii) applied research into the phenomenon of trypanotolerance and particularly on its heritability.
- (d) Develop co-operation with OAU and strengthen its capacity for regional co-ordination of control programmes.

DISCUSSION

G. NOGGE: In Sections 3.1.3 and 4.1 of your paper you mention game-ranching as an alternative to cattle production. Are there any projects in this area, sponsored by FAO or other organizations?

P. FINELLE (*Scientific Secretary*): I do not know of any current FAO project on game-ranching. There are, however, several game ranches in Kenya and other countries in eastern and southern Africa.

O.U. ALOZIE: You mentioned during your oral presentation that most scientists trained under FAO's training programme for activities in developing countries are lost to other jobs. I believe that FAO should encourage governments to lay down regulations that would ensure that those taken on for training sign a contract under which they must work in the field in which they are to be trained for at least three years.

P. FINELLE (*Scientific Secretary*): FAO can only make recommendations to governments on this important question, but every opportunity has been and will continue to be taken in order to draw their attention to it.

PATHOGENESIS OF TRYPANOSOME INFECTIONS IN CATTLE

Max MURRAY, W.I. MORRISON,
D.L. EMERY, G.W.O. AKOL, R.A. MASAKE,
S.K. MOLOO
International Laboratory for Research
on Animal Diseases,
Nairobi,
Kenya

Abstract

PATHOGENESIS OF TRYPANOSOME INFECTIONS IN CATTLE.

In trypanosome-infected cattle, dramatic structural changes occur in the lymphoid system. Initially, these are characterized by marked proliferation and germinal centre formation, whereas in long-standing infections the lymphoid organs become depleted. There is evidence that these changes are associated with immunodepression, the extent and mechanisms of which await investigation in cattle. Anaemia dominates the clinical disease syndrome in bovine trypanosomiasis. It develops with the onset of parasitaemia and is largely haemolytic, resulting from increased red blood cell destruction by phagocytosis. Several factors may be involved in this process including haemolysins produced by the trypanosome, immunological mechanisms, fever, disseminated intravascular coagulation and an expanded and active mononuclear phagocytic system. During this phase of the disease, cattle respond well to chemotherapy. However, in later phases of the disease, when trypanosomes cannot be detected, the anaemia sometimes persists and animals do not respond to treatment. The underlying mechanisms responsible for the anaemia at this time are not fully understood, but there is evidence that continued red cell destruction combined with a degree of dyshaemopoiesis, associated with a defect in iron metabolism, is responsible. Frank necrosis is not a feature of bovine trypanosomiasis but widespread tissue degeneration occurs. Organs particularly severely affected include the heart, and it has been concluded that death in bovine trypanosomiasis is due to a combination of anaemia, microcirculatory disturbances and myocardial damage. The factors incriminated in tissue damage probably vary with the species of trypanosome involved, although under natural field conditions it is common to find *T. congolense*, *T. vivax* and *T. brucei* in one animal. Likely pathogenic mechanisms operative in the bovine include anoxia as a result of anaemia, microcirculatory disorders and hypersensitivity reactions.

Trypanosoma congolense, *T. vivax* and *T. brucei* are the major trypanosomes that infect cattle. Whereas all three are pathogenic in their own right, mixed infections commonly occur in the field. The pathogenesis of African trypanosomiasis in cattle is dominated, as in other species, by the trypanosome's ability to undergo antigenic variation in the face of the host's immune response. Thus, the disease is characterized by progressive waves of parasitaemia; the outcome



FIG.1. Skin lesion in a bovine 13 days after being bitten by Glossina morsitans infected with Trypanosoma congolense. In addition to congestion and oedema, there is a diffuse cellular infiltrate of lymphoblasts and plasmablasts. Haematoxylin and Eosin. X 80.

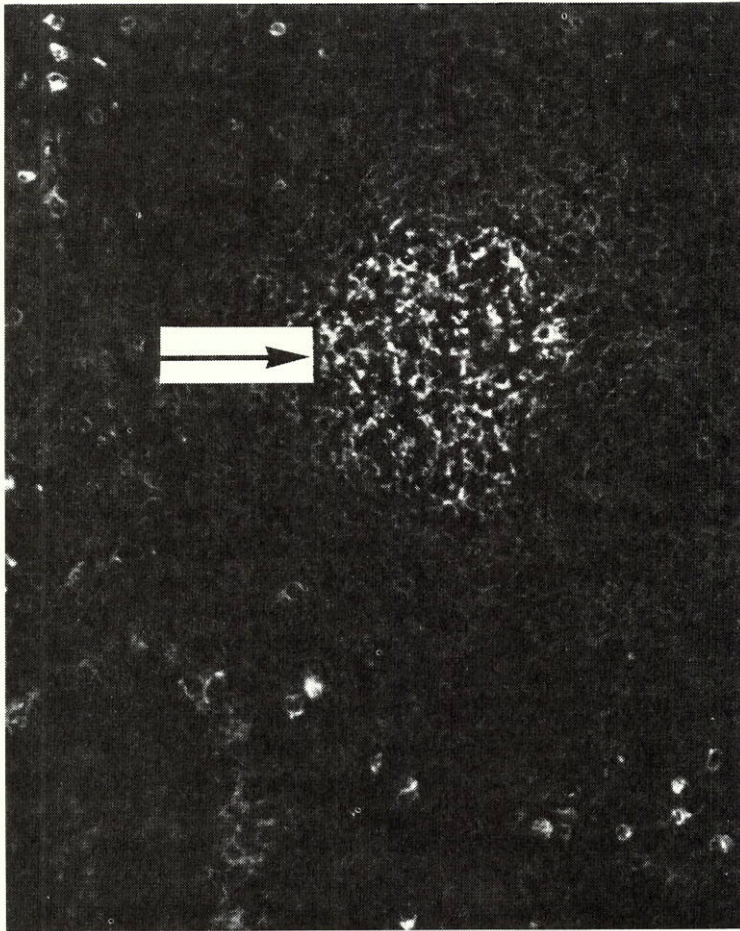


FIG. 2. Shows the follicular area of the prescapular lymph node of a bovine 21 days after inoculation with Trypanosoma vivax. Immunofluorescence staining demonstrates coarse granular deposits of IgM indicative of germinal centre activity (arrow). The tissue is stained with mouse anti-bovine IgM hybridoma followed by goat anti-mouse Ig conjugated with tetramethylrhodamine isothiocyanate. X 130.

may be death at any stage of the disease, a chronic wasting syndrome with the animals living for years or, depending on host susceptibility, spontaneous recovery. Too often, when evaluating the disease mechanisms, little consideration is given to the changing course of events involved in the progress of the disease, with the result that information on the pathogenesis of African trypanosomiasis often appears conflicting. In the present report, we have attempted to reconcile

different views and to compare what is established in laboratory animals with what is known in cattle. In cattle, we would suggest that the main areas of consideration are: (1) The early events following the tsetse fly bite and the subsequent changes in the lymphoid system; (2) anaemia; and (3) specific organ and tissue damage.

1. EARLY EVENTS AND THE LYMPHOID SYSTEM

1.1. Structural changes

Within a few days of being bitten by an infected tsetse fly, cattle develop a raised cutaneous swelling several centimetres in diameter that persists for several days. The term chancre is used to describe this lesion. The bite of an uninfected fly produces no such change. The appearance of the chancre precedes detectable parasitaemia by a few days, and is accompanied by the development of fever and marked enlargement of the draining lymph node(s).

The cellular events that accompany inoculation of metacyclic trypanosomes by tsetse flies into the dermis are striking. Following an intense inflammatory reaction characterized by congestion, oedema and extravasation of polymorphonuclear leukocytes, large numbers of lymphoblasts and plasmablasts accumulate (Fig. 1); as the lesion declines in size, increased numbers of mature plasma cells, eosinophils and mast cells are found. Thus, even at the earliest phase of the disease, there is evidence that the bovine is mounting a marked immune response to the trypanosome.

Following enlargement of the lymph node(s) draining the chancre, generalized lymph node enlargement develops. At the same time, splenomegaly becomes a consistent feature. These changes are partly the result of a marked proliferative lymphoid cell response characterized by the formation of numerous large active germinal centres (Fig. 2) and plasma cells that pack the medullary cords of lymph nodes (Fig. 3), and which are diffusely scattered throughout the red pulp of the spleen. A similar proliferative response is found in laboratory animal infections but with the important differences that germinal centre formation is not a feature [1, 2], and massive erythropoiesis and myelopoiesis occur in the spleen [1].

In the later stages of infection in laboratory rodents [2] and in longstanding field cases in cattle, both lymph nodes and spleen become depleted of lymphoid cell types. At this stage in cattle, the superficial lymph nodes are small and hard whereas the spleen is either normal in size or small and contracted.

Throughout the course of the disease in both cattle and laboratory rodents, there is marked activity of the mononuclear phagocytic system as judged histologically by the numbers and morphological appearance of the constituent cells, and by the enhanced ability to clear macromolecules from the circulation

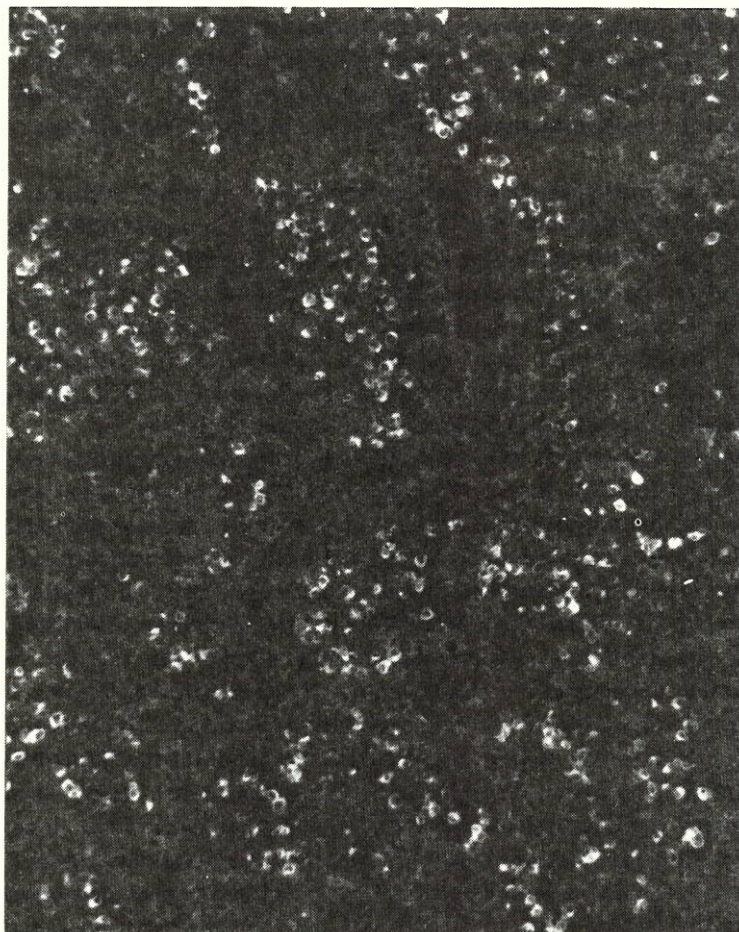


FIG.3. The medulla of the same lymph node stained as in Fig. 2. Numerous IgM-containing plasma cells are present in the medullary cords. X 80.

[2]. Macrophages are often engorged with red blood cells and occasionally trypanosomes may be found on their surface or in the cytoplasm.

1.2. Functional consequences of lymphoid changes

A consistent finding in trypanosome infections in most species, including cattle, is the development of hypergammaglobulinaemia, particularly IgM, soon after infection [3]. Although IgG₁ and IgG₂ levels are not markedly altered in

infected cattle, studies on the rates of catabolism of different immunoglobulin (Ig) classes have shown a nine- to thirteen-fold increase for IgG₁ and IgG₂ respectively, whereas there is a five-fold increase for IgM [3]. These results would suggest a marked increase in production not only of IgM but also of IgG₁ and IgG₂.

Evidence from other species indicates that much of the Ig produced is not directed against the trypanosome [4, 5] but contains heterophil antibodies, rheumatoid factor-like substances and a range of auto-antibodies [6, 7]; furthermore, an increase in background plaque-forming cells has been shown to occur in the spleen [8, 9]. As a consequence, several workers have proposed that the trypanosome may act as a polyclonal B cell mitogen [9, 10], and it has been shown that certain trypanosome fractions are mitogenic [11], although the mitogenicity reported has always been disappointingly low.

In what would appear to be a paradox regarding hypergammaglobulinaemia, it is now well recognized that the trypanosome-infected host becomes immunodepressed. The immunodepressed state is very severe in laboratory animals where it has been shown that antibody responses [8], mitogen and mixed lymphocyte reactions and graft rejection are suppressed [12], and B and T memory cells are depleted [13], although delayed-type hypersensitivity, as judged by the response to oxazolone and dinitrofluorobenzene, is apparently not affected [8, 14]. Immunodepression has also been described in man [15] and there are preliminary reports in cattle [16-19]. It would appear to us that immunodepression could be one of the most important facets of bovine trypanosomiasis and that more information is required on its mechanisms and extent. It must be determined whether infected cattle can be effectively vaccinated; it is also essential to know their susceptibility to other pathogens and, lastly, it is important to evaluate to what extent cattle can respond to each new antigenic wave of trypanosome. Some of these questions have been partly answered. It has been shown that parasitized cattle exhibit reduced antibody responses to a range of bacterial and viral vaccines [16, 17, 19]; however, it was concluded that in some cases the extent of the reduction would not have been sufficient to cause vaccine breakdown if the animals came under challenge. In view of the fact that there is a dramatic increase in catabolism of IgG in trypanosome-infected cattle [3], mere estimation of levels of specific antibody may not give a correct evaluation of the protective status of the host. It is therefore essential that such cattle be subjected to challenge with the organism against which it is being vaccinated.

There is evidence that trypanosome-infected cattle are more susceptible to other pathogens [18]. In our laboratory we have found that infected cattle may succumb to other infectious diseases such as salmonellosis. On each occasion when this occurred, we have evidence that it was the result of recrudescence of a latent infection.

The anomaly of the state of immunodepression is that hosts including the bovine [20] continue to respond immunologically to each new wave of trypanosome variant. The explanation for this is not known. Possibly it is the result of the development of a carrier-hapten situation involving different trypanosome antigens; it has been shown that certain antigens may cross-react between different variants and species [13, 21] (carrier), while the N-terminal of the variant surface antigen is highly variable [22] (hapten). In this way, the host may be primed to successive waves of parasitaemia by a common antigenic component of the trypanosome. It has been reported in *T. rhodesiense*-infected mice that immunodepression occurs with T-dependent antigens but not with T-independent antigens [23]. Thus, if an animal is primed to the carrier early in infection, the responses to the hapten may continue to be elicited despite depression of T-helper cell activity. Alternatively, variant surface antigens per se may function as T-independent antigens. However, it must be pointed out that contradictory results have been obtained in *T. brucei*-infected mice, where responses to T-dependent and T-independent antigens were found to be depressed [8]. If the ability to control and reduce the level of parasitaemia is used as an indication of the level of the immune response, it is obvious that cattle are able to mount a better response than mice (Fig. 4) [24]; this capacity varies with breed of cattle and strain of mouse [24], suggesting that the quality/quantity of the immune response to each trypanosome wave varies between and within a host species.

Numerous studies have been carried out to examine the basis of immunodepression of trypanosome-infected laboratory animals, and a number of different mechanisms have been demonstrated or incriminated. These include polyclonal B-cell mitogenicity [9], suppressor cells (T-cells and macrophages) [25–27], soluble suppressor factors produced by the trypanosome [28], and also possibly complement depletion [29]. Definitive studies remain to be done in cattle. However, one significant fact that has been demonstrated in both cattle [19] and laboratory animals [8, 30] is that the state of immunodepression depends on the continuing presence of the parasite; following treatment with trypanocidal drugs immunological competence is rapidly restored.

On the basis of histological findings, it would appear to us that the extent of lymphoid disorganization in cattle is significantly less than that found in laboratory rodents, and our current research is involved in evaluating the degree of functional immunodepression in cattle.

2. ANAEMIA

The cardinal clinical sign of African trypanosomiasis in the bovine is anaemia [31]. On the basis of the presence or possible absence of the parasite, response

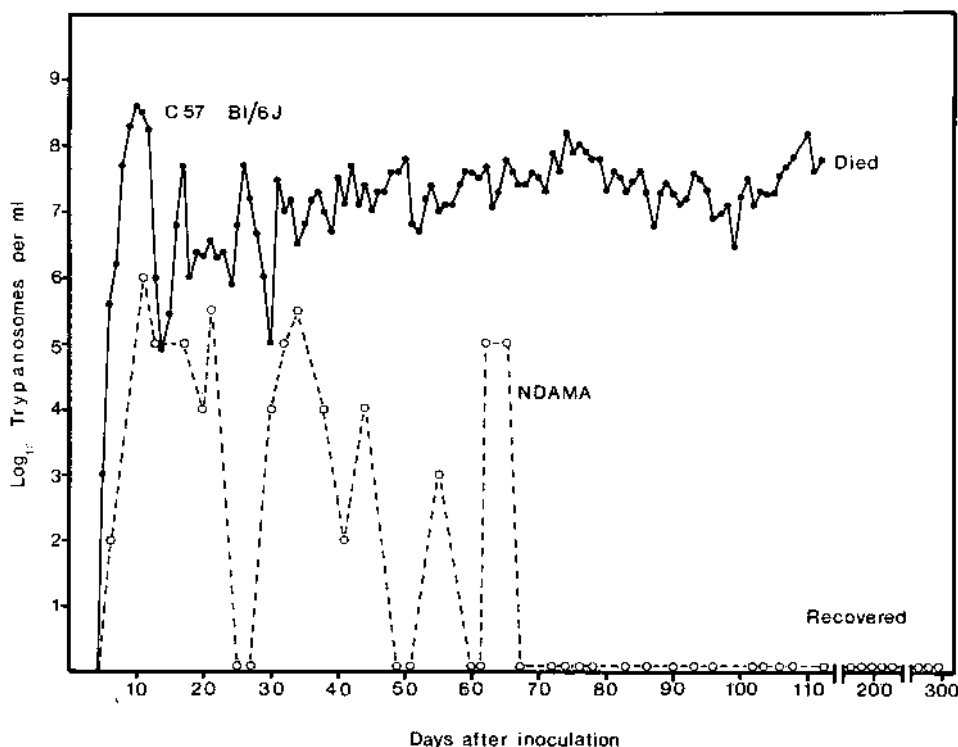


FIG.4. The level of parasitaemia found in a mouse (C57Bl/6J) and a bovine (N'Dama) inoculated with *Trypanosoma congolense*. The N'Dama has the ability, lacking in the mouse, to control, reduce and, in this particular animal, eliminate the parasite (from Ref. [24]).

to treatment, and on clinical and pathological findings, the anaemic process can be divided into at least two phases. During the initial parasitaemic phase, the anaemia is haemolytic and results from increased red cell destruction by phagocytosis; splenomegaly, which is partly due to vascular engorgement and erythrophagocytosis, is a major feature at necropsy. Several mechanisms may be involved including haemolysins produced by the trypanosome, immunological factors, fever, disseminated intravascular coagulation and an expanded and active mononuclear phagocytic system. Whereas each of these may function independently, it is likely that they interact, e.g. the haemolytic factor, fever or disseminated intravascular coagulation may damage the erythrocyte membrane which then more readily binds antigen-antibody complexes or complement, thereby facilitating erythrophagocytosis. During this phase, there is evidence that the

bone marrow is responsive as judged by ferrokinetic studies [32] and by a macrocytic response [33]. However, even in this early phase, some workers feel that the erythropoietic response is not as great as might be expected for the degree of anaemia [32]. Whereas petechiation and ecchymosis are commonly encountered, haemorrhage is not a major feature of bovine African trypanosomiasis, apart from a very striking haemorrhagic syndrome occasionally produced by *T. vivax*.

It would appear that the mechanisms responsible for anaemia during this period of the disease depend on the presence of the parasite; treatment with trypanocidal drugs usually results in a dramatic recovery [34]. Furthermore, we have found that the severity of the anaemia at this time is related to the level of parasitaemia, a parameter influenced by the weight of challenge and breed [35, 36]. Thus, animals of trypanotolerant breeds such as the N'Dama are able to limit parasitaemia to a greater extent than more susceptible breeds such as the Zebu, and the ensuing disease is less severe [36].

Later in the disease following progressively decreasing waves of parasitaemia parasites frequently cease to be detectable in some animals. Despite the apparent absence of trypanosomes, erythrocyte destruction continues and there is evidence for a progressive dyshaemopoiesis. During this phase, cattle do not respond to chemotherapy. A possible explanation for continued red cell destruction at this time comes from studies in rats which showed that, after prolonged and repeated stimulation, the mononuclear phagocytic system (MPS) remained active even after the stimulant was withdrawn [37]. An analogous situation may occur in cattle, the MPS having been stimulated initially by the massive phagocytic burden placed on it by the trypanosome. In addition, ferrokinetic studies indicate a possible defect in iron metabolism with iron retention in the MPS [32]; such a situation must lead to effective iron starvation of the bone marrow and the development of dyshaemopoiesis. These kinetics studies are complemented by histological and post-mortem findings in cattle. Thus, although spleen size is often small, erythrophagocytosis continues to be a prominent feature throughout the body and there are widespread haemosiderin deposits, possibly reflecting iron trapping. The femoral marrow is yellow, gelatinous and inactive [38]. From our field experience, we believe that this chronic anaemic trypanosomiasis syndrome is common, being characterized by stunting, wasting and lethargy as well as a persistent low grade anaemia.

What has been described for the underlying mechanisms of this later phase of the disease process in the bovine is analogous to the anaemia associated with chronic disorders of man, which are characterized by persistent red cell destruction accompanied by defective iron re-utilization and dyshaemopoiesis [32]. The fact that many of these anaemias are resolved by testosterone, cobalt or erythropoietin offers the interesting possibility that their administration may be of therapeutic value in bovine trypanosomiasis.

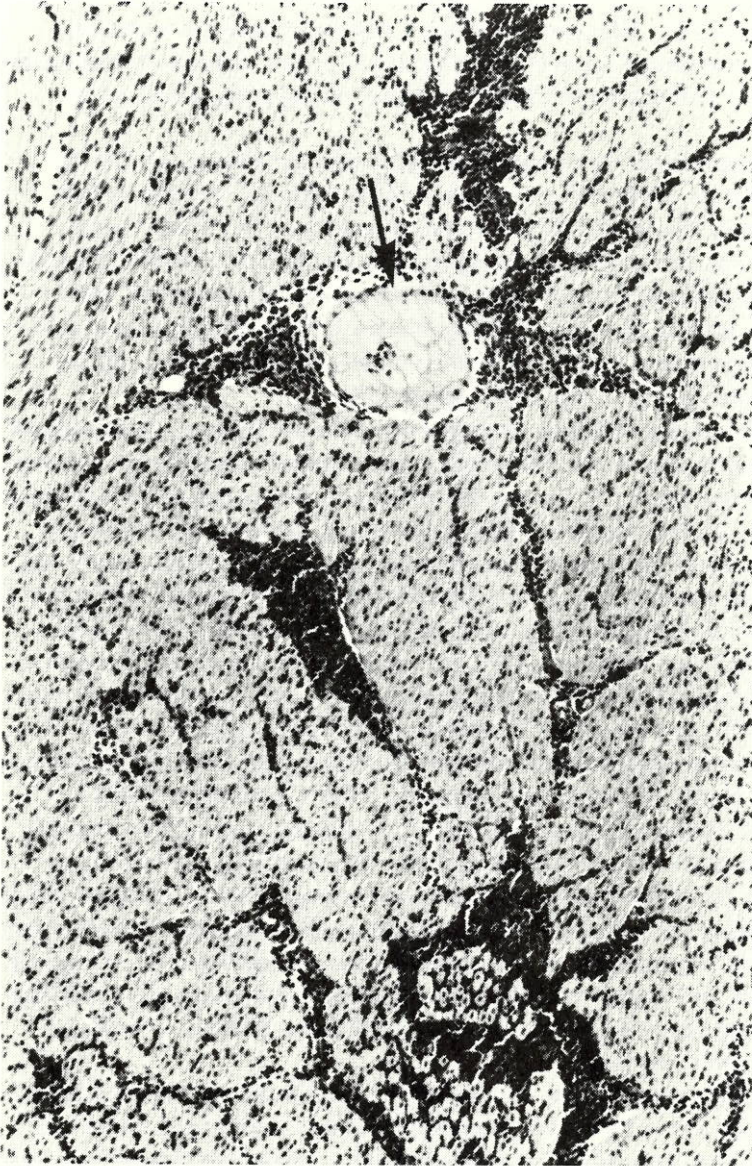


*FIG. 5. Intense cellular infiltration of the myocardium of bovine infected with *T. vivax* for six weeks. The arrow indicates a Purkinje fibre. Haematoxylin and Eosin. X 80.*

3. SPECIFIC ORGAN AND TISSUE DAMAGE

Whereas necrosis is not a feature of trypanosome infections in the bovine, widespread tissue cell damage and degeneration do occur. As might be expected from the blood-borne nature of trypanosome infections, such changes are widespread throughout the body, although certain tissues and organs are more consistently and severely affected than others. The nature of the cellular infiltrate and possibly the mechanisms involved in cell injury would appear to depend on the species of trypanosome and its tissue invasiveness. It is generally accepted that *T. congolense* parasites are confined to the circulation, particularly the microcirculation [39], *T. brucei* is distributed in both the circulation and in the tissues, whereas *T. vivax*, although predominately a vascular parasite, is found in extravascular locations [40].

One vital organ that is consistently damaged by all three species of trypanosome is the heart. Initially, lesions predominate beneath the epicardium and the endocardium in both the atria and ventricles; in advanced cases, the entire myocardium may be involved. The lesions produced by *T. brucei* and *T. vivax* are similar; the cellular infiltrate is often marked (Fig. 5), and composed of lymphoid cells, plasma cells at various stages of maturity (including Russell-Body containing cells), macrophages and occasional eosinophils (Fig. 6). These infiltrates are found in perivascular and interstitial locations and surrounding Purkinje fibres. Extravascular trypanosomes may be found in some cases. On the other hand, in *T. congolense*-infected cattle parasites have only been seen in the circulation; the interstitial cellular infiltrate is usually scanty and consists of small lymphocytes and the occasional macrophage and plasma cell. With all three species of trypanosome, perivascular and interstitial oedema is found, particularly in terminal cases.

The cardiac microvasculature is frequently distended with cells and the vessel walls are occasionally swollen and vacuolated but never necrotic, and the perivascular oedema possibly reflects increased permeability. In the bovine, thrombosis is rare although we have occasionally found thrombi in *T. vivax* infections. The lymphatics of the epicardium are often distended with cells and cell debris. Whereas cell death is uncommon, severe myocardial damage does occur in all three infections and is manifest by distortion and degeneration of myocardial fibres (Fig. 6); in addition, the conducting Purkinje fibre system is often widely separated from the myocardial fibres by oedema and cell infiltrates.

Death in cattle infected with trypanosomiasis is the result of congestive heart failure due to a combination of anaemia, microcirculatory disturbances and myocardial damage [2, 3]. This is particularly the case in cattle living under natural range conditions where, in our experience, animals may forage up to 17 miles in a day. Thus, while a packed red cell volume (PCV) of 15 or 20% is unlikely to kill an animal kept under housed conditions, a red cell deficit of this

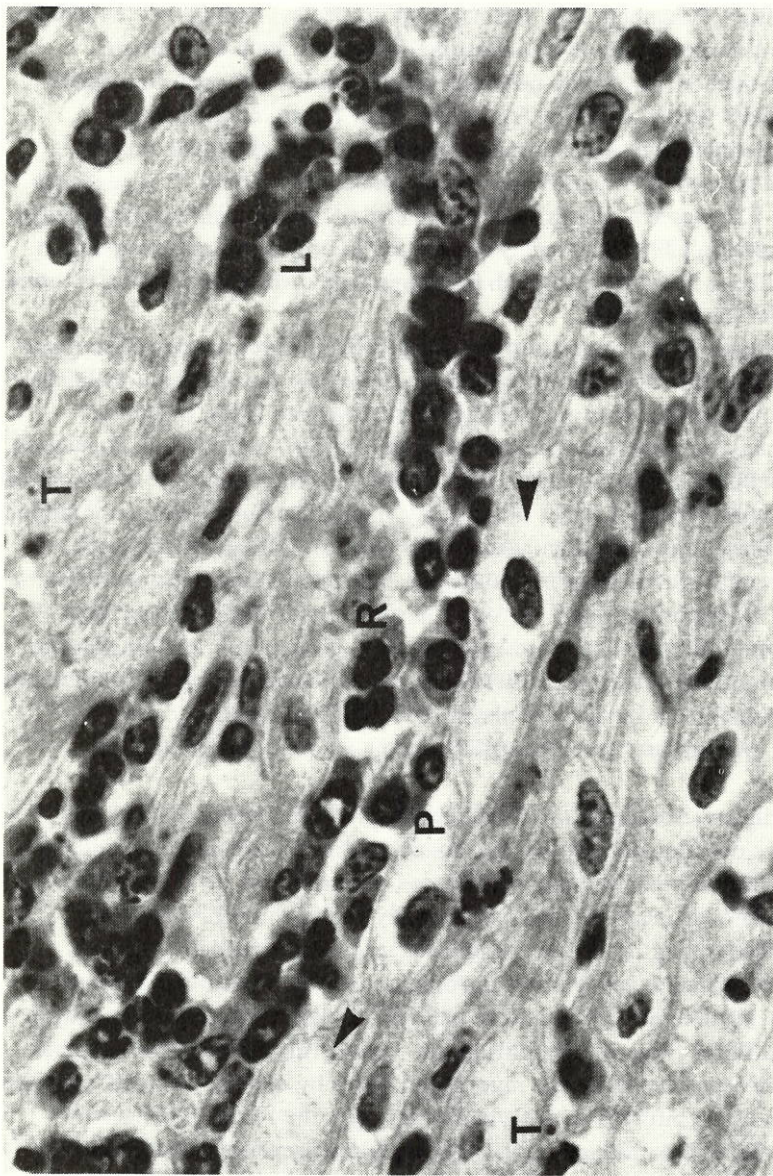


FIG. 6. The nature of the cellular infiltrate in Fig. 5. Numerous lymphocytes (L) and plasma cells (P) are present including a Russell-body containing plasma cell (R). Trypanosomes are scattered throughout the myocardium (T) and there is marked vacuolation and degeneration of myocardial muscle fibres (arrows). Haematoxylin and Eosin. X 960.

order must restrict the ability of cattle to forage over the long distances required under savanna conditions; the effort to achieve this is likely to lead to cardiac decompensation. In this respect, we have noted that cattle in a serious clinical condition will often make a dramatic improvement if moved from the range to a housed situation where they are not required to forage.

Lesions produced by trypanosome infections in cattle may be widespread throughout the body. In the present report we do not intend to catalogue these except to give a few examples that we believe to be of particular importance. Thus, the skeletal musculature may be severely damaged and degenerate resulting in wasting which is often manifest clinically. Significant effects of infection also occur in the reproductive organs, and we have observed abortion 'storms' in herds of cattle suffering from trypanosomiasis; further research is required in this area.

4. MECHANISMS OF ORGAN AND TISSUE DAMAGE

Although much descriptive work has been published on the lesions in trypanosome-infected animals, the amount of information available on the mechanisms involved is limited. This situation has been reviewed recently [41, 42], but we would like to emphasize certain aspects.

4.1. Blood and circulatory disturbances

It is likely that the anoxia caused by progressive anaemia in bovine trypanosomiasis contributes to the extensive cellular degeneration that occurs particularly in the parenchymatous organs including the heart, skeletal muscle, liver and kidneys. This situation must also be augmented by cellular infiltration and oedema which leads to structural distortion and physiological disturbance. As evidence of increased vascular permeability in trypanosome-infected animals, vessel walls are often swollen and vacuolated and show an expansion of the perivascular space. This may be due, at least in part, to the elevated kinin levels that occur in the blood of infected cattle [43] and it is thought that the activation of kinins may involve the interaction of antigen-antibody complexes with Hageman factor [44]. In addition, *T. congolense* has been shown to elaborate an inflammatory permeability factor as well as to activate complement directly [42]; at the same time, *T. congolense* has a specific affinity for the endothelial cells of the microvasculature [39], at which level both these mechanisms are likely to be operative.

Disseminated intravascular coagulation (DIC) has been implicated in the pathogenesis of both human and laboratory animal trypanosomiasis [31], but its role in cattle awaits full evaluation. Thrombosis and necrosis have been described

in cattle infected with *T. vivax* [45] and were attributed to DIC. It is possible that DIC could be important in the acute haemorrhagic syndrome of cattle caused by *T. vivax* [46].

4.2. Direct toxic damage

Until recently, the hypothesis that trypanosome 'toxins' were involved in the pathogenesis of trypanosomiasis has been out of favour, especially as the earlier reports were difficult to confirm. However, there are now several papers that have demonstrated in vitro the existence of a range of biologically active substances such as haemolysins, inflammatory and permeability factors, complement activating factors, platelet aggregating factor, immunosuppressive factors and polyclonal B lymphocyte mitogens [41, 42]. In most instances, however, these factors have been detected only by in vitro assays and their in vivo relevance remains to be determined; however, we do have evidence that a haemolytic factor may operate in vivo [31]. In this respect, it has been found in neonatal mice or sub-lethally irradiated mice infected with *T. brucei* that, although large numbers of organisms are present in the tissues, cellular infiltration is scanty and there is virtually no tissue cell degeneration or death [45]. Whereas these findings are open to a number of interpretations, it would appear unlikely that live trypanosomes by themselves produce severe tissue injury. However, it is possible, following death of large numbers of organisms in the tissues, that substances capable of initiating cell death may be released.

4.3. Immunological mechanisms

The massive and persistent immunological reaction induced by consecutive waves of trypanosomes may have adverse side effects on the host. Support for this possibility has come from recent work in mice infected with *T. brucei* [47]. In intact adult mice, *T. brucei* produces severe myocardial and skeletal muscle damage in the vicinity of extravascular trypanosomes and a cellular infiltrate composed of lymphocytes, plasma cells, macrophages and a few polymorphonuclear leukocytes. Associated with the lesions are granular deposits of Ig and trypanosome antigen. On the other hand, immunologically incompetent mice, including neonates, sub-lethally irradiated mice and athymic nude mice show only minimal tissue damage despite the presence of large numbers of trypanosomes; correspondingly there is a marked decrease in Ig deposited in the tissues. Nude mice infected with *T. brucei*, after receiving normal syngeneic spleen cells or an Ig-negative fraction of spleen cells, develop lesions as severe as those in intact mice; at the same time, heavy deposition of Ig can be detected. When antitrypanosome antibody is given to nude mice, the lesions that develop are only slightly more severe than in infected nude controls. Thus, it would appear

that the immune response, at least in mice, is important in the development of tissue lesions. Several mechanisms may be involved including antigen-antibody reactions in the circulation, antigen-antibody reactions within the tissues, cell-mediated reactions or auto-immune reactions. Whereas circulating immune complexes have been demonstrated in trypanosome-infected animals [2, 47], it is likely that the interaction of antibody with trypanosomes or trypanosome antigens within the tissues is a more important mechanism than deposition of immune complexes from the circulation; the antibody involved may enter the tissue from the circulation or may be locally produced by the large number of plasma cells in the infiltrate. It was found that the transfer of Ig-negative cells to nude mice increased the severity of the lesions, and it was suggested that this might have been due to a T-helper cell effect enhancing antibody formation and leading to Ig deposition. Whether or not conventional cell-mediated hypersensitivity reactions are important in the pathogenesis of tissue lesions remains to be determined. However, a delayed-type hypersensitivity reaction to trypanosome antigen has been demonstrated in rabbits infected with *T. brucei* [48]. Whereas a range of auto-antibodies has been detected in the sera of trypanosome-infected animals [7] and has been incriminated in tissue damage, the role played by auto-antibodies awaits evaluation and their presence may merely reflect tissue injury or polyclonal B cell activation.

It would appear to us that in the bovine the tissue invasiveness of *T. brucei*, and to a lesser extent of *T. vivax*, would make it likely that hypersensitivity mechanisms of the type described above may be operative, whereas in *T. congolense*-infected cattle we believe that most of the tissue damage can be attributed to the anoxia of the anaemia and to microcirculatory disturbances.

REFERENCES

- [1] MORRISON, W.L., MURRAY, Max, "Lymphoid changes in African trypanosomiasis", Recent Advances in the Knowledge of Pathogenicity of Trypanosomes (Proc. IDRC. Conf. Nairobi, 1978) (in press).
- [2] MURRAY, Max, "The pathology of African trypanosomiasis", Progress in Immunology II (BRENT, L., HOLBOROW, J., Eds), North-Holland Publishing Co. (1974) 181.
- [3] NIELSEN, K., et al., Immunology 35 (1978) 811.
- [4] FREEMAN, T., et al., J. Infect. Dis. 121 (1970) 401.
- [5] CORSINI, A.C., et al., Clin. Exp. Immunol. 29 (1977) 122.
- [6] HOUBA, V., BROWN, K.N., ALLISON, A.C., Clin. Exp. Immunol. 4 (1969) 113.
- [7] MACKENZIE, A.R., BOREHAM, P.F.L., Immunology 26 (1974) 1225.
- [8] MURRAY, P.K., et al., Immunology 27 (1974) 825.
- [9] HUDSON, K.M., et al., Nature (London) 264 (1976) 256.
- [10] URQUHART, G.M., et al., Trans. R. Soc. Trop. Med. Hyg. 67 (1973) 528.
- [11] HAZLETT, C.A., TIZARD, I.R., Clin. Exp. Immunol. 33 (1978) 225.
- [12] PEARSON, T.W., et al., Eur. J. Immunol. 8 (1978) 723.

- [13] BARBET, A.F., McGUIRE, T.C., Proc. Natl. Acad. Sci. U.S.A. 75 (1978) 1989.
- [14] ASKONAS, B.A., et al., Immunology (in press).
- [15] GREENWOOD, B.M., WHITTLE, H.C., MOLYNEUX, D.H., Trans. R. Soc. Trop. Med. Hyg. 67 (1973) 846.
- [16] HOLMES, P.H., et al., Vet. Rec. 95 (1974) 86.
- [17] SCOTT, J.M., et al., Trop. Anim. Hlth Prod. 9 (1977) 159.
- [18] RURANGIRWA, F.R., et al., Res. Vet. Sci. 25 (1978) 395.
- [19] WHITELAW, D.D., et al., Res. Vet. Sci. 26 (1979) 102.
- [20] WILSON, A.J., CUNNINGHAM, M.P., Exp. Parasitol. 32 (1972) 165.
- [21] CROSS, G.A.M., Nature (London) 277 (1979) 310.
- [22] BRIDGEN, P.J., CROSS, G.A.M., Nature (London) 263 (1976) 613.
- [23] MANSFIELD, J.M., BAGASRA, O., J. Immunol. 120 (1978) 759.
- [24] MURRAY, Max, MORRISON, W.I., "Parasitaemia and host susceptibility to African trypanosomiasis", Recent Advances in the Knowledge of Pathogenicity of Trypanosomes (Proc. IDRC. Conf. Nairobi, 1978) (in press).
- [25] JAYAWARDENA, A.N., WAKSMAN, B.H., EARDLEY, D.D., J. Immunol. 121 (1978) 622.
- [26] PEARSON, T.W., et al., Eur. J. Immunol. (in press).
- [27] WELLHAUSEN, S.R., MANSFIELD, J.M., J. Immunol. 122 (1979) 818.
- [28] ALBRIGHT, J.W., ALBRIGHT, J.F., DUSANIC, D.G., Proc. Natl. Acad. Sci. U.S.A. 75 (1978) 3923.
- [29] ASSOKU, R.K.G., TIZARD, I.R., NIELSEN, K.H., Lancet. ii (1977) 956.
- [30] ROELANTS, G.E., et al., Clin. Exp. Immunol. (in press).
- [31] MURRAY, Max, "The anaemia of African trypanosomiasis: an overview", Recent Advances in the Knowledge of Pathogenicity of Trypanosomes (Proc. IDRC. Conf. Nairobi, 1978) (in press).
- [32] DARGIE, J.D., et al., Parasitology (in press).
- [33] VALLI, V.E.O., FORSBERG, C.M., McSHERRY, B.J., Vet. Pathol. 15 (1978) 732.
- [34] HOLMES, P.H., JENNINGS, F.W., "The effect of treatment on the anaemia of African trypanosomiasis", Pathophysiology of Parasitic Infection (SOULSBY, E.J.L., Ed.), Academic Press, New York (1976) 199.
- [35] MURRAY, P.K., et al., ISCTRC (15th meeting, The Gambia, 1977), OAU/STRC, Publ. No. 110. 482.
- [36] MURRAY, P.K., et al., ISCTRC (15th meeting, The Gambia, 1977), OAU/STRC, Publ. No. 110. 470.
- [37] JANDL, J.H., et al., J. Exp. Med. 122 (1965) 299.
- [38] MURRAY, Max, et al., ISCTRC (15th meeting, The Gambia, 1977), OAU/STRC, Publ. No. 110. 92.
- [39] BANKS, K.L., J. Protozool. 25 (1978) 241.
- [40] BUNGENER, W., MEHLITZ, D., Tropenmed. Parasitol. 28 (1977) 8.
- [41] MORRISON, W.I., MURRAY, Max, SAYER, P.D., "The pathogenesis of tissue lesions in *Trypanosoma brucei* infections", Recent Advances in Knowledge of Pathogenicity of Trypanosomes (Proc. IDRC. Conf. Nairobi, 1978) (in press).
- [42] TIZARD, I.R., et al., Microbiol. Rev. 42 (1978) 661.
- [43] BOREHAM, P.F.L., Br. J. Pharmacol. Chemother. 32 (1968) 493.
- [44] GOODWIN, L.G., Trans. R. Soc. Trop. Med. Hyg. 64 (1970) 797.
- [45] VAN DEN INGH, T.S.G.A.M., et al., Vet. Parasitol. 2 (1976) 237.
- [46] HUDSON, J.R., J. Comp. Pathol. 44 (1944) 108.
- [47] GALVAO-CASTRO, B., HOCHMANN, A., LAMBERT, P.H., Clin. Exp. Immunol. 33 (1978) 12.
- [48] TIZARD, I.R., SOLTYS, M.A., Infect. Immun. 4 (1971) 674.

DISCUSSION

H.S. HUSSEIN: How do trypanosomes bring about immunodepression?

Max MURRAY: In Session 3 of this meeting, R.J. Terry will discuss possible mechanisms of immunodepression, several of which have been demonstrated or incriminated in laboratory animal models.¹ In cattle, however, we know little about the extent or mechanisms of immunodepression.

H.S. HUSSEIN: Is haemolytic anaemia common to both types of trypanosomes, the haematic group (*T. congolense*, *T. vivax*) and the tissue group (*T. brucei*)?

Max MURRAY: All three produce a haemolytic anaemia, although it should be emphasized that there is a virulent *T. vivax* strain that produces a haemorrhagic syndrome.

R.J. TERRY: I was very interested to hear that germinal centres developed strongly in the bovine but not in the mouse. Germinal centres are known to be the site of B-lymphocyte memory cell formation. Do you then think that perhaps one of the reasons why cattle control their infections better than mice is that they can 'remember' trypanosome antigens? This could be important if it is proved that there are cross-reacting determinants in the variant antigens.

Max MURRAY: I agree and would add that the mechanisms underlying immunodepression are unknown and require investigation.

R. GALUN: We have recently seen a very strong reaction to a non-infected bite. A woman bitten for the third time had an extremely swollen hand, lymph gland response and so on lasting for five days without antihistamines or cortisone producing any response.

Max MURRAY: A single bite by an uninfected *G. morsitans* on a susceptible bovine host produces a minimal reaction, unlike that of the infected fly. We have not examined a situation in which cattle have been subjected to multiple bites by uninfected flies.

R. GALUN: What is the pathological response of the central nervous system (CNS), and how similar it is to that found in human sleeping sickness?

Max MURRAY: CNS lesions do occur, but I am not sure of their functional significance. In general, they do not appear to be severe. The Walter Reed group working in Western Kenya have found severe CNS lesions in cattle that were possibly infected with *T. rhodesiense*. Severe lesions may also develop in *T. brucei*-infected mice. I have no personal experience of the pathology of CNS lesions in man, but the severe encephalitis described in cattle in Western Kenya would appear to be similar to that found in man.

¹ TERRY, R.J., HUDSON, K.M., FAGHIHI SHIRAZI, M., "Secondary immunodeficiencies associated with African trypanosomiasis", these Proceedings, IAEA-SM-240/27.

R.S. PHILLIPS: In Section 2 of your paper you say that some trypanosome-infected animals did not respond to treatment. What form of treatment was involved?

Max MURRAY: Berenil.

R.S. PHILLIPS: Would you like to speculate on the mechanisms involved in protecting the trypanotolerant animals?

Max MURRAY: The mechanisms of reduced susceptibility to trypanosomiasis have yet to be clarified. They have a genetic basis and are heritable, at least in mice, as a dominant trait. We believe that immunological mechanisms could be important although several other factors may be involved, such as superior heat tolerance or water conservation which could aid survival.

L.H. OTIENO: What are the pathological differences between a virulent *T. congolense* and *T. vivax* on the one hand, and chronic strains of these diseases on the other?

Max MURRAY: Many factors influence host susceptibility to trypanosomiasis, including the pathogenicity of the trypanosome. I assume that with *T. congolense* the more virulent strains produce a more rapid and severe anaemia. Very virulent strains of *T. vivax* produce a very dramatic and acute haemorrhagic syndrome. Other strains of *T. vivax* can produce the classical haemolytic anaemia of bovine trypanosomiasis, as can *T. brucei*.

GENERAL DISCUSSION

on tsetse flies

R. GALUN: Where tsetse flies are concerned, if one is aiming at control rather than eradication, one should not use sterile insects as they will increase the risk of trypanosomiasis.

D.A. LINDQUIST: I agree that use of the sterile insect technique should not be considered for control of tsetse flies. However, since tsetse eradication will probably be required to solve the trypanosomiasis problem, it will be worth while using the technique.

Max MURRAY: do tsetse develop resistance to insecticides?

D.A. LINDQUIST: There is some evidence of moderate resistance but this does not render insecticides any less effective.

Max MURRAY: With regard to the emotive question of eradication versus control, it must be emphasized that a small number of tsetse can create havoc in a herd of cattle.

O.U. ALOZIE: On behalf of the United Nations Environment Programme (UNEP), I would like to point out the need for concern about the environmental effects of control measures and for cost-effectiveness studies on such measures.

BABESIOSIS AND ANAPLASMOSIS

(Session 2)

BOVINE ANAPLASMOSIS WITH EMPHASIS ON IMMUNE RESPONSES AND PROTECTION

M. RISTIC

Department of Veterinary Pathology and Hygiene,
College of Veterinary Medicine,
University of Illinois,
Urbana, Illinois,
United States of America

Abstract

BOVINE ANAPLASMOSIS WITH EMPHASIS ON IMMUNE RESPONSES AND PROTECTION.

Anaplasmosis is an infectious and transmissible disease manifested by progressive anaemia and the appearance of other characteristic disease symptoms. It is a world-wide tick-borne disease of cattle and some wild ruminants caused by the rickettsia *Anaplasma marginale*. By drawing on information obtained from studies of plasmodial cell cultures, a method has recently been developed for short-term in vitro cultivation of *A. marginale*. An attenuated *Anaplasma* organism capable of growth in both ovine and bovine erythrocytes was used to demonstrate that the in vitro system provided the necessary requirements for active transfer of the organism from cell to cell. Organismal antigens are found in the erythrocytes of infected animals, whereas soluble antigens are derived from their erythrocytes and serum. Serums from convalescing animals interact with these antigens in agglutination, complement fixation, fluorescent antibody and precipitation tests. Passive transfer of sera from immune to susceptible cattle, however, does not seem to confer protection against the infection and development of the disease. Studies that employed various tests for measuring cell-mediated immune (CMI) responses (leukocyte migration inhibition, blast transformation and cytotoxicity), in association with information collected simultaneously on antibody activity, have shown that both humoral and cellular immune responses are needed for the development of protective immunity in anaplasmosis. It was further shown that an active replication of *Anaplasma* is essential for induction of these two types of immune responses. Consequently, live virulent and attenuated immunogens fulfil requirements for induction of protective immunity. With the virulent agent, however, development of protective immunity is preceded by induction of auto-immune responses apparently associated with pathogenesis of anaemia in anaplasmosis. Inactivated immunogens derived from blood of infected cattle and used in combination with oil adjuvants stimulate little protection and frequently induce a state of harmful isoimmunity in vaccinated animals.

Anaplasmosis is a world-wide tick-borne disease of cattle and some wild ruminants caused by the rickettsia *Anaplasma marginale*. The organism is an obligate erythrocytic parasite transmitted by arthropod vectors. Whereas the cycle of development in the tick vector remains generally unknown, newer knowledge has been acquired on the erythrocytic cycle of development of the organism and the antigenicity of certain growth forms. A method for in vitro propagation of

A. marginale, similar to that used for propagation of malaria parasites, has produced encouraging results. Further studies and perfection of this method would mean a new area in the study of anaplasmosis, ultimately providing vast possibilities for acquiring more knowledge regarding the nature of the organism and control of the disease it causes. Similarly to other infectious haemotropic diseases, anaplasmosis is also characterized by the continuous presence of the aetiological agent in the blood circulatory system. Under the circumstances, the organism must use different mechanisms to avoid destruction by an immunologically hostile host. A further interesting feature of anaplasmosis and other diseases caused by erythrocytic invaders is the mechanism of protective immunity. This immunity is referred to as 'infection immunity' or 'premunition', and is characterized by resistance established in the chronic stage of an infection that usually lasts as long as infective organisms remain in the body. More recent studies, however, indicate that a considerable degree of sterile clinical immunity does persist following elimination of the organism from a carrier cow by chemotherapy. A variety of serologically demonstrable serum antibodies appear in the course of anaplasma infection, and detection of these antibodies by various serodiagnostic procedures has been an important factor in field diagnosis and control of anaplasmosis. The role of an auto-immune-like syndrome in the pathogenesis of anaemia is clearly demonstrated by the occurrence of antibody-dependent and -independent cytotoxic effects against autologous erythrocytes. In regard to protective immunity, humoral antibodies are undoubtedly important: however, cell-mediated immunity (CMI) seems to be of at least equal importance. Consequently, the protection induced by killed vaccines, which are poor CMI stimulants, is inferior to that induced by live vaccines that are known to stimulate strong and long-lasting CMI responses. These and other aspects of anaplasmosis with emphasis on immune responses and protection are the subject of the present study.

1. THE ORGANISM AND RELATED SPECIES

In the 8th edition of Bergey's Manual, *A. marginale* is the representative species of the genus *Anaplasma*, family Anaplasmataceae, order Rickettsiales [1]. The organism (initial body) was shown to enter the erythrocyte by rhopheocytosis, a process involving invagination of the cytoplasmic membrane with subsequent formation of a vacuole. Thereafter, the initial body multiplies by binary fission and forms an inclusion body consisting generally of four to eight initial bodies. Inclusion bodies are numerous during the acute phase of the infection but low-level infections persist for several years thereafter [2]. Recent studies indicate that mature initial bodies may give rise to smaller bodies of high chromatin content. These forms may represent an actual infectious organism which enters the erythrocytes and there develops into an initial body [3]. Of the three anaplasma

species, *A. marginale* is the most pathogenic for cattle. *Anaplasma centrale* causes a relatively 'mild' form of bovine anaplasmosis in Africa, and *Anaplasma ovis* is the cause of ovine and caprine anaplasmosis.

Paranaplasma caudatum (genus *Paranaplasma*) was initially found in Oregon cattle in a mixed infection with *A. marginale*. Inclusion bodies of *P. caudatum* have appendages usually in the form of a tapering tail, a loop or a ring, demonstrated only by use of special techniques [4]. A recent study in our laboratory showed that manifestation of *P. caudatum* appendages is a function of bovine host erythrocytes, and does not occur in infected deer erythrocytes [5].

Anaplasmas morphologically resemble other haemotropic rickettsiae, i.e. *Haemobartonella* and *Eperythrozoon*, and share common antigens with these agents [4].

2. IN VITRO CULTIVATION OF *Anaplasma marginale*

Over the years, various efforts were made to maintain and/or cultivate in vitro *A. marginale*. Cell culture systems of mammalian origin included bovine bone marrow, rabbit bone marrow and bovine lymph nodes [6]. Although some successes were reported, studies were not continued and there is no information on results being reproduced by other investigators. The use of arthropod cell cultures for cultivation of *A. marginale* was reported more recently [7]. Cultures derived from the mosquito *Aedes albopictus* were used to cultivate the organism. When erythrocytes from a calf in the acute phase of anaplasmosis were introduced in the above system, cultured mosquito cells phagocytized both infected and uninfected erythrocytes. The organism remained viable in these cells, as determined by calf inoculation, for 21 days.

None of the above systems produced evidence of active replication of *A. marginale*. Nevertheless, other parasites of red blood cells have recently been cultivated in vitro [8, 9].

Recently in our laboratory a modification of these cell culture systems was developed for propagation of *A. marginale* [10, 11]. In various trials, employing different procedures for diluting the original inoculum by serial passages, the organism was maintained in vitro between 28 and 60 days. A maximum intra-erythrocytic parasitaemia increase of more than 1000% was produced. Multiple erythrocyte infections with inclusion bodies varying in size, arrangement and position were abundant. Organisms on or in proximity to erythrocytic membrane were also present. Actual invasion of new erythrocytes by the organism was demonstrated by using the attenuated *A. marginale* which transgressed from ovine into bovine erythrocytes and developed in the latter cells. Electron microscopic examination of cultures revealed a considerable quantity of extra-erythrocytic *Anaplasma* bodies being produced by the system (Fig.1). Thus the true quantum

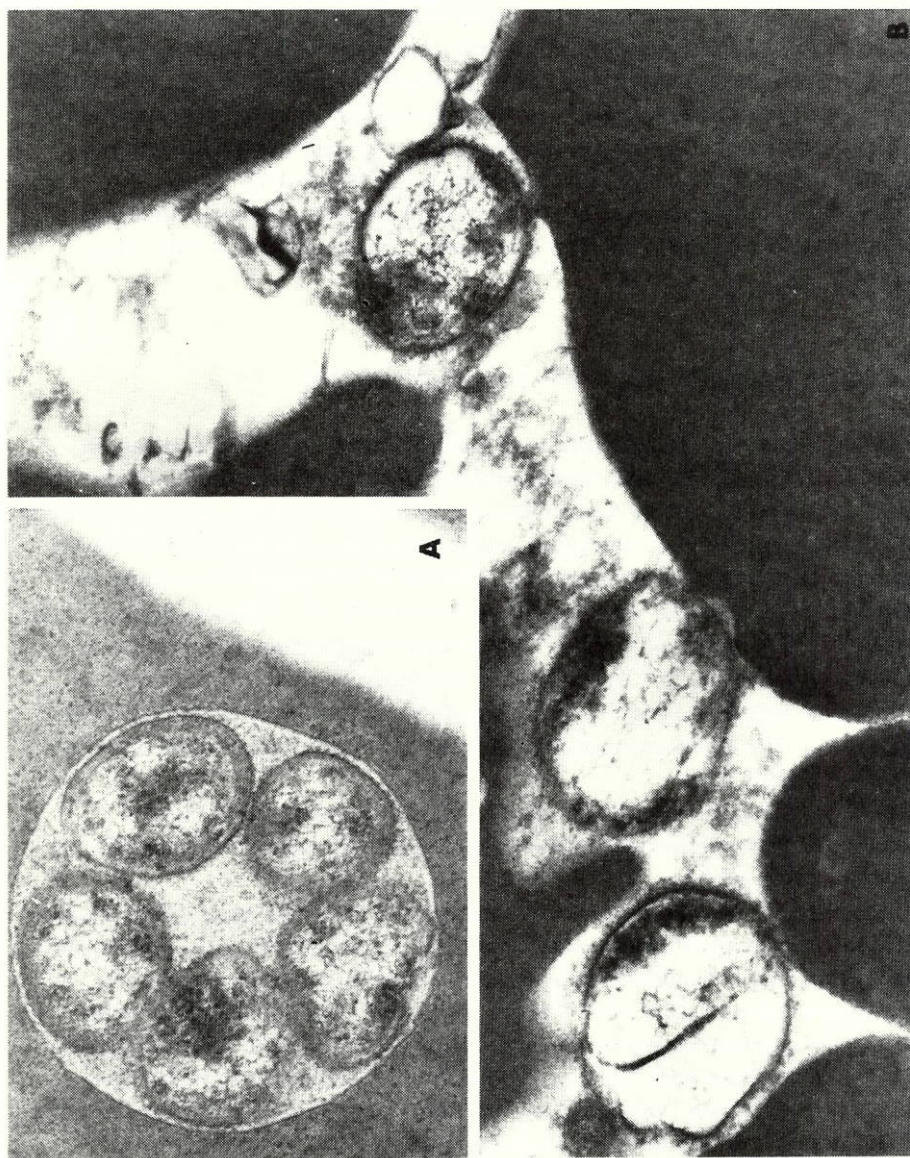


FIG. 1. *Anaplasma marginale* in cell cultures. Electron micrographs of an intra-erythrocytic inclusion body composed of five initial bodies (A) and three extra-erythrocytic initial bodies in close proximity to erythrocytic membranes (B). X 55, 746.

of parasitic mass produced by this cell culture system was greater than that revealed by enumeration of intra-erythrocytic forms. The organisms of 30- to 60-day-old cell culture consistently induced infections in susceptible cattle. In all instances, incubation periods and the virulence of the organism were similar to those produced by infections with whole blood derived from an animal in the acute phase of the disease.

3. ANTIGENIC AND SEROLOGIC STUDIES OF *A. marginale*

Organismal antigens are found in the erythrocytes of infected animals, whereas soluble antigens are found in the erythrocytes and free in the serum. These antigens are serologically related and obtained most readily from animals in the acute phase of the disease.

4. INTRA-ERYTHROCYTIC ANTIGENS

Soluble, hydrochloric acid (HCl) and protamine sulphate (PS)-precipitated *A. marginale* antigens have been found to be lipoproteins. The antigens were reacted with antibody in the micro agar gel precipitation test. Chemical resemblance of the PS antigen to a similar preparation made from anaplasma-free erythrocytes incriminated it as a possible antigen responsible for the auto-immune state proposed in anaplasmosis [2]. A soluble proteinaceous *A. marginale* antigen has been derived from lysed erythrocytes, and its reactivity studied in agar gel diffusion [12]. No antigenic differences were found between two virulent *A. marginale* isolants and the attenuated *A. marginale* when soluble antigens and specific antisera were reacted in agar gel and immunoelectrophoresis tests [13].

Various particulate antigens for diagnostic tests have been prepared from infected erythrocytes. The active component of the complement fixation (CF) antigen was found to be lipoprotein [12, 14]. Capillary tube agglutination (CA) antigen consists of structures resembling initial anaplasma bodies that reacted specifically with sera from infected animals [15]. A rapid card agglutination antigen was produced by liberating corpuscular anaplasma antigen from washed infected erythrocytes using the French pressure cell under precisely controlled conditions [16].

Those who have developed and studied agglutinating *Anaplasma* antigens considered initial anaplasma bodies (subunits of the marginal inclusion body) as the antigenic units functional in the agglutination test [15]. This concept was questioned and it was proposed that the agglutination reaction in the CA test does not involve a classic antigen-antibody reaction [17]. The same laboratory reported that their study indicated the antibody detected by the CA test is directed against

the erythrocytic stroma and not against the 'finite' purified initial anaplasma bodies produced by them. This concept is convincingly disproved by a recent immunoelectronmicroscopic study using ferritin-conjugated antibody from infected cattle to stain corpuscular antigens of *P. caudatum* [18].

5. SERUM ANTIGENS

Soluble antigens of *A. marginale* were extracted from the serum and lysed erythrocyte preparations of animals in the acute phase of anaplasmosis [19]. Cyclic emission of soluble parasite antigen was indicated by fluctuation of test results. The presence of detectable intra-erythrocytic *A. marginale* bodies did not always coincide with the presence of soluble antigens in serum. It also appeared that a soluble component of the CF antigen and the exoantigen found in serum of infected cattle were identical.

6. KINETICS OF THE SERUM RESPONSE

Fluctuations of serum proteins and lipids occurring in the course of infection with *A. marginale* have been studied. Kinetics of immunoglobulins associated with complement-fixing (CF) and agglutinating (CA) antibodies following vaccination with the attenuated *A. marginale* vaccine was one such study [20]. Complement-fixing antibodies were first detected at three weeks after vaccination, reached the maximum titre of 1:640 approximately two weeks later, and gradually subsided to a titre of 1:20 during the period of 14 to 22 months post vaccination. Agglutinating antibodies exhibited maximum titre of 1:32 approximately 2½ months after vaccination and gradually subsided to a level of 1:2 at 12 to 22 months of observation. Complement fixing and CA antibodies resided exclusively in the IgM class during at least 3 and 2 post-vaccination months, respectively. During the period of 4½ to 22 months of observation, the CF antibody was apparently equally distributed between the IgM and IgG. On the contrary, two thirds of the CA antibody resided in the IgG and one third in the IgM classes at 4 months after vaccination. During the period of approximately 7 to 22 post-vaccination months, the CA antibody was present exclusively in the IgG class.

7. IMMUNIZATION

Prophylactic immunization against anaplasmosis used naturally occurring *Anaplasma* species (premunization), laboratory-attenuated *A. marginale* of ovine origin, and inactivated *A. marginale* of bovine and ovine origin.

7.1. Naturally occurring *Anaplasma marginale* and *A. centrale*

Early use of field isolants of anaplasma for premunization consisted of injecting blood from known carriers into susceptible cattle. This procedure presented a certain risk since it was often necessary to control the initial infection with drugs so that subsequent recovery and development of the carrier state could occur. A method for premunization using a small inocula to induce protection has also been reported [21] but later results indicated that severe disease and mortality can result from even small doses of *A. marginale* [22].

Prior infection with *A. centrale* did not prevent infection with *A. marginale* but reduced the severity of the superimposed disease. On this basis, Theiler developed the method of *A. centrale* vaccination which is still used in several countries [23]. Variable resistance to *A. marginale* resulting from vaccination with *A. centrale* was later described. Furthermore, it was observed that cattle inoculated with *A. centrale* (South African) strain developed severe symptoms of the disease [24].

7.2. Laboratory attenuated *Anaplasma marginale*

A detailed description of the development of the attenuated *A. marginale* from the virulent Florida anaplasma isolant has been reported elsewhere [25, 26].

The basic methodology used for attenuation of the Florida isolant has been: (a) induction of an apparently accelerated rate of mutation of the organism by exposure to irradiation; and (b) selection of an avirulent *A. marginale* strain by serial passage of irradiated organisms in splenectomized deer (2 passages) and sheep (138 passages). Lots of sheep blood-derived seed material of established immunogenicity, growth pattern and safety were preserved by storage in liquid nitrogen. For vaccine production, the seed material is reactivated in splenectomized sheep, using four to five passages at seven- to nine-day intervals. The vaccine is dispensed in liquid nitrogen and inoculated intramuscularly in 1- to 2-ml doses. The prepatent period in inoculated animals varies between four and six weeks after which the organism may be detected in approximately 0.5–8% of the peripheral blood erythrocytes. A slight haematocrit decrease, usually not exceeding 4–15% of the pre-inoculation value, generally occurs. These manifestations are transitory and are in evidence for one to two weeks. Inoculated animals are not clinically affected.

A series of laboratory and field experiments conducted in the United States of America, Peru, Venezuela, Mexico and Colombia have shown that the immune response induced by this vaccine protects adult susceptible cattle against challenge with virulent endemic strains of *Anaplasma* [26].

8. INACTIVATED *Anaplasma marginale* OF BOVINE AND OVINE ORIGIN

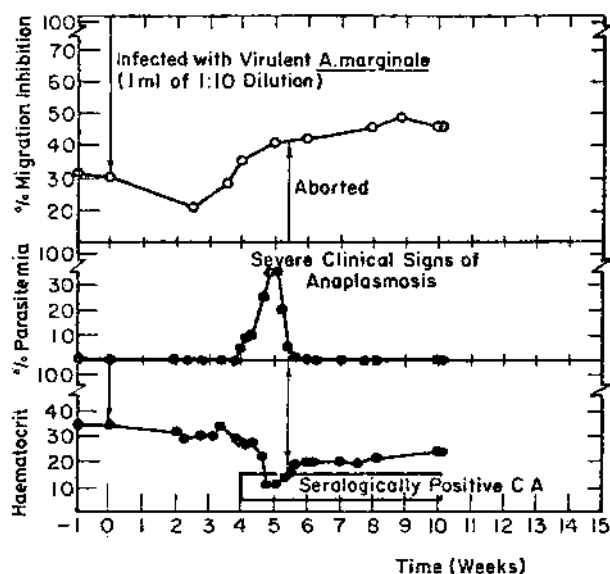
Use of a vaccine containing killed anaplasma organisms has been described in Africa [27] and the United States of America [28]. The United States vaccine at present used commercially, 'Anaplaz', was prepared from blood of infected animals collected at the peak of parasitaemia. Blood cells were washed, lysed and lyophilized. The desiccated material was reconstituted with an oil adjuvant. Two doses of the vaccine were given subcutaneously at an interval of about six weeks. A degree of protection from the clinical signs of anaplasmosis has been reported to be afforded two weeks after the second injection. Vaccinated animals become carriers after field challenge. An annual booster injection is also recommended. The greatest drawback of this vaccine is induction of iso-immunization and erythrolysis due to the bovine blood group components which it contains.

A preparation similar to Anaplaz was developed in our laboratory using ovine erythrocytes infected with the attenuated *A. marginale*. The Anaplaz adjuvant has been used to reconstitute the lyophilized material and the aforementioned vaccination regimen followed. Protection obtained after challenge of cattle vaccinated with Anaplaz and this sheep-derived inactivated vaccine was principally manifested in lower parasitaemia but to a lesser degree with reference to the ensuing anaemia.

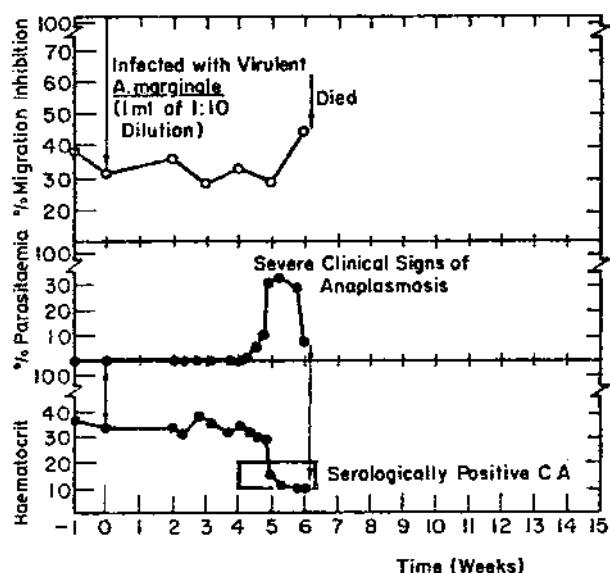
The effect of blood group substances versus parasitic components on induction of delayed cutaneous hypersensitivity and isoimmunity was examined by using test antigens derived from normal and parasitized erythrocytes, virulent *A. marginale* in bovine erythrocytes and attenuated *A. marginale* in ovine erythrocytes. The use of inactivated vaccine of bovine origin induced production of isoantibodies and cutaneous hypersensitivity to both bovine and ovine blood group factors, whereas a similar vaccine preparation of ovine origin stimulated antibodies and cutaneous reactivity only against ovine blood group factors [29].

9. IMMUNE RESPONSE TO VACCINES: RELATIONSHIP BETWEEN CELL-MEDIATED IMMUNE RESPONSES AND PROTECTION

Immunity to many infective agents is known to involve more than specific antibody interaction with the invading organism. Development of protection against various intracellular parasites of viral, rickettsial, bacterial and protozoan nature has been reported to be related to cell-mediated immunity (CMI) [30]. Recent reports have indicated the presence of CMI in anaplasmosis [20, 31]. The cell-mediated response has been studied with respect to various *Anaplasma* immunogens, including virulent and attenuated *A. marginale* in live and inactivated forms [32, 33].



a



b

FIG.2. Leukocyte migration inhibition, haematocrit, and capillary tube agglutination (CA) results on two four-year-old Holstein cows following inoculation with the virulent *Anaplasma marginale*. Note, that the animal (b) which failed to develop a LMIT response at six weeks after infection died. The animal which responded, aborted, but survived the infection (a).

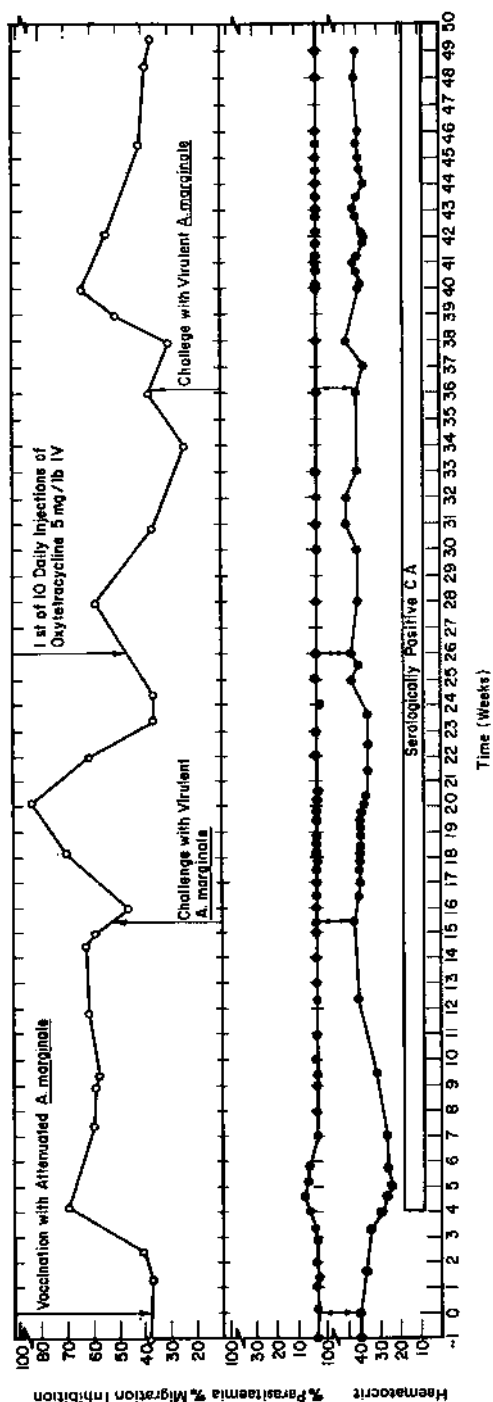


FIG. 3. Leukocyte migration inhibition, parasitaemia, haematocrit and capillary tube agglutination (CA) results for a four-year-old Holstein cow. The animal was initially injected with the attenuated *Anaplasma marginale* vaccine, resisted challenge with virulent *A. marginale*, was treated with oxytetracycline to destroy the *Anaplasma* and rechallenged with the virulent organism approximately 10 weeks after treatment. The animal fully resisted challenge. Note the rapid LMIT response following challenge.

Cattle inoculated with virulent *A. marginale* had a marked and prolonged leukocyte migration inhibition test (LMIT) response which began either early or late in the prepatent period. Cattle that received the attenuated *A. marginale* generally showed an early LMIT response after inoculation. The characteristically mild parasitaemia produced by the attenuated *A. marginale* causes no clinical disease, and the concomitant early cellular response may further moderate the infection. Animals given two doses of commercial vaccine¹ (inactivated virulent organisms, bovine origin) had a transient, low-level LMIT response. Cattle that received the inactivated attenuated *A. marginale* of ovine origin responded as did the animals given the commercial vaccine. Susceptible cattle injected with virulent *A. marginale* provided information on the relationship of the CMI response to clinical illness. When the LMIT response was increased early in the prepatent period, the cattle survived the infection even though acute signs of anaplasmosis were evident. In situations where the LMIT response did not increase markedly until the end of the prepatent period or increased only after the onset of parasitaemia, the cattle became critically ill or died (Fig.2).

Lymphocyte-transformation studies indicated that the degree of sensitization of leukocytes reached much higher levels in cattle given inactivated *A. marginale* in adjuvant compared with those in cattle given viable *A. marginale*. The degree of increased cellular activity indicated by a high degree of radioisotope incorporation, even in the absence of test antigen, was indicative of nonspecific stimulation of the reticulo-endothelial system, possibly caused by the use of an adjuvant-*A. marginale* preparation.

Recovery from acute virulent *A. marginale* infection was coincident with evidence of a strong CMI response and clinical protection against challenge inoculation. Cattle vaccinated with the attenuated *A. marginale* were also protected against challenge with virulent *A. marginale*. These findings indicate a correlation between CMI (measured by the LMIT) and protection. The CMI response seems to parallel antibacterial cellular immunity in the requirement of living cells to produce a continuous stimulus with concomitant residual sensitivity of leukocytes subject to an anamnestic secondary response. Complete protection against both parasitaemia and anaemia was demonstrated only in cattle which had clinically recovered from anaplasmas or had been vaccinated with live attenuated *A. marginale*. Subsequent elimination of the carrier state has been reported to render the host susceptible to anaplasmosis, but this concept has recently been questioned, since cattle rendered free of *Anaplasma* organisms seemed immune to challenge inoculation [34]. To further examine this observation, we subjected two four-year old cows, previously vaccinated with the attenuated agent and subsequently resisting challenge of the virulent organism, to systemic therapy. The animals received ten daily doses of oxytetracycline at 5 mg/lb administered intravenously. Subinoculation

¹ Anaplaz, Fort Dodge Laboratories, Fort Dodge, Iowa, United States of America.

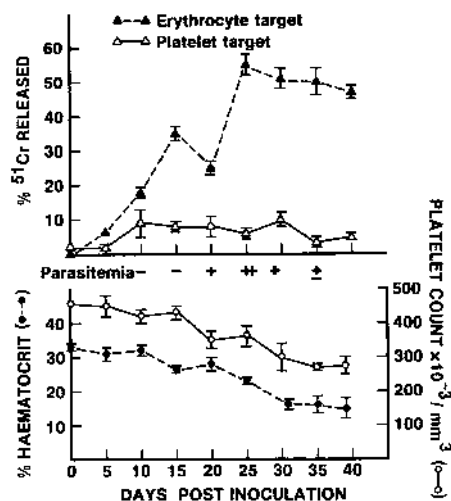


FIG.4. Antibody independent cell-mediated cytotoxicity in cows inoculated with virulent *Anaplasma marginale*. Points represent mean values of four animals.

of 100 ml of whole blood from these cows into two splenectomized cows failed to produce infection in the recipient animals. Approximately 10 weeks after treatment the cows were rechallenged with virulent *A. marginale*. A prompt LMIT response was noted after the challenge and the animals demonstrated full clinical resistance (Fig.3).

10. CELL-MEDIATED CYTOTOXICITY

Tests for cytotoxicity used peripheral blood leukocytes from *Anaplasma*-infected cattle with ⁵¹Cr-labelled autologous erythrocytes and platelets as target cells. The assays examined the cytotoxic effects of leukocytes per se, leukocytes and autologous serum and serum with complement [35].

Antibody-independent cytotoxicity to erythrocytes was first demonstrated five days post inoculation before any detectable parasitaemia (Fig.4). The highest activity of this effector system was observed at day 25 post inoculation coinciding with parasitaemia, drop in haematocrit and moderate reduction in thrombocyte count. This form of cytotoxicity persisted at significant levels even after there was no detectable parasitaemia.

Antibody and complement-mediated cytotoxicity against erythrocytes was first observed 10 days after inoculation (Fig.5). This system was coincident with antibody-dependent lymphocyte-mediated cytotoxicity. In general, the most

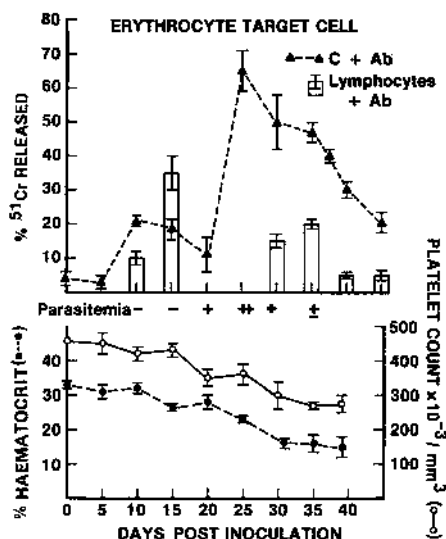


FIG.5. Antibody dependent target cell injury in cows inoculated with virulent *Anaplasma marginale*. Points represent mean values of four animals.

potent cytodestructive mechanism was due to complement and antibody which caused 65% or greater specific ^{51}Cr release. This cytotoxicity persisted at significant levels even after parasitaemia was no longer evident. Similar effector mechanisms and magnitude of cytotoxicity were demonstrated when platelets were used as target cells.

Other work on the cell-mediated cytotoxic assay utilized ^{51}Cr -labelled chicken erythrocytes in the presence of soluble *Anaplasma* antigen [36]. In this test, sensitized lymphocytes after interacting with specific *Anaplasma* antigen are believed to release a soluble substance, lymphotoxin, which causes lysis of the labelled target cells. In intact calves infected with *Anaplasma*, this assay showed a moderate degree of antigen-specific target cell lysis which occurred transiently during the early phase of the disease.

Analysis of data indicates that at least three immunologic mechanisms may exert a cytotoxic effect on autologous erythrocytes and platelets in bovine anaplasmosis. These are (a) antibody-complement mediated cytotoxicity, (b) antibody-dependent cell-mediated cytotoxicity, and (c) antibody-independent cell-mediated cytotoxicity. Such mechanisms could explain the hypothesis that protection in anaplasmosis is partially mediated by immune elimination of parasitized erythrocytes by activated leukocytes and partially by the opsonizing antibody. The ^{51}Cr release assay appears to be related to the pathogenesis of anaemia, and elevated levels in this test coincide with the acute clinical disease.

The observation that LMIT tests remained positive for more extended periods in animals exposed to replicating anaplasma suggests that at least two lymphoid cell types were activated in the process of an infection with *A. marginale*. The early immune lymphoid cells may have cytotoxic activity and the late immune lymphoid cells are related to the LMIT and protection during the chronic carrier state.

11. IMMUNOLOGIC BASIS OF PROTECTION AND PATHOGENESIS OF ANAEMIA

It is evident from the findings described above and those acquired in earlier studies that combined effects of humoral and cell-mediated immune responses are instrumental in acquisition of protective immunity in anaplasmosis. It is further indicated that an infection with a virulent *A. marginale* may incite similar immune elements to contribute to development of an immunologic anaemia. We shall first discuss the mechanisms of protective immunity induced by the attenuated *A. marginale* vaccine, and then elaborate on the immunologic basis of anaemia resulting from infections with the virulent organism.

It is proposed that initial exposure of cattle to replicating attenuated *A. marginale* vaccine presents the host reticulo-endothelial system with sufficient initial antigenic stimulus to induce a cell-mediated response that may serve as the protective mechanism in concert with the humoral response simultaneously induced. Continuous antigenic stimulation results from the perpetual low-level infection. Correlation of protection and cell-mediated immunity, as measured by the LMIT, implies the presence of an *in vivo* mechanism directed towards neutralization of the invading organism. Humoral antibody may serve as the opsonic function in promoting the phagocytosis of *A. marginale*-infected erythrocytes by macrophages that have been 'stimulated' and are attracted to that particular area by lymphokines released from sensitized lymphocytes. Re-exposure to *A. marginale* stimulates memory T lymphocytes to multiply and afford a magnified protective response due to the increased numbers of sensitized lymphocytes capable of specific response (Fig.6).

It has been known for many years that the severity of anaemia in infected animals was not always proportional to the intensity of parasitaemia. Fixed and free-serum autohaemagglutinins have been demonstrated in anaplasma-infected cattle and it has been proposed that anaemia might be, in part, of immunologic aetiology [37-39]. It was then shown that autohaemagglutinins apparently contributed to the onset of erythrophagocytosis in the spleen and bone marrow of infected animals [40]. Opsonins were found in the serum of infected cattle and an *in vitro* test demonstrated that these induce phagocytosis of normal bovine autologous and homologous erythrocytes [41]. Further studies showed that the

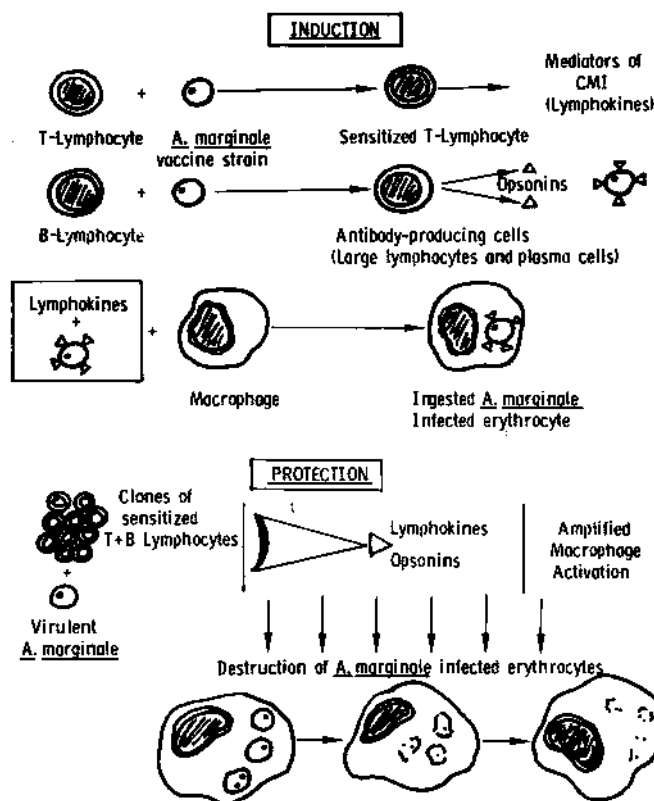


FIG. 6. Proposed mechanism of protection induced by the attenuated *Anaplasma marginale* vaccine.

opsonins could be eluted from erythrocytes of infected animals before they were detected in the serum [42]. The detection of opsonins in the erythrocytic eluate and serum preceded the appearance of parasites in the peripheral blood and the onset of anaemia. Maximal opsonic titres coincided with the anaemic crisis. These findings of an apparent role of immunologic elements in the pathogenesis of anaemia in anaplasmosis are further strengthened by more recent data (see above) in which cell-mediated cytotoxicity to ^{51}Cr -labelled autologous erythrocytes was demonstrated [35].

An analysis of all immunologic data in anaplasmosis indicates that an infection with the virulent *A. marginale* incites a dual immune response. In the sequences of their appearance following infection, immune responses contributing to the development of anaemia appear first to be followed by protective immune elements that become most pronounced after recovery of the acute phase of the disease.

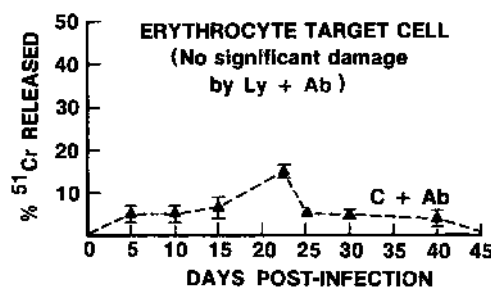


FIG. 7. Anti-erythrocytes cytotoxicity effects in cows inoculated with the attenuated *Anaplasma marginale* vaccine. Note a mild and transitory effect induced by antibody complement. No significant damage was caused by lymphocyte-antibody combination. Points represent mean values of four animals.

With the attenuated *A. marginale* vaccine, the later protective immune responses are as equally pronounced as with the virulent agent but the mild nature of the agent seems to instigate development of little, if any, anaemia-associated immune factors. Transient and low levels of anti-erythrocyte cytotoxicity mediated by lymphocytes and humoral elements following inoculation with the attenuated *A. marginale* is shown in Fig. 7.

REFERENCES

- [1] RISTIC, M., KREIER, J.P., "Family Anaplasmatidae", Bergey's Manual of Determinative Bacteriology (BUCHANAN, R.E., GIBBONS, N.E., Eds), Williams and Wilkins, Baltimore, Maryland (1974) 906.
- [2] RISTIC, M., "Anaplasmosis" Infectious Blood Diseases of Man and Animals (WEINMAN, D., RISTIC, M., Eds), Academic Press, New York (1968) 478.
- [3] KOCAN, R.M., et al., Ultrastructure of *Anaplasma* inclusions (Pawhuska Isolate) and their appendages in intact and hemolyzed erythrocytes and in complement-fixation antigen, Am. J. Vet. Res. 39 (1978) 1123.
- [4] KREIER, J.P., RISTIC, M., Definition and taxonomy of anaplasma species with emphasis on morphologic and immunologic features, Z. Tropenmed. Parasitol. 23 (1972) 88.
- [5] CARSON, C.A., et al., Appendage-related antigen production by *Purvanaplasma caudatum* in deer erythrocytes, Am. J. Vet. Res. 35 (1974) 1529.
- [6] HIDALGO, R.J., Propagation of *Anaplasma marginale* in bovine lymph node cell culture, Am. J. Vet. Res. 36 (1975) 635.
- [7] MAZZOLA, V., et al., Survival of *Anaplasma marginale* in *Aedes albopictus* cells, Am. J. Vet. Res. 37 (1976) 978.
- [8] TRAGER, W., JENSEN, J.B., Human malaria parasites in continuous culture, Science 193 (1976) 673.

- [9] ERP, E.E., et al., Growth of *Babesia bovis* in bovine erythrocyte cultures, Am. J. Trop. Med. Hyg. 27 (1978) 1061.
- [10] KESSLER, R., et al., *In vitro* cultivation of *Anaplasma marginale*: I. Growth pattern and morphologic appearance of virulent strain, Am. J. Vet. Res. (in press).
- [11] KESSLER, R., RISTIC, M., *In vitro* cultivation of *Anaplasma marginale*: II. Invasion of and development in previously non-infected erythrocytes, Am. J. Vet. Res. (in press).
- [12] AMERAULT, T.E., ROBY, T.O., Preparation and characterization of a soluble *Anaplasma marginale* antigen, Am. J. Vet. Res. 28 (1967) 1067.
- [13] CARSON, C.A., et al., An antigenic and serologic composition of two virulent strains and an attenuated strain of *Anaplasma marginale*, Am. J. Vet. Res. 31 (1970) 1071.
- [14] FRANKLIN, T.E., et al., Anaplasmosis-complement fixation antigen production, Am. J. Vet. Res. 24 (1963) 483.
- [15] RISTIC, M., A capillary tube agglutination test for anaplasmosis - A preliminary report, J. Am. Vet. Med. Assoc. 141 (1962) 588.
- [16] AMERAULT, T.E., ROBY, T.O., A rapid agglutination test for bovine anaplasmosis, J. Am. Vet. Med. Assoc. 153 (1968) 1829.
- [17] BUDDEN, J.R., DIMOPOULLOS, G.T., Finite purification of *Anaplasma marginale*: Serologic activity of the *Anaplasma* body, Am. J. Vet. Res. 38 (1977) 633.
- [18] KOCAN, K.M., et al., Ultrastructural localization of *Anaplasma* antigens (Pawhuska Isolate) with ferritin-conjugated antibody, Am. J. Vet. Res. 39 (1978) 1131.
- [19] AMERAULT, T.E., ROBY, T.O., An exo-antigen of *Anaplasma marginale* in serum and erythrocytes of cattle with acute anaplasmosis, Am. J. Vet. Res. 25 (1974) 1642.
- [20] RISTIC, M., NYINDO, M.B.A., "Mechanisms of immune response to an attenuated *Anaplasma marginale* vaccine" (Proc. 6th Natl. Anaplasmosis Conf., Las Vegas, 1973) 60.
- [21] FRANKLIN, T.E., HUFF, J.W., A proposed method of preimmunizing cattle with minimum inocula of *Anaplasma marginale*, Res. Vet. Sci. 8 (1967) 415.
- [22] KLEWER, I.O., et al., "The preservation and minimum infective dose of *A. marginale*" (Proc. 6th Natl. Anaplasmosis Conf., Las Vegas, 1973) 39.
- [23] THEILER, A., Further Investigations into Anaplasmosis in South Africa, Rep. Director Veterinary Research, Union of South Africa (1911) 1.
- [24] RANATUNGA, P., WANDURAGALA, L., Reactions and haematology in imported Jersey cattle preimmunized in Ceylon, Br. Vet. J. 128 1 (1972) 9.
- [25] RISTIC, M., et al., "An attenuated *Anaplasma marginale* vaccine" (Proc. 72nd Annual Meeting U.S. Livestock Sanitary Association, 1968) 56.
- [26] RISTIC, M., CARSON, C.A., "Methods of immunoprophylaxis against bovine anaplasmosis with emphasis on use of the attenuated *Anaplasma marginale* vaccine", Immunity to Blood Parasites of Animals and Man (MILLER, L.H., PINO, J.A., McKELVEY, J.J., Eds), Plenum Publ. Corp., New York (1977) 151.
- [27] McHURDY, N., SIMPSON, R.M., Attempts at immunizing cattle against anaplasmosis using killed vaccine, Trop. Anim. Health Prod. 5 (1973) 166.
- [28] BROCK, W.E., et al., A vaccine for anaplasmosis, J. Am. Vet. Med. Assoc. 147 (1965) 948.
- [29] CARSON, C.A., et al., Cutaneous hypersensitivity and isoantibody production in cattle injected with live or inactivated *Anaplasma marginale* in bovine and ovine erythrocytes, Am. J. Vet. Res. 37 (1976) 1059.
- [30] MacKANESE, G.B., "Cell-mediated immunity to infection", Immunobiology (GOOD, R.A., FISHER, D.W., Eds), Sinauer Assoc., Inc., Stanford (1971) 45.
- [31] BUENING, G.M., Cell-mediated immune responses in calves with anaplasmosis, Am. J. Vet. Res. 34 (1973) 757.

- [32] CARSON, C.A., et al., Cell-mediated immune response to virulent and attenuated *Anaplasma marginale* administered to cattle in live and inactivated forms, Am. J. Vet. Res. 38 (1977) 173.
- [33] CARSON, C.A., et al., Cell-mediated immunity related to challenge exposure of cattle inoculated with virulent and attenuated strains of *Anaplasma marginale*, Am. J. Vet. Res. 38 (1977) 1167.
- [34] ROBY, T.O., et al., Immunity in bovine anaplasmosis after elimination of *Anaplasma marginale* infection with imidocarbs, Am. J. Vet. Res. 25 (1974) 993.
- [35] KAKOMA, I., RISTIC, M., Mechanisms of target cell injury in anaplasmosis (in preparation).
- [36] BUENING, G.M., Cell-mediated immune response in anaplasmosis as measured by a micro cell-mediated cytotoxicity assay and leucocyte migration-inhibition test, Am. J. Vet. Res. 37 (1976) 1215.
- [37] RISTIC, M., Studies in anaplasmosis: III. An autoantibody and symptomatic macrocytic anaemia, Am. J. Vet. Res. 22 (1961) 871.
- [38] MANN, D.K., RISTIC, M., Anaplasmosis: XIII. Studies concerning the nature of auto-immunity, Am. J. Vet. Res. 24 (1963) 703.
- [39] MANN, D.K., RISTIC, M., Anaplasmosis: XIV. The isolation and characterization of an autohemagglutinin, Am. J. Vet. Res. 24 (1963) 709.
- [40] KREIER, J.P., et al., Anaplasmosis: XVI. The pathogenesis of anemia produced by infection with anaplasma, Am. J. Vet. Res. 25 (1964) 343.
- [41] SCHROEDER, W.F., RISTIC, M., Blood serum factors associated with erythrophagocytosis in calves with anaplasmosis, Am. J. Vet. Res. (1968) 1991.
- [42] MORRIS, H., et al., Characterization of opsonins eluted from erythrocytes of cattle infected with *Anaplasma marginale*, Am. J. Vet. Res. 32 (1971) 1221.

DISCUSSION

H.S. HUSSEIN: Have you studied the infectivity of your attenuated vaccine strain of *Anaplasma marginale* to ticks (and hence its possible danger to other animals) in the field?

M. RISTIC: Yes, we have. We used *Dermocentor andersoni* ticks to transfer infection from infected (vaccinated) to non-infected (non-vaccinated) cattle. However, no transfer of the infection took place.

R.E. PURNELL: In your comparison of cell-mediated immunity (CMI) serological tests for the assessment of live and dead vaccines, the tests were not carried out on coinciding days. Only day 14 was significant for dead vaccine and there was no test on the animals receiving the live vaccine between days -7 and +25.

M. RISTIC: Two tests for CMI response in bovine anaplasmosis were used. The first test, the leukocyte migration inhibition test, indicated protective immunity to the disease. The blast transformation test, on the other hand, did not do this but produced strong reactions in animals inoculated with killed vaccine, which is known to induce little protective immunity, and weak reactions in animals inoculated with a live vaccine, which is known to induce good protective immunity.

R.S. PHILLIPS: What was the source of the lymphocytes used in the cytotoxicity tests with ^{51}Cr -labelled erythrocytes?

M. RISTIC: Peripheral blood.

R.S. PHILLIPS: In one of the graphs in your Fig.3 the macrophage migration inhibition response in a challenged animal showed a short rise and then rapidly dropped. What was the significance of this decline?

M. RISTIC: Macrophage migration inhibition usually rose after challenge.

R.S. PHILLIPS: With regard to the attenuated *Anaplasma*, was the attenuation produced by irradiation, passage through deer and sheep or passage through splenectomized animals?

M. RISTIC: I would rather think that it was the result of selection of a mutant by continuous passage through deer and sheep.

I would like to report briefly on some results we have obtained in our work on babesiosis. Cell-culture-derived merozoites of *B. bovis* contain surface coat antigen. This antigen can be liberated by extraction in cold saline. Cattle immunized with the antigen fortified by an adjuvant failed to develop clinical signs of the disease when challenged by *Boophilus microplus* ticks infected with *B. bovis*.

STUDIES ON THE PREFERENCE OF *Babesia microti* AND *Babesia hylomysci* FOR ERYTHROCYTES OF A PARTICULAR AGE GROUP AND ON THEIR PATHOGENESIS USING IRON-59

H.S. HUSSEIN

Department of Microbiology and Parasitology,
Faculty of Veterinary Science,
University of Khartoum,
Khartoum-North,
Sudan

Abstract

STUDIES ON THE PREFERENCE OF *Babesia microti* AND *Babesia hylomysci* FOR ERYTHROCYTES OF A PARTICULAR AGE GROUP AND ON THEIR PATHOGENESIS USING IRON-59.

A tracer technique utilizing ^{59}Fe was employed to investigate the preference of *Babesia microti* and *B. hylomysci* for mouse erythrocytes of a particular age group. The technique entailed in vivo cohort labelling of mouse erythrocytes. One group was used as a young cohort and the other was allowed to age before inoculation with either parasite. This was followed by scintillation counting on erythrocytes, serum and urine of both infected and uninfected mice. *Babesia microti* was found to prefer young erythrocytes, and *B. hylomysci* preferred older mature ones. The anaemia produced during both infections came mainly from the direct destruction of infected erythrocytes. An autoimmune reaction has also been incriminated as a possible factor in the pathogenesis of the anaemia of both infections, but was of a lesser magnitude and its effects were masked by the direct destruction of infected erythrocytes especially during the *B. hylomysci* infection.

INTRODUCTION

The preference for erythrocytes of a particular age group is well established among blood sporozoan parasites [1–5]. Such preference varies with the particular species of the parasite, *Plasmodium berghei* and *Babesia bigemina* both preferring young erythrocytes [3, 4, 6] whereas *P. chabaudi* and *B. hylomysci* preferentially invade older mature erythrocytes [5, 6].

In a previous publication [5], the preference of the rodent parasite *B. hylomysci* was determined using a tracer technique. The same technique is used here to study the preference of another rodent parasite, *B. microti*, and compare it with that of *B. hylomysci*, together with an investigation of the pathogenesis of the anaemia produced by both parasites.

MATERIALS AND METHODS

Male, laboratory-bred LACA mice, 5–8 weeks old and weighing 20–30 g, were used in all experiments.

Babesia microti (King's strain) and *B. hylomysci* (Antwerp strain) were maintained by periodical syringe passage through the mice and as stabillates stored in liquid nitrogen (-196°C). The original strains were obtained from F.E.G. Cox of the University of London and J.M. Bafort when he was at the Liverpool School of Tropical Medicine.

Iron-59 (^{59}Fe) was obtained from the Radiochemical Centre, Amersham, United Kingdom, as an isotonic ferric chloride solution containing 1% sodium citrate, with a radioactivity of 3–15 $\mu\text{Ci}/\mu\text{g}$. The preparation was inoculated into five groups of mice (30 mice per group), each mouse receiving 1 μCi intraperitoneally every other day for six days, i.e. a total of 3 μCi per mouse. Mice of groups A and B were inoculated with *B. microti*, those of groups C and D with *B. hylomysci* and group E were controls.

Group B and group D mice (old cohort) were inoculated with the isotope 14 days before those of groups A and C (young cohort); half of group E were inoculated with the isotope together with each set of groups. Another group of mice (group F – 15 mice) were not injected with the isotope and counts on their blood were used as background controls.

A radioactive standard equivalent to 0.05 μCi was provided from the inoculum, diluted with 0.1N HCl to prevent hydrolysis of the ferric chloride, and counted daily to determine the percentage decay of the isotope.

Group A and group C mice were inoculated with *B. microti* and *B. hylomysci* respectively eight days following the isotope treatment (young cohort). Those in groups B and D were inoculated with the parasites on day 22 to allow the cohort of labelled erythrocytes to age naturally for three weeks. The reticulocyte response in infected animals was determined daily by vital staining with brilliant cresyl blue according to the method of Baker and co-workers [7].

Two mice from each infected group together with one mouse from each corresponding control (group E) as well as one mouse from group F were killed daily following parasite inoculation in the case of *B. hylomysci*, and every other day in the case of *B. microti* infection. The mice with the highest parasitaemia at the time were chosen in order to lessen the likely losses through death. Aliquots of heart blood (0.5–1.0 ml) from each animal were placed in 3.0-ml centrifuge tubes each containing approximately 10 mg of heparin. The specimens were centrifuged at 3000 rev/min for 10 minutes. An amount of 0.1 ml of the serum was removed with a siliconized pipette to a standard 7-ml sample bottle, diluted with 7 ml distilled water. The bottle was then tightly capped ready for counting. The remainder of the serum was discarded together with the buffy leucocyte layer. The packed cells were then re-suspended in Kreb's bicarbonate

buffer (pH 7.4) and centrifuged at 3000 rev/min for 10 minutes after which the supernatant and buffy layer were again removed and the process repeated with a fresh buffer. Then 95 μg of packed erythrocytes were weighed into a 7-ml sample bottle. These were then lysed with 7 ml distilled water and the bottle tightly stoppered ready for counting.

Urine (0.2 ml) was obtained using a sterile Pasteur pipette from the bladder of each mouse into a 7-ml sample bottle. The volume was made up to 7 ml with distilled water and the bottle tightly stoppered ready for counting.

The samples were counted in a well-type gamma scintillation counter (Panax Ltd., Redhill, Surrey, United Kingdom). The radiation counts per minute (counts/min) were recorded and corrected for background radiation using counts on samples from mice in group F, and then for the standard decay rate, i.e. the percentage decay rate of the isotope during the period of the experiment. However, no corrections were made for the self absorption of the gamma rays as these were found to be minimal in an isotope such as ^{59}Fe [8].

RESULTS

The background counts throughout the experiments were in the range of 125–145 counts/min whereas the erythrocyte counts in normal mice (group C) ranged from 5.04×10^3 to 14.2×10^3 counts/min, serum counts from 60 to 160 counts/min and urine counts from 80–152 counts/min.

Group A, *B. microti* in young cohort

The erythrocyte radioactivity dropped progressively following the sixth day of infection and was lowest (4.3×10^3 counts/min) during peak parasitaemia. Then the radioactivity rose progressively from day 12 of the infection and was within normal limits by day 18 (Fig.1). A moderate increase in serum radioactivity occurred around peak parasitaemia, but during the rest of the infection both serum and urine radioactivity were within normal limits. These counts were, however, somewhat elevated in one mouse whose urine showed haemoglobinuria around peak parasitaemia.

Group C, *B. microti* in old cohort

Erythrocyte radioactivity of mice in group C fell steadily in a similar fashion to that of the controls, but was consistently lower than in controls (Fig.2). Serum and urine radioactivity were always within normal limits.

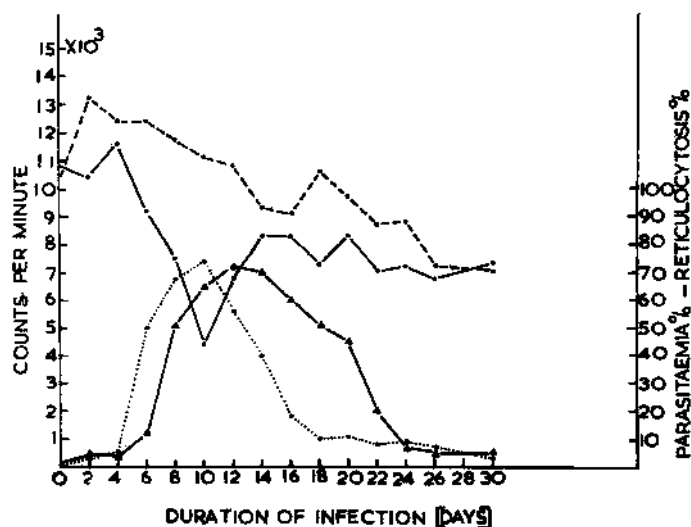


FIG.1. Erythrocyte radioactivity in *B. microti*-infected mice with a young labelled cohort: $\bullet$ $\bullet$, parasitaemia; $\bullet$ — $\bullet$, erythrocyte radioactivity in infected mice; $\bullet$ ----- $\bullet$, erythrocyte radioactivity in controls; $\blacktriangle$ — $\blacktriangle$, reticulocytosis in infected mice.

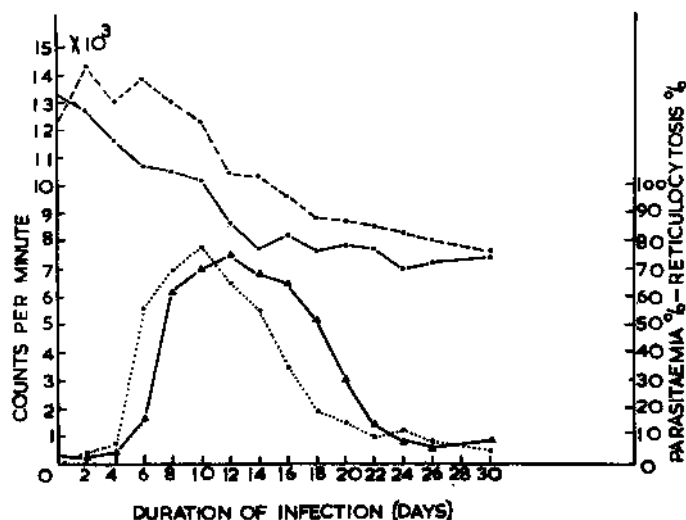


FIG.2. Erythrocyte radioactivity in *B. microti*-infected mice with an old labelled cohort: $\bullet$ $\bullet$, parasitaemia; $\bullet$ — $\bullet$, erythrocyte radioactivity in infected mice; $\bullet$ ----- $\bullet$, erythrocyte radioactivity in controls; $\blacktriangle$ — $\blacktriangle$, reticulocytosis in infected mice.

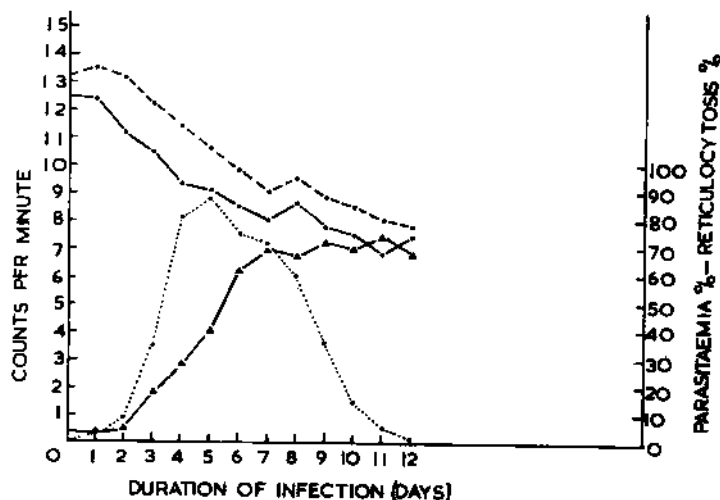


FIG.3. Erythrocyte radioactivity in *B. hylomysci*-infected mice with a young labelled cohort:•, parasitaemia; —•, erythrocyte radioactivity in infected mice; - - -•, erythrocyte radioactivity in controls; —▲, reticulocytosis in infected mice.

Group B, *B. hylomysci* in young cohort

The fall in erythrocyte radioactivity in group B was similar to that in the controls, but the counts were consistently lower than in the controls (Fig.3). The serum and urine counts were similar to those in the controls despite clear signs of intravascular haemolysis (red serum) and haemoglobinuria (dark coloured urine).

Group D, *B. hylomysci* in old cohort

Erythrocyte radioactivity in this group fall rapidly following the first day of infection to reach a low level of 600 counts/min on day 8. This was followed by a sharp rise which coincided with the reticulocyte peak where the counts were very close to those of the controls (Fig.4).

Both serum and urine radioactivity of mice in this group were very much elevated, especially around peak parasitaemia (Fig.5).

DISCUSSION

The results indicate that the bulk of *B. microti* infection occurred in the young erythrocytes as there was a steady but considerable fall in erythrocyte

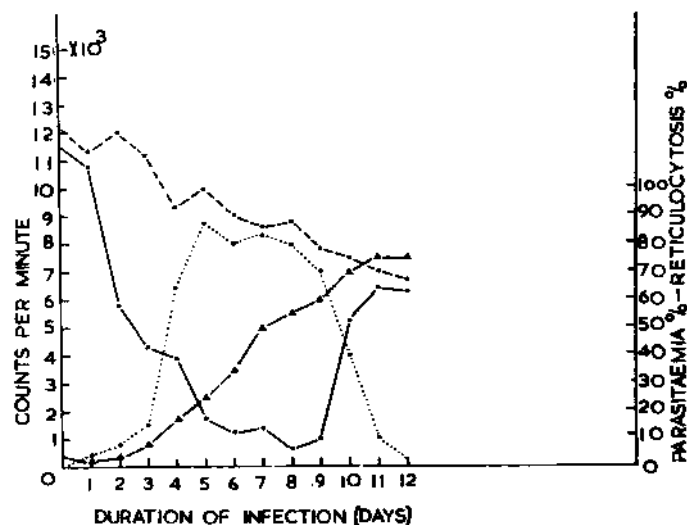


FIG. 4. Erythrocyte radioactivity in *B. hyalomysci*-infected mice with an old labelled cohort: $\bullet$ $\bullet$, parasitaemia; $\bullet$ — $\bullet$, erythrocyte radioactivity in infected mice; $\bullet$ ----- $\bullet$, erythrocyte radioactivity in controls; $\blacktriangle$ — $\blacktriangle$, reticulocytosis in infected mice.

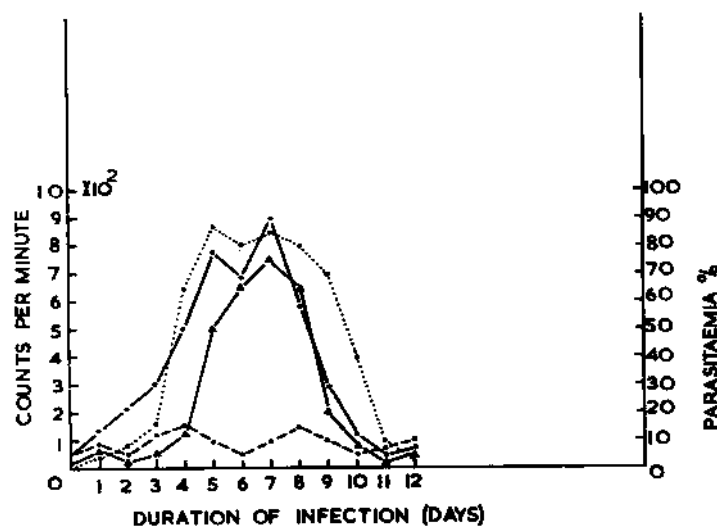


FIG. 5. Serum and urine radioactivity during *B. hyalomysci* infection in mice with an old labelled cohort: $\bullet$ $\bullet$, parasitaemia; $\bullet$ ----- $\bullet$, serum and urine radioactivity in controls; $\bullet$ — $\bullet$, serum radioactivity in infected mice; $\blacktriangle$ — $\blacktriangle$, urine radioactivity in infected mice.

radioactivity in infected mice of group A. Also the lowest figure of erythrocyte radioactivity coincided with peak parasitaemia. A further evidence is provided by the moderate rise in serum radioactivity of infected mice, and this too coincided with peak parasitaemia when *B. microti* is known to cause a moderate intravascular haemolysis [9]. Moreover, the elevated urine radioactivity of the mouse with haemoglobinuria indicates that the haemoglobin in the urine is radioactive, and radioactive haemoglobin in this mouse occurs in young erythrocytes only.

The fall in erythrocyte radioactivity in mice with an old cohort and infected with *B. microti* was steady and similar to that in normal mice, though consistently lower, and there were no particular changes coinciding with peak parasitaemia. This might indicate that the bulk of the infection occurs in young erythrocytes whose haemoglobin is not radioactive.

The changes in erythrocytes, serum and urine radioactivity are more marked during *B. hylomysci* infection, which is known to produce a severe infection in mice with spectacular clinical and pathological manifestations [10, 11]. The results obtained with this parasite clearly demonstrate its preference to older mature erythrocytes. The fall in erythrocyte radioactivity of mice in group C was steady and similar to that in the controls whereas *B. hylomysci* is known to eliminate large numbers of infected erythrocytes [10]. Therefore the destruction of older, non-radioactive erythrocytes is greater than that of radioactive young ones, and consequently the bulk of the infection must have occurred in the older erythrocytes. A further proof is given by mice in group D whose erythrocyte radioactivity fell drastically during the infection, the lowest being during peak parasitaemia and whose serum (intravascular haemolysis) and urine (haemoglobinuria) were highly radioactive, especially during peak parasitaemia. This might indicate that *B. hylomysci* prefers older mature erythrocytes, which is in line with most other species in the genus *Babesia* [2, 12], whereas the preference of *B. microti* for young erythrocytes confirms the observations of Nowell [13] and Hussein [9] who used far less sensitive techniques for this purpose. This follows *B. rodhaini* and *B. bigemina* as the species in the genus that prefer young erythrocytes [4, 13].

These results might throw some light on the pathogenesis of the anaemia produced by these parasites. It was suggested that the plasma acquires some lytic properties during the infection and brings about haemolysis of both infected and uninfected erythrocytes [14]. Moreover, lysis of both normal and infected erythrocytes during similar infections was attributed to a buildup of metabolites inside both infected and uninfected erythrocytes as a result of a toxin elaborated during the infection [15]. This does not seem to be the case with *B. microti* and *B. hylomysci* infections as the present results, and those of Hussein [5], showed that only infected erythrocytes were haemolysed. This is also similar to the situation in malaria reported by George and co-workers [16] and Kreier and Laste [17].

However, uninfected erythrocytes during both infections might be removed by other means, possibly through phagocytosis of both infected and uninfected erythrocytes [18]. This might also be deduced from erythrocyte radioactivity of mice in groups B and C which was consistently lower than that of the controls (Figs 2 and 3). Evidence has just been furnished that this fall in radioactivity is not due to the lysis of uninfected radioactive erythrocytes. This could therefore be due to the removal of some of the uninfected erythrocytes, possibly through phagocytosis by cells of the reticuloendothelial system [18]. Such erythrocytes may, therefore, have been altered during the infection in such a way as to become 'non-self' to the immune mechanisms of the body and thus removed. Similar observations have been attributed to an autoimmune response during the infection [19, 20]. Therefore, an autoimmune response might be incriminated, in a way, as a possible factor in the pathogenesis of *B. hylomysci* and *B. microti* anaemia. However, this factor might be of a lesser intensity than the direct injury to infected erythrocytes and hence might be masked by the latter, especially during *B. hylomysci* infection which is known to cause massive destruction of erythrocytes.

ACKNOWLEDGEMENTS

The author wishes to thank members of the Parasitology Section of the Department of Microbiology and Parasitology, University of Khartoum, for their valuable suggestions and help during the work.

REFERENCES

- [1] HEGNER, R., ESKRIDGE, L.C., Susceptibility of young red cells to the merozoites of malaria, *Am. J. Hyg.* 27 (1938) 471.
- [2] LANDSBERG, J.W., ESKRIDGE, L.C., The erythrocyte in *Babesia canis*, *J. Parasitol.* 26 (1940) 377.
- [3] THEAKSTON, R.D.G., FLETCHER, K.A., The use of isotopic tracer techniques for examining the preference of *Plasmodium berghei* for a particular age-group of erythrocytes and for studying the dynamics of haemolysis in malaria, *Ann. Trop. Med. Parasitol.* 65 (1971) 441.
- [4] WRIGHT, I.G., KERR, J.D., The preferential invasion of young erythrocytes in acute *Babesia bigemina* infections of splenectomized calves, *Z. Parasitenkd.* 43 (1974) 63.
- [5] HUSSEIN, H.S., *Babesia hylomysci* in mice: Preference for erythrocytes of a particular age-group and pathogenesis of the anaemia, *Z. Parasitenkd.* 50 (1976) 103.
- [6] OTT, K.J., Influence of reticulocytosis on the course of infection of *Plasmodium chabaudi* and *P. berghei*, *J. Protozool.* 15 (1968) 365.

- [7] BAKER, F.J., SILVERTON, R.E., LUCKOCK, E.D., Introduction to Medical Laboratory Technology, Butterworths, London (1966).
- [8] FRANCIS, G.E., MULLIGAN, W., WORMALL, A., Isotopic Tracer, Athlone Press, University of London (1959).
- [9] HUSSEIN, H.S., Studies on rodent babesiosis caused by *Babesia hyalomysci* (Bafort, Timperman and Molyneux, 1970) and *Babesia microti* (Franca, 1910), PhD Thesis, University of Liverpool (1973).
- [10] HUSSEIN, H.S., The pathology of *Babesia hyalomysci* infection in mice: I. Clinical signs and liver lesion, J. Comp. Pathol. 87a (1977) 161.
- [11] HUSSEIN, H.S., The pathology of *Babesia hyalomysci* infection in mice: II. Kidney lesion, J. Comp. Pathol. 87b (1977) 169.
- [12] SIMONS, H., Sur les rapports des piroplasmodies paludiques et des piroplasmoses avec les réticulocytes, Ann. Parasitol. 17 (1939) 72.
- [13] NOWELL, F., The blood picture resulting from *Nuttallia* (= *Babesia*) *rodhaini* and *Nuttallia* (= *Babesia*) *microti* infections in rats and mice, Parasitology 61 (1969) 425.
- [14] MAEGRAITH, B.G., GILLES, H.M., DEVAKUL, K., Pathological processes in *Babesia canis* infections, Z. Tropenmed. Parasitol. 8 (1957) 485.
- [15] DUNN, M.J., Alterations of red blood cell metabolism in simian malaria: Evidence for abnormalities of non-parasitized cells, Mil. Med. (special issue) 134 (1969) 1100.
- [16] GEORGE, J.N., STOKES, E.F., WICKER, D.J., CONRAD, M.E., Studies of the mechanism of haemolysis in experimental malaria, Mil. Med. (special issue) 131 (1966) 1217.
- [17] KREIER, J.P., LESTE, J., Parasitaemia and erythrocyte destruction in *Plasmodium berghei*-infected rats: II. Effect of infected host globulins, Exp. Parasitol. 23 (1968) 198.
- [18] HUSSEIN, H.S., *Babesia microti* and *Babesia hyalomysci*: Spleen and phagocytosis in infected mice, Exp. Parasitol. (in press).
- [19] ZUCKERMAN, A., Autoimmunization and other types of indirect damage to host cells as factors in certain protozoan diseases, Exp. Parasitol. 15 (1964) 138.
- [20] SCHROEDER, W.F., COX, H.W., RISTIC, M., Anaemia, parasitaemia, erythrophagocytosis and haemagglutinins in *Babesia rodhaini* infections, Ann. Trop. Med. Parasitol. 60 (1966) 31.

DISCUSSION

F.E.G. COX: The type of cell invaded by *Babesia microti* varies according to the strain of mouse and the number of parasites injected. What strain did you use and how many parasites were injected? What route of infection did you use?

H.S. HUSSEIN: The LACA mouse strain was used. The infection dose was 5×10^6 infected cells, and the mice were inoculated intraperitoneally.

F.E.G. COX: Were the mice you used free of *Eperythrozoon* and *Haemobartonella*? These might affect the type of cell invaded. They might also affect the phagocytosis of uninfected red blood cells by macrophages. I have never seen red cells taken up in this way in *B. microti*-infected mice but it does occur in those infected with *Eperythrozoon*.

H.S. HUSSEIN: All mice used in the experiments were pretreated with Neoarsphenamine as recommended by Kreier and Ristic in order to clear *Eperythrozoon* infection. However, phagocytosis in the blood-stream during *B. microti* infection in mice is very rare, although it is quite common in the bone marrow and the spleen.

J.D. DARGIE (*Scientific Secretary*): Are the different preferences of the species you used a reflection of different penetration sites?

H.S. HUSSEIN: I have not carried out any experiments to determine the reasons for the different preferences of the two parasites used. The difference may be a reflection of different penetration sites, but it seems more likely that it is caused by the different metabolic requirements of each species, which are met by young erythrocytes in one cell and by older erythrocytes in the other.

IMMUNE MECHANISMS IN *Babesia*-INFECTED ANIMALS

R.S. PHILLIPS
Department of Zoology,
University of Glasgow,
Glasgow,
United Kingdom

Abstract

IMMUNE MECHANISMS IN *Babesia*-INFECTED ANIMALS.

The course of a *Babesia* infection depends on the species of host and parasite involved. Animals infected with virulent babesias may need chemotherapy before acquired immunity develops. Maintenance of immunity is not dependent on the presence of the parasite. *Babesia* infections are characteristically of long duration. The immune response to babesias includes both humoral and cellular components. Antibody levels detected in serodiagnostic tests do not relate to levels of resistance to the parasite. Some antibodies, however, appear to be protective. Antiparasitic antibodies may damage parasites in or outside the red cell and act as opsonins. T-cell-deficient and anti-lymphocyte-serum-treated rodents are more susceptible to rodent piroplasms indicating a role for T-cells as either helper cells and/or as mediators of cell-mediated immunity (CMI). There is indirect evidence of CMI, but the cell-mediated mechanisms involved in parasite killing are not known. In domestic animals immunity is largely species- and strain-specific. Antigenic variation by babesias occurs. In rodents, however, there is cross-immunity between different species of rodent piroplasms and between rodent piroplasms and some malaria parasites. Prior infection with agents such as BCG, and *Corynebacterium parvum*, gives mice non-specific resistance to rodent piroplasms possibly mediated through a soluble non-antibody factor. This factor may also be liberated during piroplasm infections and by being toxic to malaria parasites account for heterologous immunity. Active immunization against babesias has been achieved with avirulent strains, irradiated parasites and dead parasites in adjuvant. During *Babesia* infections the primary and, to a lesser degree, the secondary immune response to heterologous antigens can be depressed. Maximum depression coincides with peak parasitaemia when antigen priming may be abolished completely. Immunosuppression during *Babesia* infections can prolong or exacerbate concurrent parasitic infections. The effect of this immunosuppression on the immune response to the babesias themselves has not been fully investigated.

INTRODUCTION

Immunity to *Babesia* parasites in the vertebrate host could be directed both against the infective stages (sporozoites) introduced by the feeding tick and also the erythrocytic stages. Little is known about natural or acquired immunity to the infective stages and is an area worthy of investigation. This review will be

concerned with recent investigations into both mechanisms of acquired immunity to the erythrocytic stages and the occurrence of and possible mechanisms involved in the depression of immune responsiveness to heterologous antigens during *Babesia* infections. Earlier work is well summarized in the reviews of Mahoney [1] and Aragon [2].

In different combinations of host-parasite species, including both natural and experimental infections, the pattern of acquired immunity varies considerably. In some hosts a *Babesia* infection is rapidly fatal with the host apparently making no effective immune response, e.g. *Babesia rodhaini* in the mouse. Control of an acute and rapidly fatal parasitaemia with babesiacidal drugs can enable the host to acquire an effective immunity [3]. In other hosts, babesias are rapidly eliminated, and a strong acquired immunity of long duration ensues, e.g. *B. microti* in the mouse [3, 4]. For most *Babesia* infections, however, survival of the host from the acute phase of the disease signifies the start of a long-lasting chronic and usually subpatent infection. The pattern of infection in the host species can depend on the age of the host. In some studies older cattle were more susceptible than young calves [5]. Adult rats are more resistant to *B. rodhaini* than weanling rats [6, 7]. Thus analysis of acquired immunity in these different situations should shed light both on the mechanisms involved in control and elimination of babesias and, equally important, on those circumstances under which the parasite survives in the semi-immune host.

There are now numerous reports to show that maintenance of acquired immunity to homologous parasites in cattle and rodents is not dependent on the continued presence of the parasite, i.e. on a state of premunition [7, 8].

ROLE OF ANTIBODY

The role of humoral antibody and hence B cells in acquired immunity has been established by passive transfer studies [9–14] and transfer of protection in colostrum from immune dams to their offspring [15, 16]. The class of immunoglobulins involved has not been elucidated. In a few passive transfer experiments no protection could be transferred with serum, and occasionally serum had the effect of exacerbating the infection [17, 18]. Antibody levels detected by various serological techniques [19, 20] do not usually reflect levels of resistance to re-infection [21]. The mode of action of antibodies in vivo is not fully determined. Phagocytosis of both free parasites, parasitized and non-parasitized cells by monocytes and neutrophils [22] has been reported, and for *B. rodhaini* in the rat opsonizing antibody has been demonstrated [23]. By analogy with malaria parasites [24] it would be expected that the *Babesia* parasite in passing from one red cell to another would be exposed to serum antibodies and would be damaged in the process. Recent work with *B. rodhaini* in mice [14] suggests that the free

parasite, removed from the red cell, is indeed more vulnerable to serum antibodies although the same workers also suggest that antibodies may pass through the red cell membrane to the parasite within or combine with babesial antigens in the red cell membrane. Although babesiasis may be accompanied by a drop in complement levels [25], its role in protective immune mechanisms is not clear.

CELL-MEDIATED IMMUNITY

In nude mice and thymectomized mice, *B. microti* and *B. rodhaini* infections are more severe [26, 27]. Treatment of hamsters [28] and cattle [29] with antilymphocyte and antithymocyte serum respectively exacerbated *Babesia* infections. These observations indicate a role for T-cells in acquired immunity to *Babesia* parasites. T-cells may act as helper cells in protective antibody production and/or as mediators of various cell-mediated immune mechanisms. A role of protective antibodies has already been described. The only evidence (and this is indirect evidence) for cell-mediated immune mechanisms in immunity to babesias comes from the results of delayed skin tests [30] and macrophage migration inhibition tests [31]. It is not known which of the various cellular mechanisms, e.g. activated macrophages, cytotoxic lymphocytes, might contribute to babesial immunity, at which stage of an infection such mechanisms might be operative and whether humoral and cellular mechanisms act synergistically. The fact that immunity to *B. rodhaini* in the rat was more effectively transferred with spleen cells than serum [18] does not necessarily imply cell-mediated immune mechanisms: the transferred cells may subsequently promote antibody production as in the adoptive transfer of immunity to *Plasmodium berghei* in the rat [32].

For many *Babesia* infections the spleen is necessary for the development of acquired immunity and its maintenance in the presence of a subclinical infection [33–35], although in some species, e.g. *B. argentina (bovis)* in cattle, extra splenic sites appear to be able to take over the role of the spleen. It is possible to induce a degree of immunity in splenectomized rats with irradiated *B. rodhaini* [36]. The observed necessity for the spleen does not help to delineate accurately immune mechanisms to babesias although it is accepted that the spleen is a major site of antibody production and phagocytosis.

SURVIVAL OF BABESIAS IN THE SEMI-IMMUNE HOST

Babesia infections are frequently of long duration with the parasites surviving in the semi-immune host. Survival of the parasite may reflect its ability to evade the host's immune response, e.g. by undergoing antigenic variation, and/or because the level of the immune response is insufficient to effect complete

eradication of the parasite. Two babesias, *B. rodhaini* [37–39] and *B. argentina* [40, 41] undergo antigenic variation, the latter having been shown to undergo repeated antigenic variation in a single animal and to revert to a basic antigenic type after passage through the tick. In chronically infected or primed animals the response to new variants [37] appears to be rapid suggesting a role for common antigens in the immune response. Antigenic differences between field isolates of the same species are now well documented [42, 43]. It remains to be shown that antigenic variation is a feature of most or all babesias.

IMMUNIZATION AGAINST *Babesia*

Experimental studies on immunization against babesias have not added greatly to our knowledge of immune mechanisms in babesia infections. Immunity has been induced by infection with avirulent strains [44], injection of irradiated parasitized cells [36, 45–47] and injection of dead parasites in complete Freund's adjuvant (CFA) [48]. The effective use of CFA could indicate a role for cell-mediated immune mechanisms in these immunized animals. It may be significant that in cattle immunized with dead *B. argentina* parasites in CFA the immunity induced protected against heterologous and homologous strains [49]: in this latter study dead parasites alone gave no protection.

NON-SPECIFIC RESISTANCE TO BABESIAS

In babesias of domestic animals acquired immunity is usually species and strain specific. For babesias of rodents, however, cross-immunity between different species has been reported [3, 4]. Further, mice immune to *B. microti* show some resistance to rodent malarias [50]. Mice previously infected with BCG (*Mycobacterium bovis*) [51], *M. lepraemurium* [52], *Corynebacterium parvum* [53], or *Brucella* [54] all showed non-specific resistance to rodent babesias. The damaged parasites, crisis forms, seen in mice previously infected with BCG and the other agents are said to be similar to the crisis forms seen in mice in which the primary parasitaemia is going into remission [55]. It has been suggested that soluble non-antibody factors mediate killing of the *Babesia* parasite in the red cell (although it is likely that the parasites are initially damaged when exposed in the plasma in making the passage from one red cell to another), and that these factors are liberated as a result of infection with BCG and the other agents and also during the course of *Babesia* infections in mice not pretreated in any way. Such soluble factors may also damage malaria parasites and could therefore be responsible for non-specific resistance between rodent piroplasms and rodent malaria parasites. Clark [56] has hypothesized that these soluble non-antibody

factors are liberated as the result of endotoxin shock. The interaction between non-specific antiparasitic factors generated during infection and acquired immunity is not clear, nor whether the non-antibody factors are liberated during chronic infections as well as acute primary parasitaemias.

IMMUNOSUPPRESSION DURING *Babesia* INFECTIONS

During the course of acute *Babesia* infections it has been shown that the immune response to heterologous antigens is depressed. It was first demonstrated that during acute *B. hyalomysci* and *B. microti* infections in mice the primary antibody response to sheep red cells was depressed, and the immune expulsion of an intestinal nematode, *Trichuris muris*, was delayed [57]. Purvis [58] investigated the immunodepressive effect of *B. microti* in mice in more detail. She reported that the IgM and IgG responses to sheep red cells was maximally depressed seven days after peak parasitaemia and as the parasitaemia declined there was a slow return of immune responsiveness. Cell-mediated responses as exemplified by oxazolone sensitivity and allograft rejection were not significantly diminished during the *B. microti* infection. Gray [59] and Gray and Phillips [60] have examined both primary and secondary responses to sheep red cells in *B. microti* and *B. hyalomysci* infections in mice. Unlike Purvis they found peak suppression of the primary antibody response to coincide with peak parasitaemia. Both the total antibody levels, levels of mercaptoethanol resistant antibody, and numbers of IgM and IgG plaque-forming cells were depressed. Kinetic studies showed the immunosuppression to be a truly reduced response and not merely a delayed response. Gray and Phillips also found that at peak parasitaemia the secondary response was reduced to the level of a primary response although the levels of mercaptoethanol resistant antibody and the ratio of IgM to IgG plaque-forming cells present in the spleen showed that the reduced secondary response had the characteristics of a secondary response. Mice primed with sheep cells at peak parasitaemia on subsequent challenge with sheep cells after the parasitaemia had subsided gave a normal primary response. The processes which might be responsible for the immunosuppression in *Babesia* infections are not known although possibilities have been discussed [58, 60]. Work on other protozoal infections has indicated increased suppressor cell activity and loss of B-cell potential in mice infected with *Trypanosoma brucei* [61] and in malarious mice dysfunction of macrophages [62]. It is equally important to know the influence of these immunosuppressive factors on the immune response to the babesia infection itself, especially with regard to the survival of the parasite in the semi-immune host, and to the ticks which the infected animal may be carrying. This latter aspect was examined by Callow and Stewart [63] who found that the burden of ticks carried by cattle infected with *Babesia bovis* was significantly greater than non-infected controls.

CONCLUSION

The outcome of a *Babesia* infection depends on the host-parasite species involved. In animals surviving the acute phase the parasite may be rapidly eliminated or the infection may persist albeit at subpatent level in a host which can be shown to have developed a level of acquired immunity. Persistence of the parasite in chronically infected animals may be the result of antigenic variation by the parasite and/or the result of some constraints on the immune response of the host perhaps generated by the parasite itself.

There is still much to be learnt of the specific immune and non-specific antiparasitic mechanisms in babesia infections. A role for antibodies is demonstrable, perhaps a thymus dependent antibody response: they may combine with *Babesia* infected red cells or extra cellular babesias passing from one red cell to another. Of the cellular elements phagocytic cells are involved, their activity being promoted by opsonins. Nothing is known of any cell-mediated processes which might be operating although indirect evidence points to their involvement. We still need to assess the significance of the activity of soluble non-antibody factors, which non-specifically kill babesias and malaria parasites, in both the acute and chronic stages of *Babesia* infections, and how these non-specific antiparasitic mechanisms act and how they interact with acquired immunity. Little is known about the nature of protective babesial antigens.

Much of the recent work on the immunology of malaria infections, particularly in rodent systems, could be readily applicable to *Babesia* infections, and hence more detailed knowledge can be obtained of the class of immunoglobulins with antibody activity, and of the role of different lymphoid cells in immunity to babesias. The use of adjuvants in immunization studies needs more detailed investigation. The recent adaptation of a bovine *Babesia*, *B. divergens*, to gerbils [64] may enable the immune responses to this parasite to be more readily investigated. The introduction of techniques to grow babesias in vitro [65] should make analysis of the activity of antibodies and other factors on parasites more practicable.

All the work reviewed above has been concerned with the erythrocytic stages of babesias, and in conclusion it is stressed that the immune response to the infective stages injected by the tick merits investigation.

REFERENCES

- [1] MAHONEY, D.F., "Immune response to Hemoprotozoa: II. *Babesia* spp.", Immunity to Animal Parasites (SOULSBY, E.J.L., Ed.), Academic Press, London (1972) 301.
- [2] ARAGON, R.S., Bovine babesiosis: A review, Vet. Bull. 46 (1976) 903.
- [3] COX, F.E.G., YOUNG, A.S., Acquired immunity to *Babesia microti* and *Babesia rodhaini* in mice, Parasitology 59 (1969) 257.

- [4] HUSSEIN, H.S., The nature of immunity against *Babesia hyalomysci* and *B. microti* infections in mice, Ann. Trop. Med. Parasitol. 71 (1977) 249.
- [5] RIEK, R.F., "Immunity to babesiosis", Immunity to the Protozoa (GARNHAM, P.C.C., PIERCE, A.E., ROITT, I., Eds), Blackwell, Oxford (1963) 160.
- [6] BEVERIDGE, E., *Babesia rodhaini*: a useful organism for the testing of drugs designed for the treatment of piroplasmosis, Ann. Trop. Med. Parasitol. 47 (1953) 134.
- [7] PHILLIPS, R.S., Studies on *Babesia rodhaini* in laboratory animals, PhD Thesis, University of Cambridge (1968).
- [8] CALLOW, L.L., MCGREGOR, W., PARKER, R.J., DALGLIESH, R.J., The immunity of cattle to *Babesia argentina* after drug sterilization of infections of varying duration, Aust. Vet. J. 50 (1974) 6.
- [9] NOCORDER, E., MOTAS, C.S., Contribution à l'étude de la piroplasmose canine, Ann. Inst. Pasteur 16 (1902) 257.
- [10] MATSON, B.A., Tick transmission in *Babesia divergens* and immunity in *Babesia rodhaini*, PhD Thesis, University of Cambridge (1964).
- [11] MAHONEY, D.F., Bovine babesiosis: the passive immunization of calves against *Babesia argentina* with special reference to complement fixing antibodies, Exp. Parasitol. 20 (1967) 119.
- [12] PHILLIPS, R.S., The protective activity of serum from immune rats against *Babesia rodhaini*, Parasitology 59 (1969) 357.
- [13] ROBERTS, J.A., TRACEY-PATTE, P.D., A study of the effects of immune serum, Int. J. Parasitol. 5 (1975) 577.
- [14] ABDALLA, H.S., HUSSEIN, H.S., KREIER, J.P., *Babesia rodhaini*: passive protection of mice with immune serum, Tropenmed. Parasitol. 29 (1978) 295.
- [15] HALL, W.T.K., The immunity of calves to *Babesia argentina* infection, Aust. Vet. J. 36 (1960) 361.
- [16] HALL, W.T.K., The immunity of calves to tick transmitted *Babesia argentina* infection, Aust. Vet. J. 39 (1963) 386.
- [17] MITCHELL, G.F., HANDMAN, E., HOWARD, R.J., Protection of mice against Plasmodium and Babesia infections: attempts to raise host-protective sera, Aust. J. Exp. Biol. Med. Sci. 56 (1978) 553.
- [18] ROBERTS, J.A., Adoptive transfer of immunity to *Babesia rodhaini* by spleen cells from immune rats, Aust. J. Exp. Biol. Med. Sci. 46 (1968) 807.
- [19] TODOROVIC, R.A., Serological diagnosis of babesiosis: a review, Trop. Anim. Health Prod. 7 (1975) 1.
- [20] BIDWELL, D.E., JOYNER, L.P., PAYNE, R.C., PURNELL, R.E., Comparisons of serological tests for babesia in British cattle, Vet. Rec. 103 (1978) 446.
- [21] CALLOW, L.L., MCGREGOR, W., PARKER, R.J., DALGLIESH, R.J., Immunity of cattle to *Babesia bigemina* following its elimination from the host, with observations on antibody levels detected by the indirect fluorescent antibody test, Aust. Vet. J. 50 (1974) 12.
- [22] NEITZ, W.O., The destruction of *Piroplasma canis* by the neutrophils and large mono-nuclear leucocytes, Onderstepoort J. Vet. Sci. Anim. Ind. 10 (1938) 33.
- [23] ROGERS, R.J., Serum opsonins and the passive transfer of protection in *Babesia rodhaini* infections in rats, Int. J. Parasitol. 4 (1974) 197.
- [24] COHEN, S., BUTCHER, G.A., CRANDALL, R.B., Action of malarial antibody in vitro, Nature (London) 223 (1969) 368.
- [25] CHAPMAN, W.E., WARD, P.A., The complement profile in babesiosis, J. Immunol. 117 (1976) 935.

- [26] CLARK, I.A., ALLISON, A.C., *Babesia microti* and *Plasmodium berghei yoelii* in nude mice, *Nature* (London) **252** (1974) 328.
- [27] MITCHELL, G.F., Studies on immune responses to parasite antigens in mice: V. Different susceptibilities of hypothyroid and intact mice to *Babesia rodhaini*, *Int. Arch. Allergy Appl. Immunol.* **53** (1977) 385.
- [28] WOLF, R.E., Effects of antilymphocyte serum and splenectomy on resistance to *Babesia microti* infection in hamsters, *Clin. Immunol. Immunopathol.* **2** (1974) 381.
- [29] CALLOW, L.L., Vaccination against bovine babesiosis, *Adv. Exp. Med. Biol.* **93** (1977) 121.
- [30] BANERJEE, D.P., SINGH, B., GAUTAM, O.P., SARUP, S., Cell-mediated immune responses in equine babesiosis, *Trop. Anim. Health Prod.* **9** (1977) 153.
- [31] PEREZ, M., CARSON, C.A., RISTIC, M., Cell-mediated responses in hamsters infected with *Babesia microti*, *Vet. Parasitol.* **3** (1977) 161.
- [32] PHILLIPS, R.S., *Plasmodium berghei*: passive transfer of immunity by antisera and cells, *Exp. Parasitol.* **24** (1970) 479.
- [33] PHILLIPS, R.S., The role of the spleen in relation to natural and acquired immunity to infections of *Babesia rodhaini* in the rat, *Parasitology* **59** (1969) 637.
- [34] LEGG, J., The Occurrence of Bovine Babesiosis in Northern Australia, Council for Scientific and Industrial Research of Australia, Pamphlet No.56 (1935) 1.
- [35] UILENBERG, G., Notes sur les babésioses et l'anaplasmoses des bovins à Madagascar: II. Influence de la splénectomie, *Rev. Elev. Méd. Vét. Pays Trop.* **22** (1969) 237.
- [36] PHILLIPS, R.S., "Review of studies on protective immunization against piroplasmosis with irradiated parasitized cells", *Isotopes and Radiation in Parasitology III* (Proc. Research Co-ordination Meeting, Kabete, 1971), IAEA, Vienna (1973) 173.
- [37] PHILLIPS, R.S., Antigenic variation in *Babesia rodhaini* demonstrated by immunization with irradiated parasites, *Parasitology* **63** (1971) 315.
- [38] ROBERTS, J.A., TRACEY-PATTE, P., *Babesia rodhaini*: immuno-induction of antigenic variation, *Int. J. Parasitol.* **5** (1975) 573.
- [39] THOONGSUWAN, S., COX, H.W., Antigenic variants of the haemosporidian parasite, *Babesia rodhaini*, selected by *in vitro* treatment with immune globulin, *Ann. Trop. Med. Parasitol.* **67** (1973) 373.
- [40] CURNOW, J.A., *In vitro* agglutination of bovine erythrocytes infected with *Babesia argentina*, *Nature* (London) **217** (1968) 267.
- [41] CURNOW, J.A., Studies on antigenic changes and strain differences in *Babesia argentina* infections, *Aust. Vet. J.* **49** (1973) 279.
- [42] CALLOW, L.L., Strain immunity in babesiosis, *Nature* (London) **204** (1964) 1213.
- [43] CALLOW, L.L., Sterile immunity, coinfections, immunity and strain differences in *Babesia bigemina* infections, *Parasitology* **57** (1967) 455.
- [44] CALLOW, L.L., MELLORS, L.T., A new vaccine for *Babesia argentina* infection prepared in splenectomized calves, *Aust. Vet. J.* **42** (1966) 464.
- [45] PHILLIPS, R.S., Immunity of rats and mice following injection with ⁶⁰Co irradiated *Babesia rodhaini* infected red cells, *Parasitology* **62** (1971) 221.
- [46] BISHOP, J.P., ADAMS, L.G., *Babesia bigemina*: immune response to cattle inoculated with irradiated parasites, *Exp. Parasitol.* **35** (1974) 35.
- [47] PURNELL, R.E., BROCKLESBY, D.W., STARK, A.J., Protection of cattle against *Babesia major* by the inoculation of irradiated piroplasms, *Res. Vet. Sci.* **25** (1978) 388.
- [48] PHILLIPS, R.S., Resistance of mice and rats to challenge by *Babesia rodhaini* after inoculation with irradiated red cells infected with *B. rodhaini*, *Nature* (London) **227** (1970) 1255.

- [49] MAHONEY, D.F., WRIGHT, I.G., *Babesia argentina*: immunization of cattle with a killed antigen against infection with a heterologous strain, Vet. Parasitol. 2 (1976) 273.
- [50] COX, F.E.G., Protective immunity between malaria parasites and piroplasms in mice, Bull. World Health Organ. 43 (1970) 325.
- [51] CLARK, I.A., ALLISON, A.C., Protection of mice against *Babesia* and *Plasmodium* with BCG, Nature (London) 259 (1976) 309.
- [52] BROWN, I.N., PHILLIPS, R.S., Comparison of the resistance to *Plasmodium chabaudi* and *Babesia microti* in mice infected with *Mycobacterium lepraemurium* (in preparation).
- [53] CLARK, I.A., COX, F.E.G., ALLISON, A.C., Protection of mice against *Babesia* spp. and *Plasmodium* spp. with killed *Corynebacterium parvum*, Parasitology 74 (1977) 9.
- [54] HEROD, E., CLARK, I.A., ALLISON, A.C., Protection of mice against haemoprotozoan *Babesia microti* with *Brucella abortus* strain 19, Clin. Exp. Immunol. 31 (1978) 518.
- [55] COX, F.E.G., Heterologous immunity between piroplasms and malaria parasites: the simultaneous elimination of *Plasmodium vinckei* and *Babesia microti* from the blood of doubly infected mice, Parasitology 76 (1978) 55.
- [56] CLARK, I.A., Does endotoxin cause both the disease and parasite death in acute malaria and babesiosis, Lancet i (1978) 75.
- [57] PHILLIPS, R.S., WAKELIN, D., *Trichuris muris*: effect of concurrent infections with rodent piroplasms on immune expulsion from mice, Exp. Parasitol. 39 (1976) 95.
- [58] PURVIS, A.C., Immunodepression in *Babesia microti* infections, Parasitology 75 (1977) 197.
- [59] GRAY, G.D., The effect of *Babesia microti* on the immune response of mice to sheep erythrocytes, Parasitology 77 (1978) xlv.
- [60] GRAY, G.D., PHILLIPS, R.S., The primary and secondary response to sheep red cells in mice infected with rodent babesias (in preparation).
- [61] CORSINI, A.C., CLAYTON, C., ASKONAS, B.A., OGILVIE, B.M., Suppressor cells and loss of B-cell potential in mice infected with *Trypanosoma brucei*, Clin. Exp. Immunol. 29 (1977) 122.
- [62] LOOSE, L.D., DiLUZIO, N.R., A temporal relationship between reticuloendothelial system phagocytic alterations and antibody responses in mice infected with *Plasmodium berghei* (NYU-strain), Am. J. Trop. Med. Hyg. 25 (1976) 221.
- [63] CALLOW, L.L., STEWART, N.P., Immunosuppression by *Babesia bovis* against its tick vector, *Boophilus microplus*, Nature (London) 272 (1978) 818.
- [64] LEWIS, D., WILLIAMS, H., Infection of the Mongolian gerbil with the cattle piroplasm *Babesia divergens*, Nature (London) 278 (1979) 170.
- [65] ERP, E.E., GRAVELY, S.M., SMITH, R.D., RISTIC, M., OSORNO, B., CARSON, C.A., Growth of *Babesia bovis* in bovine erythrocyte cultures, Am. J. Trop. Med. Hyg. 27 (1978) 1061.

DISCUSSION

H.S. HUSSEIN: You do not say anything in your paper about immunity against the tick stages of the *Babesia*, such as the sporozoites. Has any work been done on this?

R.S. PHILLIPS: As far as I am aware there is no information available on the immune response to tick stages. I certainly think this is an area which merits investigation.

R.J. TERRY: Has anyone looked at germinal centre formation in *Babesia*-infected mice? For example, do mice infected with avirulent species form germinal centres and do those infected with virulent species fail to do so?

R.S. PHILLIPS: To my knowledge this has not been studied, but, in view of your comment during the discussion of paper SM-240/19¹ (Session I), it clearly would be of value.

¹ MURRAY, Max, et al., "Pathogenesis of trypanosome infections in cattle", these Proceedings, IAEA-SM-240/19.

BOVINE BABESIOSIS

Protection of cattle by the inoculation of irradiated piroplasms

R.E. PURNELL, D. LEWIS,
D.W. BROCKLESBY*
Agricultural Research Council,
Institute for Research on Animal Diseases,
Compton, Newbury,
Berks.,
United Kingdom

Abstract

BOVINE BABESIOSIS: PROTECTION OF CATTLE BY THE INOCULATION OF IRRADIATED PIROPLASMS.

A series of experiments are described in which the effects of irradiation on the infectivity of intra-erythrocytic piroplasms of *Babesia divergens* and *B. major* for cattle were investigated. Initially blood was taken from infected animals and stabulates of strains of the parasites were made and cryopreserved in an ultra-low deep-freeze. For each experiment, an aliquot of stabulate was inoculated into a splenectomized pre-donor calf and when this animal had a patent parasitaemia, a donor calf was infected by subinoculation. Blood was taken from the donor calf when 5 to 10% of its erythrocytes contained parasites. In the first two experiments, the parasites were titrated in groups of three splenectomized calves and it was seen that the strains maintained their pathogenic identities. Increasing dilution of the infective blood simply resulted in linear extension of the prepatent period. In the next pair of experiments, infected blood from the donor calves was irradiated at doses of 24 to 40 kilorads before inoculation into splenectomized calves. In these experiments irradiation had a marked effect on the pathogenic identity of the parasites. Not only were the prepatent periods extended, but animals receiving piroplasms irradiated at 24 and 28 kilorads had mild reactions whereas those receiving piroplasms irradiated at 36 and 40 kilorads had no overt reactions. Some animals receiving blood irradiated at 32 kilorads had mild reactions, others had none. On challenge the animals which had mild reactions were solidly immune and those having no reactions had limited protection. In the final pair of experiments intact calves received parasites irradiated at these same dose levels and the results were virtually identical. It was difficult to provoke severe reactions in intact animals to inoculation of infected blood, but the protection engendered by inoculation of irradiated piroplasms was clearly demonstrable.

* Present address: Centre for Tropical Veterinary Medicine, Roslin, Midlothian, United Kingdom.

INTRODUCTION

In 1970, Phillips [1] reported that the inoculation of irradiated *Babesia rodhaini* parasites into mice and rats conferred some protection against subsequent homologous challenge. Phillips suggested that the possibility of extending his experiments to economically important piroplasms of cattle merited investigation. While the senior author was on home leave from Kenya in 1971, the opportunity arose to carry out a pilot experiment on protecting splenectomized calves against *B. major* by the inoculation of irradiated infective blood. The results of this experiment were presented at the Research Co-ordination Meeting on the Use of Isotopes and Radiation in Control of Parasitic and Associated Diseases in Domestic Animals in Kabete, Nairobi, in November 1971 [2] and resulted in strong recommendations from the meeting that more work should be carried out on this subject.

When the senior author finally returned to the Institute for Research on Animal Diseases in December 1974, a research programme was initiated which was hoped to lead to irradiated vaccines against *B. divergens* and *B. major*, the cattle piroplasms identified in the European Community.

The results of the laboratory investigations are presented here.

MATERIALS AND METHODS

Cattle

The cattle used were Friesian crossed bull calves, born and reared under tick-free conditions and infected with *Babesia* at three to six months of age. Where splenectomy supervened, this was carried out when the animals were two to three months of age and they were then put on experiment a month or so later.

Parasites

B. major

The stabilate used is of the strain known as *B. major* (Essex) since the parasite was originally isolated by Brocklesby and Barnett [3] from ticks collected in Essex. The stabilate, IRAD 88, is cryopreserved using glycerol as a cryoprotectant [4] and the parasite revived by subcutaneous inoculation of 5 ml of the stabilate into a susceptible splenectomized calf and one further intravenous (iv) syringe passage.

B. divergens

The stabilate used is of the strain known as *B. divergens* (Eire 2) since the parasite was originally isolated by Purnell, Brocklesby, Hendry and Young [5] from blood from a heifer in Co. Wicklow, Eire. The stabilates, IRAD 67 and IRAD 98, result from the cryopreservation of the second and fourth laboratory passages of this parasite. They are cryopreserved using dimethyl sulphoxide as a cryoprotectant [4].

Observations

Observations were made on the calves before inoculation and daily afterwards for about three weeks. Animals were challenged four to six weeks post inoculation and observations were continued afterwards until reactions subsided. A quantity of 2.5 ml of blood was taken daily from the jugular vein into EDTA and rectal temperatures were recorded. Giemsa-stained thin blood smears were examined daily for parasites, and in the challenge of Experiment 3a thick blood smears were stained using the technique described by MacLennan [6] and examined. Haematology was carried out using a Coulter counter.

The following parameters were used to indicate the severity of response of splenectomized calves to infection [7]: prepatent periods to a parasitaemia of at least 1 parasite/1000 erythrocytes, maximum parasitaemia, total parasitaemia, prepatent period to a febrile response of 39.4°C or above, maximum febrile response, total febrile response in excess of 39.4°C, highest mean corpuscular volume (MVC) and lowest packed cell volume (PCV). Where intact calves were used, the following parameters were taken [8]: prepatent period to an overt parasitaemia, maximum parasitaemia, prepatent period to a febrile response, total febrile response, and lowest PCV expressed as a percentage of the pre-inoculation PCV.

Serum was prepared from 20 ml blood samples taken from calves before inoculation and at seven-day intervals until the end of the experiment. Serum antibodies against babesial antigen were assayed using the microplate enzyme linked immunosorbent assay (micro-ELISA) [9]. Results of the micro-ELISA test were particularly useful to detect susceptibility to challenge of intact calves in Experiments 3a and 3b, where the ratio of the micro-ELISA reading before and after challenge gave a clear indication of the animals' immune status after primary inoculation.

Inoculations

Three pairs of experiments were carried out. In Experiments 1a and 1b, *B. divergens* and *B. major* piroplasms were titrated in splenectomized calves.

In Experiments 2a and 2b, infected blood containing 10^9 – 10^{10} *B. divergens* or *B. major* piroplasms was irradiated at doses of 0, 24, 28, 32, 36 or 40 krad and then inoculated into splenectomized calves, which were subsequently challenged with approximately 10^4 homologous parasites. In Experiments 3a and 3b, infected blood containing 10^9 – 10^{10} *B. divergens* or *B. major* piroplasms were irradiated as before, but were subsequently inoculated into intact calves. These calves were homologously challenged with 10^9 – 10^{10} parasites.

Experiment 1a

This experiment was in two parts. In the first part, blood was taken from a donor splenectomized calf when its *B. divergens* parasitaemia was rising and had reached 70 parasites/1000 erythrocytes. The blood was taken into Anticlot (Clinton Laboratories, Santa Monica, California) and diluted with phosphate-buffered saline (PBS) (pH 7.2). Ten-fold dilutions were then made so that parasite numbers from 10^9 to 10^5 were present in 2 ml aliquots for iv inoculation into five groups of three calves.

In the second part, blood for titration was taken and pooled from two donor calves when their parasitaemias had risen to 54 and 46 parasites/1000. The blood was then diluted to give parasite numbers from 10^6 to 10^2 in 2 ml aliquots for iv inoculation into five groups of three calves. Calves which did not become parasitaemic were homologously challenged, together with a susceptible control animal, 28 days after infection.

Experiment 1b

Blood was taken from a donor calf with a rising *B. major* parasitaemia of 34 parasites/1000. Ten-fold dilutions in PBS were made so that 2 ml aliquots for injection contained 10^7 to 10^2 parasites. Six groups of three calves were then inoculated iv.

Experiment 2a

500 ml of blood taken from a calf with a *B. divergens* parasitaemia of 210 parasites/1000 was dispensed into 20 ml aliquots in plastic Universal bottles and taken to the MRC Unit at Harwell for irradiation. Four aliquots were then subjected to gamma irradiation from a ^{60}Co source at each of the irradiation levels, 24.1, 27.8, 32.4, 36.1 and 39.7 kilorad. Groups of three calves were inoculated

iv with 20 ml of blood containing 1.2×10^{10} parasites irradiated at each irradiation level. Two control groups of three animals were inoculated with non-irradiated blood diluted with PBS to produce inocula of 1.2×10^7 and 1.2×10^4 parasites.

All the animals, together with three control calves, were challenged 28 days later by iv inoculation of infected blood diluted in PBS to give an inoculum of 1.1×10^4 parasites.

Experiment 2b

400 ml of blood taken from a calf with a rising *B. major* parasitaemia of 80 parasites/1000 was dispensed into 20 ml aliquots in plastic Universal bottles. Three aliquots were then irradiated at 24, 28, 32, 36 and 40 kilorad and then inoculated iv into groups of three calves, each animal receiving 1.2×10^{10} parasites. Two control groups of three animals were inoculated with non-irradiated blood diluted with PBS to produce inocula of 1.2×10^7 and 1.2×10^4 parasites.

Six weeks later, all the surviving calves, together with three control calves, were challenged iv with 2.6×10^4 parasites.

Experiment 3a

A quantity of 500 ml of blood taken from a calf with a rising *B. divergens* parasitaemia of 62 parasites/1000 was dispensed into 20 ml aliquots as before and three aliquots irradiated at levels of 0, 23.9, 27.8, 31.8, 35.7 and 39.6 kilorad.

Groups of three calves were then inoculated iv with 20 ml aliquots of irradiated blood containing 5.9×10^9 parasites.

Six weeks later, all the animals, together with three control calves, were challenged iv with 7.5×10^9 parasites of a strain of *B. divergens* recently isolated from the field in Northern Ireland (Ulster 3 strain).

Experiment 3b

A quantity of 500 ml of blood taken from a calf with a rising *B. major* parasitaemia of 36 parasites/1000 was dispensed into 20 ml aliquots, three of which were irradiated at the following levels: 0, 23.3, 27.3, 31.4, 35.4 and 39.5 kilorad. Groups of three calves were then inoculated iv with 20 ml aliquots of irradiated blood containing 5.3×10^9 parasites.

Four weeks later, all the animals, together with three control calves, were challenged iv with 6.0×10^9 parasites of the homologous strain.

TABLE 1. THE MEAN VALUES FOR THE REACTIONS OF GROUPS OF THREE SPLENECTOMIZED CALVES TO INOCULATION OF TITRATED NUMBERS OF *B. divergens*-INFECTED ERYTHROCYTES (10^5 – 10^9), TOGETHER WITH THE APPROPRIATE STANDARD ERRORS

Number of parasites inoculated	Parasitaemia		Febrile response			Haematology	
	Prepatent period to paras 1/1000 +	Highest parasitaemia $\times 10^3/\text{mm}^3$	Prepatent period to temp. $39.5^\circ\text{C} +$	Highest fever ($^\circ\text{C}$)	Total fever above $39.4^\circ\text{C}(\text{degC})$	Lowest PCV	Highest MCV
10^9	2.00	636	3.33	41.0	4.30	8.3	48.7
10^8	3.00	676	4.00	40.9	4.50	12.4	40.0
10^7	3.67	761	5.00	40.9	4.70	10.7	49.7
10^6	4.33	648	5.33	40.8	3.87	13.1	44.3
10^5	5.33	566	5.33	40.5	2.80	11.4	44.7
Standard error for difference of two means (experiment 1)	0.385	254.9	0.544	0.35	0.921	2.77	5.94

TABLE II. THE MEAN VALUES FOR THE REACTIONS OF GROUPS OF THREE SPLENECTOMIZED CALVES TO INOCULATION OF TITRATED NUMBERS OF *B. divergens*-INFECTED ERYTHROCYTES (10^3 – 10^6), TOGETHER WITH THE APPROPRIATE STANDARD ERRORS

Number of parasites inoculated	Parasitaemia		Febrile response		Haematology	
	Prepatent period to paras 1/1000 +	Highest parasitaemia $\times 10^3/\text{mm}^3$	Prepatent period to temp. $39.5^\circ\text{C} +$	Highest fever ($^\circ\text{C}$)	Lowest PCV	Highest MCV
10^6	4.67	1010	5.67	40.9	10.8	50.5
10^5	7.67	804	7.33	40.8	11.7	45.3
10^4	6.67	1006	7.67	41.3	9.0	57.3
10^3	8.00	689	8.00	40.3	17.2	47.0
Standard error for difference of two means (experiment 2)	0.976	404.4	0.707	0.36	4.45	5.91

TABLE III. REACTIONS OF GROUPS OF THREE SPLENECTOMIZED CALVES TO THE INOCULATION OF TITRATED NUMBERS OF *Babesia major*-INFECTED ERYTHROCYTES

Number of parasites inoculated	Parasites		Febrile response		Reaction ^a
	Mean prepatent period to parasites in days	Mean maximum parasitaemia /1000 rbc	Mean prepatent period to fever in days	Mean maximum febrile response (°C)	
10 ⁷	3.0	251	4.0	41.2	1D 2SR
10 ⁶	4.3	169	5.3	41.2	3SR
10 ⁵	4.7	200	6.0	41.3	3SR
10 ⁴	5.7	259	7.3	41.2	2D 1SR
10 ³	7.7	311	8.7	41.3	2D 1SR
10 ²	7.3	291	8.3	40.9	1D 2SR

^a D = died; SR = severe reaction.

RESULTS

Experiment 1a

The results of the first part of the experiment are shown in Table I. Analysis of this data indicated that the only significant differences in the responses of the calves to inoculation with decreasing numbers of parasites were in the prepatent periods before appearance of parasites ($p < 0.001$) and detection of a febrile response ($p < 0.01$). Both of these were inversely related to the numbers of parasites inoculated. In the case of prepatent period to parasites, the relationship was linear. One animal, J497, inoculated with 10^8 parasites, failed to undergo a normal disease reaction.

The results of the second part of the experiment are shown in Table II. Since one of the animals inoculated with 10^3 parasites and all three inoculated with 10^2 parasites failed to develop patent parasitaemias, analysis of the data in this part of the experiment was less fruitful. However, a significant inverse linear relationship between the number of parasites inoculated and the prepatent period to detection of a febrile response was observed ($p < 0.05$). There were no other significant effects in this part of the experiment. On challenge the four animals having no detectable parasitaemia were fully susceptible. The data for the animals inoculated with 10^5 and 10^6 parasites in the two parts of the experiment were compared. The only significant difference between the reactions was in the prepatent period to parasitaemia. This was significantly extended ($p < 0.05$) in the animals in the second part of the experiment which had been inoculated with 10^5 parasites. This suggested the necessity of stabilizing the linear relationship between prepatent period to onset of disease and inoculated dose by always using two dosage levels of normal parasites as controls for the interpretation of the effect of other factors on the reactions of inoculated calves.

Experiment 1b

The results of the experiment are shown in Table III. Calves in all groups had severe reactions and a third of them died. There was no difference in the severity of the reactions between calves in different groups, but increasing dilution resulted in a linear increase in the prepatent period before parasites were seen in blood films and a febrile response occurred ($p < 0.001$).

Experiment 2a

The results of the experiment are summarized in Table IV. All the animals receiving blood irradiated at approximately 24, 28 and 32 kilorad had mild reactions and all but one were immune to challenge. One animal in the group receiving

TABLE IV. REACTIONS OF GROUPS OF THREE SPLENECTOMIZED CALVES TO THE INOCULATION OF IRRADIATED *Babesia divergens* PIROPLASMS AND TO THEIR SUBSEQUENT HOMOLOGOUS CHALLENGE

Number of parasites inoculated	Irradiation dose (krad)	Primary inoculation						Challenge			
		Parasitaemia			Febrile response			Parasitaemia		Febrile response	
		Mean prepatent period to parasites (d)	Mean maximum parasitaemia /1000 rbc	Mean prepatent period to fever (d)	Mean maximum febrile response (°C)	Reaction ^a	Reaction ^a	Mean prepatent period to parasites (d)	Mean maximum parasitaemia /1000 rbc	Mean prepatent period to fever (d)	Mean maximum febrile response (°C)
1.2×10^{10}	24	7.7	18.0	7.7	39.9	3MR	3MR	—	—	—	—
1.2×10^{10}	28	9.0	18.7	8.0	40.4	3MR	3MR	12.5(2/3)	1.0	—	—
1.2×10^{10}	32	11.0	13.3	11.0(2/3)	40.1(2/3)	3MR	3MR	9.7	1.0	—	—
1.2×10^{10}	36	10.0(2/3)	89.0(2/3)	9.3	40.8	1MR 1SR	1MR 1SR	7.0(2/3)	42.5(2/3)	5.0	40.0
1.2×10^{10}	40	—	—	11.0(1/3)	39.7(1/3)	3NR	3NR	7.7	54.0	3.7	40.6
1.2×10^7	0	4.0	207.7	4.3	40.7	3SR	3SR	—	—	—	—
1.2×10^4	0	6.0	286.0	5.7	41.0	3SR	3SR	—	—	—	—
1.1×10^4	0	—	—	—	—	—	—	6.0	193.0	7.0	41.1

^a SR = severe reaction; MR = mild reaction; NR = no reaction.

TABLE V. REACTIONS OF GROUPS OF THREE SPLENECTOMIZED CALVES TO THE INOCULATION OF IRRADIATED *Babesia major* PIROPLASMS AND TO THEIR SUBSEQUENT HOMOLOGOUS CHALLENGE

Number of parasites inoculated	Irradiation dose in (krad)	Primary inoculation						Challenge			
		Parasitaemia			Febrile response			Reaction ^a	Parasitaemia		Reaction ^a
		Mean prepatent parasites (d)	Mean maximum parasitaemia /1000 rbc	Mean prepatent fever (d)	Mean maximum febrile response (°C)	Mean prepatent fever (d)	Mean maximum febrile response (°C)		Mean prepatent fever (d)	Mean maximum febrile response (°C)	
1.2 X 10 ¹⁰	24	9.7	3	—	—	—	—	3NR	—	—	3NR
1.2 X 10 ¹⁰	28	11.0	8	—	—	—	—	3NR	—	—	3NR
1.2 X 10 ¹⁰	32	10(1/3)	1(1/3)	—	—	—	—	1MR	7.5(2/3)	40.9(2/3)	1NR
								2NR			2SR
1.2 X 10 ¹⁰	36	—	—	—	—	—	—	3NR	7.0	40.8	3SR
1.2 X 10 ¹⁰	40	—	—	—	—	—	—	3NR	8.0	40.7	3SR
1.2 X 10 ⁷	0	3.0	359	3.3	40.9	—	—	3D	—	—	—
1.2 X 10 ⁴	0	6.3	220	6.3	41.1	—	—	2D	—	—	1NR
								1SR			
2.6 X 10 ⁴	0	—	—	—	—	—	—	—	5.7	41.5	3SR

^a D = died; SR = severe reaction; MR = mild reaction; NR = no reaction.

TABLE VI. SUMMARIZED REACTIONS OF GROUPS OF THREE INTACT CALVES TO THE INOCULATION OF 5.9×10^9 IRRADIATED *B. divergens* PIROPLASMS

Group	Animal	Irradiation dose (krad)	Max. parasitaemia /1000 rbc	Fever	Mean % age Lowest / Pre-inoc. PCV	Summarized reaction ^b
A	M612	0	1	+	86.8	MR
	M869		1	+		MR
	M906		1	+		MR
B	M610	24	-	-	90.4	NR
	M871		<1	-		VMR
	M905		-	-		NR
C	M409	28	-	+	93.1	VMR
	M872		-	-		NR
	M903		-	-		NR
D	M22	32	<1	+	84.6	VMR
	M870		<1	-		VMR
	M904		-	+		VMR
E	M411	36	-	-	91.7	NR
	M607		-	+		VMR
	M876		<1	+		VMR
F	M611	40	^a	-	93.5(2/3)	NR
	M874		-	+		VMR
	M907		-	+		VMR

^a Died of intercurrent disease.

^b MR = mild reaction = parasitaemia 1/1000 + and fever; VMR = very mild reaction = parasites or fever recorded; NR = no overt reaction observed.

blood irradiated at 32 kilorad experienced a mild reaction on challenge. Animals receiving blood irradiated at 36 kilorad exhibited varying responses to the primary inoculation: one had a mild reaction, the second a severe reaction and the third experienced no reaction. The reactions of this group to challenge reflected their initial response and were mild, nil and severe respectively. All the animals receiving blood irradiated at 40 kilorad exhibited no reaction on primary inoculation and two of these had severe reactions on challenge. The third animal from this group experienced a mild reaction on challenge.

Control animals in both parts of the experiment had severe reactions.

Statistical analysis of the reactions to challenge of animals that had received parasites subjected to the higher doses of irradiation showed significant differences between them and those of the challenge control group. Mean maximum temperatures, lowest PCVs and highest MCVs of the group receiving 36-kilorad irradiated blood were significantly different ($p < 0.05$) from those of the challenge animals. The mean prepatent periods to parasitaemia and fever of the 40-kilorad group were also significantly different from those of the challenge animals ($p < 0.05$).

The immune status of the animals was assessed by a comparison of the micro-ELISA readings before and after challenge. The only significant difference ($p < 0.01$) in the ratio obtained was between animals shown to be immune and those shown to be susceptible to challenge.

Experiment 2b

The results of the experiment are summarized in Table V. All animals receiving blood irradiated at 24 or 28 kilorad had mild reactions and were immune to challenge, as was one of the animals receiving blood irradiated at 32 kilorad. The remaining two animals in the latter group, and all the animals receiving blood irradiated at 36 or 40 kilorad, had no reactions initially and severe reactions to challenge. On the day following the original inoculations, low numbers of morphologically abnormal parasites were detected in blood smears from animals in all groups receiving irradiated blood. Subsequent statistical analysis of the reactions to challenge of all the animals which had received parasites subjected to the higher doses of irradiation compared with those of animals in the challenge control group showed significant differences in the prepatent period to parasites, in the highest MCV and in the lowest PCV ($p < 0.05$).

Experiment 3a

The results of inoculating the calves with blood containing irradiated *B. divergens* piroplasms are summarized in Table VI. All the animals that received irradiated material had either very mild reactions, where parasitaemias never reached 1 parasite/1000 and febrile responses did not necessarily occur, or no overt

TABLE VII. SUMMARIZED REACTIONS OF GROUPS OF THREE INTACT CALVES TO HETEROLOGOUS CHALLENGE WITH 7.5×10^9 *B. divergens* PIROPLASMS

Group	Animal	Irradiation dose (krad)	Total parasites/ml		Fever	Mean % age Lowest / Pre-inoc. PCV / PCV	Summarized reaction ^a
			Thin smear	Thick smear			
A	M612	0	(<1/1000)	2.8×10^5	-	93.8	VMR
	M869		(<1/1000)	3.4×10^5	-		VMR
	M906		(<1/1000)	4.4×10^5	-		VMR
B	M610	24	6.2×10^6	2.4×10^6	-	84.6	MR
	M871		-	1.9×10^5	+		VMR
	M905		7.2×10^6	-	-		VMR
C	M409	28	6.6×10^6	7.8×10^6	+	87.5	MR
	M872		-	-	-		NR
	M903		-	3.2×10^5	-		VMR
D	M22	32	1.4×10^7	1.6×10^6	+	86.9	MR
	M870		8.1×10^6	3.8×10^6	-		MR
	M904		(<1/1000)	4.3×10^5	-		VMR
E	M411	36	2.8×10^6	5.2×10^7	+	81.5	SR
	M607		5.7×10^7	3.0×10^7	+		SR
	M876		-	1.7×10^5	+		VMR
F	M611	40				79.1	
	M874		6.2×10^7	3.0×10^7	-		SR
	M907		3.7×10^7	3.1×10^7	+		SR
H	M71	0	2.6×10^7	1.5×10^7	+	83.4	SR
	M613		(<1/1000)	3.6×10^6	-		MR
	M614		3.0×10^6	1.6×10^6	+		SR

^a SR = severe reaction = parasitaemia 10^7 +/ml recorded; MR = mild reaction = parasitaemia 10^6 +/ml recorded; VMR = very mild reaction = parasitaemia 10^5 +/ml recorded; NR = no overt reaction observed.

reaction. In contrast, the animals that received untreated infective blood all had mild reactions, where parasitaemias rose to 1/1000 and febrile responses occurred. One animal, which had received blood irradiated at 40 kilorad, died of intercurrent disease.

The results of challenging the calves with the heterologous strain of *B. divergens* are summarized in Table VII. Animals which had received blood irradiated at 24, 28 or 32 kilorad or non-irradiated blood all had mild, very mild, or no overt reaction. In contrast four of five surviving animals that had received blood irradiated at 36 or 40 kilorad and two of three control animals had severe reactions, where parasitaemias in excess of 10^7 parasites/ml of blood were recorded. The severity of these reactions was further deduced from the lower PCV ratios recorded from animals in these groups.

Assessment of the immune status of the animals before challenge by examination of their micro-ELISA ratios showed that only the four animals that had received irradiated blood but subsequently had severe reactions to challenge had ratios in excess of 1.50 : 1. This was further confirmation of their susceptibility and of the immunity of the other animals.

Experiment 3b

The results of the experiment are summarized in Table VIII. Calves receiving non-irradiated blood soon reacted to the inoculations. They had low level but persistent parasitaemias, parasites being detected for approximately one week. Parasitaemia was accompanied by a febrile response and resulted in a significant drop in PCV. The calves proved immune to challenge. Animals receiving blood irradiated at doses of 23.3 – 31.4 kilorad had delayed and minimal reactions, parasitaemias becoming briefly patent (except in one case), and febrile responses being generally absent or ephemeral. The reactions had no apparent effect on the calves' haematology, but all animals had marked rises in serum antibody titre, comparable with the control group, and were immune to challenge. Two of the three animals receiving blood irradiated at 35.4 kilorad had similar minimal reactions, but the third calf, and the three receiving blood irradiated at 39.5 kilorad, had no detectable parasitaemic reaction. On challenge these animals proved susceptible and had significant rises in serum antibody titre.

DISCUSSION

The results of experiments 1a and 1b indicate that titration of *B. divergens* and *B. major* parasites in splenectomized Friesian calves results in a linear increase in the length of the prepatent period to onset of disease. They are thus in agreement with those of Overdulve and Antonisse [10] for *B. rodhaini* in mice and of

TABLE VIII. REACTIONS OF GROUPS OF THREE INTACT CALVES TO THE INOCULATION OF 5.3×10^9 IRRADIATED *Babesia major* PIROPLASMS AND TO THEIR SUBSEQUENT HOMOLOGOUS CHALLENGE WITH 6.0×10^9 IRRADIATED *B. major* PIROPLASMS

Irradiation dose (krad)	Animal	Parasitaemia		Febrile response			Minimum PCV as % age of pre-inoculation	Micro-ELISA test ratio day 7 post-chall. Day 28 p.i.	Immune status after primary inoculation ^a
		Prepatent period (d)	Maximum parasitaemia /1000 rbc	Prepatent period (d)	Maximum febrile response (°C)	Total febrile response over 39.4°C (degC)			
0	L493	2	4	2	39.7	0.8	63.6	1.42	I
	L671	2	14	3	39.8	0.7	66.6	1.31	I
	L686	1	10	3	39.8	0.9	64.6	1.32	I
23.3	L243	9	<1	—	—	—	70.8	1.36	I
	L254	13	<1	6	39.4	—	89.0	1.17	I
	L492	9	<1	3	40.1	1.1	74.0	1.59	I
27.3	L221	16	<1	4	39.5	0.1	74.6	1.14	I
	L250	—	—	2	39.7	0.3	87.8	1.35	I
	L693	9	<1	3	39.4	—	78.1	1.37	I
31.4	L231	10	<1	—	—	—	73.7	1.51	I
	L273	14	<1	—	—	—	83.6	1.27	I
	L478	10	<1	—	—	—	83.7	1.09	I
35.4	L233	13	<1	—	—	—	76.4	1.17	I
	L477	10	<1	—	—	—	82.6	1.09	I
	L494	—	—	8	39.6	0.2	82.4	3.75	S
39.5	L244	—	—	11	39.5	0.1	77.0	2.67	S
	L495	—	—	—	—	—	81.3	3.22	S
	L673	—	—	—	—	—	78.2	2.92	S

^a I = immune to homologous challenge; S = susceptible to homologous challenge.

Mahoney, Wright and Ketterer [11] for *B. argentina* in splenectomized calves. In each part of the experiment, the results showed that there were no differences in the severity of the calves' reactions to the varying doses. The parasites thus retained their pathogenic identities and this emphasized the need to explore irradiation as a means of decreasing the pathogenicity of the parasites while retaining their immunogenicity.

The results of experiments 2a and 2b indicate that pathogenicity of these parasites can indeed be reduced by irradiation of intra-erythrocytic piroplasms at doses of 24 and 28 kilorad, but that the immune response which such piroplasms provoked in splenectomized calves protected them against homologous challenge. Where parasites were subjected to higher doses of irradiation there were indications that the presence of large numbers of dead or dying parasites had stimulated limited reactions in the inoculated animals and had provided some protection against challenge.

The results of experiments 3a and 3b demonstrate that intact calves can be protected against heterologous *B. divergens* challenge or homologous *B. major* challenge by the prior inoculation of 10^9 – 10^{10} piroplasms irradiated at the same doses, 24 or 28 kilorad. The protection induced by inoculation of *Babesia* piroplasms irradiated at 24 or 28 kilorad may result from a variety of effects of irradiation on the piroplasms. It is likely either that a high proportion of the parasites have been killed, or that there has been an alteration in the virulence of parasites surviving irradiation. It is tempting to speculate that the atypical reactions of inoculated calves may have resulted from the simultaneous inoculation of a small number of viable parasites and a large number of dead or dying ones.

Although protection of calves demonstrated in these experiments is encouraging, it remains to be seen if such calves are capable of withstanding unlimited challenge with infected ticks in the field. Similar initial results with *B. bovis* in Australia [11] and *B. bigemina* in Colombia [12] have not apparently been followed by field trials of irradiated 'vaccines' suggesting that there are problems which we have yet to encounter.

ACKNOWLEDGEMENTS

We are most grateful to E. Young, A. Brabazon and L. Nelson for technical assistance, to P. Adams of the MRC Radiobiological Unit at Harwell for irradiating the blood, to A. Stark for statistical analyses, and to M. Hoare, D. Davies and D. Baggott for splenectomizing the calves.

We are grateful to the Ministry of Overseas Development for financial support (Research Project R3270).

The work described in this paper forms part of a co-ordinated programme of research on tick-borne diseases of livestock under the auspices of the International Atomic Energy Agency.

REFERENCES

- [1] PHILLIPS, R.S., Nature, London 227 (1970) 1255.
- [2] PURNELL, R.E., BROCKLESBY, D.W., SELLWOOD, S.A., "⁶⁰Co-irradiation of bovine erythrocytes infected with *Babesia major*", Isotopes and Radiation in Parasitology III (Proc. Research Co-ordination Meeting, Kabete, 1971), IAEA, Vienna (1973) 169.
- [3] BROCKLESBY, D.W., BARNETT, S.F., Nature, London 228 (1970) 1215.
- [4] PURNELL, R.E., BROCKLESBY, D.W., HARRADINE, D.L., Vet. Parasitol. 1 (1975) 199.
- [5] PURNELL, R.E., et al., Vet. Rec. 99 (1976) 415.
- [6] MACLENNAN, K.J.R., Trans. R. Soc. Trop. Med. Hyg. 51 (1957) 301.
- [7] PURNELL, R.E., et al., Vet. Rec. 100 (1977) 4.
- [8] PURNELL, R.E., LEWIS, D., BROCKLESBY, D.W., Int. J. Parasitol. (in press).
- [9] PURNELL, R.E., et al., Vet. Rec. 99 (1976) 102.
- [10] OVERDULVE, J.P., ANTONISSE, H.W., Exp. Parasitol. 27 (1970) 310.
- [11] MAHONEY, D.F., WRIGHT, I.G., KETTERER, P.J., Int. J. Parasitol. 3 (1973) 209.
- [12] BISHOP, J.P., ADAMS, L.G., Exp. Parasitol. 35 (1974) 35.

DISCUSSION

A.D. IRVIN: Would you like to speculate on the mechanism of the protection conferred by irradiated blood? Do you think, for example, that the balance between parasites killed by irradiation and living parasites is fortuitous?

R.E. PURNELL: In the presence of such eminent immunologists I hesitate to speculate on this matter. However, I do feel that a fortuitous balance of this type is likely to have effected protection.

NON-SPECIFIC IMMUNIZATION AGAINST BABESIOSIS

F.E.G. COX

Department of Zoology,
King's College,
University of London,
London,
United Kingdom

Abstract

NON-SPECIFIC IMMUNIZATION AGAINST BABESIOSIS.

The rodent babesias, *Babesia rodhaini* and the less virulent *B. microti*, are useful models with which to study immunity to and immunization against babesiosis. In contrast with the difficulty in inducing specific immunity to these parasites it is comparatively easy to induce non-specific immunity by prior exposure to related and unrelated intra-erythrocytic protozoa, micro-organisms such as *Mycobacterium bovis* (BCG) and *Corynebacterium parvum*, microbial extracts and muramyl dipeptide. This non-specific immunity is long lasting and extremely effective. It is characterized by the facts that (a) it occurs early in the infection at the height of the first peak of parasitaemia, and (b) it involves the intra-erythrocytic death of the parasites. After the primary parasitaemia has resolved, some parasites continue to persist at a low level and when introduced into clean mice produce only low-level 'attenuated' infections in these. Non-specific immunity is not equally effective in all strains of mice. It is suggested that immunity to babesiosis, and infections caused by other intra-erythrocytic protozoa, involves two mechanisms, the first non-specific and the second specific. The actual balance between these two mechanisms varies from parasite to parasite and from host to host. An effective vaccine would have to be based on an understanding of the roles of non-specific and specific immunity in the actual disease under consideration, and would ideally combine an adjuvant that would also stimulate non-specific immunity and an attenuated strain of parasite that would induce a specific response.

INTRODUCTION

Three genera of intra-erythrocytic protozoa cause diseases of major importance, *Plasmodium*, causing malaria in man, *Babesia*, causing babesiosis, including red water fever, in cattle, horses and dogs, and *Theileria*, causing theileriosis, in particular East Coast Fever, in cattle. Recovery from infection and resistance to re-infection occur in all these diseases but, as is characteristic of protozoal infections in general, the immunity is slow to develop and usually incomplete. It is

possible to induce artificial immunity experimentally but there are no immediate prospects of producing a widely acceptable vaccine against any of these parasites nor is it clear what kind of vaccine should be developed. One major problem is that the particular parasites involved in these diseases are either host-specific or have narrow host ranges. *Babesia* and *Plasmodium* spp. can be maintained in standard experimental animals but this leads to the investigation of particular laboratory host-parasite systems and to the extrapolation from these to entirely different systems. This contrasts with the situation that exists, for example, in trypanosomiasis in which the host can be varied whereas the parasite is still the one that actually causes the disease under investigation.

This problem of host specificity has imposed severe limitations on the study of specific immunization but, partly as a result of intensive work on the immunology of tumours in mice, has drawn attention to the possibilities that non-specific mechanisms may play a part in the natural acquisition of immunity to both malaria and babesiosis, that these mechanisms may be similar, if not the same, in both kinds of infection and that non-specific immunization may be a feasible proposition. In the context of experimental babesiosis, the first kind of non-specific immunity to be described was heterologous immunity following recovery from infections with related or unrelated intra-erythrocytic protozoa. The avirulent piroplasm, *Babesia microti*, protects mice against the virulent *B. rodhaini* [1], and certain *Plasmodium* spp. also protect mice against *B. microti* [2-4], *B. rodhaini* [3-5] and *B. hylomysci* [6]. Another piroplasm, *Anthemiosoma garnhami*, protects against *B. microti* and *B. rodhaini* [7]. Heterologous immunity also occurs in rats [5]. That this kind of immunity is non-specific and does not involve cross-reacting antigens is discussed elsewhere [8].

Protection against *B. microti* and *B. rodhaini* can also be induced by pre-treating mice with *Mycobacterium bovis* (BCG) [9], *Corynebacterium parvum* [10], *Brucella abortus* [11, 12], *Coxiella burnetii* extract [13] and chlorite-oxidized oxyamylose (COAM) [14], and against *B. microti* by pretreating mice with *Salmonella enteritidis*, *Listeria monocytogenes*, trehalose 6'-6, dimycolate (cord factor), zymosan and glucan [14]. In all cases the actual mechanism of immunity involves the death of the parasites within the red blood cells. These observations have led Clark [15] to postulate that the mechanism of killing is akin to the effects of endotoxin shock.

Whatever the mechanism involved, the facts that non-specific immunity does occur, is so effective and follows pretreatment with so many different agents warrant further investigation of its role both in its own right and in conjunction with conventional specific immune mechanisms. In this study it is proposed to expand upon what is known about non-specific immunity in rodent babesiosis, and to relate what is known to bovine and equine babesiosis.

EXPERIMENTAL METHODS AND RESULTS

Babesia infections in laboratory mice

The two parasites used were *B. microti* (King's strain) and *B. rodhaini* (Antwerp strain). Both strains were maintained by the intraperitoneal injection of infected blood or kept deep frozen in liquid nitrogen. TO Swiss female mice were the most useful experimental hosts. In such mice, the patterns of infections following the intravenous injection of known numbers of parasites were quite predictable, and the prepatent periods were directly related to the number of parasites injected, thus the patterns of infection could be varied at will and any variations from normal easily detected. The increase in parasitaemia was logarithmic until the infected mouse died in the case of *B. rodhaini* or a crisis was reached after which the parasitaemia gradually declined in the case of *B. microti*. This recovery was invariable in our laboratory and this made *B. microti* an ideal subject for immunological studies. During the declining phase of the infection, the parasites within the red blood cells were seen to be degenerate and similar to those seen after non-specific immunization (see below). This suggests that natural recovery and recovery after non-specific immunization involve similar mechanisms.

Immunization with *B. microti*, *Mycobacterium bovis* (BCG) and *Corynebacterium parvum*

Mice were routinely immunized intravenously with 1×10^4 *B. microti*, 1×10^6 *Mycobacterium bovis* (BCG, Glaxo) or 1.0 mg *Corynebacterium parvum* (Coparvax, Wellcome). Challenge with *B. microti* or *rodhaini* was carried out four weeks after injection with BCG or *C. parvum* or 10 weeks after infection with *B. microti*, four weeks after recovery. Under these experimental conditions, protection against *B. microti* and *B. rodhaini* was almost absolute (Table I). However, even in mice apparently free from parasites, subpatent infections persisted for some time after challenge and the blood was infective to clean mice.

Influence of strain and sex of mice on protection against *Babesia* spp. following pretreatment with *C. parvum*

Preliminary experiments indicated that the patterns of infection of *B. microti* and *B. rodhaini* and the degree of protection against these parasites following pretreatment with BCG or *C. parvum* varied between strains of mice. Two strains were selected for more detailed study, Swiss TO and BSVS (Bacteria Susceptible Virus Susceptible). The BSVS mice were originally bred for susceptibility to bacteria and viruses [16] and have been found to be particularly susceptible to infection with *B. microti* [17]. Mice pretreated with *C. parvum*

TABLE I. PROTECTION OF DIFFERENT STRAINS OF MICE AGAINST *Babesia microti* AND *B. rodhaini* FOLLOWING PRETREATMENT WITH *Corynebacterium parvum*

Strain	<i>B. microti</i>		<i>B. rodhaini</i>	
	No. protected	Maximum parasitaemia (%)	No. protected	Maximum parasitaemia (%)
TO ♀	24/24	0.01	12/12	0.01
TO ♂	12/12	0.2	12/12	0.8
BSVS ♀	12/12	26	12/12	28
BSVS ♂	0/24	55	8/12	56
TO/BSVS ♀	12/12	24	12/12	25
TO/BSVS ♂	12/12	26	12/12	22
TO ♀ controls	0/24	47	0/12	dead

as described above were challenged with 1×10^4 *B. rodhaini* or 1×10^5 *B. microti* intravenously. The results obtained, summarized in Table I, indicate that there are only slight differences between the sexes in TO mice but in BSVS mice the males are less well protected by pretreatment with *C. parvum* than females. In other experiments it was also shown that BSVS mice in general were less well protected non-specifically against several intra-erythrocytic protozoa than TO mice, and crosses between TO and BSVS mice were intermediate between the two parent groups.

Influence of age of mice on protection against *Babesia* spp. following pretreatment with *C. parvum*

Groups of six TO male and female mice were injected with 0.35 mg *C. parvum* shortly after weaning when 28 days old. Four weeks later they were challenged with 1×10^6 *B. microti* or 1×10^5 *B. rodhaini*. None of the 12 female mice became infected whereas the males experienced parasitaemias of up to 0.2%. In the controls, the infections reached 61% in the case of *B. microti* and killed all the mice infected with *B. rodhaini*.

Twelve TO female mice were injected with *C. parvum* when 18 months old and challenged with *B. microti* and *B. rodhaini* four weeks later. All 12 mice showed solid protection.

Duration of protection against *Babesia* spp. following pretreatment with *C. parvum*

Groups of TO female mice were injected with 1 mg *C. parvum* and challenged with *B. microti* or *B. rodhaini* at various intervals over a period of 12 months. At six months all the mice were solidly protected, at nine months approximately half the treated mice were protected and after 12 months there was no protection. Mice pretreated with *C. parvum* and challenged with *B. microti* four weeks later resisted subsequent homologous or heterologous challenge for over 12 months at which time the experiment was discontinued.

Effect of pregnancy on protection against *Babesia* spp. following pretreatment with *C. parvum*

Eighteen TO female mice were injected with *C. parvum*, mated 14 days later and challenged with *B. microti* or *B. rodhaini* after another 14 days. Litters were produced seven-nine days after infection. Only the occasional parasite was seen in the blood of the pretreated mice while three controls challenged with *B. microti* experienced high parasitaemias and the three controls challenged with *B. rodhaini* died.

Protective effects of microbial extracts against challenge with *B. microti*

Certain well-characterized microbial extracts are known to substitute for BCG or *C. parvum* as immunostimulants [18], and some of these were used to investigate their potential in protection against *Babesia* spp. In these preliminary experiments, *B. microti* was selected for study as it is a less virulent parasite than *B. rodhaini*. The amounts of microbial extracts used and the interval between pretreatment and challenge were chosen as a result of comparisons with other systems.

Groups of six TO female mice were injected intravenously with 250 µg cell wall of *Mycobacterium smegmatis*, cell wall of *Corynebacterium parvum*, mycobacterial cord factor (trehalose-6, 6'-dimycolate) or muramyl dipeptide (MDP) suspended in saline with the aid of an ultrasonic disintegrator with or without arachis oil. Eight weeks later, the mice were challenged with 1×10^6 *B. microti*. The results obtained are shown in Fig. 1.

Further groups of four mice were given injections of each of the substances listed above plus a second injection of *M. smegmatis* cell wall 26 weeks later, and after a further four weeks were challenged with 1×10^6 *B. microti*. All the mice in the group given cord factor and three in each of the other groups were protected. The results are shown in Fig. 2. The protection was as good as that following pretreatment with BCG or *C. parvum*.

Further groups of 12 mice were injected subcutaneously or intravenously with 0.4 ml 'Polidin', a bacterial extract known to have immunostimulatory

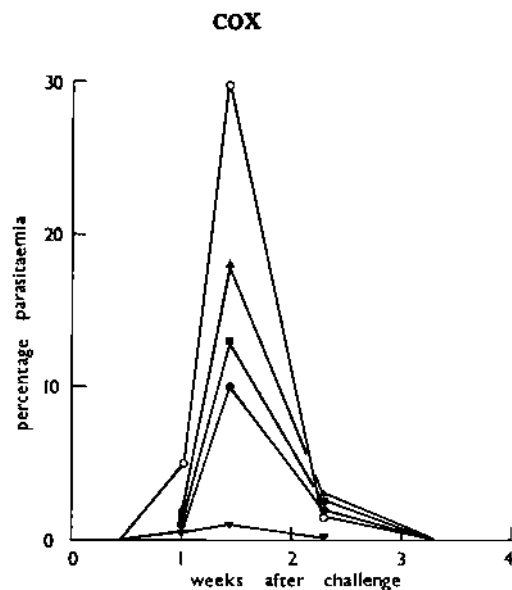


FIG.1. Mean percentage parasitaemia in mice protected against *Babesia microti* following pretreatment eight weeks previously with muramyl dipeptide (●), cord factor (■), *Corynebacterium parvum* cell wall (▲) and *Mycobacterium smegmatis* cell wall (▼); controls (○).

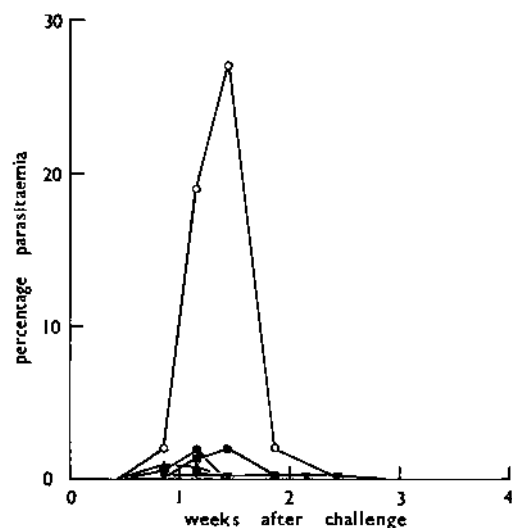


FIG.2. Mean percentage parasitaemia in mice protected against *Babesia microti* following pretreatment with *Mycobacterium smegmatis* cell wall four weeks previously and 26 weeks previously with muramyl dipeptide (●), cord factor (■), *Corynebacterium parvum* cell wall (▲) and *Mycobacterium smegmatis* cell wall (▼); controls (○).

properties [19]. Four weeks later these mice were challenged with *B. microti* or *B. rodhaini* but there was no protection.

The isolation of attenuated strains of *B. microti* and *B. rodhaini* from mice pretreated with *C. parvum*

Mice were pretreated with *C. parvum* and challenged with *B. microti*. Seven days later, 10 μ l blood from each of these mice was injected into two untreated mice in which the resulting parasitaemias were very low. Blood from these recipient mice also produced low parasitaemias when passaged into further untreated mice. Similar results were obtained with *B. rodhaini*. These 'attenuated' strains were frozen.

Groups of mice were injected with the 'attenuated' *B. microti* and challenged with *B. rodhaini* or *B. microti*. The parasitaemias in these mice rose to near normal levels and the mice challenged with *B. rodhaini* died. In the *B. microti* infected mice, on the other hand, the post-crisis infections were considerably curtailed.

DISCUSSION

Non-specific immunity to rodent babesiosis is relatively easy to induce even in strains of mice particularly susceptible to such infections. This non-specific immunity is long lasting, is effective in both young and old mice and is unaffected by pregnancy. A variety of micro-organisms are able to induce non-specific immunity against rodent babesiosis, and non-specific immunity also follows the injection of microbial extracts and the synthetic substance muramyl dipeptide (MDP) which is widely regarded as representing the smallest unit capable of replacing mycobacterial cell wall as an adjuvant [18]. These micro-organisms, extracts and synthetic substances have well established immunostimulatory roles particularly as adjuvants, but their potential as non-specific stimulators of immunity against parasitic diseases in their own right is only just beginning to be realized [18]. At present, none of these can be regarded as a realistic alternative to specific immunization. However, the fact that so many of them are effective justifies further investigation of their potential particularly as their effects are apparently not evaded by antigenic variation or other escape mechanisms evolved by parasites.

The present needs are threefold, to understand the mechanisms of non-specific immunity, to develop safe and effective alternatives to the crude micro-organisms and extracts now available and to evaluate the methods, potential and limitations of any possible immunization procedures particularly with respect to diseases of economic importance. Several suggestions as to the nature of the

mechanism of non-specific immunity in rodent babesiosis have already been made [13–15], but a final answer will probably only come from work on tumour immunology where the need for non-specific immunization is considerable.

In both rodent babesiosis and malaria, all the evidence suggests that non-specific immunity acts at the height of the first wave of parasitaemia causing a crisis during which parasites die within the red blood cells but this immunity is not complete and, after the crisis, parasites persist for a considerable time at a sub-patent or very low level.

The evidence presented in this study and elsewhere suggests that the primary target cells in the non-specific immune response are the rapidly dividing parasites within the red cells, and that a population of less rapidly dividing cells survives and slowly elicits a specific response that eventually eradicates the infection and prevents re-infection. That two populations of parasites evoking different kinds of immune responses may be involved is not difficult to understand and could result from switches in gene activity [20]. It follows, therefore, that immunity to intra-erythrocytic protozoa involves two mechanisms, one non-specific and one specific. The actual balance between the two varies from species to species, or even strain to strain, of host, and from species to species of parasite. The non-specific mechanism presumably involves the primary sensitization and the final triggering of the immune response. Primary sensitization may occur as a result of the natural progression of an infection or artificially as a result of prior exposure to an unrelated agent such as BCG. The final triggering is probably dependent on the actual parasitaemia experienced by the host. In the results presented in this study it appears that TO mice respond at a lower threshold than BSVS mice, and it is of interest to note that here the strain of host is more important than the species of parasite. Non-specific mechanisms also occur in *Plasmodium vinckei* but not in *P. berghei* infections in mice [21] so it is likely that, when considering all intra-erythrocytic infections, there will be a spectrum of immunity from almost wholly specific to almost wholly non-specific with every possible combination of intermediate states.

In equine and cattle babesiosis, little is known about the role of non-specific immunity. Prior exposure to BCG does not appear to protect the bovine against *B. divergens* [22], but this need not hold true for all species of *Babesia* or all breeds of cattle. The single most important indication of non-specific immunity is the intra-erythrocytic death of the parasites, and it is essential that this should be observed during natural recovery before an important role be assigned to non-specific immunity. Conversely, if intra-erythrocytic death is observed then non-specific immunization should be possible.

Immunization against babesiosis (and malaria) needs to be based on an appreciation of the respective roles of non-specific and specific immunity. Ideally, the non-specific immunity should be stimulated by a synthetic immunostimulant and the specific part by an attenuated strain of the parasite. There are several ways

of producing and isolating such strains including the use of irradiation techniques and the method described in this study and elaborated elsewhere [21]. The future role for non-specific immunization is likely to be its use as an alternative to long-lasting chemotherapeutic substances for the control of the initial parasitaemia and clinical disease while sterilizing specific immunity is allowed either to develop naturally or to develop after exposure to attenuated parasites. Non-specific immunization is not likely to be an alternative to specific immunization but an adjunct to it, and should not be considered in isolation. The possibility of developing a vaccine consisting of a specific antigen and an adjuvant that both enhances a specific immune response and separately stimulates a non-specific one is a very exciting prospect.

ACKNOWLEDGEMENTS

I should like to express my gratitude to L. Chedid of the Institut Pasteur, Paris, for providing me with *M. smegmatis* cell wall, cord factor and MDP, the Wellcome Laboratories for providing *C. parvum* cell wall and I. Pencea of the Cantacuzino Institute, Jassy, Romania, for providing me with samples of 'Polidin'.

REFERENCES

- [1] COX, F.E.G., YOUNG, A.S., *Parasitology* **59** (1969) 257.
- [2] COX, F.E.G., *Nature (London)* **219** (1968) 646.
- [3] COX, F.E.G., *Bull. World Health Organ.* **43** (1970) 325.
- [4] COX, F.E.G., *Parasitology* **65** (1972) 379.
- [5] COX, H.W., MILAR, R., *Am. J. Trop. Med. Hyg.* **17** (1968) 173.
- [6] BAFORT, J.M., TIMPERMAN, G., MOLYNEUX, D.H., *Ann. Soc. Belg. Med. Trop.* **50** (1970) 301.
- [7] COX, F.E.G., *Parasitology* **65** (1972) 389.
- [8] COX, F.E.G., *Symp. Soc. Exp. Biol.* **29** (1975) 429.
- [9] CLARK, I.A., ALLISON, A.C., COX, F.E.G., *Nature (London)* **259** (1976) 309.
- [10] CLARK, I.A., COX, F.E.G., ALLISON, A.C., *Parasitology* **74** (1977) 9.
- [11] HEROD, E., CLARK, I.A., ALLISON, A.C., *Clin. Exp. Immunol.* **31** (1978) 518.
- [12] COX, F.E.G., DANCZAK, J.C., MILLOTT, S.M. (in preparation).
- [13] CLARK, I.A., *Infect. Immun.* (in press).
- [14] CLARK, I.A., *Parasite Immunology* (in press).
- [15] CLARK, I.A., *Lancet* **ii** (1978) 75.
- [16] BRILES, D.E., KRAUSE, R.M., DAVIE, J.M., *Immunogenetics* **4** (1977) 381.
- [17] IRVIN, A.D., BROCKLESBY, D.W., *J. Parasitol.* **55** (1969) 1190.
- [18] CHEDID, L., "Therapeutic potential of immunoregulating synthetic compounds", *Immunity in Parasitic Diseases*, INSERM, Paris (1978) 249.
- [19] PENCEA, I., et al., *Arch. Roum. Pathol. Exp. Microbiol.* **23** (1964) 477.

- [20] BEALE, G.H., "Genetics of antigenic variation in *Paramecium*: a model system", Parasites in the Immunized Host: Mechanisms of Survival, Ciba Foundation Symposium 25 (1974) 21.
- [21] COX, F.E.G. (in preparation).
- [22] BROCKLESBY, D.W., PURNELL, R.E., Nature (London) 265 (1977) 343.

DISCUSSION

H.S. HUSSEIN: Is non-specific immunity against babesiosis applicable to bovine babesiosis? Brocklesby and Purnell recently published a paper in *Nature* in which they say they are unable to demonstrate this in the bovine host.

F.E.G. COX: Similar unsuccessful results have also been reported from groups working in Kenya. This does not necessarily mean that non-specific immunization will be impossible in all breeds of cattle or with all species of *Babesia*. It will be necessary to discover more about the nature of the non-specific mechanism and more about both the way in which the bovine host responds and the way in which the bovine parasites are affected before we can be definite about this.

R.E. PURNELL: I was a co-author with Mr. Brocklesby of the paper referred to by Mr. Hussein. We did find intra-erythrocytic death in naturally recovering animals (injected with *Babesia divergens*).

R.J. TERRY: Has any progress been made with the identification of soluble factors that are involved in non-specific immunity to *Babesia*?

F.E.G. COX: Progress is certainly being made but as yet no single factor has been identified. The mediator may be one of those involved in the non-specific response to tumours. If so, we shall probably have to wait until the tumour immunologists have identified and characterized it. On the other hand, it may be that a soluble mediator is not involved. At present it is probably safest to keep all alternatives open.

R.J. TERRY: In our experience, prior treatment with BCG had only a marginal effect on the course of an S42 *T. brucei* infection.

A.D. IRVIN: May I comment on the role of NK cells? I believe it is true to say that NK activity reaches a peak in *Babesia*-infected mice just before intra-erythrocytic death of parasites, that A-strain mice which cannot mount an NK response easily succumb to infection, and that removal of the spleen, the main reservoir of NK cells, greatly exacerbates infection.

F.E.G. COX: Yes, this is all true, but the evidence for the involvement of NK cells still remains circumstantial. There is a need to determine a correlation between NK cell activity and parasite killing throughout the life of the host. The actual role of the spleen also needs to be clarified.

A.D. IRVIN: I was interested to hear that you found male bacteria-susceptible, virus-susceptible (BSVS) mice much more susceptible to babesiosis than females. We have obtained similar results with Dh/+ mice in which higher levels of parasitaemia and more frequent deaths occurred in the males. Can you think of any reason why the males should be more susceptible?

F.E.G. COX: Males tend to be more susceptible to a variety of infections. The importance of this finding is that it should enable us to identify differences between male and female mice of the same strain and between males or females of different strains. At present there is no widely accepted explanation.

Max MURRAY: It would appear that sex plays a major role in the susceptibility of the host to a variety of parasitic infections including a range of helminth parasites, such as *N. brasiliensis*. In the same way, male adult mice are more susceptible than female mice to infection with African trypanosomes.

I would also like to make a comment on non-specific immunity: the susceptibility of mice to *T. congolense* can be significantly reduced by the use of a variety of immunostimulants before or on the day of challenge. Immunostimulant-treated mice are able to limit parasitaemia to a significantly greater extent than untreated mice and as a result they survive longer. It must be emphasized that protection has not been achieved, only reduced susceptibility.

M.J. MUTINGA: At what stage of malaria (*Plasmodium*) infection in animals were the infections with *Babesia* species induced? Were they induced simultaneously?

F.E.G. COX: In the heterologous immunity experiments (see References [3] and [4] in the paper), mice were infected, cured with an appropriate drug and challenged 4–36 weeks later.

I.R. TIZARD (*Chairman*): Do interferon inducers influence the course of *Babesia* infections and does interferon itself influence *Babesia*?

F.E.G. COX: There is no evidence of any significant effect of interferon inducers on *Babesia* infections. However, the nature of interferon and of the various types of interferon is being actively investigated in many laboratories, and it may be that the soluble mediator postulated is a kind of interferon. Tumour necrosis factor, for example, has been suggested by Clark as being a possible mediator of intracellular killing.

UPTAKE OF ^3H PURINES AS AN IN VITRO TEST TO MEASURE VIABILITY OF *Babesia* PARASITES FOLLOWING CHEMOTHERAPY, IRRADIATION AND OTHER TREATMENTS

A.D. IRVIN*, E.R. YOUNG, R.E. PURNELL
Agricultural Research Council,
Institute for Research on Animal Diseases,
Compton, Newbury,
Berks.,
United Kingdom

Abstract

UPTAKE OF ^3H PURINES AS AN IN VITRO TEST TO MEASURE VIABILITY OF
Babesia PARASITES FOLLOWING CHEMOTHERAPY, IRRADIATION AND OTHER
TREATMENTS.

Babesia rodhaini and *B. microti* from mice, and *B. divergens* and *B. major* from cattle, were maintained in Eagles minimal essential media to which different radioactive purines and pyrimidines were added. Parasites selectively incorporated several purines, particularly ^3H hypoxanthine, at high levels. Incorporation of ^3H hypoxanthine was directly related to the metabolic activity of parasites. Treatments which suppressed or abolished metabolic activity proportionately depressed ^3H hypoxanthine uptake. The treatments applied to parasitized cultures included drug therapy, irradiation, storage and growth in different media. It is suggested that these findings could form the basis for simple, rapid and inexpensive in vitro tests for drug screening of babesicides or assay of stored blood vaccines.

INTRODUCTION

Parasites of the genus *Babesia* infect most species of domestic animal as well as many wild species. Babesiosis is of particular economic importance in cattle, horses and dogs, and recent reports indicate that it may also be of zoonotic importance [1].

An understanding of the biochemistry of these parasites could have important implications in disease control, especially in drug development and therapy. In this respect we have studied the biosynthesis of nucleic acids by four species of *Babesia*: *B. divergens* and *B. major* of cattle, and *B. microti* and *B. rodhaini* of mice.

* Present address: International Laboratory for Research on Animal Diseases,
Nairobi, Kenya.

Studies on the synthesis of nucleic acids by intra-erythrocytic protozoa have been confined mainly to malaria parasites [2–10]. It appears that, in most cases, these parasites are unable to synthesize the purine ring and are therefore dependent on the host for a source of pre-formed purine [11]. This is in contrast to most cells in the body which can synthesize purines *de novo*.

Studies on the uptake of tritiated (^3H) nucleic acid precursors by *Babesia* parasites have revealed a similar dependence on pre-formed purines for nucleic acid synthesis [12–13]. These findings, together with their possible practical applications, are reviewed in this study.

MATERIALS AND METHODS

Full details are given elsewhere [12–14], but in general blood was taken from *Babesia*-infected animals, washed and counted. Duplicate aliquots of approximately 1×10^8 parasitized erythrocytes per millilitre were incubated in Eagles minimal essential medium (MEM) with 10% foetal bovine serum (FBS) in the presence of ^3H nucleic acid precursors (approximately $2 \mu\text{Ci/ml}$). The amount of precursor incorporated by infected blood was measured (usually after 24 hours) by liquid scintillation counting, and counts compared with those obtained from uninfected control blood samples treated identically. Various physical and chemical treatments were applied to the blood samples to study their effects on the ability of parasites to incorporate nucleic acid precursors.

RESULTS

Viability of cultured blood

B. rodhaini-infected blood was maintained in culture for 24 hours and then 1×10^7 parasitized erythrocytes inoculated into each of seven mice. A similar group of mice was inoculated on the same day each with 1×10^7 parasitized erythrocytes freshly collected from an infected donor mouse. There was no significant difference in the mean times to death in the two groups, indicating that the viability of parasites maintained in culture had not waned over 24 hours [12].

Uptake of different ^3H nucleic acid precursors by *Babesia*

Aliquots of infected and uninfected blood were maintained in culture in the presence of different nucleic acid precursors for 24 hours and then counted. The results expressed as counts for infected blood minus counts for control

TABLE I. THE UPTAKE OF ^3H PYRIMIDINES AND PURINES BY *Babesia*
 Figures represent the mean of duplicate counts per minute for infected blood
 minus counts per minute for uninfected blood

	Counts per minute ^a			
	<i>B. microti</i> (7%) ^b	<i>B. rodhaini</i> (20%)	<i>B. major</i> (23%)	<i>B. divergens</i> (22%)
Pyrimidines				
Thymine	ND	5	0	0
Thymidine	48	337	34	103
Uridine	0	984	31	625
Orotic acid	6	23	165	785
Purines				
Adenine	3 090	11 116	8 643	11 492
Adenosine	ND	2 408	7 326	1 863
Hypoxanthine	13 375	12 573	84 612	30 463

^a Counts per minute are adjusted to assume a specific activity of 5 Ci/mmol for all ^3H precursors.

^b % parasitaemia.

TABLE II. UPTAKE OF ^3H HYPOXANTHINE BY *Babesia*-INFECTED
 AND UNINFECTED BLOOD, BEFORE AND AFTER FILTRATION TO
 REMOVE LEUCOCYTES

Sample	Counts per minute		% difference
	Unfiltered	Filtered	
Control blood (C)	128	100	21.6
<i>Babesia</i> -infected (B)	8346	8183	2.0
B-C	8218	8083	1.6

blood are given in Table I. This shows that purines, particularly hypoxanthine, were readily incorporated by all four species of *Babesia*, whereas pyrimidines were not [12].

A subsequent experiment confirmed these findings and also showed that precursor incorporation was highest in the early stages of infection, indicating that nucleic acid synthesis was most active at this stage [13].

The effect of leucocytes on the uptake of ^3H hypoxanthine by *B. major*

B. major-infected and uninfected blood was collected from two cattle. Each sample was subdivided into two aliquots. One of each aliquot was filtered [15] to remove the leucocytes and the other was untreated. All aliquots were then maintained for 24 hours in culture in the presence of ^3H hypoxanthine. The amount of ^3H hypoxanthine taken up by the aliquots is shown in Table II. The removal of leucocytes from control blood resulted in a substantial fall in the amount of ^3H hypoxanthine incorporated. In the case of *Babesia*-infected blood this fall was insignificant when compared with the massive uptake of ^3H hypoxanthine by the parasites [13]. In subsequent experiments therefore no attempt was made to remove leucocytes from test samples.

Uptake of ^3H hypoxanthine by *B. microti* after different times in culture

B. microti-infected blood and uninfected control mouse blood were incubated in culture in the presence of ^3H hypoxanthine for different periods of time before measurement of ^3H hypoxanthine incorporation. The results are shown in Table III. Hypoxanthine became incorporated very rapidly by parasites, reached a peak at 12 hours and remained at this level for 24 hours [12]. The 24-hour incorporation of ^3H hypoxanthine was subsequently chosen as a convenient parameter for measuring metabolic activity of *Babesia*.

In subsequent experiments it was shown that parasites rapidly lost their ability to incorporate ^3H hypoxanthine in cultures maintained for more than 24 hours [13, 16].

Uptake of ^3H hypoxanthine by *B. major*-infected blood stored at 4°C for different times

B. major-infected blood and uninfected control bovine blood were collected as before and each divided into seven equal aliquots. These were stored at 4°C for different lengths of time and then cultured for 24 hours in the presence of ^3H hypoxanthine. The results are shown in Table IV. After seven days storage the ability of parasites to incorporate ^3H hypoxanthine was negligible. A steer injected with blood stored for 13 days failed to become infected [13].

TABLE III. UPTAKE OF ^3H HYPOXANTHINE BY *Babesia*-INFECTED BLOOD AFTER DIFFERENT PERIODS OF INCUBATION

Figures represent the mean of duplicate counts per minute for infected blood minus counts per minute for uninfected blood

Period of incubation	Uptake of ^3H hypoxanthine (counts/min)
1 min	13
5 min	84
10 min	133
30 min	401
1 hour	778
2 hours	1580
4 hours	1990
6 hours	2496
12 hours	4663
24 hours	4945

TABLE IV. UPTAKE OF ^3H HYPOXANTHINE BY *Babesia*-INFECTED BLOOD AFTER DIFFERENT TIMES OF STORAGE AT 4°C

Figures represent the mean of duplicate counts per minute for infected blood minus counts per minute for uninfected blood

Time of storage at 4°C (days)	Counts per minute	% uptake compared with day 0 sample
0	9 951	100
1	10 482	105.3
2	6 403	64.3
3	3 870	38.9
7	46	0.4
10	10	0.1
13	10	0.1

TABLE V. DIFFERENT TREATMENTS APPLIED TO IN VITRO CULTURES OF *Babesia*

Treatments given are arranged in descending order of efficacy

Experiment 1 Media	Experiment 2 Supplement	Experiment 3 Culture conditions
Eagle's MEM	Glutamine	Control
RPMI 1640	Control	Addition of fresh RBCs
Medium 199	Adenosine	Medium change + fresh RBCs
Dulbecco's	Asparagine	Medium change
BHK-21	Glucose	Roller tube
	Vitamin B12	Spleen feeder layer
	20% FBS	Stationary tube
	Bovine plasma albumin	
	Lactalbumin hydrolysate	
	Tryptose phosphate broth	

Uptake of ^3H hypoxanthine by *B. rodhaini* maintained in different media and under different culture conditions

In three separate experiments (Table V) *B. rodhaini*-infected blood and control blood were maintained in 25-cm² Falcon flasks containing:

- (1) different culture media
- (2) Eagles MEM plus 10% FBS with different supplements
- (3) Eagles MEM plus 10% FBS under different culture conditions.

The 24-hour incorporation of ^3H hypoxanthine by the parasites was measured after culturing for one, two and three days.

Eagles MEM supplemented with 10% FBS provided the best short-term maintenance medium for *Babesia*, as judged by the ability of the parasites to incorporate ^3H hypoxanthine. However, none of the media was able to support the metabolic activity of *Babesia* for more than 24 hours, since after this time there was a rapid decline in the ability of the parasites to incorporate ^3H hypoxanthine. None of the additives with the possible exception of glutamine resulted in improved parasite maintenance. Stationary 25-cm² Falcon flasks provided the best physical conditions for parasite maintenance over 24 hours.

The effect of drugs on the uptake of ^3H hypoxanthine by *Babesia* maintained in culture

Control blood and blood from animals infected with *B. rodhaini*, *B. divergens* and *B. major* were incubated for 24 hours in medium containing ^3H hypoxanthine and a variety of drugs. The effect of these drugs was assessed by the ability of the parasites to incorporate ^3H hypoxanthine. Any compound that reduced the uptake of ^3H hypoxanthine by 75% or more was considered to be active against the *Babesia* [14]. The results are shown in Table VI. Several purine analogues were active against *Babesia* in vitro, but when tested against *B. rodhaini* in vivo they showed no activity [17]. This contrasted with the babesicides that were active both in vitro [14] and in vivo [17]; the in vitro activity corresponding very closely to the different in vivo activities previously reported [18–21]. The anti-malarial drug, chloroquine, was not active against *Babesia* in vitro; this also agrees with in vivo findings [22].

The effects of irradiation on the uptake of ^3H hypoxanthine by *Babesia* maintained in culture

In a series of experiments blood infected with *B. rodhaini*, *B. major* and *B. divergens*, together with appropriate controls, was irradiated at different doses from 0 to 120 krad. After irradiation the blood was maintained in culture in the presence of ^3H hypoxanthine for 24 hours. The effects of irradiation were measured by the ability of the parasites to incorporate ^3H hypoxanthine. In the case of *B. rodhaini* the ability of the parasites to infect mice was also recorded [23]. Results are given in Table VII. With increasing doses of irradiation there was a progressive fall in the ability of *B. rodhaini* to incorporate ^3H hypoxanthine. There was also a similar reduction in the infectivity of irradiated blood for mice. *B. divergens* and *B. major* appeared slightly more radioresistant than *B. rodhaini*, but otherwise showed a similarly reduced ability to incorporate ^3H hypoxanthine as radiation dose increased. At about 40 krad ability to incorporate ^3H hypoxanthine had been virtually abolished in all three parasites [23].

Low doses of irradiation (<5 krad) sometimes resulted in increased ^3H hypoxanthine uptake by *Babesia* [23]. The reason for this was not determined.

DISCUSSION

The early experiments established a number of facts which were later exploited in developing an in vitro technique to measure metabolic activity of *Babesia* and the effects of different treatments on this activity. It was found that parasites retained their viability in culture for at least 24 hours, and that

TABLE VI. PERCENTAGE UPTAKE OF ^3H HYPOXANTHINE BY *Babesia* SUBJECTED TO DIFFERENT DRUG TREATMENTS IN VITRO

Drug and type	<i>B. rodhaini</i> (19%) ^a	<i>B. divergens</i> (18%)	<i>B. major</i> (9%)
Control	100	100	100
Purine			
Hypoxanthine	5.3	6.5	4.3
Purine analogues			
2-Mercaptopurine	—	8.7	16.2
6-Mercaptopurine	22.9	42.1	32.2
8-Azaguanine	90.1	68.1	81.0
6-Thioguanine	41.1	34.9	39.5
8-Azaadenine	60.8	84.1	—
Allopurinol	55.7	34.3	—
Pyrimidine analogues			
Fluorodeoxyuridine	82.1	102.4	—
Bromodeoxyuridine	73.4	122.7	—
Antifolate			
Aminopterin	86.5	120.8	72.1
Babesicides			
Trypan blue	—	36.7	67.7
Quinuronium SO_4	28.2	65.6	10.2
Imidocarb	14.6	13.5	—
Diminazene	39.5	72.1	9.0
Amicarbalide	10.1	20.8	21.1
Anti-malarial			
Chloroquine SO_4	—	81.0	113.4

^a Parasitaemia.

during this time they incorporated high levels of purines, particularly hypoxanthine. The presence of leucocytes did not affect the level of incorporation.

The rapid movement of purines across erythrocyte membranes is well documented [24, 25], and it now appears that *Babesia*, in common with *Plasmodium* [3, 4, 11, 26], are dependent on these pre-formed compounds for synthesis of nucleic acids.

TABLE VII. PERCENTAGE UPTAKE OF ^3H HYPOXANTHINE BY IRRADIATED *B. Rodhaini*, AND PERCENTAGE DEATH IN MICE INOCULATED WITH IRRADIATED BLOOD

Radiation dose (krad)	% uptake of ^3H hypoxanthine	% of mice killed on 1 $^{\circ}$ inoculation
0	100	100
5	65.6	100
10	40.8	96
15	26.5	80
20	18.0	38
25	12.4	10
30	8.3	15
35	5.6	5
40	4.2	0

The failure of parasites to incorporate significant amounts of pre-formed pyrimidines probably relates to the relative inability of these compounds to cross the erythrocyte membrane [25, 27].

The ability of parasites to incorporate ^3H hypoxanthine appeared to be directly related to their metabolic activity, and this in turn could be closely correlated with parasite viability. This finding was exploited in the experiments that examined the effects of refrigeration, culture conditions, drug treatment and irradiation on *Babesia* in vitro. In all cases ^3H hypoxanthine uptake provided a reliable measure of the effect of these different treatments on *Babesia*, which could be closely correlated with similar in vivo studies. Based on these findings it is suggested therefore that a test similar to that used for *Plasmodium* [28] could have valuable application in in vitro screening of potential babesicides. Such a test would have a number of advantages over current in vivo screening methods, particularly in terms of rapidity, cost and the fact that it is species specific. Also viability of parasites in potential blood vaccines could be conveniently measured in vitro in both refrigerated material [29] and in irradiated blood [30].

ACKNOWLEDGEMENTS

We would like to thank the following for their advice, encouragement or technical assistance during various stages of the work: Professor D.W. Brocklesby, T.A. Connors, D.L. Hughes, M. Corp, P.J.V. Adams, P.D. Luther and C. Mallinson.

REFERENCES

- [1] RISTIC, M., LEWIS, G.E., *Parasitic Protozoa IV* (KREIER, J.P., Ed.), Academic Press, London (1977) 53.
- [2] BUNGENER, W., NIELSON, G., *Z. Tropenmed. Parasitol.* 18 (1967) 456.
- [3] BUNGENER, W., NIELSON, G., *Z. Tropenmed. Parasitol.* 19 (1968) 185.
- [4] VAN DYKE, K., *Z. Tropenmed. Parasitol.* 26 (1975) 232.
- [5] VAN DYKE, K., TREMBLAY, G.C., LANTZ, C.H., SZUSTKIEWICZ, C., *Am. J. Trop. Med. Hyg.* 19 (1970) 202.
- [6] POLET, H., BARR, C.F., *Am. J. Trop. Med. Hyg.* 17 (1968) 672.
- [7] GUTTERIDGE, W.E., TRIGG, P.I., *J. Protozool.* 17 (1970) 89.
- [8] SCHMIDT, G., WALTER, R.D., KONIGK, E., *Z. Tropenmed. Parasitol.* 25 (1974) 301.
- [9] WALTER, R.D., KONIGK, E., *Z. Tropenmed. Parasitol.* 25 (1974) 227.
- [10] WALSH, C.J., SHERMAN, I.W., *J. Protozool.* 15 (1968) 185.
- [11] GUTTERIDGE, W.E., COOMBS, G.H., *Biochemistry of Parasitic Protozoa*, Macmillan, London (1977).
- [12] IRVIN, A.D., YOUNG, E.R., PURNELL, R.E., *Int. J. Parasitol.* 8 (1978) 19.
- [13] IRVIN, A.D., YOUNG, E.R., *Int. J. Parasitol.* (in press).
- [14] IRVIN, A.D., YOUNG, E.R., *Nature (London)* 269 (1977) 407.
- [15] BAGGALEY, C.V., ATKINSON, E.M., *Trans. R. Soc. Trop. Med. Hyg.* 66 (1972) 4.
- [16] IRVIN, A.D., YOUNG, E.R., LUTHER, P.D. (submitted for publication).
- [17] IRVIN, A.D., YOUNG, E.R., *Res. Vet. Sci.* 25 (1978) 211.
- [18] BEVERIDGE, E., *Res. Vet. Sci.* 10 (1969) 534.
- [19] JOYNER, L.P., BROCKLESBY, D.W., *Adv. Pharmacol. Chemother.* 11 (1970) 321.
- [20] JOYNER, L.P., DAVIES, S.F.M., KENDALL, S.B., *Exp. Chemother.* 1 (1963) 603.
- [21] ZWART, D., VAN DEN ENDE, M.C., KOUWENHOVEN, B., BUYS, J., *Tijdschr. Diergeneesk.* 93 (1968) 126.
- [22] TAYLOR, A.E.R., TERRY, R.J., GODFREY, D.G., *Br. J. Pharmacol.* 11 (1956) 71.
- [23] IRVIN, A.D., YOUNG, E.R., ADAMS, P.J.V., *Res. Vet. Sci.* (in press).
- [24] MAGER, J., HERSHKO, A., ZEITLIN-BECK, R., SHOSHANI, T., RAZIN, A., *Biochim. Biophys. Acta.* 149 (1967) 50.
- [25] BERLIN, R.D., OLIVER, J.M., *Int. Rev. Cytol.* 42 (1975) 286.
- [26] OELSHLEGEL, F.J., BREWER, G.J., *The Red Blood Cell II* (SURGENOR, D.M., Ed.), Academic Press, London (1975) 1263.
- [27] LIEU, T.S., HUDSON, R.A., BROWN, R.K., WHITE, B.C., *Biochim. Biophys. Acta.* 241 (1971) 884.
- [28] VAN DYKE, K., SZUSTKIEWICZ, C., CENEDELLA, R., SAXE, L.H., *Chemotherapy* 15 (1970) 177.
- [29] CALLOW, L.L., MELLORS, L.T., *Aust. Vet. J.* 42 (1966) 464.
- [30] PURNELL, R.E., BROCKLESBY, D.W., STARK, A.J., *Res. Vet. Sci.* (in press).

DISCUSSION

R.S. PHILLIPS: I noticed that in your time course experiments the uptake of ^3H hypoxanthine was the same after 12-hour and 24-hour culture. Would you like to comment on this in relation to (1) the time between divisions of the

Babesia and (2) the condition of the parasites in your culture after 24 hours compared with their condition after 12 hours?

A.D. IRVIN: It is very difficult to comment usefully on the time between divisions in our system. I think this could be done if we were able to work with synchronized parasites and if we knew when the parasites synthesized their nucleic acids. At the moment, we cannot synchronize *Babesia* and we know virtually nothing about the parasite's reproductive cycle in relation to the synthesis of nucleic acids. Parasites maintained for 24 hours in culture retained their normal morphology and the same infectivity as that of freshly collected parasites (see Experiment 1 described in Table V).

C. MELLA LIZAMA: When you carried out the tritiated-base incorporation experiments, did you take into account the availability of adenosine triphosphate (ATP)?

A.D. IRVIN: We did not, but I should stress we were not trying to develop or to investigate culture systems as such but rather the use of a simple system for measuring parasite viability following various treatments in vitro.

H.S. HUSSEIN: Is the inability of *Babesia* to incorporate purines following irradiation due to radiation-induced death or mutation among the parasites?

A.D. IRVIN: I feel sure that the inability to incorporate purines following irradiation is due to the death of the parasites. However, what do we mean by the term death? One definition applicable in the case of the radiation doses we used would be inability of the parasites to synthesize nucleic acids for replication. This would of course mean inability to incorporate purines. If mutations had been induced there need be no reason why nucleic acid synthesis, and hence purine incorporation, should be inhibited. Only at very low doses would one expect mutations to occur; the doses needed to inhibit purine incorporation in our work were very much greater than potential mutagenic doses.

TRYPANOSOMIASIS AND LEISHMANIASIS
(Session 3)

PATHOPHYSIOLOGY OF TRYPANOSOMIASIS IN THE BOVINE

J.D. DARGIE*

Department of Veterinary Physiology,
Glasgow University Veterinary School,
Glasgow,
United Kingdom

Abstract

PATHOPHYSIOLOGY OF TRYPANOSOMIASIS IN THE BOVINE.

Results are presented of a comparative study of the pathogenesis of the anaemia, hypoalbuminaemia and bodyweight changes occurring in N'dama and Zebu cattle infected with *Trypanosoma congolense*. The kinetics of the anaemia were examined through measurements of plasma and red cell volumes and rates of haemoglobin breakdown and synthesis, and measurements of albumin pools and catabolic rate provided an insight into the cause of the hypoalbuminaemia. The possible impact of reduced feed intake on both features, as well as on the pathogenesis of the accompanying changes in bodyweight, was assessed indirectly through measurements of water turnover.

INTRODUCTION

Trypanosomiasis is still one of the most serious obstacles to livestock development in Africa and, despite much research, there remain large gaps in our knowledge of the basic mechanisms involved in the disease. In particular, there is still considerable doubt about the cause of the three major clinical signs — anaemia, hypoalbuminaemia and weight loss or impaired growth [1]. There does, however, seem to be general agreement on the existence of trypanotolerant livestock in many areas of West and Central Africa [2] but, again, the basis of this tolerance and the economic benefits it imparts are largely unknown. Radioisotopic tracer techniques when combined with more conventional procedures offer the opportunity of obtaining some of this information. This report is an attempt to summarize the findings of a study conducted in the Gambia with previously trypanosome-free N'dama and Zebu cattle which were artificially infected with the same number of *T. congolense* (3×10^6 each) and maintained under natural grazing conditions. The experimental protocol and techniques employed to make the measurements are given elsewhere [3], and full details of the mechanisms involved in producing many of the changes observed are reviewed by Murray [4], and Murray and co-workers [5].

* Present address: Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, International Atomic Energy Agency, Vienna.

TABLE 1. BLOOD PARASITAEMIAS AND PCV VALUES OF N'DAMA AND ZEBU CATTLE INFECTED WITH *Trypanosoma congolense*

Breed	Time to 1st parasitaemic peak (days)	First peak (log ₁₀ tryps/ml)	Pre-infection	Weeks post-infection						
				4	6	8	10	12	14	
N'dama	13	4.0	PCV (%)	28	26	28	28	28.5	28.5	28.5
			Parasitaemia	3.3	1.7	0.5	0.2	0	0	0
Zebu	10	4.8	PCV (%)	21	20	21	22	22.5	22.5	22.5
			Parasitaemia	4.4	3.7	1.5	0.9	2.5	2.5	3.0

ANAEMIA OF TRYPANOSOMIASIS

Development of anaemia and its relationship to parasitaemia

The information given in Table I illustrates the average blood parasitaemias of the cattle and the corresponding changes in their PCV values. Infections became patent in animals of both breeds, and at about the same time (i.e. day 4), but in the N'dama peak parasitaemias were significantly lower and attained later, fell sharply between the 4th and 7th weeks, and after week 11 no parasites were detected. By contrast, the levels of parasitaemia in the Zebu remained elevated until week 6, declined progressively until about week 11 and then increased again. In association with the first parasitaemic peak, the PCVs of both groups deteriorated rapidly, but this was much more pronounced in the Zebu. During this early stage there was therefore a reasonably close relationship between the onset and severity of the anaemia and the appearance, level and duration of the parasitaemia. Later, however, this was not so, because even when parasites were apparently absent from the blood, the PCVs of the N'dama showed little tendency to recover; conversely, the second peak of parasitaemia in the Zebu did not produce any further fall in PCV although this was little less than the first.

Blood volumes and capillary permeability

Independent measurements of plasma and circulating red cell volumes on the 7th week of the study showed that the plasma volumes of both infected groups were significantly higher, and their circulating red cell volumes significantly lower than those of the controls (Table II). Both changes were more marked in the Zebu, but in neither breed was the total blood volume altered by infection. This indicates that the expansion of plasma volume was simply a normal homeostatic response for maintaining the blood volume and pressure in the face of the drop in red cell mass. Consequently, haemodilution which has been suggested as an important cause of anaemia [6] was not observed in these animals – at least at this time. Also shown in Table II is an assessment of the transcapillary exchange rate of albumin. This was essentially the same in all animals, indicating that the high plasma volumes recorded in the infected cattle were not simply a reflection of increased leakage of labelled albumin from the circulation during the period of measurement.

Haemoglobin breakdown and synthesis

To determine the cause of the lower red cell volumes in the infected cattle, their erythrokinetics were compared with those of the controls between the 7th and 15th weeks of the study. The results show several features of interest

TABLE II. BLOOD COMPARTMENT VOLUMES AND TRANSCAPILLARY EXCHANGE OF ALBUMIN IN NORMAL CATTLE AND CATTLE INFECTED WITH *Trypanosoma congolense* (Mean $\pm$ SD)

Breed	Group	Plasma ^a (ml/kg)	Red cells (ml/kg)	Blood ^b (ml/kg)	TER (alb) ^c (%/hour)
N'dama	Infected	46.9 $\pm$ 5.6	15.6 $\pm$ 2.1	62.5 $\pm$ 5.6	3.97
	Control	38.8 $\pm$ 3.4	21.4 $\pm$ 3.0	60.2 $\pm$ 3.4	4.12
	p	<0.02	<0.01	N.S.	N.S.
Zebu	Infected	48.7 $\pm$ 5.3	10.9 $\pm$ 2.9	59.6 $\pm$ 7.7	3.41
	Control	34.4 $\pm$ 2.0	20.8 $\pm$ 4.2	55.2 $\pm$ 4.4	3.21
	p	<0.002	<0.002	N.S.	N.S.

^a Measured with ¹²⁵I-albumin.

^b Calculated as sum of plasma volume measured with labelled albumin, and red cell volume measured with ⁵¹Cr-labelled red cells.

^c TER (alb) — transcapillary exchange of albumin.

TABLE III. ERYTHROKINETICS OF NORMAL CATTLE AND CATTLE INFECTED WITH *T. congolense* (Mean $\pm$ SD)

Breed	Group	Red cell lifespan (days)	Red cell iron utilization (mg/kg daily)	⁵⁹ Fe red cell half-life (days)
N'dama	Infected	34.3 $\pm$ 3.8	0.66 $\pm$ 0.08	86.3 $\pm$ 8.4
	Control	86.2 $\pm$ 15.9	0.33 $\pm$ 0.06	198 $\pm$ 19.9
	p	<0.01	<0.05	<0.001
Zebu	Infected	17.8 $\pm$ 1.8	0.80 $\pm$ 0.03	61.9 $\pm$ 6.0
	Control	72.7 $\pm$ 6.4	0.32 $\pm$ 0.03	223 $\pm$ 20.0
	p	<0.001	<0.001	<0.001

(Table III). Firstly, in both infected groups, but particularly in the Zebu, the average red cell lifespan was greatly shortened, indicative of accelerated rates of erythrocyte breakdown. In fact, it could be calculated that these rates corresponded to the removal of about 38 g haemoglobin/day in the N'dama and 56 g/day in the Zebu compared with only 26 g/day in both groups of controls. Secondly, judging by their rates of red cell iron incorporation, infected animals also synthesized more haemoglobin than the controls, i.e. 42, 58 and 28 g/day respectively. In effect, animals within all groups produced slightly more haemoglobin than was degraded, a finding consistent with the slight increases in PCV observed over this period. However, it should be emphasized that while this was the general type of response observed, in two of the infected Zebu there was clear-cut suppression of erythropoiesis. These animals were hypoferraemic and had very low total iron-binding capacities and red cell iron utilization rates (i.e. about 0.45 mg/kg daily). Both died before the termination of the study with PCVs of 9–12%. Some insight into why erythropoiesis was so low in these animals, and why in general the erythropoietic response was not particularly marked despite the low PCVs of the infected cattle, can be gained by examining the changes in red cell radioactivity with time (Table III). In all infected cattle, but again particularly in the Zebu, red cell activity fell very sharply, indicating that much of the iron from degraded red cells was not available for further haemoglobin synthesis.

TABLE IV. ALBUMIN METABOLISM OF NORMAL AND *T. congolense*-INFECTED CATTLE

Breed	Group	Serum albumin (g %)	CA (g/kg)	EA (g/kg)	K ⁺	Albumin catabolized (g/kg daily)
N'dama	Infected	1.47 ± 0.11	0.69 ± 0.06	1.01 ± 0.11	0.065 ± 0.003	0.045 ± 0.005
	Control	2.08 ± 0.14	0.81 ± 0.02	1.21 ± 0.05	0.073 ± 0.005	0.059 ± 0.003
	p	<0.02	N.S.	N.S.	N.S.	N.S.
Zebu	Infected	1.54 ± 0.12	0.75 ± 0.08	1.12 ± 0.14	0.066 ± 0.004	0.049 ± 0.004
	Control	2.73 ± 0.05	0.95 ± 0.04	1.84 ± 0.37	0.092 ± 0.011	0.087 ± 0.013
	p	<0.001	N.S.	<0.05	<0.005	<0.01

K⁺ = Fraction of the intravascular albumin pool catabolized daily.

Although the kinetics of the early drop in PCV values was not examined directly, it can be calculated that even without any increase in erythropoiesis, the daily rates of haemoglobin breakdown which would have been necessary to produce anaemias of the severity recorded in the infected N'dama and Zebu were about 65 g and 104 g respectively, i.e. about 1.75 times greater than later. If the ability of the bone marrow to increase its output of red cells during this period also increased, then the amounts involved would clearly have been correspondingly greater.

Collectively, these results provide a framework for defining the development of anaemia in trypanosome-infected cattle. They suggest that shortly after infection when the animal shows high parasitaemia, there is a greatly accelerated rate of haemoglobin breakdown that is not compensated by a corresponding increase in erythropoiesis because the synthetic machinery is slow to respond. Consequently large amounts of haemoglobin are lost from the circulation without significant replacement and the animal becomes severely anaemic. The extent of this anaemia is primarily dependent upon the rate of red cell destruction and this in turn on the level of parasitaemia. Later, as the levels of parasitaemia decline, rates of haemoglobin degradation also fall, and this combined with increased synthesis allows the PCV values to stabilize. It should be stressed, however, that even when parasites are not detectable in the blood, haemoglobin breakdown remains higher than normal, suggesting that after the initial parasitaemic peaks, trypanosomes may not be necessary for red cell destruction; rather this could be due to a continuously active mononuclear phagocytic system. Also, following the initial waves of parasitaemia, subsequent peaks may not necessarily cause any further drop in PCV. Presumably this is because the erythropoietic system has become geared to a higher rate of red cell synthesis and this in turn is capable, for a time at least, of counteracting any increase in red cell destruction. Finally, one of the crucial consequences of trypanosomiasis is the presence of haemosiderosis, i.e. large amounts of iron from degraded erythrocytes are 'locked up' in the form of iron storage compounds. There is evidence from the work presented here that in severe or long-standing infections, the quantities involved become sufficient to impair the erythropoietic response and lead to the development of an iron-deficiency type of anaemia. Under these circumstances it can be readily envisaged that even where parasitaemias and hence rates of haemoglobin degradation are low, animals will nevertheless become progressively anaemic and will not respond to trypanocidal therapy.

HYPOALBUMINAEMIA

In Table IV the results are presented of a study of albumin kinetics 7–15 weeks after infection of N'dama and Zebu cattle. Both groups were hypoalbuminaemic,

and had markedly lower albumin reserves than their corresponding controls. In general, the extravascular pool (EA) was depleted to a greater extent than the intravascular pool (CA), and consequently EA:CA ratios were abnormally low. However, in none of the infected cattle were these changes associated with increased albumin catabolism, at least after the 7th week. In fact, this was lower than in the corresponding control groups, and in the case of the Zebu, the differences were significant. It would therefore appear that the hypoalbuminaemia of this disease is primarily a reflection of reduced albumin synthesis, and the expansion of plasma volume which attends the development of anaemia. Although it was not possible to measure albumin synthesis directly, there is good evidence that this was reduced as a result of protein deficiency. In the first place, hypocatabolism is an acknowledged feature of adaptation to protein depletion [7], and its occurrence in the present series of trypanosome-infected cattle is therefore strongly suggestive of inappetence and consequential reduction in crude protein intake. Secondly, although it was also not possible to measure feed intake directly, the infected cattle had markedly lower water turnover rates than their corresponding controls (i.e. 360 compared with 446 ml/kg^{0.82} in the N'dama and 295 compared with 471 ml/kg^{0.82} in the Zebu). This is further indirect evidence of reduced voluntary feed intake, particularly in the Zebu animals, and hence that albumin synthesis was suppressed by inappetence.

WEIGHT CHANGES

The reason for the weight loss or poor weight gain associated with trypanosomiasis is still largely a matter of conjecture. In this study, there were large pre-infection differences in bodyweight between the Zebu and N'dama animals, the former as a group being about 24 kg heavier. However, during the first seven weeks of infection, i.e. coincident with the initial waves of parasitaemia, both groups lost weight and the Zebu more so than the N'dama. Subsequently, the weights of the N'dama increased although at a slower rate than those of their uninfected counterparts (i.e. 1.4 kg/week and 2.4 kg/week respectively; $p < 0.05$), whereas those of the infected Zebu remained virtually static compared with control animals of this breed (i.e. 0.25 kg/week compared with 2.0 kg/week; $p < 0.02$). The greater impact of the disease on the Zebu cattle is further demonstrated by the fact that the initial weight advantage held over the N'dama was reduced to only 12 kg by the end of 15 weeks.

The major factors causing weight loss or poor growth in parasitized animals have recently been reviewed [8], and it would appear from this study that in many respects the pathogenesis of the phenomenon is very similar to that associated with helminth infections, i.e. reduced feed intake, and preferential utilization of available amino acids and energy for immunoglobulin and haemoglobin synthesis,

at the expense of muscle protein anabolism. However, as yet there is a conspicuous lack of definitive data to support this hypothesis, and clearly this is an area worthy of further and more detailed study. What does seem to be clear is the existence of genetically determined variations in the responses of different breeds of cattle to pathogenic trypanosomes, that these appear to operate at the level of parasite establishment, and that this in turn determines the severity and ultimate outcome of the host-parasite relationship.

REFERENCES

- [1] FIENNES, R.N.T.-W., "Pathogenesis and pathology of animal trypanosomiasis", *The African Trypanosomiasis* (MULLIGAN, H.W., Ed.), George Allen and Unwin, London (1970) 729.
- [2] INTERNATIONAL LIVESTOCK CENTRE FOR AFRICA, 'Trypanotolerant Livestock in West and Central Africa 1: General Studies, International Livestock Centre for Africa, Addis Ababa, Ethiopia (1979).
- [3] DARGIE, J.D., MURRAY, P.K., MURRAY, Max, GRIMSHAW, W.R.T., MCINTYRE, W.I.M., Bovine trypanosomiasis: the red cell kinetics of Ndama and Zebu cattle infected with *Trypanosoma congolense*, *Parasitology* 78 (1979) 271.
- [4] MURRAY, Max, "The pathology of African trypanosomiasis", *Progress in Immunology* (BRENT, L., HOLBOROW, J., Eds), North Holland Publishing Company, Amsterdam and London (1974) 181.
- [5] MURRAY Max, MORRISON, W.I., EMERY, D.L., AKOL, G.W.O., MASAKE, R.A., MOLOO, S.K., "Pathogenesis of trypanosome infection in cattle", these Proceedings, IAEA-SM-240/19.
- [6] HOLMES, P.H., "Use of radioisotopic tracer techniques in the study of the pathogenesis of trypanosomiasis", *Nuclear Techniques in Animal Production and Health* (Proc. Symp. Vienna, 1976), IAEA, Vienna (1976) 463.
- [7] DARGIE, J.D., BERRY, C.I., The hypoalbuminaemia of ovine fascioliasis: the influence of protein intake on the albumin metabolism of infected and of pair-fed control sheep, *Int. J. Parasitol.* 9 (1979) 17.
- [8] DARGIE, J.D., "The pathophysiological effects of gastrointestinal and liver parasites in sheep", *Digestion and Metabolism in the Ruminant* (RUCKEBUSCH, Y., THIVEND, P., Eds) (in press).

DISCUSSION

R.E. PURNELL: Were all the animals grazed in an area in which they are normally found? I am concerned about the possibility of stress (particularly nutritional stress) influencing the comparative results.

J.D. DARGIE (*Scientific Secretary*): No, the Zebu were purchased in Senegal. They were, however, allowed to graze the experimental area for several months

before the study began and were performing very satisfactorily in terms of growth. I therefore doubt whether they were under any more stress than the N'dama prior to infection.

Max MURRAY: The Zebu used in these studies were purchased from an area where conditions were similar to those found on the ranch where the experiment was carried out.

F.E.G. COX (*Chairman*): Were the N'dama cattle you used from an area of endemic trypanosomiasis? Is it possible that such cattle continuously subjected to trypanosomiasis challenge are at an advantage from the point of view of natural selection and might be different from cattle never exposed to such challenge?

J.D. DARGIE (*Scientific Secretary*): Anything is possible. Trypanosomiasis is endemic in many parts of the Gambia and consequently there's always a chance that the N'dama had been infected earlier. We did, however, select (in so far as is possible in this sort of situation) animals reared in areas considered tsetse-free. These animals were screened by a variety of parasitological and immunological methods for the presence of trypanosomes (or earlier infection) and in all cases were found to be negative. The Zebu were subjected to the same vigorous examination. We therefore feel that we were dealing with primary infections in both breeds.

Max MURRAY: There is circumstantial epidemiological evidence that previous exposure may reduce the susceptibility of cattle to trypanosomiasis. However, both the N'dama and the Zebu were obtained from tsetse-free areas.

M.E. LEEGWATER-VAN DER LINDEN: You say that the imported Zebu animals were not under stress. However, different grasses and shrubs can have different nutritional values. Was the diet of the Zebu in the experiment different from that in the place from which they were imported, and could this cause the animals to be under stress?

J.D. DARGIE (*Scientific Secretary*): I don't know anything about the botanical make-up of the pastures in Senegal and Gambia, but I doubt whether they differed significantly and, for the reasons given earlier, I can't see that there was any difference between the types or degrees of stress to which the Zebu and N'dama were subjected.

D.H. MOLYNEUX: Of all cattle in West Africa, Zebu carry out the most extensive transhumance. They are therefore very likely to be capable of eating most of the grasses available, so I don't think that this factor would be important in your work.

J. VERCOE: It is most unlikely that there was any difference in the relative nutritional 'status' of the Zebu from Senegal and the N'dama from Gambia. Mr. Dargie has emphasized that the animals were grazing together and had been acclimatized for a time before the experimental measurements were made; it is difficult to do more than this in a field experiment. There can be little doubt that the results presented by Mr. Dargie are due to genetic differences in susceptibility

to trypanosome infection. However, more information is certainly needed on the manner in which the interaction between genotype and nutrition influences the pathogenicity of the parasite.

F.E.G. COX (*Chairman*): This study is very important because it represents an attempt to quantify differences between N'dama and Zebu cattle. The evidence on which trypanotolerance as a concept is based is inadequate, by and large, and this work must be regarded as establishing base lines rather than as a definitive and final explanation of the phenomenon. Much more work with cattle will be required, however.

J.D. DARGIE (*Scientific Secretary*): I couldn't agree more. I'm sure trypanotolerance is part of a 'package deal' and will be influenced by a number of environmental variables. I would like to see this kind of work broadened to include studies on the relative heat and water tolerances of the breeds concerned as well as the influence of nutrition on bovine and ovine trypanosomiasis.

SECONDARY IMMUNODEFICIENCIES ASSOCIATED WITH AFRICAN TRYPANOSOMIASIS

R.J. TERRY, K.M. HUDSON,
M. FAGHIHI SHIRAZI, D. MAY
School of Biological Sciences,
Brunel University,
Uxbridge,
Middlesex,
England

Abstract

SECONDARY IMMUNODEFICIENCIES ASSOCIATED WITH AFRICAN TRYPANOSOMIASIS.

Infection with various species of African trypanosomes have been shown to depress immune responses in experimental animals, in domestic cattle and in man. The mechanisms underlying this immunodepression are currently the subject of much research, and are now known to be diverse and complex. Mechanisms positively identified are: (1) the polyclonal activation of B-lymphocytes, leading eventually to clonal exhaustion, and (2) the excessive stimulation of cells with suppressor properties. Other putative mechanisms under investigation include the development of auto-antibodies cytotoxic for autologous lymphocytes, the effects of polyunsaturated fatty acids and the activation of complement. All these investigations rely heavily on the use of radioisotopes and/or whole-body ionizing irradiation.

INTRODUCTION

During the last decade there have been many reports which suggest that parasitic infections induce secondary immunodeficiencies (1). Because of the high burden of parasitism in tropical developing countries, there is concern that parasites may reduce the effectiveness of current immunization programmes, as well as increasing susceptibility to secondary infections (2). It has also been speculated that the induced state of immunodeficiency may extend to responses against the homologous parasite as well as unrelated antigens (1, 3). Thus, a parasite, which could at least partially depress immune responses aimed at itself, might avoid immune rejection by the host, and set up a chronic infection.

The secondary immunodeficiency induced by African trypanosomes particularly those of the brucei group is especially striking and has been demonstrated in experimental

animals (1), in cattle (4) and in men (5). The full significance of this condition remains to be determined, but it is well recognized that trypanosome-infected cattle often succumb to secondary infections, and it is reported that vaccines are less effective in infected animals (4).

Not surprisingly, there have been a number of studies designed to elucidate the mechanisms underlying the immunodeficiency state. It is argued that if we understood these mechanisms we might be able to inhibit or reverse them. It is the primary purpose of this review to explore putative mechanisms; to attempt to assess their contributions to the overall immunodeficiency; and to consider the possibilities of intervention.

MECHANISMS UNDERLYING TRYPANOSOME-INDUCED SECONDARY IMMUNODEFICIENCY

1. Polyclonal B-cell activation and clonal exhaustion

It is something of a paradox that at a time when antibody responses are severely depressed, trypanosome-infected hosts have high levels of immunoglobulins, particularly IgM (6). Much of this increased immunoglobulin appears not to be parasite specific, but rather multi-specific (7,8). These observations have led Hudson and co-workers (3) to suggest that immunodepression and hyperimmunoglobulinaemia are linked phenomena, both brought about by polyclonal B-cell activation. Using a haemolytic plaque cell assay it was demonstrated that T.brucei-infected mice showed much reduced plaque-forming cell (PFC) responses to injections of sheep cells. Nevertheless, these mice showed elevated "background" PFC responses (in the absence of antigen) not only to sheep cells but also to a variety of unrelated antigens. Thus polyclonal B-cell activation undoubtedly occurs and explains the high levels of multi-specific immunoglobulins. But it was further argued that B-cell commitment, in a polyclonal fashion, would eventually lead to clonal exhaustion, and that this would make a major contribution to the immunodeficiency.

This hypothesis gains support from the experiments of Corsini and co-workers (9) who studied the *in vitro* synthesis of immunoglobulins by spleen cells from T.brucei-infected mice. Immunoglobulin synthesis, as measured by the incorporation of ³H-labelled leucine, was markedly

increased early in infection but thereafter rapidly declined, even when stimulated with the B cell mitogen lipopolysaccharide (LPS). Thus the in vivo and in vitro observations accord well and suggest that intense B cell activation is followed by B-cell exhaustion.

A search has been made in several laboratories for a putative trypanosome-associated polyclonal B-cell activator. Investigations using the in vitro incorporation of ^3H -thymidine into lymphocyte DNA have shown weak mitogenic activity in soluble extracts of trypanosomes (10, 11, 12). More recently, a stronger in vivo effect has been demonstrated to occur when insoluble trypanosome membrane fractions are injected into normal mice (13).

2. Polyclonal B-cell activation and the induction of auto-antibodies to lymphocytes

There have been a number of reports of the presence of autoantibodies in trypanosome infections (8, 14); these findings accord well with the hypothesis that trypanosomes provide a stimulus for polyclonal B-cell activation. It has been argued that numerous self-reactive B-cell clones are present in the lymphon, but that these are not normally triggered, perhaps because of the absence of helper T-cells, or perhaps because of active T-cell suppression. Direct activation of B-cells might bypass the requirement for helper T-cells, or overcome suppression, and autoantibodies would be produced.

Our interest in this area was stimulated by a report that intravenous injection of the well-recognized polyclonal B-cell activator LPS induced, in mice, autoantibodies which reacted with syngeneic spleen cells (15). We have sought for and found similar autoantibodies in mice infected with T.brucei. These antibodies, which appear 20-25 days after infection, are assayed in cytotoxic tests using ^{51}Cr -labelled spleen cells as targets in the presence of rabbit complement (16). Work is now proceeding to determine the class of antibodies involved and the exact nature of the target antigens.

This observation is significant in that it affords another parallel between the action of LPS, a well-recognized polyclonal B-cell activator, and the effects of trypanosome infections. It may also relate to the immunopathology of these infections. As these autoantibodies

do not appear until relatively late in infection, it is unlikely that they contribute much to the generalized immunodepression which is apparent 6-7 days after infection (3). Nevertheless, if these autoantibodies are active in vivo they may contribute to the marked lymphoid depletion which characterizes the late stages of trypanosome infections (17, 18).

3. Stimulation of non-specific suppressor T-cells

It is now well recognized that both antibody-mediated immunity and cell-mediated immunity is subjected to complex control by T-lymphocytes. For example, production of many antibodies may be sequentially helped by specific helper T-cells and then turned off by specific suppressor T-cells. Helper and suppressor cells may be recognized by them having a different distribution of the Ly series of surface antigens; thus specific helper cells are $Ly\ 1^+, 2^- 3^-$ whereas specific suppressor cells are $Ly\ 1^-, 2^+ 3^+$. There also appears to be a population of T-cells which suppresses responses non-specifically rather than specifically. These cells share some of the characteristics of immature T-cells and have the phenotype $Ly\ 1^+ 2^+ 3^+$.

Because the immunodepression associated with trypanosomiasis is known to affect responses to many different antigens (1) it has been suggested that non-specific suppressor T-cell activity is exaggerated in this infection, and there is evidence for this view. For example, it has been demonstrated that the addition of T-cells from trypanosome-infected mice will inhibit the uptake of ^3H -thymidine by normal lymphocytes in culture, stimulated by a variety of mitogens (19).

The fullest exposition of the role of T-cells in trypanosome-associated immunodepression is that of Jayawardena and colleagues (20). Very early in trypanosome infections, antibody responses to antigens such as sheep red blood cells (SRBC) are enhanced rather than diminished (3). The Yale group (20) have shown that if cells from mice infected with *T.brucei* for 2 days are added to normal cells an enhanced response to SRBC is obtained in vitro. The enhancing cells are T cells with an $Ly\ 1^+, 2^- 3^-$ phenotype and are thus helper cells. If, however, cells from a 6-day infection are added to normal cells the response is much diminished. In this case the suppressive effect is mediated by $Ly\ 1^+ 2^+ 3^+$ cells, i.e. non-specific suppressor T-cells.

These findings are incorporated into a hypothesis which also attempts to link high immunoglobulin levels with immunodepression, but here relating to T-cells rather than B-cells. It is postulated that the first effect of a trypanosome infection is to activate a population of $Ly\ 1^+$, $2^-\ 3^-$ helper cells which then non-specifically (more correctly multi-specifically) stimulate B-cells to produce their pre-programmed antibodies. This will explain polyclonal B-cell activation. Following this, and perhaps related, there is a stimulation of non-specific $Ly\ 1^+\ 2^+\ 3^+$ T-cells which bring about immunodepression.

The view that T-cells are the primary target for trypanosome-induced immunodepression has also been expressed by Mansfield and Bagasra (21). Using a strain of T.rhodesiense in mice, they found that infected mice had an enhanced response to thymus-independent antigens until shortly before death, although they showed a depressed response against thymus-dependent antigens throughout most of the infection. It is suggested that intrinsic B-cell function is intact and indeed elevated throughout most of the infection, only failing shortly before death. It is further suggested that immunodepression arises because of a failure of helper T-cell function, and that high levels of immunoglobulin results from a failure of suppressor T-cell function.

Thus, whilst agreeing with Jayawardena and colleagues (20) that T-cells are primarily affected in trypanosomiasis, Mansfield and Bagasra (21) attribute immunodepression and polyclonal B-cell activation to T-cell failure rather than to excessive T-cell activity.

That B-cells are affected directly in trypanosomiasis has been recently demonstrated in experiments on nude mice, which possess few, if any, T-cells. Clayton and colleagues (22) have shown that both increased immunoglobulin production and non-specific immunodepression can be demonstrated during infection with the S42 strain of T.brucei in these mice.

It is our view that trypanosome infections initially stimulate both T- and B-cells, but that later in infection all lymphocyte functions are impaired. The exact contributions to the overall immunodepression which may result from the malfunctions of different lymphocyte populations may well vary according to the strain of trypanosome under investigation, the duration of the infection and to the level of parasitaemia.

4. The role of macrophages in trypanosome-associated immunodepression

It is now well recognized that in order to stimulate B-cells to produce many kinds of antibodies, not only helper T-cells but also other accessory cells are required. These accessory cells are generally described as macrophages, but they may well represent a particular sub-population of these cells. One explanation of the well-recognized but poorly understood phenomenon of "antigenic competition" is that the competition takes place at the surface of antigen presenting cells of the macrophage type (23). Some investigators of the immunodepression associated with experimental malaria have claimed that macrophages from infected animals may be defective in antigen handling and presentation (24, 25), but there are no convincing reports that this is also true for trypanosomiasis.

A different role for macrophages in immunodepression has also been suggested. In the non-specific suppression seen in animals undergoing graft versus host reactions (26), or in animals treated with the T-cell mitogen Con A (27), the primary effector of suppression is a T-cell, but the cell which is actually suppressive may be a macrophage. Whether macrophages from trypanosome-infected animals have suppressor properties or not is in dispute. Corsini et al. (9) found peritoneal macrophages from *T.brucei*-infected mice to have suppressor properties, but Jayawardena et al. (20) state that they have presently no evidence implicating macrophages in the mediation of suppression.

5. Complement depletion and immunodepression

Complement has long been known as a powerful effector arm of the immune response, but more recently C3 has also been shown to have a role in the induction of antibody synthesis (28) and in the generation of B-memory cells (29). There are reports that trypanosome infections may activate C3 either by the classical (30) or alternative pathways (31), and cattle infected with *T.congolense* showed reduced circulating C3 levels (32).

Accordingly, Nielsen and colleagues (32) have suggested that reduced C3 levels may contribute to the immunodepression associated with trypanosomiasis. This may be true for some hosts but is unlikely to apply in murine trypanosomiasis. Our group, in collaboration with

G.B. Klaus and M. Holman (NIMR-Mill Hill), has recently been studying C3 levels in mice infected with a chronic strain of T.brucei. In this infection, there is an early and rapid increase in C3 to twice or three times normal. At about 10 days, C3 levels return to normal and remain there for some weeks. From other work (33) we know that mice infected with this strain of trypanosome are severely immunodepressed from day 10 onwards, when adequate levels of C3 would appear to be present.

Why C3 levels are not reduced in infected mice probably relates to the independent observation that mouse IgM-antigen complexes are very poor activators of complement (34). As T-deprived mice control this infection just as well as normal mice, it is reasonable to assume that the effective anti-trypanosome responses are of the IgM class. Interestingly, T-deprived mice chronically depleted of C3 by repeated injections of cobra venom were still capable of controlling the blood parasitaemia, even though they had only about 10% of normal C3 levels (35).

In summary, reduction of C3 levels may well contribute to trypanosome-associated immunodepression, but at least in the mouse there can be severe immunodepression concomitant with normal C3 levels.

6. Polyunsaturated fatty acids (PUFA) and immunodepression

It has long been known that the lipid metabolism of trypanosome infected animals is disturbed. This, taken with the observation that certain of the higher PUFA (C18:2; C20:4) depress the immune functions of mice (36), has led to the suggestion that they play a role in trypanosome-associated depression. Tizard and colleagues (37) showed that the pathogenic T.congolense on autolysis released free PUFA whilst the non-pathogenic T.lewisi did not, and suggested that the PUFA might contribute markedly to immunosuppression (38). Repeating this experiment with T.brucei and T.lewisi we found that autolysates of both yielded free PUFA but only T.brucei yielded the higher (C18:2; C20:4) immunosuppressive PUFA (39).

Serum levels of free PUFA fall rather than rise during infection (39, 40), but it is possible that local

concentrations of PUFA following trypanolysis in lymph nodes and spleen may alter lymphocyte function and contribute to the immunodepression.

7. Increased catabolism of immunoglobulins and immunodepression

A very early report indicated that the half-life of a ^{131}I -labelled preparation of what is now known to be IgG was less in monkeys infected with T.gambiense than in normal monkeys (41). More recently Nielsen and colleagues have obtained similar findings with radio-labelled immunoglobulins and complement components in cattle infected with T.congolense (42). These workers suggest that an increased catabolic rate of antibodies might contribute to an apparent reduction in antibody response.

It should be noted, however, that plaque-forming cell assays in mice indicate a reduced synthesis of antibody following antigenic stimulation. Nevertheless, increased catabolism coming on top of impaired synthesis may well worsen the situation.

8. Unexplored mechanisms

In view of the many data and hypotheses relating to trypanosome-associated immunodeficiency set out above, it may seem foolhardy to search for more. Yet two areas come readily to mind which are currently unexplored but which may well repay further study.

There is firstly a vast literature relating malnutrition to immunodeficiency (2). This is unlikely to be of much importance in the immunodepression seen in trypanosome-infected mice; up until a few days of death they appear to eat well and look healthy although they show severe immunodepression. Malnutrition may, however, play a role in the field where man and his domestic animals both become malnourished when chronically infected.

Secondly, there may be a relationship between trypanosomiasis, endocrine dysfunction and immunodepression.

Apted [43] reports several kinds of endocrine disturbances in human trypanosomiasis which seem to relate to steroid hormones. It is of course well known that certain corticosteroids have powerful immunosuppressive functions so that work on this aspect might prove interesting and useful.

DOES TRYPANOSOME-ASSOCIATED IMMUNODEFICIENCY EXTEND TO RESPONSES AGAINST THE HOMOLOGOUS PARASITE?

African trypanosomiasis is characterized by successive waves of parasitaemia, each wave consisting of trypanosomes whose major surface antigens differ from those of preceding waves. This phenomenon is known as antigenic variation and the surface antigens as variant specific surface antigens (VSSA). Hudson and co-workers (3) have argued that the immunodepression demonstrated to occur with unrelated antigens should, in theory, extend to late appearing VSSA. This should result in the infected host making less than fully optimal responses to the trypanosomes themselves.

The data of Hudson et al. (3) using the semi-acute S42 strain of *T.b.brucei* in mice would seem to support this hypothesis. In this infection, the first wave of parasitaemia is well-controlled, but the second wave, appearing at a time when responses to heterologous antigens are much reduced, is poorly controlled and the mice die with an overwhelming parasitaemia.

Many trypanosome infections are, however, more chronic than the S42 *T.brucei*, giving several waves of parasitaemia. Such an infection was studied by Mansfield and Bagasra (21), using a strain of *T.rhodiense* which gave six or seven successive waves of parasitaemia in mice before death around 35 days. In these experiments, antibody responses to thymus-independent antigens appear to be enhanced although responses to T-dependent antigens are markedly impaired. It is argued therefore that the generalised immunodepression does not extend to responses against trypanosomes, at least until the terminal stage of infection, because anti-trypanosome responses are thymus-independent.

This argument may not apply in all situations. Jayawardena et al. (20) find that responses to the T-independent antigen DNP-Ficoll are much impaired in culture

when spleen cells from mice infected with S42 *T.brucei* are added. Hudson and Terry (33), after investigating a chronic strain of *T.brucei* which lasts about 80 days in mice, suggest that although T-independent responses to trypanosome antigens may be less than optimal, they can in some circumstances still serve to control the blood parasitaemia. They point out, however, that the pivotal event linking immunodepression with course of infection may not relate to the degree of IgM antibody produced against blood parasites, but rather to possible failure of the host to produce IgG antibody against the tissue parasites. There is evidence that antigenic variation may occur primarily in the tissues (44) and such parasites are likely to be unaffected by IgM responses. In the absence of specific IgG responses they will survive to reseed the blood each time a blood stream variant is destroyed.

At this time then it is not resolved whether trypanosome-associated immunodepression also affects the homologous parasite. There are indications that it may do so, but much further work is required before any clear ideas can emerge.

GENERAL CONCLUSIONS

It is apparent from the foregoing that the relationships between the activities of African trypanosomes and the immune responses of their hosts are extraordinarily complex. Indeed these infections seem to offer the investigator a "Complete Compendium of Immunological Puzzles". There is good evidence that multiple mechanisms contribute to the overall immunodepression, but what is the extent of the several contributions and are they in any way related? Is there, for example, any early induced malfunction of a key mechanism from which all of the other disturbances follow? There is at least much evidence for a massive activation of lymphocytes, although there is dispute as to whether T-cells or B-cells are primarily affected. Do the trypanosomes activate lymphocytes directly or indirectly by altering the host physiology? Is there indeed a true trypanosome mitogen and, if so, what is its nature and mode of action?

Answers to these questions are hard to come by for they probably lie beyond the limits of our present overall understanding of immunological mechanisms. Nevertheless, it is encouraging to see a number of groups throughout the

world attacking these problems with skill and vigour, and all the techniques available to modern Immunology and Cell Biology. It is not possible to forecast that if we understood the basic mechanisms of trypanosome associated deficiency, we would be able to use this knowledge to interfere meaningfully in the disease. What is certain, is that we cannot do so at the present time.

ACKNOWLEDGEMENTS

We are grateful to the Ministry of Overseas Development of the United Kingdom for their continued support for our own work on the immunology of trypanosomes through Research Grant R 3080.

We all thank Ann Johnston for her skilled help in preparing the manuscript.

REFERENCES

- [1] TERRY, R.J., "Immunodepression in parasitic infections", Les Colloques de l'Institut National de la Santé et de la Recherche Médicale 72 (1977) 161.
- [2] WORLD HEALTH ORGANIZATION, Immunodeficiency: Report of a WHO Scientific Group, Technical Report Series 630, WHO, Geneva (1978).
- [3] HUDSON, K.M., BYNER, C., FREEMAN, J.C., TERRY, R.J., Immunodepression, high IgM levels and evasion of the immune response in murine trypanosomiasis, Nature (London) 264 (1976) 256.
- [4] SCOTT, J.M., PEGRAM, R.G., HOLMES, P.H., PAY, T.W.F., KNIGHT, P.A., JENNINGS, F.W., URQUHART, G.M., Immunosuppression in bovine trypanosomiasis: field studies using foot-and-mouth disease vaccine and clostridial vaccine, Trop. Anim. Hlth Prod. 9 (1977) 159.
- [5] GREENWOOD, B.M., WHITTLE, H.C., MOLYNEUX, D.H., Immunosuppression in gambian trypanosomiasis, Trans. R. Soc. Trop. Med. Hyg. 63 (1973) 846.
- [6] CLARKSON, M.J., "Immunoglobulin M in trypanosomiasis", Pathophysiology of Parasitic Infections (SOULSBY, E.J.L., Ed.), Academic Press, New York (1976).

- [7] HOUBA, V., BROWN, K.N., ALLISON, A.C., Heterophile antibodies, M-antiglobulins and immunoglobulins in experimental trypanosomiasis, *Clin. Exp. Immunol.* 4 (1969) 113.
- [8] MACKENZIE, A.R., BOREHAM, P.F.L., Autoimmunity in trypanosome infections: 1. Tissue auto-antibodies in *Trypanosoma (Trypanozoon) brucei* infections of the rabbit, *Immunology* 26 (1974) 1225.
- [9] CORSINI, A.C., CLAYTON, C., ASKONAS, B.A., OGILVIE, B.M., Loss of B cell potential in mice infected with *Trypanosoma brucei*, *Clin. Exp. Immunol.* 29 (1977) 122.
- [10] ESUOROSO, G.O.E., The demonstration *in vitro* of the mitogenic effects of trypanosomal antigen on the spleen cells of normal athymic and cyclophosphamide treated mice, *Clin. Exp. Immunol.* 23 (1976) 314.
- [11] GREENWOOD, B.M., ODULOJO, A.J., Mitogenic activity of an extract of *Trypanosoma gambiense*, *Trans. R. Soc. Trop. Med. Hyg.* 72 (1978) 408.
- [12] MANSFIELD, J.M., CRAIG, S.A., STELZER, G.T., Lymphocyte function in experimental African trypanosomiasis: mitogenic effects of trypanosome extracts *in vitro*, *Infect. Immunol.* 14 (1976) 976.
- [13] CLAYTON, C.E., SACKS, D.L., OGILVIE, B.M., ASKONAS, B.A., Membrane fractions of trypanosomes mimic the immunosuppressive and mitogenic effects of living parasites on the host, *Parasite Immunology* 1 (1979) (in press).
- [14] MANSFIELD, J.M., KREIER, J.P., Autoimmunity in experimental *Trypanosoma congolense* infections of rabbits, *Infect. Immunol.* 5 (1972) 648.
- [15] PRIMI, D., EDVARD SMITH, C.I., HAMMARSTROM, L., MOLLER, G., Sera from LPS-injected mice exhibit complement-dependent cytotoxicity against syngenic and autologous spleen cells, *Cell. Immunol.* 32 (1977) 232.
- [16] MITRY-MARTIN, L., HUDSON, K.M., TERRY, R.J., Autoantibodies to splenocytes in murine trypanosomiasis, *Trans. R. Soc. Trop. Med. Hyg.* 73 (1979) 98.
- [17] HUDSON, K.M., BYNER, C., Changes in the lymphoid architecture of trypanosome infected mice, *Trans. R. Soc. Trop. Med. Hyg.* 67 (1973) 265.
- [18] MURRAY, M., MURRAY, P.K., JENNINGS, F.W., FISHER, E.W., URQUHART, G.M., The pathology of *Trypanosoma brucei* in the rat, *Trans. R. Soc. Trop. Med. Hyg.* 67 (1973) 276.
- [19] JAYAWARDENA, A.N., WAKSMAN, B.M., Suppressor cells in experimental trypanosomiasis, *Nature (London)* 265 (1977) 539.
- [20] JAYAWARDENA, A.N., WAKSMAN, B.H., EARDLEY, D.D., Activation of distinct helper and suppressor T cells in experimental trypanosomiasis, *J. Immunol.* 121 (1978) 622.
- [21] MANSFIELD, J.M., BAGASRA, O., Lymphocyte function in experimental African trypanosomiasis: 1. B cell responses to helper T cell-independent and -dependent antigens, *J. Immunol.* 120 (1978) 759.
- [22] CLAYTON, C.E., OGILVIE, B.M., ASKONAS, B.A., *Trypanosoma brucei* infection in nude mice: B lymphocyte function is suppressed in the absence of T lymphocytes, *Parasite Immunology* 1 (1979) (in press).
- [23] SCHRADER, J.W., FELDMANN, M., The mechanism of antigenic competition: 1. The macrophage as a site of a reversible block to T-B lymphocyte cooperation, *Eur. J. Immunol.* 3 (1973) 711.
- [24] LOOSE, L.D., DI LUZIO, N.R., A temporal relationship between reticulo-endothelial system phagocytic alterations and antibody responses in mice infected with *Plasmodium berghei* (NYU-strain), *Am. J. Trop. Med. Hyg.* 25 (1976) 271.
- [25] WARREN, H.S., WEIDANZ, W.P., Malarial immunodepression *in vitro*: adherent spleen cells are functionally defective as accessory cells in the response to horse erythrocytes, *Eur. J. Immunol.* 6 (1976) 816.

- [26] BYFIELD, P., CHRISTIE, G.H., HOWARD, J.G., Alternative potentiating and inhibitory effects of GVH reaction on formation of antibodies against a thymus-independent polysaccharide (SII), *J. Immunol.* 111 (1973) 72.
- [27] PIERCE, C.W., KAPP, J.A., Regulation of immune responses by suppressor T cells, *Contemp. Top. Immunobiol.* 5 (1976) 91.
- [28] PEPYS, M.B., Role of complement in the induction of immunological responses, *Transpl. Rev.* 32 (1976) 93.
- [29] KLAUS, G.G.B., HUMPHREY, J.H., The generation of B memory cells, *Immunology* 33 (1977) 31.
- [30] JARVINEN, J.A., DALMASSO, A.P., Complement in experimental *Trypanosoma lewisi* infections of rats, *Infect. Immunol.* 14 (1976) 894.
- [31] NAGLE, R.B., WARD, P.A., LINDSLEY, H.B., SADUN, E.H., JOHNSON, A.J., BERKOW, R.E., HILDEBRANDT, P.K., Experimental infections with African trypanosomes: iv. Glomerulonephritis involving the alternative pathway of complement activation, *Am. J. Trop. Med. Hyg.* 33 (1974) 15.
- [32] NIELSEN, K., SHEPPARD, J., HOLMES, W., TIZARD, I., Experimental bovine trypanosomiasis: Changes in serum immunoglobulins, complement and complement components in infected animals, *Immunology* 35 (1978) 817.
- [33] HUDSON, K.M., TERRY, R.J., Immunodepression and the course of infection of a chronic *Trypanosoma brucei* infection in mice, *Parasite Immunology* 1 (1979) (in press).
- [34] KLAUS, G.G.B., PEPYS, M.B., KITAJIMA, K., ASKONAS, B.A., Activation of mouse complement, *Eur. J. Immunol.* (in press).
- [35] FAGHIHI SHIRAZI, M., HOLMAN, M., HUDSON, K.M., KLAUS, G.G.B., TERRY, R.J., Complement and murine trypanosomiasis (in preparation).
- [36] MERTIN, J., HUGHES, D., Specific inhibitory action of PUFA on lymphocyte transformation induced by PHA and PPD, *Int. Arch. Allergy. Appl. Immunol.* 48 (1975) 203.
- [37] TIZARD, I.R., NIELSEN, K., MELLORS, A., ASSOKU, R.K., Free fatty acids and pathogenesis of African trypanosomiasis, *Lancet* i (1977) 750.
- [38] ASSOKU, R.K.G., TIZARD, I.R., NIELSEN, K.H., Free fatty acids, complement activation and polyclonal B cell activation as factors in the immunopathogenesis of African trypanosomiasis, *Lancet* ii (1977) 956.
- [39] TAI, P.F., McDONALD GIBSON, R., TERRY, R.J., Polyunsaturated fatty acids (PUFA) and trypanosomiasis, *Trans. R. Soc. Trop. Med. Hyg.* 73 (1979) 99.
- [40] ROBERTS, C.J., CLARKSON, M.J., Free fatty acids, lysophosphatidylcholine and pathogenesis of trypanosomes, *Lancet* i (1977) 952.
- [41] SMITHERS, S.R., TERRY, R.J., Changes in the serum proteins and leucocyte counts of rhesus monkeys in the early stages of infection with *Trypanosoma gambiense*, *Trans. R. Soc. Trop. Med. Hyg.* 53 (1959) 336.
- [42] NIELSEN, K., SHEPPARD, J., HOLMES, W., TIZARD, I., Experimental bovine trypanosomiasis: changes in the catabolism of serum immunoglobulins and complement components in infected cattle, *Immunology* 35 (1978) 811.
- [43] APTED, F.I.C., "Clinical manifestations and diagnosis of sleeping sickness", *The African Trypanosomiasis* (MULLIGAN, H.W., Ed.), George Allen and Unwin, London (1970).
- [44] SEED, J.R., EFFRON, H.G., Simultaneous presence of different antigenic populations of *Trypanosoma brucei gambiense* in *Microtus montanus*, *Parasitology* 66 (1973) 269.

DISCUSSION

R.S. PHILLIPS: You mentioned in your oral presentation that malaria-induced immunodepression appears to be, in part at least, the result of some macrophage dysfunction; Weidanz has recently suggested that this is T-cell-mediated.

R.J. TERRY: Macrophage dysfunction may also be involved in trypanosome-induced immunodepression, but the more popular view seems to be that macrophages can have a more active suppressor function.

R.S. PHILLIPS: I would like to ask you, in connection with your remarks about IgG levels and trypanosomes coming from the tissues into the circulation, what the current thinking is about the relationship between the occurrence of specific variant types in the tissues and their appearance in the blood.

R.J. TERRY: Your question is difficult to answer with certainty at present. Evidence from Seed's and from Jenni's laboratories suggests that the new variants appear first in the tissues and then recolonize the blood. On the other hand, work carried out in Vickerman's laboratory suggests a considerable heterogeneity of antigenic types throughout infection. It is important that further critical investigations be carried out in this area.

J.D. DARGIE (*Scientific Secretary*): Reduced numbers of plaque-forming cells in infected mice indicate reduced Ig synthesis. In cattle, Ig levels in the serum are normal but catabolism is greatly enhanced, which is indicative of increased synthesis. How do we explain these differences?

R.J. TERRY: We have to distinguish between B-cell activation by specific antigens introduced into trypanosome-infected animals and the polyclonal B-cell activation induced by the infection. In mice there is at the same time a decrease in plaque-forming cells producing antibody to any introduced antigen and a marked increase in the total number of antibody-producing cells. So synthesis of antibodies of many specificities increases, but the contribution of antibody of any given specificity is small.

H.S. HUSSEIN: Does immunosuppression during trypanosomiasis occur only in the mouse? We have found in Professor Kreier's laboratory at Ohio State University that the ability of the mouse to mount a good immunological response against malaria parasites and *Babesia* is very slight.

R.J. TERRY: It is true that the mouse probably shows the most severe immunodepression, but it also occurs in rats, in cattle and in man. The mouse is as widely used as it is because we understand its immune system better than that of any other host. For example, only in the mouse are we able to differentiate easily the several T-cell sub-sets. I believe, however, that what we find in mice will apply to other hosts, although perhaps to a lesser degree.

F.E.G. COX (*Chairman*): Immunodepression is a general phenomenon and occurs with many infections in several different hosts, including man and

domesticated animals. It should be possible to extrapolate from other infections to trypanosomiasis, although the actual mechanisms of immunodepression may vary considerably.

R.J. TERRY: I agree with you that parasite-associated immunodepression is a general phenomenon (see Ref. [1]), and that similar underlying mechanisms may operate in different parasitic infections. But I also believe that some of the mechanisms seen in other parasites, particularly parasitic helminths, will not apply to trypanosomes and vice-versa. It is important, though, that we should continuously be aware of work being done on other host-parasite systems.

ROLE OF BIOLOGICALLY ACTIVE SUBSTANCES IN THE PATHOGENESIS AND IMMUNOLOGY OF TRYPANOSOMIASIS

I.R. TIZARD, A. MELLORS, K. NIELSEN

University of Guelph,
Guelph,
Ontario,
Canada

Abstract

ROLE OF BIOLOGICALLY ACTIVE SUBSTANCES IN THE PATHOGENESIS AND IMMUNOLOGY OF TRYPANOSOMIASIS.

Pathogenic trypanosomes are toxigenic organisms. Living trypanosomes release surface components such as exoantigen or filopodia. These factors are capable of activating complement and can participate in immune-complex derived lesions. Actively metabolizing trypanosomes generate biologically active tryptophan metabolites of which indole-3-ethanol (tryptophol) and indole-3-acetic acid are probably most important. These compounds are capable of inducing nervous system depression and are immunosuppressive. Factors released from dead, dying and fragmented trypanosomes include lysosomal proteases and membrane-associated phospholipases. These latter enzymes which appear to be largely restricted to pathogenic trypanosomes increase significantly in activity following trypanosome disruption. They act on endogenous trypanosome phospholipids to generate lysophospholipids and free fatty acids. There is also evidence to suggest that trypanosome free fatty acids may function as B-cell mitogens and thus provoke a polyclonal response which may result in hypergammaglobulinaemia. Autolysing trypanosomes also generate factors which, depending on the species of trypanosome involved, activate complement by either the classical and/or alternate pathways. The nature of these factors is unclear. There are reports of factors which inhibit erythropoiesis, which inhibit macrophage migration, which lyse erythrocytes, and factors which are hepatotoxic or which cause inflammation. Most of these are uncharacterized, and their significance undetermined.

INTRODUCTION

Because animals and humans dying from uncomplicated trypanosomiasis show relatively few and unspectacular gross pathological lesions, it was assumed by many early investigators that trypanosomes produced lethal toxins. Unfortunately, experiments designed to demonstrate these toxins tended to yield negative results and it was eventually concluded that trypanosomes were not toxigenic organisms [1]. Nevertheless a few investigators were successful in demonstrating trypanosome toxic activity. Perhaps the most important of these were Landsteiner

and Raubitschek [2] who, while working in Vienna, showed that *Trypanosoma equiperdum* suspensions could become haemolytic if left to autolyse overnight in the cold. The haemolytic activity appeared to be lipid in nature but its precise composition was not ascertained.

Both before and after Landsteiner's studies, individual workers [3–6] succeeded in producing tentative evidence for the existence of trypanosome toxins but none was sufficiently persuasive to cause acceptance of a toxin hypothesis for the pathogenesis of trypanosomiasis.

THE LESIONS OF AFRICAN TRYPANOSOMIASIS

As pointed out earlier, humans and animals dying of uncomplicated trypanosomiasis show few gross lesions. There is generally severe emaciation with loss of body fat and muscle. The viscera tend to be enlarged and wet, and although the spleen and lymph nodes are generally enlarged they and the thymus may atrophy following prolonged infections [7, 8].

Histologically there is evidence of generalized vascular endothelial damage. This endothelial damage may be associated with accumulations of trypanosomes within capillaries and local perivascular inflammatory cell infiltration. If severe this damage can lead to oedema or haemorrhage. It may also result in local platelet aggregation and thrombus formation which may proceed to disseminated intravascular coagulation [8]. In infections due to tissue invasive micro-organisms such as *T. brucei*, accumulations of trypanosomes may be found within connective tissue.

Infected humans and domestic animals suffer from a profound anaemia. The signs of this anaemia include extensive erythrophagocytosis by mononuclear phagocytic cells and, provided the animal is not severely debilitated, evidence for a bone marrow erythropoietic response [8].

The third group of lesions appear to relate to the immunological changes occurring in infected animals. Firstly, there may be extensive immune-complex formation, evidence for which may be seen as deposits on glomerula basement membranes [9]. Secondly, there may be a massive rise in serum immunoconglutinin levels, a reflection of such immune complex formation [10]. Morphologically there is severe disruption of spleen and lymph node architecture associated with a depletion of functional T-cells, and functionally this is reflected in a profound immunosuppression [11]. This immunosuppression involves not only the cell-mediated immune responses but also antibody-mediated immunity. It may be considered to be the single most important pathogenic mechanism in trypanosomiasis since infected individuals generally die, not as a direct result of trypanosome activity, but as a result of secondary bacterial and viral infections [8].

Immunological disturbances are also reflected in a marked increase in serum IgM and a drop in serum IgA and IgE [12]. The excess IgM is directed against a variety of antigens, only some of which are on the trypanosomes. Catabolic studies have shown that the half lives of all the immunoglobulins including IgM are greatly decreased [13].

Serum complement levels are also severely depressed in trypanosomiasis. The most severely affected components being the early ones in the classical pathway such as C1 and C3. The alternate pathway is only minimally affected [14].

TRYPANOSOME FACTORS

Clinically it is well recognized that trypanosome-infected individuals show fever peaks, haemolytic crises and increased malaise at a time related to trypanosome destruction. This is a frequent occurrence in chronic trypanosome infections being related to the cyclical parasitaemia [15]. When trypanosomes are disrupted in this way they can release a variety of factors which are potentially capable of causing vascular endothelial damage and erythrocyte destruction. These factors include proteolytic enzymes, phospholipases and free fatty acids as well as a number of other, uncharacterized factors.

Proteases

Trypanosome lysosomes contain proteases such as cathepsin D which may be released on autolysis [16]. This enzyme can act on plasminogen to generate plasmin and on certain kininogens to generate kinins. The plasmin may be responsible for the increased quantities of fibrinogen degradation products seen in *T. brucei*-infected rabbits [17], while kinins are known to be elevated in several forms of trypanosomiasis [18].

Phospholipases

Pathogenic trypanosomes also generate phospholipases [15]. Thus *T. brucei* and *T. congolense* are, when freshly isolated, relatively low in phospholipase activity. However, if they are permitted to autolyse at 20°C for several hours or if the phospholipase is released by detergents large quantities of active phospholipase may be detected. In both *T. congolense* and *T. brucei* this enzyme hydrolyses phosphatidyl choline at the 1 acyl position and is therefore a phospholipase A1. Phospholipase released from fresh trypanosomes by detergent treatment has a Vmax of 0.145 μ mole/min per mg for phosphatidyl choline substrates and a pH optimum of 6.5.

T. congolense also possesses a low level of lysophospholipase activity when freshly isolated. The significance of this enzyme and its relation to the phospholipase A1 is unclear. The presence of an activatable phospholipase appears to be very closely correlated with the pathogenic potential of an organism. Thus neither *T. lewisi*, *T. musculi* nor *T. theileri* possess an activatable phospholipase and all are non-pathogenic.

It has been suggested [15] that the phospholipase may play a role in mitochondrial re-organization such as occurs when trypanosomes are ingested by tsetse flies. Within infected animals phospholipases would be expected to be released following trypanosome destruction and act on phospholipids to yield lysophospholipids and free fatty acids. Lysophospholipids are potent cytotoxins but are probably rapidly broken down to glycerylphospholipids and more free fatty acids by the trypanosome lysophospholipase.

Phospholipases can destroy cell membrane phospholipids and in this way provoke endothelial damage [19]. In the case of invasive organisms, such as *T. brucei*, active phospholipases within connective tissue could kill or damage fibroblasts, break down connective tissue, increase vascular permeability and provoke oedema. This type of reaction has been reported to occur following intradermal inoculation of *T. gambiense*, *T. brucei* and *T. congolense*.

Trypanosome phospholipases can also provoke mast cell degranulation which would also contribute to this inflammatory process [15].

Free fatty acids

The action of trypanosome phospholipase on endogenous phospholipid results in vivo in the generation of relatively large quantities of free fatty acids. These free fatty acids are surface-active and hence both haemolytic and cytotoxic. It is most probable that the haemolytic activity discovered by Landsteiner and Raubitschek [2] was in fact due to these fatty acids. Of the many fatty acids released, the major ones in both *T. congolense* and *T. brucei* are linoleic, palmitic and stearic which together account for about 75% of the total and a lesser amount of oleic, gamma linolenic and arachidic acids. When the properties of individual fatty acids are tested, it is clear that linoleic acid is primarily responsible for the haemolytic properties of the mixture. Although the massive release of free fatty acids is a major cause of some anaemias such as blackwater fever [20], there is no evidence to suggest that this is the case in trypanosomiasis. One reason for this is that serum albumin, by binding to free fatty acid, blocks its haemolytic activity. Thus bovine serum albumin in very low concentrations can block trypanosome-induced haemolysis in vivo. Calculations also reveal that insufficient free fatty acid could be released to saturate bovine albumin in vivo and enable free fatty acids to reach haemolytic levels in bovine plasma. In addition pretreatment of bovine erythrocytes with sub-haemolytic doses of free fatty acids in vitro does not

influence their subsequent survival in vivo. Although it is therefore clear that trypanosome-derived fatty acids are not haemolytic in vivo, they remain, at least in theory, capable of inducing thrombosis, myocardial lesions, hypoglycaemia and thrombocytopenia [15], all of which have been observed in trypanosomiasis.

UNCHARACTERIZED HAEMOLYTIC FACTORS

In 1954 Fiennes reported on a *T. congolense*-infected cow whose serum had the capacity to lyse normal bovine erythrocytes [6]. Unfortunately this material was not characterized. Later, Huan and his colleagues [21] reported that they had found a slow-acting haemolysin in suspensions of *T. brucei*, *T. gambiense*, *T. vivax* and *T. congolense*. This activity has been associated with a protein with a molecular weight of about 10 000 daltons. The relation of this factor to the phospholipases and free fatty acids is unclear.

INFLAMMATORY FACTORS

A local inflammatory skin reaction occurs in response to the bite of infected tsetse flies. Whereas some of the factors described above such as the proteases, phospholipases or free fatty acids may mediate such a reaction, other vascular permeability increasing factors have been described. Thus Seed [22] reported on a protein extracted from *T. gambiense* which increased vascular permeability on intradermal inoculation into rabbit. Tizard and Ringleberg [23] reported on material from *T. congolense* which provoked inflammation around intraperitoneal diffusion chambers. Neither of these activities have been fully characterized.

Finally, as will be discussed below, several of the trypanosome-derived complement activating factors can, probably by provoking anaphylatoxin generation, cause local acute inflammation.

IMMUNOMODULATING FACTORS

In studying the pathogenesis of trypanosomiasis, investigators are faced with the problem of accounting for a simultaneous profound immunosuppression and enhanced immunoglobulin production. There are currently two major hypotheses that seek to account for these. In one, it is suggested that B-cells are polyclonally stimulated and thus cause excess immunoglobulin production and immunosuppression by clonal exhaustion [24]. In the other theory, it is suggested that immunosuppression is mediated by suppressor T-cells and that hypergamma-globulinaemia M occurs as a response to some form of loss of T-cell control [25].

It is likely that both hypotheses are correct at least in part and complementary. Insofar as trypanosome immunomodulating factors are concerned it is clear that they possess immunosuppressive factors. Thus the tryptophane metabolite indole-3-ethanol (tryptophol) can suppress the response of mice to sheep erythrocytes [26].

Of possibly more importance than tryptophol are the free fatty acids mentioned previously. Linoleic acid which constitutes 22–23% of the fatty acids liberated by *T. brucei* or *T. congolense* has been shown to be capable of prolonging skin allograft survival in mice and of reducing the severity of rejection episodes in human renal allografts [15]. We have shown that when mice are given a daily injection of a free fatty acid mixture calculated to be identical in dose and composition with that from *T. congolense*, their ability to mount an immune response to sheep erythrocytes is profoundly depressed (unpublished observations).

It has been shown that these fatty acids exert their suppressive effect directly on lymphocytes. Thus linoleic acid can suppress the mitogenic responses of lymphocytes to a variety of phyto-mitogens and antigens whereas trypanosome suspensions have also been shown to possess this property. Linoleic acid can produce structural changes in splenic and lymphoid architecture similar to that observed in trypanosomiasis. There is also some preliminary evidence to suggest that linoleic acid may stimulate suppressor cell activity [27].

A number of authors have claimed to have demonstrated mitogenic activity in trypanosome preparations and in general these appear to be B-cell mitogens [28–30]. In our own experiments mitogenic activity was present in autolysates of *T. congolense* which could be demonstrated on cells from nude (T-cell deficient) mice but not on cells from cyclophosphamide-treated (B-cell deficient) mice. The mitogenic activity was extractable in chloroform:methanol, and on subsequent fractionation was found to be associated with the saturated fatty acids, most notably palmitic acid. The unsaturated acids in these preparations appeared to exert an antimitogenic effect.

In contrast to the relatively potent mitogenic activity of the pathogenic trypanosomes, similar preparations from *T. musculi*, *T. lewisi* or *T. theileri* were found to be ineffective.

The occurrence of these mitogens tends to support the polyclonal activation hypothesis but certainly does not exclude a role for suppressor T-cells in this process.

COMPLEMENT ACTIVATING FACTORS

Nielsen and his co-workers [12] have shown that both *T. congolense* and *T. lewisi* contain internal factors which activate complement both in vivo and in vitro. The substance(s) is not exposed on living organisms but requires their disruption to be effective. Its physicochemical nature is unclear, all attempts to

characterize it have been unsuccessful but preliminary data indicate that it might be lipopolysaccharide in nature. It can act on bovine, human, swine, guinea pig and rat complements by interfering with the classical pathway at the C1S stage. Rats de complemented with this material are rendered highly susceptible to pathogenic organisms such as *Salmonellae*. At least some of the apparent susceptibility of trypanosome-infected animals to secondary invaders may be due to this effect.

Nielsen, Sheppard, Tizard and Holmes [14] have also described the complement-activating activity of trypanosome proteases. These are also only released from disrupted trypanosomes but may be of significance following intravascular trypanosome destruction. They accounted for only about 10% of the total complement-activating activity in the preparations tested.

The surface variant-specific glycoproteins of *T. brucei* are capable of activating the classical complement pathway [31]. Whereas they are generally bound to the trypanosome surface they may be shed into plasma and bind to host erythrocytes. This may contribute to erythrocyte destruction. Purified glycoprotein, on intradermal injection will cause extravasation of capillary fluid which can be inhibited by antihistamines, and the reaction is probably mediated through anaphylatoxin generation.

ANAESTHETICS

Perhaps the most dramatic clinical feature of human gambian trypanosomiasis is the 'sleeping sickness' that affects infected individuals. *T. gambiense* has been shown to metabolize tryptophane to indole-3-ethanol (tryptophol), indole-3-acetaldehyde and indole-3-acetate. These factors, but especially tryptophol, can produce a coma-like syndrome in cats, mice, rats and chicks [32]. This coma closely resembles clinical 'sleeping sickness' and it has been suggested that tryptophol functions as a toxin generated by trypanosomes. However, as with most of the other factors described here, accurate quantitative studies will be required to determine whether the amount of tryptophol generated by trypanosomes in vivo is sufficient to produce the clinical signs observed.

OTHER POSSIBLE TRYPANOSOME TOXINS

Whereas the major trypanosome toxic products discovered to date are described above, a number of other, primarily metabolic, products may be associated at least in part with some pathogenic changes. For example, both tyrosine and phenylalanine are catabolised to toxic compounds such as p-hydroxy-phenylethanol and p-hydroxyphenylpyruvate. Trypanosomes produce large

quantities of pyruvic acid which may be cytotoxic. There is some slight evidence for the existence of hepatotoxins, for example Kawamitsu [33] showed that *T. gambiense* emulsions could cause hypoglycaemia and decrease liver glycogen when injected into rats. In cattle the serum of trypanosome-infected animals has been shown to inhibit bone marrow myeloid colony-forming cells [34], and to inhibit blood monocyte migration [35]. In mice, trypanosome products from infected spleens appear to be immunosuppressive [36]. None of these have been characterized and their significance can only be guessed at.

CONCLUSIONS

Given the wide variety of biologically active factors which have been reported to exist in African trypanosomes, it is tempting to suggest that they can account fully for the pathogenicity of these organisms. This would be a very premature assumption, however. Whereas the factors described are potentially capable of causing the lesions observed, in most cases a direct connection has not been made. For example, trypanosome phospholipases have not been shown to be active in vitro, nor do we know whether sufficient tryphophol is produced in vivo to account for the clinical signs of sleeping sickness.

It is likely that at least some of the factors described above contribute to some of the pathogenesis of trypanosomiasis, but it is also probable that they represent only pieces of a jigsaw and that many other processes and mechanisms have to be identified before the puzzle is completed.

REFERENCES

- [1] GOODWIN, L.G., "Mechanisms of pathogenesis in trypanosomiasis", Trypanosomiasis and Leishmaniasis with Special Reference to Chagas' Disease, Ciba Foundation Symposium, No. 20, Elsevier, Amsterdam (1974).
- [2] LANDSTEINER, K., RAUBITSCHKE, H.H., Beobachtungen über Hämolyse und Hamagglutination, Centralbl. Bakt. Abb. Orig. 45 (1907) 660.
- [3] LAVERAN, A., MESNIL, F., Recherches morphologiques et expérimentales sur le trypanosome du nagana ou maladie de la mouche tsétsé, Ann. Inst. Pasteur 16 (1902) 1.
- [4] LAVERAN, A., PETTIT, A., Des trypanotoxines, Bull. Soc. Path. Exot. 4 (1911) 42.
- [5] NOVY, F.G., DE KRUIF, P.H., NOVY, R.L., Anaphylatoxin and anaphylaxis: I. Trypanosome anaphylatoxin, J. Infect. Dis. 20 (1917) 461.
- [6] FIENNES, R.N.T.-W., Hematological studies in trypanosomiasis of cattle, Vet. Rec. 66 (1954) 423.
- [7] FIENNES, R.N.T.-W., "Pathogenesis and pathology of animal trypanosomiasis", The African Trypanosomiasis (MULLIGAN, H.W., Ed.), Allen and Unwin, London (1970).
- [8] LOSOS, G.J., IKEDE, B.O., Review of pathology of disease of domestic animals caused by *Trypanosoma congolense*, *T. vivax*, *T. brucei*, *T. rhodesiense* and *T. gambiense*, Vet. Path. Suppl. 9 (1973) 1.

- [9] NAGLE, R.B., WARD, P.A., LINDSEY, H.B., SADUN, E.H., JOHNSON, A.J., BERKAW, R.E., HILDEBRANDT, P.K., Experimental infections with African trypanosomes: VI. Glomerulonephritis involving the alternate pathway of complement activation, *Am. J. Trop. Med. Hyg.* 23 (1974) 15.
- [10] INGRAM, D.G., SOLTYS, M.A., Immunity in Trypanosomiasis: IV. Immunoconglutinin in animals infected with *Trypanosoma brucei*, *Parasitology* 50 (1960) 231.
- [11] GOODWIN, L.G., GREEN, D.G., GUY, M.W., VOLLER, A., Immunosuppression during trypanosomiasis, *Br.J.Exp. Pathol.* 53 (1972) 40.
- [12] NIELSEN, K.H., SHEPPARD, J., TIZARD, I.R., HOLMES, W.L., Experimental bovine trypanosomiasis: Changes in serum immunoglobulin, complement and complement component levels in infected cattle, *Immunology* 35 (1978) 811.
- [13] NIELSEN, K.H., SHEPPARD, J., HOLMES, W.L., TIZARD, I.R., Experimental bovine trypanosomiasis: Changes in the catabolism of serum immunoglobulins and complement components in infected cattle, *Immunology* 35 (1978) 817.
- [14] NIELSEN, K.H., SHEPPARD, J., TIZARD, I.R., HOLMES, W.L., Direct activation of complement by trypanosomes, *J. Parasitol.* 64 (1978b) 544.
- [15] TIZARD, I., NIELSEN, K.H., SEED, J.R., HALL, J.E., Biologically active products from African Trypanosomes, *Microbiol. Rev.* 42 (1978) 661.
- [16] VENKATESEN, S., BIRD, R.G., ORMEROD, W.E., Intracellular enzymes and their localization in slender and stumpy forms of *Trypanosoma brucei rhodesiense*, *Int. J. Parasitol.* 7 (1977) 139.
- [17] BOREHAM, P.F.L., FACER, C.A., Fibrinogen degradation products in African trypanosomiasis, *Trans. R. Soc. Trop. Med. Hyg.* 67 (1973) 279.
- [18] BOREHAM, P.F.L., Kinin release and the immune reaction in human trypanosomiasis caused by *Trypanosoma rhodesiense*, *Trans. R. Soc. Trop. Med. Hyg.* 64 (1970) 394.
- [19] CARNE, H.R., ONON, E.O., Action of *Corynebacterium ovis* exotoxin on endothelial cells of blood vessels, *Nature (London)* 271 (1978) 246.
- [20] LASER, H., KEMP, P., MILLER, H., LANDER, D., KLEIN, P., Malaria, quinine and red cell lysis, *J. Parasitology* 71 (1975) 167.
- [21] HUAN, C.N., Pathogenesis of the anemia in African Trypanosomiasis: Characterization and purification of a hemolytic factor, *J. Suisse Med.* 47 (1975) 1582.
- [22] SEED, J.R., *Trypanosoma gambiense* and *T. lewisi* increased vascular permeability and skin lesions in rabbits, *Exp. Parasitol.* 26 (1969) 214.
- [23] TIZARD, I., RINGLEBERG, C.P., Release of biologically active material from dying *Trypanosoma congolense* in rats, *Trans. R. Soc. Trop. Med. Hyg.* 72 (1973) 198.
- [24] ASSOKU, R.K.G., TIZARD, I.R., NIELSEN, K.H., Free fatty acids, complement activation and polyclonal B-cell stimulations as factors in the immunopathogenesis of African trypanosomiasis, *Lancet* ii (1977) 956.
- [25] JAYAWARDENA, A.N., WAKSMAN, B.H., Suppressor cells in experimental trypanosomiasis, *Nature (London)* 265 (1977) 539.
- [26] ACKERMAN, S.B., SEED, J.R., The effects of Tryphophol on immune responses and its implications towards trypanosome-induced immunosuppression, *Experientia* 32 (1976) 645.
- [27] MERTIN, J., MEADE, C.J., HUNT, R., SHEENA, J., Importance of the spleen for the immunoinhibitory action of linoleic acid in mice, *Int. Arch. Allergy Appl. Immunol.* 53 (1977) 469.
- [28] ESURUOSO, G.O., The demonstration *in vitro* of the mitogenic effects of trypanosomal antigen on the spleen cells of normal, athymic and cyclophosphamide-treated mice, *Clin. Exp. Immunol.* 23 (1976) 314.
- [29] ASSOKU, R.K.G., TIZARD, I.R., Mitogenicity of autolysates of *Trypanosoma congolense*, *Experientia* 34 (1978) 127.

- [30] GREENWOOD, B.M., ODULOJU, J., Mitogenic activity of an extract of *Trypanosoma gambiense*, Trans. R. Soc. Trop. Med. Hyg. 72 (1978) 408.
- [31] MUSKOKKE, A.J., BARBET, A.F., Activation of complement by variant-specific surface antigen of *Trypanosoma brucei*, Nature (London) 270 (1977) 438.
- [32] SEED, J.R., SEED, T.M., SECHELSKI, J., The biological effects of tryptophol (indole-3-ethanol): hemolytic, biochemical and behaviour modifying activity, Comp. Biochem. Physiol. (in press).
- [33] KAWAMITSU, K., The relation of several hormones to the hypoglycemic action of Trypanotoxin from *T. gambiense*, Nagasaki Med. J. 33 (1958) 115.
- [34] KAAYA, G., Thesis, University of Guelph, Ontario, Canada, 1979.
- [35] RURANGIRWA, F.R., Thesis, University of Guelph, Ontario, Canada, 1979.
- [36] ALBRIGHT, J.W., ALBRIGHT, J.F., DUSANIC, D.G., Mechanisms of trypanosome-mediated suppression of humoral immunity in mice, Proc. Natl. Acad. Sci. U.S.A. 75 (1978) 3923.

DISCUSSION

L.H. OTIENO: The number of blood trypanosomes (*T. congolense*) that you use to elicit the haemolytic reaction in your systems is enormous. In my experience, the numbers of metacyclic *T. congolense* or *T. vivax* secreted by infected flies are very small. Would you therefore expect the metacyclic trypanosomes to be more able to secrete the free fatty acids you describe than the blood form trypanosomes?

I.R. TIZARD: I do not know.

Max MURRAY: We have detected haemolytic activity not only in preparations of dying trypanosomes but also in fresh-freeze and thawed *T. brucei*, *T. congolense* and *T. vivax*. Evidence that trypanosome haemolysins may be operative in vivo comes from the fact that haemolytic activity can be detected in the plasma of infected cattle (the plasma was heated at 60°C for 1 hour before testing). The level of haemolytic activity appeared to wax and wane with parasitaemia. Furthermore, activity was also found in the plasma of trypanosome-infected rats which had been subjected to total-body irradiation (950 rad).

D.H. MOLYNEUX: Could you explain why phospholipase by itself does not produce haemolysis but the combination of *T. lewisi* autolysate and phospholipase does?

I.R. TIZARD: The ability of phospholipases to cause haemolysis of erythrocytes varies. It depends both on the phospholipases and on the surface pressure of the target erythrocyte. The bee venom phospholipase used in our *T. lewisi* experiment was non-haemolytic for sheep erythrocytes.

D.H. MOLYNEUX: How compatible are *T. theileri* culture forms with *T. congolense* blood stream forms?

I.R. TIZARD: Our *T. theileri* culture forms were trypomastigotes grown in primary bovine spleen cell cultures. Although we do not know whether they were exactly the same as blood stream forms, they were morphologically compatible.

R.J. TERRY: It may be that fatty acids could have important effects on the immune system even if they were present in only small amounts. They could perhaps trigger an effect which then might become greatly amplified by other mechanisms.

I was particularly interested in your preliminary finding, reported in your oral presentation, that infected mice treated with phospholipase inhibitor had improved survival. Do you know whether immune responses are restored in treated mice?

I.R. TIZARD: No, they definitely are not; it was originally to establish this that the work was performed, but the results were unequivocally negative with respect to both immunodepression and polyclonal activation. In considering the significance of free fatty acids and phospholipases in immunosuppression in trypanosomiasis, the working hypothesis we used was that it worked through the prostaglandin pathway. For this reason we sought to modify this suppression with indomethacin, which inhibits prostaglandin synthetase and phospholipases. We found that, although indomethacin had no effect on either immunosuppression or polyclonal stimulation, it did reduce mortality in *T. congolense*-infected mice significantly.

Max MURRAY: It would appear that the presence of the trypanosome in close proximity to lymphoid cells may be required to induce depression. For example, work at the International Laboratory for Research on Animal Diseases (ILRAD) has shown that in *T. congolense*-infected mice severe depression of cellular responses had occurred in the spleen whereas no depression was found in the lymph nodes; *T. congolense* is a blood-borne intravascular parasite and large numbers are found in the spleens of infected mice.

MICROBIAL STASIS OF *Leishmania enriettii* IN ACTIVATED GUINEA PIG MACROPHAGES

C.M. GROOCCOCK*, E.J.L. SOULSBY†

Department of Pathobiology,
School of Veterinary Medicine,
University of Pennsylvania,
Philadelphia,
United States of America

Abstract

MICROBIAL STASIS OF *Leishmania enriettii* IN ACTIVATED GUINEA PIG MACROPHAGES.

Peritoneal exudate cells (PEC) from *Leishmania*-sensitized guinea pigs were cultured in vitro in the presence (activated) or absence (non-activated) of leishmanial antigen for 24 or 48 hours. These were then labelled with ^{51}Cr and challenged with ^{125}I -labelled promastigotes. The changing relationship between the macrophage and the parasite was monitored by observing changes in the ratio of the cell-associated isotopes. Highly significant differences in the ratio change developed during culture. These differences were a result of the activated cultures showing a higher release of ^{51}Cr and a lower release of ^{125}I when compared with the non-activated cells, at 12 hours the percentage release of ^{125}I from the parasite within the activated macrophage was fourfold less than that released by parasites within non-activated cells (9.2% versus 38.3%) and tenfold less than that released from glutaraldehyde-killed organisms phagocytosed by activated macrophages (91.6%). These studies indicate that stasis rather than killing of leishmaniae occurs in the activated macrophage in vitro. Parallel experiments evaluated by the visual counting of leishmaniae within the macrophages support these data. PEC from tuberculin-sensitized guinea pigs activated in vitro by purified protein derivative showed little or no activity against leishmaniae, indicating a specific requirement for this microbial stasis by activated macrophages. As a corollary of this, peritoneal exudate lymphocytes separated from the same preparations of PEC were shown to be specifically reactive to leishmanial antigen by transformation and incorporation of ^3H -thymidine.

INTRODUCTION

The compelling clinical evidence led Garnham and Humphrey [1] to postulate an important role for cell-mediated immunity (CMI) in leishmaniasis. Immunological responses in cutaneous leishmaniasis are characterized by strong delayed hypersensitivity (DH) skin reactions to the leishmanin (Montenegro) skin

* Present address: Plum Island Animal Disease Center, SEA/USDA, Greenport, N.Y., United States of America.

† Present address: School of Veterinary Medicine, University of Cambridge, Cambridge, United Kingdom.

test [2]. Certain patients, however, suffer from a bizarre spreading form of the disease, diffuse cutaneous leishmaniasis (DCL); these persons show evidence of immunological unresponsiveness [3,4] and this anergy is a specific deficiency of CMI to Leishmania antigens. In response to chemotherapy the patient may convert from leishmanin-negative to leishmanin-positive DH response and, in such cases, the prognosis is good [5].

The clear manifestation of CMI events in forms of human leishmaniasis which undergo spontaneous cure are also found in several animal models including Leishmania enriettii infections of the guinea pig. Bryceson et al. [6] reported the appearance of strong DH reactions, antigen-induced blast transformation of lymphocytes and the production of macrophage migration inhibition and mitogenic lymphokines during the course of infection of guinea pigs. Similar studies by Blewett et al. [7] confirmed the important role of CMI in this model.

Leishmania are obligate parasites of macrophages and their dependence on this host cell is indicated by the increased protection conferred on the host when the macrophage population is depleted [8,9]. It is to be expected that immune effector mechanisms might act through the macrophages by their acquiring a resistant state; such a state against Listeria monocytogenes was described by Mackaness [10] in which macrophage activation occurred concurrently with the appearance of DH. Specifically activated macrophages have been reported to acquire the ability to kill a variety of diverse organisms [11]. Miller and Twohy [12] studying the fate of Leishmania donovani in explanted peritoneal macrophages from L. donovani superinfected mice, showed a decrease in parasite numbers in "immune" macrophages; this finding was confirmed by Bradley [13] who reported up to 90% of intracellular parasites destroyed. Various strains of mice show differing susceptibilities to Leishmania, some having a resistance that is considered to be non-immunological in origin. Manuel et al. [14] using the L. enriettii-guinea pig model showed no destruction of leishmaniae in macrophages from immune animals, thus showing that this activated macrophage may not have a significant role in the natural host-parasite relationship.

There are serious limitations in quantifying the in vitro growth of leishmaniae within macrophages by visual means. Macrophages, particularly from the guinea pig, survive poorly in vitro and the assessment of remaining adherent cells may not reflect the total population. Isotope labelling of the cells provides a reliable means of monitoring the health of both parasite and macrophage and an objective evaluation of their interaction. Using these sensitive techniques an effect of activated guinea pig macrophages against L. enriettii can be demonstrated.

MATERIALS AND METHODS

Animals and preparation of guinea pig cells

Outbred Hartley strain female guinea pigs, weighing 300-350 gms were used. Peritoneal exudate cells (PEC) were elicited by the injection, into the peritoneum, of 6 ml of heavy mineral oil and were harvested by trochar and cannula 4 to 6 days later. Cultures of macrophages were prepared by the method described by Van Furth [15]. Minimum essential medium—Eagles suspension cell modification (SMEM) supplemented with 0.22% sodium bicarbonate and 5% inactivated fetal calf serum (IFCS) was used as a cell washing fluid. PEC were passed through adherent columns similar to those described by Rosenstreich and Rosenthal [16] for the separation of peritoneal exudate lymphocytes (PEL).

Organisms and antigen preparation

The strain of Leishmania enriettii was kindly provided by Dr. D. C. Dumpde, Kennedy Institute of Rheumatology, London. Amastigote forms, maintained by animal passage, and low passage promastigote culture forms were stored as stabulates in liquid nitrogen. Amastigotes were isolated from guinea pig ear lesions by the method described by Herrer [17]. Promastigotes were grown in Grace's insect tissue culture medium (GITC) supplemented with 20% fetal calf serum (FCS) and the preparation of single and double cell suspensions for in vitro infection of PEC has been described [18].

Listeria monocytogenes, serotype 36 19115, was obtained from the American Type Culture Collection. It was maintained at 4°C in colony form on NNN medium overlaid with sterile mineral oil. Bacteria were grown in a brain heart infusion broth (BHI) in preparation for in vitro infection of PEC [15] and bacteria were quantified by a combination of a drop plate method and colorimetry [18].

A crude soluble antigen (CSA) was prepared from disrupted promastigotes [19] and its protein content determined by the Lowry method. Solutions of CSA were tested for potency by comparison to a standard intradermal leishmanin test and also titrated for lymphocyte stimulatory activity [18]. Preservative-free purified protein derivative (PPD) was obtained from Parke Davis & Co.

Infection and immunization of animals

Actively growing promastigotes of 4 to 10 passages were harvested from GITC, single cell suspensions prepared and 1×10^6 promastigotes in 0.1 ml Hanks balanced salt solution

(HBSS) were injected intradermally into the dorsal surface of the left ear of normal animals. Guinea pigs were sensitized to PPD by footpad inoculation of Freund's complete adjuvant (FCA) enriched with 0.5 mg of heat-killed Mycobacterium tuberculosis, strain H 37 Ra [18].

Isotope labelling of organisms and PEC

Optimum methods of isotope labelling and measuring for the conditions of these experiments are described in detail elsewhere [18]. Briefly, actively dividing promastigotes, grown for 24 h in fresh GITC containing 5 $\mu\text{Ci/ml}$ of ^{125}I -iodo-deoxyuridine ($^{125}\text{IUdR}$) and 1×10^{-6} M fluoro-deoxyuridine (FUdR), were subsequently washed and kept at 4°C for up to 8 h to allow unbound label to be released. Cultures of L. monocytogenes in fresh BHI broth were incubated with the same concentrations of $^{125}\text{IUdR}$ and FUdR. However, the bacteria were grown for only 1 h before washing and overnight storage at 4°C . PEC and promastigotes (where indicated in text) were suspended in SMEM containing 0.1 $\mu\text{g/ml}$ of ^{51}Cr as sodium chromate and cultured for 1 h at 37°C on a rocking platform in a 5% CO_2 atmosphere. After washing the PEC were allowed to stand at 4°C for at least 30 min to allow unbound label to diffuse away.

Antigen-induced lymphocyte transformation

Lymphocyte stimulation was assessed by the incorporation of tritiated thymidine ($^3\text{HTdR}$) into DNA [20]. CSA and PPD were titrated for both optimum concentration of antigen and optimum time of culture [18]. Two $\times 10^6$ PEL in 2 ml aliquots were cultured for 4 days with antigen at which time 1 μCi of $^3\text{HTdR}$ in 0.1 ml HBSS was added; 18 h later the cells were harvested and the incorporated HTdR was measured by liquid scintillation. Concentrations of 50 $\mu\text{g/ml}$ CSA and 10 $\mu\text{g/ml}$ PPD were used to demonstrate lymphocyte reactivity in vitro.

Activation and infection of PEC in vitro

PEC were harvested from convalescent and normal guinea pigs. All animals were tested for delayed skin sensitivity; convalescent animals were judged acceptable donors if they showed high sensitivity and normal animals if they had negative reactions. PEC were divided into two parts, one part incubated with antigen (activated), the other without antigen (non-activated) for 24 or 48 h; antigens used were CSA at 50 $\mu\text{g/ml}$ and PPD at 10 $\mu\text{g/ml}$. The cells were then labelled with ^{51}Cr and infected with $^{125}\text{IUdR}$ labelled organisms. In all experiments aliquots of labelled organisms were incubated separately in the same medium.

An optimum multiplicity of infection (MOI) of 1 or less promastigote per PEC was determined [18]. Promastigotes and PEC were incubated together for 1 h at 37°C after which they were washed and centrifuged at 110 X g for 5 min to obtain a macrophage-rich population. These populations were counted and dispensed as 2-ml aliquots containing 1×10^6 PEC into 12-20 replicate tubes. Samples for scintillation counting were taken from these tubes after incubation of 1½ to 18 h. For optical microscope evaluation of macrophage activity, infected PEC were prepared in the same way but 5×10^5 cells were dispensed into the chambers of a 24-well culture plate¹, the resulting adherent cells were washed and stained with Giemsa at 1½, 24 and 48 h. For these experiments, a MOI of 3 to 1 was used, for lower MOI's produced, in many cases, a visually undetectable infection. A MOI of 10 to 1 L. monocytogenes to PEC was chosen, normal PEC generally resisted this level of infection over a period of 12 h [18].

In all experiments the final suspensions of organisms and PEC including periods of activation and infection were made in RPMI 1640 medium supplemented with 25 mM Hepes buffer, 20% IFCS and 200 u of potassium penicillin and 200 µg streptomycin sulphate per ml. Antibiotics were excluded during L. monocytogenes infection and in initial experiments with the bacteria (see text).

Data presentation

The data relating to the release of isotope from cells were treated by two means. The first was a simple proportion representing the amount released over the period of time during which the cells were being monitored and was expressed as the percentage of the total amount of isotope which was initially associated with the cells.

The second method applied to the parasitized cell in which the parasite and the host cell were labelled with the different isotopes. The amount of cell-associated isotopes were measured and were expressed as the following ratio:

$$\frac{\text{CPM cell associated } ^{125}\text{I}}{\text{CPM cell associated } ^{51}\text{Cr}}$$

The initial ratio was determined by sampling at 1½ h and later samplings indicated any change in the value of this ratio. The amounts of cell associated ^{125}I and ^{51}Cr were quantitative measurements of the number of parasites and host cells respectively therefore, if the value of the ratio decreased during

¹ Falcon Labware, Oxnard, CA 93030, United States of America.

incubation, it meant that isotope associated parasite material was being preferentially lost from the cells and, conversely, if the ratio increased, isotope-associated host cell material was preferentially released. The real values of the $1\frac{1}{2}$ h ratio varied between experiments for they were dependent on the relative numbers of parasites and host cells, degree of isotope labelling of each, and the specific activity of the isotopes used. All of these would be expected to differ between different cell populations and different isotope batches. In order to compare different experiments the $1\frac{1}{2}$ h value was considered as unity and all subsequent values were expressed as the factor by which unity has changed. This is achieved mathematically, by dividing all ratio values in each experiment by their respective $1\frac{1}{2}$ h value, thus the $1\frac{1}{2}$ h value becomes 1 and subsequent sample values show the relative change from 1.

Statistical analysis

Student t-test was used to evaluate the significance of the data.

RESULTS

Series I. Isotope release from labelled *Leishmania* and *Listeria* within unlabelled macrophages

In order to determine the fate of leishmaniae within activated macrophages PEC from guinea pigs were infected in vitro with isotope-labelled promastigotes and the subsequent release of isotope measured.

Three replicate pairs of animals comprised of one convalescent (C) animal recovering from a leishmanial infection and one normal (N) animal, were used (Expt. 1). PEC obtained from these animals were incubated with (+) or without (-) CSA for 24 h. After infection the following mean percentages of total ^{51}Cr were released over a period of 18 h:

N	PEC + CSA	50.0% (47.3-53.3%) ²
N	PEC - CSA	50.7% (49.1-52.5%)
C	PEC + CSA	43.6% (39.9-47.1%)
C	PEC - CSA	50.8% (45.9-52.9%)

These results showed no marked differences in release of ^{51}Cr from the leishmaniae within the different populations of macrophages.

² Range in parenthesis.

Failure to show enhanced lysis and excretion of leishmanial components by stimulated macrophages raised the question of whether the macrophages were, in fact, activated. In Experiment 2 macrophages, from 3 further pairs of guinea pigs, were additionally infected with ^{125}I -labelled Listeria monocytogenes as a positive control for intracellular killing. Similar percentages of ^{51}Cr Leishmania label were released at 18 h as in Experiment 1, in addition the excretion of ^{125}I -L. monocytogenes label was of the same order and was as follows:

N	PEC + CSA	40.5% (36.6-45.1%)
N	PEC - CSA	39.4% (35.8-43.1%)
C	PEC + CSA	46.1% (39.4-53.9%)
C	PEC - CSA	42.3% (36.7-47.5%)

These results failed to show activated macrophages releasing significantly greater amounts of isotope from Leishmania, however there was a slightly enhanced release from Listeria.

In order to determine whether a longer period of activation would enhance lysis and isotope release PEC, from two convalescent animals, were incubated with CSA for 48 h. In this experiment (3) both leishmaniae and L. monocytogenes were labelled with ^{125}I and equal aliquots of the activated and nonactivated PEC were infected separately with one of the two organisms. Separate infection was adopted because Experiment 2 had shown that doubly infected activated macrophages failed to kill and release significantly larger amounts of labelled L. monocytogenes components. This failure might be a result of overloading of the macrophages or of interference by intracellular leishmania with the normal destruction of L. monocytogenes. In addition, antibiotics were added to the culture medium to prevent extracellular replication of Listeria. Samples were taken at 12 h; 48-h activation also did not enhance the release of leishmania-associated isotope. In contrast, the effect on L. monocytogenes isotope release was marked:

C	PEC + CSA	69.0% (76.1-61.9%)
C	PEC - CSA	55.8% (51.6-59.9%)

These data showed that, irrespective of the state of activation, macrophages released similar amounts of labelled organism components at 12 and 18 h with the exception of L. monocytogenes within stimulated cells cultured in the presence of antibiotics.

The presence of antibiotics may have affected the intracellular Listeria enhancing the release of isotope shown in Experiment 3. Consequently an experiment (4) was designed to

TABLE I. TWENTY-FOUR HOUR STIMULATION OF MACROPHAGE ACTIVITY AGAINST ^{125}I -LABELLED *Leishmania* WITHIN ^{51}Cr -LABELLED MACROPHAGES BY CRUDE SOLUBLE *Leishmania* ANTIGEN (CSA)

Experiment No. (hours sampled)	<i>Leishmania</i> -infected macrophages					¹²⁵ I- <i>Leishmania</i> Change in ratio ⁵¹ Cr-macrophage				
	Mean %	¹²⁵ I-release		Mean %	Cr-release	Mean	± se	Mean	± se	p < ^a
	<i>Leishmania</i> control	+ CSA	- CSA	+ CSA	- CSA					
(6-8)										
1	10.4	36.1	54.4	34.0	24.3	0.84	0.05	0.55	0.02	0.001
2	5.4	25.6	33.5	36.0	27.1	0.96	0.13	0.78	0.01	0.01
3	11.9	27.8	43.7	37.5	26.5	0.81	0.03	0.62	0.02	0.001
4	12.5	21.4	31.9	36.5	25.5	0.85	0.04	0.62	0.06	0.001
(12-15)										
1	17.6	49.7	65.3	40.4	32.4	0.73	0.05	0.51	0.03	0.001
2	12.5	37.1	44.6	43.8	34.4	0.91	0.15	0.76	0.02	0.05
3	24.2	54.2	58.9	43.4	32.1	0.39	0.06	0.37	0.06	NS ^b
4	36.3	46.4	68.6	42.5	35.5	0.5	0.03	0.42	0.01	0.001

^a p = Probability -- Student t test.^b NS = Not significant.

find how the macrophages digested killed organisms. ^{125}I -labelled promastigotes and *L. monocytogenes* were killed by treatment with glutaraldehyde, PEC stimulation time was reduced to 24 h and samples taken at 12 h after the addition of the dead organisms. Lysis of the organisms was rapid, from the killed leishmaniae these were;

C	PEC + CSA	91.6%
C	PEC - CSA	75.4%

and from killed *Listeria*:

C	PEC + CSA	93.6%
C	PEC - CSA	89.7%

These percentages are approximately twice the amounts released from live organisms (Experiments 1 and 2).

Activity by stimulated macrophages against *L. monocytogenes* was variable, however in all experiments there was a consistently lower release of isotope from leishmaniae within activated macrophages than from non-activated or normal macrophages.

Series II. Isotope release from ^{125}I -labelled *Leishmania* and ^{51}Cr -labelled macrophages

Previous work (Grocock, unpublished) had indicated that guinea pig macrophage populations in vitro could be very labile and failure to demonstrate microbicidal activity might be a result of loss of function of the cells prior to death. Thus it was necessary to monitor the health of the cells, and this was done by the routine labelling of PEC with ^{51}Cr . PEC were again prepared from single convalescent animals, incubated with or without CSA for 24 h or 48 h and then challenged with ^{125}I -labelled *L. monocytogenes* or leishmaniae. Isotope release was measured from both PEC and organism.

The results, presented in Tables I and II, are expressed as the percentage of isotope release and as the change in the cell-associated ratio of the two isotopes over a period of incubation of 1½ to 15 h.

Experiments 1-4, Table I, are representative of ten experiments. In these four experiments aliquots of PEL were also separated from the PEC and prepared for culture with CSA for in vitro lymphocyte transformation studies. These sensitized PEL were reactive to CSA showing a mean stimulation index of 7.1, range 5.6 - 8.3. The change in ratio of cell-associated isotopes shown in Table I is significantly less between PEC incubated with CSA and PEC incubated without CSA.

TABLE II. FORTY-EIGHT-HOUR STIMULATION OF MACROPHAGE ACTIVITY AGAINST ^{125}I -LABELLED *Leishmania* WITHIN ^{51}Cr -LABELLED MACROPHAGES BY CRUDE SOLUBLE *Leishmania* ANTIGEN (CSA)

Experiment No. (hours sampled)	<i>Leishmania</i> -infected macrophages					Change in ratio $\frac{^{125}\text{I}-\text{Leishmania}}{^{51}\text{Cr-macrophage}}$				p < ^a	
	Mean %		^{125}I -release		Mean %	^{51}Cr -release		+ CSA	- CSA		
	<i>Leishmania</i> control		+ CSA	- CSA	+ CSA	- CSA					
(12)								Mean	± se	Mean	± se
1	29.6	65.1	71.2	28.2	26.1	0.46	0.04	0.51	0.09	NS ^b	
2	20.6	58.7	73.1	36.4	26.8	0.65	0.08	0.44	0.02	0.001	
3	2.8	9.2	38.3	36.5	32.2	1.42	0.04	0.8	0.05	0.001	
4	3.0	13.5	59.8	37.8	40.2	1.3	0.1	0.47	0.08	0.001	

^a p = Probability — Student test.^b NS = Not significant.

In 15 out of 16 samplings stimulated macrophages excreted greater percentages of ^{51}Cr but, more importantly, they excreted consistently smaller percentages of ^{125}I leishmania label. Table II shows the results of similar experiments, in which the prior stimulation period was increased to 48 h. These results are essentially similar to those of Table I although the difference in percentage ^{125}I release is greater and is fourfold in Experiments 3 and 4. In addition these populations of stimulated and unstimulated macrophages were challenged under identical conditions with ^{125}I -labelled L. monocytogenes. At 12 h the release of ^{125}I was similar in all populations (76.7 - 85.4%) and the differences in change of ratio of cell-associated isotopes were nonsignificant.

Series III. Isotope release from ^{125}I -labelled Leishmania within Mycobacteria-activated macrophages

To achieve a high state of macrophage activation in order to kill intracellular leishmania, macrophages from tuberculin (PPD) sensitive guinea pigs were used; animals were sensitized by injection of killed Mycobacterium tuberculosis and their PEC stimulated in vitro by incubation with PPD. Table III shows the results of three representative experiments. Similar percentages of ^{125}I were excreted by activated and non-activated PEC and the differences in change of ratio of cell-associated isotopes were nonsignificant. PEL prepared from these PEC were, nevertheless, highly reactive to PPD, showing a mean lymphocyte stimulation index of 16.3 (range 10.4 - 19.6). Further stimulation of PEC for 48 h with PPD did not effect the percentage release (39.2 and 42.1%) of ^{125}I nor were the change in ratios significant. All these populations of 24 and 48 h-treated PEC were challenged with ^{125}I L. monocytogenes, PEC incubated with or without PPD excreted similar amounts of ^{125}I at 12 h (67.1 - 71.8%) and changes in ratio were again nonsignificant.

Series IV. Microscopic evaluation of Leishmania survival in macrophages

The preceding experiments indicated that leishmaniae survived in activated macrophages in a changed state, rather than being lysed and their associated isotopes being rapidly released. In order to confirm this survival, isotope-labelled leishmaniae and macrophages from four convalescent animals were evaluated by visual counting. Table IV shows the survival of the organism in all populations of macrophages; in addition there is an apparent two to threefold increase in non-activated macrophages.

TABLE III. TWENTY-FOUR-HOUR STIMULATION OF MACROPHAGE ACTIVITY AGAINST ^{125}I -LABELLED *Leishmania* WITHIN ^{51}Cr -LABELLED MACROPHAGES BY PURIFIED PROTEIN DERIVATIVE (PPD)

Experiment No. (hours sampled)	<i>Leishmania</i> -infected macrophages				Change in ratio $\frac{^{125}\text{I}-\text{Leishmania}}{^{51}\text{Cr-macrophage}}$			
	Mean %	^{125}I -release	Mean %	^{51}Cr -release				
	<i>Leishmania</i> control	+ PPD	- PPD	+ PPD	+ PPD	- PPD	+ PPD	- PPD
(12)					Mean	Mean	Mean	Mean
1	20.0	46.6	43.4	27.7	31.9	31.9	0.17	0.16
2	15.4	33.1	34.2	28.8	26.7	26.7	0.22	0.11
3	16.5	15.3	15.1	25.2	20.9	20.9	0.02	0.21
								NS

^a p = Probability -- Student t test.^b NS = Not significant.

TABLE IV. TWENTY-FOUR-HOUR STIMULATION OF MACROPHAGE ACTIVITY AGAINST THE GROWTH OF *Leishmania*^a WITHIN MACROPHAGES BY CRUDE SOLUBLE *Leishmania* ANTIGEN (CSA)

Experiment No.	CSA	Number of leishmaniae per 100 macrophages ^b					
		1½ M	hour ^c range	24 M	hour ^c range	48 M	hour ^c range
1	+	2	1-2	2	1-3	3	1-5
	-	2	1-3	4	1-5	5	3-8
2	+	2	1-2	3	2-4	4	2-8
	-	2	1-3	2	1-5	4	1-8
3	+	2	2-3	3	1-5	2	1-3
	-	3	1-4	5	4-8	6	4-7
4	+	2	1-2	3	1-6	2	1-2
	-	2	1-2	1	1-2	4	2-8

^a Leishmaniae and PEC were labelled with isotopes as in previous experiments.

^b Data presented to the nearest whole number. Mean (M) and range of counts of 400 to 500 PEC in each of three replicate cultures.

^c Time sampled after infection.

DISCUSSION

The role of activated macrophages in the destruction of many phylogenetically diverse micro-organisms is firmly established [21] and this immune effector mechanism is of major importance in the control of obligatory intracellular protozoa such as *Toxoplasma* [22,23] and *Trypanosoma cruzi* [24,25]. Miller and Twohy [12] noted a marked difference in the number of surviving *L. donovani* amastigotes within "immune", compared to normal, macrophages. This was interpreted as either destruction or inhibition of replication; the latter interpretation was confirmed by Bradley [26]. More recently the in vitro destruction of *L. tropica* within mouse macrophages has been reported [27]. *L. enriettii* is also destroyed in activated mouse macrophages [28] but not in activated macrophages of its natural host [14]. This destruction however is nonspecific as it can be induced by mitogen stimulated lymphocytes [29] and it is suggested that this is a manifestation of the natural

resistance of the mouse to L. enriettii rather than an acquired cellular immune response.

In this report the excretion of significantly different amounts of parasite-associated isotope label indicates that activated guinea pig macrophages inhibit the metabolism of L. enriettii, rather than killing the parasite. Previous work by the authors (unpublished data) has shown that actively dividing ^{125}I UdR-labelled promastigote cultures will rid themselves of isotope over a period of 6 to 7 days as their initial DNA is progressively catabolized. The characteristics of release of label from Leishmania added to unstimulated macrophages are essentially the same [18]. In the first few hours there is a burst of release due to the death of organisms in the inocula which are unable, either through damage in preparation or biological unfitness, to maintain themselves within the macrophage. This early death has also been reported for Toxoplasma, in which 50 to 80% of the inoculum is destroyed in the first few hours [22,30] and for L. tropica promastigote inocula in vivo [31]. When L. enriettii is deliberately compromised, i.e. by glutaraldehyde, this initial rate continues with 70 to 90% release at 12 h (Series I, Expt. 4); these rates are comparable to the digestion and excretion of ^{125}I -iodinated human serum albumin and bovine gamma globulin in mouse macrophages [32]. Isotope also continues to be released from a live inoculum but at a lower rate of 30-40% at 12 h (Table I, Expts. 1-4). Within the activated macrophage, the rate is even less and may be as low as 9-14% (Table II, Expts. 3 and 4); this is significantly different from the release from unstimulated macrophages and very different from the release from killed parasites thus indicating that the organisms are static rather than killed. The visual enumeration of similar macrophage cultures (Table IV) strengthens this interpretation. Though there is considerable overlap in the data, the results show an increasing number of parasites within unstimulated macrophages over a period of 48 h indicating that they are dividing and hence metabolically active. More importantly, however, parasites also survive within the activated macrophage during the period of observation but in approximately constant numbers.

It is possible that the level of macrophage activation was insufficient to kill L. enriettii. Experiments using Mycobacterium-sensitized PEC were designed to test this point (Series III, Table III), however such PPD-stimulated PEC had no significant affect against L. enriettii even though sensitized PEC were highly reactive with a stimulation index of 10.4 - 19.6. Though serological cross reactivity has been reported between Leishmania and Mycobacterium [33], the inhibitory effect shown by these data required specific

induction by CSA presumably mediated by a specific arming factor [34,35].

The use of Listeria monocytogenes as a positive control of microbicidal activity was inconclusive. No significant differences were seen in release of ^{125}I -Listeria label (Series I, Expt. 2) and, if antibiotics were added to the cultures in order to prevent extracellular replication (Series I, Expt. 3), rates of release were similar to that from glutaraldehyde-treated bacteria; parallel studies (unpublished data) enumerating Listeria by colony counts showed little survival in any macrophage cultures. Hoff and Frenkel [36] suggest that Listeria may be unsuitable as an indicator of macrophage activation due to its nonspecific and high susceptibility to macrophage activity. In addition it has been reported that intracellular killing of Listeria by macrophages in vitro is greatly influenced by antibiotics in the culture medium [14,37].

In spite of failure to show activation of macrophages independently of their effect on L. enriettii there is additional evidence that they were, in fact, activated. PEL were shown to be reactive to CSA and presumably, under identical conditions, would release macrophage arming or activating factors. PEC previously incubated with CSA showed higher initial ratios of cell associated ^{125}I to ^{51}Cr indicating increased phagocytosis [6,18]. These macrophages also excreted greater amounts of ^{51}Cr from themselves and from killed organisms when compared to unstimulated macrophages indicating increased rates of metabolism and digestion (Series I, Expt. 4). These observations, in conjunction with the marked difference in effect on the parasite, show that specifically activated macrophages have a microbiostatic effect on intracellular L. enriettii. This view of inhibition of multiplication being a major role of the activated macrophage has been proposed in the control of other microorganisms for instance, vaccinia virus [38,39], mycobacterium [40,41] and *Toxoplasma* [42,43].

REFERENCES

- [1] GARNHAM, P.C.C., HUMPHREY, J.H., Curr. Top. Microbiol. Immunol. 48 (1969) 29.
- [2] DOSTROWSKY, A.F., SAGHER, F., Am. Trop. Med. Parasitol. 40 (1945) 265.
- [3] DESTOMBES, P., Bull. Soc. Path. Exot. 53 (1960) 299.
- [4] CONVIT, J., KERDEL-VEGAS, F., Arch. Dermatol. 91 (1965) 439.
- [5] BRYCESON, A.D.M., Trans. R. Soc. Trop. Med. Hyg. 64 (1970) 369.
- [6] BRYCESON, A.D.M., BRAY, R.S., WOLSTENCROFT, R.A., DUMONDE, D.C., Clin. Exp. Immunol. 7 (1970) 301.
- [7] BLEWETT, T.M., KADIVAR, D.M., SOULSBY, E.J.L., Am. J. Trop. Med. Hyg. 20 (1971) 546.

- [8] BRYCESON, A.D.M., PRESTON, P.M., BRAY, R.S., DUMONDE, D.C., Clin. Exp. Immunol. **10** (1972) 305.
- [9] SHAW, J.J., VOLLER, A., Am. Trop. Med. Parasitol. **62** (1968) 174.
- [10] MACKANESS, G.B., J. Exp. Med. **116** (1962) 381.
- [11] NELSON, D.S., "Macrophages as effectors of cell-mediated immunity", Critical Reviews in Microbiology (LASKIN, A.I., LECHEVALIER, M., Eds), The Chemical Rubber Co., Cleveland (1972) 353.
- [12] MILLER, H.C., TWOHY, D.W., J. Parasitol. **55** (1969) 200.
- [13] BRADLEY, D.J., Trans. R. Soc. Trop. Med. Hyg. **65** (1971) 366.
- [14] MAUEL, J., BEHIN, R., BIROUM-NOERJASIN, ROWE, D.S., Clin. Exp. Immunol. **20** (1975) 339.
- [15] VAN FURTH, R., VAN ZWET, T., "In vitro determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes", Handbook of Experimental Immunology, 2nd Edn (WEIR, D.M., Ed.), Blackwell Scientific Publications, Oxford (1973) 36.1.
- [16] ROSENSTREICH, D.L., ROSENTHAL, A.S., J. Immunol. **110** (1973) 934.
- [17] HERRER, A., THATCHER, V.E., JOHNSON, C.M., J. Parasitol. **52** (1966) 954.
- [18] GROOCCOCK, C.M., Immune Mechanisms of Resistance of the Guinea Pig infected with *Leishmania enriettii*, PhD thesis, University of Pennsylvania (1976).
- [19] BRAY, R.S., LAINSON, R., Trans. R. Soc. Trop. Med. Hyg. **60** (1966) 605.
- [20] OPPENHEIM, J.J., WOLSTENCROFT, R.A., GELL, P.G.M., Immunology **12** (1967) 89.
- [21] BLANDEN, R.V., HAPPEL, A.J., DOHERTY, P.C., ZINKERNAGEL, R.M., Immunobiology of the Macrophage (NELSON, D.S., Ed.), Academic Press, New York (1976) 367.
- [22] ANDERSON, S.E., REMINGTON, J.S., J. Exp. Med. **139** (1974) 1154.
- [23] McLEOD, R., REMINGTON, J.S., Am. J. Trop. Med. Hyg. **26** (1977) 170.
- [24] HOFF, R., J. Exp. Med. **142** (1975) 299.
- [25] NOGUEIRA, N., COHN, Z., Am. J. Trop. Med. Hyg. **26** (1977) 194.
- [26] BRADLEY, D.J., Personal communication; Cell-mediated and Humoral Immunity to Protozoan Infections (MAUEL, J., BEHIN, R., Eds), Transplant Rev. **19** (1974) 129.
- [27] HANDMAN, E., SPIRA, D.T., Z. Parasitenkd. **53** (1977) 75.
- [28] BEHIN, R., MAUEL, J., BIROUM-NOERJASIN, ROWE, D.S., Clin. Exp. Immunol. **20** (1975) 351.
- [29] MAUEL, J., BUCHMÜLLER, Y., BEHIN, R., J. Exp. Med. **148** (1978) 393.
- [30] JONES, T.C., YEH, S., HIRSCH, J.G., J. Exp. Med. **136** (1972) 1157.
- [31] ZUCKERMAN, A., Acta Med. Orient. **12** (1953) 238.
- [32] EHRENREICH, B.A., COHN, Z.A., J. Exp. Med. **126** (1967) 941.
- [33] SENGUPJA, P.C., Bull. Calcutta School Trop. Med. **16** (1968) 126.
- [34] EVANS, R., COX, H., ALEXANDER, P., Proc. Soc. Exp. Biol. Med. **143** (1973) 256.
- [35] LINDBERG, R.E., FRENKEL, J.V., Infect. Immun. **15** (1977) 855.
- [36] HOFF, R.L., FRENKEL, J.K., J. Exp. Med. **139**.
- [37] COLE, P., BROSTOFF, J., Nature (London) **256** (1975) 515.
- [38] SILVERSTEIN, S.C., Sem. Hemat. **7** (1970) 185.
- [39] MORAHAN, P.S., GLASGOW, L.A., CRANE, J.L., KERN, E.R., Cell Immunol. **28** (1977) 404.
- [40] SUTER, E., J. Exp. Med. **97** (1953) 235.
- [41] KLUN, C.L., YOUNG, G.P., J. Reticuloendothel. Soc. **13** (1973) 263.
- [42] JONES, T.C., MASUR, H., LEN, L., FU, T.L.T., Am. J. Trop. Med. Hyg. **26** (1977) 187.
- [43] BORGES, J.S., JOHNSON, W.D., Jr., J. Exp. Med. **141** (1975) 483.

DISCUSSION

I.R. TIZARD: Did you observe any morphological or other changes in your activated macrophages?

C.M. GROOCKOCK: No, all populations of macrophages were elicited with oil and were consequently non-specifically stimulated. They all appeared well spread with foamy cytoplasm. The activated macrophages had higher amounts of *Leishmania* isotope initially associated with them, which indicates increased phagocytosis; they also released greater amounts of isotope from killed organisms, which indicates faster breakdown of phagocytosed material.

R.S. PHILLIPS: How did you measure the release of isotope from the macrophages and parasites?

C.M. GROOCKOCK: After sampling the tubes were centrifuged gently (110 g for 5 min) and half the supernatant withdrawn. This and the tube containing the other half of the supernatant and cells were both measured. Thus, both total release and cell-associated isotopes were measured.

R.S. PHILLIPS: Why do you think there was stasis of the *Leishmania* in macrophages activated by culturing sensitized peritoneal cells with leishmanial antigen but not in macrophages activated by the incubation of sensitized cells with purified protein derivative?

C.M. GROOCKOCK: There may be a specific macrophage arming factor, perhaps similar to that which has been demonstrated to inhibit the growth of tumour cells [1] or, in a more closely related situation, to inhibit the multiplication of *Toxoplasma* within mouse macrophages [2]. The factor behaves rather like cytophilic antibody. In the invagination and formation of the parasitophorous vacuole (PV) the membranes of the parasite and PV might remain linked, thus altering their normal association. In other work we have shown that pretreatment of promastigotes with convalescent serum compromises their survival within macrophages. In the experiments under discussion the possibility of cytophilic antibody activity is not excluded.

R.S. PHILLIPS: Did you check the viability of the amastigotes in a condition of stasis by putting them into culture and seeing whether they changed into promastigotes?

C.M. GROOCKOCK: No, this was not done. It would have been interesting to know whether they were permanently compromised or not.

J.D. DARGIE (*Scientific Secretary*): Could you describe your ^{51}Cr -release assay? Were your precipitates not washed?

C.M. GROOCKOCK: No, the volume of the cell suspension in each tube was known. After centrifuging, half the volume was sampled and with its companion was assayed for radioactivity. Twice the number of counts per minute (counts/min) for the first sample gave the amount released; the counts/min for the second sample minus the first gave cell-associated counts/min. After a period of culture

the macrophages were increasingly susceptible to mechanical damage, and any advantage gained by washing the cells at this stage would have been countered by the isotope release resulting from washing; consequently all samples were treated in the same way, whether harvested at $1\frac{1}{2}$ hours or 12 hours.

PATTERNS OF DEVELOPMENT OF TRYPANOSOMES AND RELATED PARASITES IN INSECT HOSTS

D.H. MOLYNEUX
Department of Biology,
University of Salford,
Salford,
United Kingdom

Abstract

PATTERNS OF DEVELOPMENT OF TRYPANOSOMES AND RELATED PARASITES IN INSECT HOSTS.

The trypanosome parasites of man and his domestic animals and the closely related *Leishmania* parasites pathogenic to man have widely different patterns of development in their various vector species. However, certain common features of the development of these parasites are exhibited when they develop in insects. These features include temporary storage in the crop; transformation from mammalian forms to primary multiplicative forms; avoidance of digestion by host enzymes; association with the peritrophic membrane; establishment of infection and, associated with this, attachment and colonization of surfaces; migration to different areas of gut to sites of development; formation of a reservoir of forms to ensure sufficient organisms are available for transformation to forms infective to the vertebrate host; subsequent transmission by bite or by contamination of host surfaces. The different features of development outlined above are discussed in relation to trypanosomes and related parasites. The utilization of different model systems for use in this type of study are discussed in view of difficulties in obtaining adequate numbers of infected flies (e.g. *Glossina*, or sandflies), and the costs and frequent problems of maintaining such colonies. Recent studies (1) on *Glossina*-transmitted Salivarian trypanosomes are described which indicate possible behavioural differences between infected and uninfected flies that have a bearing on epidemiology and epizootiology; (2) on the fluid mechanics of the *Glossina* labrum infected and uninfected with trypanosomes; and (3) on attachment of trypanosomes and *Leishmania* to insect gut wall surfaces.

INTRODUCTION

The mammalian pathogenic trypanosomes and the *Leishmania* parasites are members of a group of organisms comprising the order Kinetoplastida, the characteristic feature of which is the presence of an extra nuclear DNA-containing body, the kinetoplast. Although many members of this group do have an economic or social significance, most of the parasitic forms (family Trypanosomatidae) have no such importance and are of apparent significance only in comparative terms or in pure research. This situation probably reflects the

antiquity and success of the group and the wide range of invertebrate (probably insects) parasites which have subsequently adopted digenetic life cycles.

The variety of life cycles and vectors displayed by the group has been reviewed in [1] a paper which represented an attempt to synthesize available knowledge on host-parasite relationships in both digenetic and monoxenous trypanosomatid parasites. This report concentrates on work which has been published since 1977 and presents results obtained recently which may have significance in transmission of trypanosomiasis and have a bearing on epidemiology or epizootiology of the disease. An attempt is also made to generalize about life cycle patterns in these organisms using examples from both non-pathogenic and pathogenic species; although this may appear difficult bearing in mind the wide range of vector species belonging to different groups under consideration, certain features enable valuable parallels to be drawn and it is these common features on which this study will concentrate.

In the development of trypanosomes and *Leishmania* parasites in invertebrate hosts common features include (1) temporary storage in crop; (2) transformation from mammalian form to primary multiplicative form; (3) avoidance of digestion by host enzymes; (4) association with a peritrophic membrane; (5) establishment of infection and attachment and colonization of surfaces; (6) migration to different areas of gut and to other development sites; (7) formation of a reservoir of forms to ensure transmission of forms infective to vertebrate host; (8) successful transmission by bite or contamination. Some of the above features also apply to monoxenous insect flagellates of the genera *Herpetomonas*, *Leptomonas*, *Crithidia* and *Blastocrithidia* which have been used as model systems to enable a greater understanding of host-parasite relationships [2-4]. Discussion of the above common features will draw on examples from all appropriate host-parasite systems to emphasize similarities in development patterns.

STORAGE IN CROP: PERITROPHIC MEMBRANE GROWTH

It has been known for some years [5] that if *Glossina* was to have the highest chance of becoming infected with trypanosomes of the subgenus *Trypanozoon* then it must feed within 24 hours of emergence from the pupa. It was believed that this was associated with the ability of parasites to penetrate the peritrophic membrane [6]. However, studies [7] have recently indicated that the barrier to establishment, although related to the peritrophic membrane, is a result of the time the blood is stored by *Glossina* in the crop. In newly emerged flies the peritrophic membrane is not fully formed nor does it extend for its full length [7]. Harmsen studying *Glossina pallidipes* found that the peritrophic membrane grew at the rate of 1 mm/h for 30 hours; after 30 hours the growth rate declined. If the fly fed between 30-80 hours the original growth

rate of the peritrophic membrane was resumed. Harmsen [7] suggested that the amount of blood reaching the midgut was dependent on its length as breaking of the peritrophic membrane never occurred. In young flies, where the peritrophic membrane was not fully formed, more blood would be stored for longer periods in the crop until it could be transferred to the midgut. Harmsen [7] suggested that either the slow flow of blood from the crop enabled penetration of the soft region of the peritrophic membrane, or that the time parasites spend in the crop enabled a biochemical pre-adaptation to occur so that survival in the fly is more likely. Harmsen favoured the latter explanation after finding Glucose-6-phosphatase levels changed after 1 h in the crop. This sojourn in the crop could only occur when the peritrophic membrane was poorly developed; this means within 24 hours after emergence from the pupa. Harmsen's view is borne out by more recent work of Jenni [8] who found that by reducing the temperature at which flies were kept, immediately after the first blood meal, then increased infection rates of various *Trypanozoon* isolates could be obtained; however, Jenni also fed flies on mice whose skin had been sterilized to reduce bacterial infection in experimental flies, and on mice whose infection had just passed the peak of parasitaemia. These were factors which may have also raised the infection rates in addition to the technique of cooling the flies after feeding.

Although further studies would seem to be required, it is clear that the 24-h period after emergence is critical if a *Glossina* is to become infected. It is important that a feed should be obtained within this period and from an infected host, if a *Trypanozoon* infection is to have the best chance of further development involving establishment, migration and finally transmission. The ability of *Glossina* to become infected with *Duttonella* and *Nannomonas* is not recognized as being related to the time of infective feed.

TRANSFORMATION

The successful establishment of a parasite in a vector is dependent on the physiological ability of the organism to transform under the conditions pertaining. A situation that illustrates this is described above but in *Trypanozoon* in *Glossina* where mature infection rates are so low that barriers seem to be present which require a complex of changes and suitable factors to operate before the infections become established, and are capable of being transmitted. However, in other trypanosome or *Leishmania*/insect relationships high levels of infection and infection rates are obtained. This is particularly the case in the Stercoraria section of mammalian trypanosomes (subgenera *Schizotrypanum*, *Herpetosoma* and *Megatrypanum*) where infection rates of up to 100% are obtained if the appropriate vector feeds on an infected mammal [1, 9]. However, all these successful and long-standing host-parasite relationships involve physiological,

accompanied by morphological changes by the parasite, on its uptake by the insect. These are an essential prerequisite for cyclical transmission where multiplication of epimastigotes, in the case of trypanosomes, or promastigotes in *Leishmania*, are essential for colonization of the gut, and if subsequent retransformations to forms infective to the vertebrate host are to occur. Such transformations have been studied intensively in *Trypanozoon* in *Glossina* where the biochemical pathways have been elucidated which indicate marked physiological differences between mammalian and insect forms. These differences reflect the different conditions found in the two hosts and emphasize the adaptability of these parasites to rapidly changing micro-environments.

In *Trypanozoon* the change is from a non-Krebs cycle respiratory pathway in the mammalian host where the mitochondrial structure is regressed and only partially functional. In the vector, however, the mitochondrion of *Trypanozoon* is well developed and is capable of functioning via a Krebs cycle. This is associated with the need to utilize as efficiently as possible the available oxygen in the insect whereas in the mammalian tissues oxygen is more freely available. Bohringer and Hecker [10] have found the mean mitochondrial volume of trypomastigotes and epimastigotes in the vector to be $12 \mu\text{m}^3$ whereas in the metacyclic infective trypomastigotes and in slender and stumpy haematozoic trypomastigotes the mean volume of the mitochondrion is 1.4, 0.3 and $3.3 \mu\text{m}^3$ respectively. Vickerman [11, 12] first associated the change during transformation of morphological form with mitochondrial changes in *Trypanozoon*. Subsequently, associated changes in the cell surface coat have been observed [13]; this cell coat, which in the mammalian forms is associated with the outer unit membrane, is made up of glycoproteins of molecular weight around 65 000 and represents the variable antigen [14, 15], and is lost in the insect host until it is re-acquired by the metacyclic trypomastigotes in the salivary glands [16]. Similar mitochondrial changes are observed in *Glossina*-transmitted subgenera *Nannomonas* and *Duttonella* but less information is available on the changes which occur [13, 15]. In addition, in *Stercoraria* trypanosomes and *Leishmania*, although some evidence is available of structural changes associated with transformation, physiological changes, although they occur, do not seem to be as extreme or as well documented as in *Trypanozoon*.

EFFECTS OF HOST ENZYMES IN PARASITES

Although evidence is available on the enzymes present in the midgut of vectors of trypanosomes and *Leishmania* [17], particularly *Glossina*, little if any information is available on the effects of these enzymes on the parasites themselves. It is clear that if a parasite is to be successful in a particular host it must have adopted methods of avoiding the action of the digestive enzymes which are

responsible for digesting the very medium which, in the mammal it parasitizes, serves as its chosen environment. It is possible that these enzymes act as triggers for parasite transformations to forms which are capable of avoiding digestion or stimulate behavioural responses on the part of the parasite, e.g. penetration or circumvention of the peritrophic membrane, entry into gut walls or migration to the hind or fore gut where digestive activity will not affect it. Whatever the mechanisms involved, further studies on these aspects are required.

ASSOCIATION WITH PERITROPHIC MEMBRANE

Much attention has been focused (see above) on the peritrophic membrane of *Glossina* in its role in the life cycle of *Trypanozoon*. Recent studies [18] have shown that, although early workers [6] considered that trypanosomes acquired access to the ectoperitrophic space in the soft part near the point of its secretion [16], evidence [18] suggests that penetration can occur in the middle section of the midgut halfway between the proventriculus and midgut-hindgut junction. Ellis and Evans [18] found trypanosomes trapped in folds and pockets and penetration took place most commonly at the apex of membrane folds. Penetration took place throughout the central two thirds of its length but not in the posterior or anterior part. Passage of trypanosomes across the membrane took from 9–11 days after the infective feed (on cultured procyclic forms). The membrane was observed to be split into two layers in the presence of trypanosomes and it was suggested that enzymes are involved in this process. These observations have changed our views on the role of the peritrophic membrane and the way in which it functions in *Glossina*/trypanosome relationships. In *Leishmania* parasites the peritrophic membrane has been regarded as important [19] in determining the vector capacity of certain sandfly species. In *P. chinensis* and *P. mongolensis* the membrane behaves differently. In *Le. donovani*-infected *P. chinensis* the membrane breaks after 48 hours releasing parasites to colonize other parts of the gut, but in *P. mongolensis* the peritrophic membrane remains as a complete sac and is voided intact together with digested blood and parasites, thus the fly does not become infected. Recent studies on the ultrastructure of the peritrophic membrane of sandflies have been undertaken [20, 21] which show it to be less of a membrane but more a secretion from the microvillar surface of the midgut which encases the bloodmeal. Ashford and co-workers [22] found the bloodmeal of *P. longipes* infected with *Le. aethiopica* broke up after 48 hours releasing parasites to colonize the gut; this behaviour resembles that described in Ref. [19], and would seem to be an important characteristic of a good vector species of sandfly.

Some host insects do not seem to possess a peritrophic membrane but remain good vectors of trypanosomes. Molyneux and co-workers [23] described

the Scanning Electron Microscope (SEM) appearance of *T. (M.) melophagium* in the sheep ked *Melophagus ovinus* where in the midgut no peritrophic membrane has been found but where extensive parasite colonization occurs.

ESTABLISHMENT OF INFECTION AND ATTACHMENT TO SURFACES

If transformation has occurred and multiplication of the transformed types (epimastigotes or promastigotes in *Leishmania*) is occurring, establishment is assured and this implies few barriers remain to successful completion of the life cycle. However, established infections must be maintained and provide a reservoir of infective forms. In addition parasites are generally living in a hostile environment where fluid is constantly moving. They must therefore maintain their position in their favoured developmental sites.

Such sites are usually the midgut, foregut or hindgut, and in the case of *Salivaria* trypanosomes the *Glossina* food canal, hypopharynx and salivary glands (*Trypanozoon*). The surfaces of the midgut and salivary glands are lined with microvilli whereas the foregut and hindgut are lined with a cuticular intima. Attachment to these different sites has been reviewed by Molyneux [1], and recent studies [23] on a model system by the SEM study have confirmed the transmission electron microscope studies carried out in many systems by different workers. In microvillar lined surfaces of the midgut parasites (*Leishmania* and trypanosomes) insert the flagella between the microvilli. No junction between flagella and microvilli (hemidesmosomes) are found in the midgut of insects, but in salivary glands of *Glossina Trypanozoon* form hemidesmosomes with microvilli [16], and recently Desser [24] has found similar junctions in the microvilli-lined gastric epithelium of leeches infected with amphibian trypanosomes.

Hemidesmosome junctions are characteristic of all known attachments between kinetoplastid flagellates and the cuticle-lined foregut and hindgut development sites. The attached parasites show a modified flagellum with an expanded distal tip closely applied to the cuticular surface of the insect gut wall. Hemidesmosomes are dense masses of condensed material which appear in the inner unit membrane of the flagella where they are in association with the cuticle. The axonemes terminate close to the hemidesmosomes with their axonemes at 90 degrees to the epithelium. The distance between the outer leaflet of the flagellar membrane and the host surface is approximately 12–15 nm and the hemidesmosome extends some 30–40 nm from the inner flagellar membrane towards the expanded intraflagellar area. In some attached parasites strands of material in the form of filaments can be seen to be orientated at 90 degrees to the attachment site. These junctions have been produced in *in vitro* systems [4, 25] which seem to emphasize that such a phenomenon is non-specific. However, it does seem that attachment will only occur in certain sites of an insect,

and such sites are associated with establishment of certain parasites which itself suggests specificity. This is exemplified by the inability of *Nannomonas* and *Duttonella* to attach to *Glossina* hindgut but they can attach to the labrum [26] whereas *T. grayi* attaches to the hindgut [27] but not elsewhere. In *Le. mexicana amazonensis* the attachment to the hindgut of *Lu. longipalpis* is observed but hemidesmosomes are formed with the cuticle lining the foregut [21]; *Le. b. brasiliensis* however can attach to the hindgut of this vector [28].

These mechanisms of attachment are a characteristic feature of trypanosomes and *Leishmania* in their vectors, and play a most important role in the survival of the organism in the vector. More work, however, needs to be carried out to understand the biochemical basis of these associations; this type of study should reveal information on host-vector specificity, a subject which at present is little understood.

MIGRATION TO DIFFERENT SITES

The attached forms of epimastigotes of trypanosomes and promastigotes of *Leishmania* have been referred to as 'haptomonad' forms [1, 21]; forms responsible for colonization of other parts of the gut are not attached, are freeswimming, and described as 'nectomonads'. Colonization of different parts of the insect is clearly essential for survival, and migration of parasites will involve sometimes difficult movements through gut cells (e.g. *T. (h) rangeli* in *Rhodnius*; possibly in some stocks of *Trypanozoon* in *Glossina*) with perhaps subsequent invasion of the salivary glands. The search for an alternative life cycle pathway in *Trypanozoon* in *Glossina* has revealed mid-gut wall cell infection [29] after haemocoelic stages had been earlier described [30]. This has prompted the suggestion that salivary gland infection can occur via the haemocoel in *Trypanozoon* as it appears to do in *T. rangeli* in *R. prolixus*; this pathway remains to be proven, but the tortuous route to the salivary glands via proventriculus, labrum and hypopharynx has seemed to some authors unlikely.

The significance of midgut cell infection with some parasites (trypanosomes and *Leishmania* [31]) requires to be further investigated. Molyneux [1] has reviewed the cases where this occurs, but as yet no firm significance can be attached to such observations which provide a basis, where multinucleate parasite forms are found, to suggest a mechanism for genetic exchange.

RESERVOIR OF FORMS FOR TRANSMISSION

The successful establishment of large numbers of parasites in the selected site, their multiplication and retransformation is indicative of a successful cycle

of development. This cycle of development is completed after an effective transmission. However, the infected insect remains infected throughout its life. This is a clear advantage to the parasite, and there is continual replenishment of epimastigote forms for transformation to later infective stages but multiplication of the former stages.

TRANSMISSION BY BITE OR CONTAMINATION

This process is the termination of a cycle of development. The transmission by bite is likely to be the most efficient method of transfer, but surprisingly it has only developed in Salivarian trypanosomes, *T. (H.) rangeli* and *Leishmania* parasites. Elsewhere transmission is by contamination, a more biologically hazardous method, which may involve the death of the vector in crushing or ingestion; for this reason it is important that a high percentage of infection occurs in Stercoraria trypanosomes compared with Salivaria where transmission by bite in a well-adapted haematophagous insect is less likely to result in death of the vector.

FEATURES OF THE LIFE CYCLE OF KINETOPLASTIDA IN INSECTS: HAEMOCOELIC INFECTIONS

Haemocoelic infections in *Glossina* with *Trypanozoon* species have been identified in both laboratory and the field in recent years [30, 32, 33]. It should however be remembered that similar infections have been known for some time in other systems, e.g. *T. rangeli* in *Rhodnius*. In experimental haemocoelic infections it has been shown that trypanosomes and *Leishmania* can survive for considerable periods in a variety of hosts [34, 35]. In view of this it is important to obtain more information about such infections in the field and what significance they have, if any, in relation to invasion of the salivary glands and thus in transmission; the stage in the life cycle of the infection in which they occur, and if associated with such invasion, whether pathological changes occur as in *T. rangeli* [36]. Recent studies indicate more frequent haemocoelic infections than hitherto believed [37–39], particularly in ticks.

PATHOLOGICAL CHANGES IN INSECTS WITH TRYPANOSOMES AND *Leishmania*

Watkins [36] has described the pathology of *T. rangeli* infections in *R. prolixus*; this study emphasizes the complexity of the interaction that will

result from haemocoelic invasion. The means by which such infections may be controlled remains to be studied, and this is particularly important in *Glossina*; The role of the insect defence system in such infection in *T. rangeli* was studied by Zeledon and de Monge [40] and from Refs [36, 30] it appears that certain strains of the same parasite behave differently. *Leishmania* parasites may also invade the haemocoel [22, 31], but the significance of the infection in sandflies remains in doubt [41]. Mortality of *Hippelates* has been associated with haemocoelic invasion by *Herpetomonas* which allow bacterial infections to set in [42]. Pathology associated with midgut wall invasion was also suggested by these authors [42].

Pathological response to mixed *Leishmania* infections induced experimentally has been demonstrated by Safjanova and Alexseev [43], but the basis of this pathology is not known. Williams [44] has described heavy flagellate infections of *Lutzomyia beltrani* which disrupted the gonotrophic cycle of this sandfly. The parasite was probably a bat trypanosome. However, this finding may be extended to other insects heavily infected with flagellates. It seems likely that such massive infections could disrupt the insects' physiology by causing digestive or absorptive problems by occluding functional surfaces [23, 44].

HOST-PARASITE RELATIONSHIPS: EPIDEMIOLOGY AND TRANSMISSION

Killick Kendrick and co-workers [45] have suggested that when sandflies are infected with *Leishmania* in the cibarium the attachment of parasites will cover the cibarial sensilla and render them inoperative, implying full engorgement would not occur although the unimpaired external sense organs would mean probing would continue. It is known that *Leishmania*-infected sandflies have difficulty obtaining a blood meal and continue to probe frequently producing a group of small lesions when the disease later develops at points where probing has occurred. Recent studies [46] have suggested that a similar phenomenon occurs in the labra of *Glossina* infected with *Salivaria* trypanosomes, in particular *Duttonella* and *Nannomonas*. LC1 and LC2 mechanoreceptive sensilla [47] responsible for monitoring fluid flow rate are blocked by (1) attachment of parasites to bases of the sensilla, (2) entanglement of sensilla tips with rosettes of parasites and (3) the modification of fluid flow by the parasites distal to the receptor thus altering the flow rate perceived by the sensilla in infected compared with uninfected *Glossina*.

Livesey and Molyneux [48], using principles of fluid dynamics, have established that the flow through the labrum of *Glossina* of blood is a laminar or viscous flow regime with 'entrance effects' confined to a length of less than $D/3$. This type of flow is obtained from Uchida [49] which establishes a pulsating viscous flow in which the velocity distribution is in phase with pressure fluctuations and retains

parabolic velocity distribution of Hagen-Poiseuille flow. A nearly exact theory yields for Hagen-Poiseuille flow

$$\bar{Q} = \frac{\pi D^{-4}}{8\mu} \left(-\frac{\Delta p}{L} \right)$$

Thus the suck required to obtain the same flow rate is proportional inversely to D^{-4} and directly to the length if the viscosity is constant. Calculations using the above formula show that if the labrum diameter is occluded by 50% by trypanosome colonies for 10% of its length, a factor of 2.5 is required by which either the flow rate is reduced or the cibarial pump is required to increase Δp to maintain the same flow rate; if 60% of the length is infected the factor is 10. This suggests parasites influence considerably the conditions which the LC1 and LC2 mechanoreceptors must monitor. This will result in increased chances of transmission by more frequent probing, longer feeding times and possibly disturbed feeding due to inability to become engorged and thus the movement of the fly to another site or host. The epidemiological and epizootiological consequences are, we believe, profound [46], and such a phenomenon of parasite-receptor interaction is a distinct biological advantage to the parasite.

REFERENCES

- [1] MOLYNEUX, D.H., *Adv. Parasitol.* 15 (1977) 1.
- [2] WALLACE, F.G., *Exp. Parasitol.* 18 (1966) 124.
- [3] BROOKER, B.E., *Protoplasma* 73 (1971) 191.
- [4] BROOKER, B.E., *Protoplasma* 72 (1971) 19.
- [5] WIJERS, D.J.B., *Ann. Trop. Med. Parasitol.* 52 (1958) 385.
- [6] FREEMAN, J.L., *Acta Trop.* 30 (1973) 347.
- [7] HARMSSEN, R., *Trans. R. Soc. Trop. Med. Hyg.* 67 (1978) 364.
- [8] JENNI, L., *Acta Trop.* 34 (1977) 35.
- [9] HOARE, C.A., *Trypanosomes of Mammals*, Blackwell, Oxford (1972).
- [10] BOHRINGER, S., HECKER, M., *J. Protozool.* 22 (1975) 463.
- [11] VICKERMAN, K., *Trans. R. Soc. Trop. Med. Hyg.* 56 (1962) 487.
- [12] VICKERMAN, K., *Nature (London)* 208 (1965) 762.
- [13] VICKERMAN, K., *J. Cell Sci.* 5 (1969) 163.
- [14] CROSS, G.A.M., *Parasitology* 71 (1975) 393.
- [15] VICKERMAN, K., *Ciba Foundation Symposium No. 25* (1974) 53.
- [16] STEIGER, R.F., *Acta Trop.* 30 (1973) 64.
- [17] GOODING, R.H., *Acta Trop.* 32 (1975) 96.
- [18] ELLIS, D.S., EVANS, D.A., *Nature (London)* 267 (1977) 834.
- [19] FENG, L.C., *Peking Nat. Hist. Bull.* 19 (1951) 327.
- [20] GEMETCHU, T., *Ann. Trop. Med. Parasitol.* 68 (1974) 111.
- [21] KILLICK KENDRICK, R., et al., *Proc. R. Soc. (London), Ser. B* 187 (1974) 409.

- [22] ASHFORD, R.W., et al., Trans. R. Soc. Trop. Med. Hyg. 67 (1973) 568.
- [23] MOLYNEUX, D.H., et al., Acta Trop. 35 (1978) 319.
- [24] DESSER, S.S., Can. J. Zool. 54 (1976) 1712.
- [25] HOMMEL, M., ROBERTSON, E.B., Experientia 32 (1976) 464.
- [26] VICKERMAN, K., J. Protozool. 20 (1973) 394.
- [27] MOLYNEUX, D.H., Protozoology 3 (1977) 83.
- [28] KILLICK KENDRICK, R., et al., Proc. R. Soc. (London), Ser. B 198 (1977) 191.
- [29] EVANS, D.A., ELLIS, D.S., Nature (London) 258 (1975) 231.
- [30] MSHELBWALA, A.S., Trans. R. Soc. Trop. Med. Hyg. 66 (1972) 637.
- [31] MOLYNEUX, D.H., et al., Proc. R. Soc. (London) Ser. B. 190 (1975) 341.
- [32] OTIENO, L.H., et al., Acta Trop. 33 (1976) 143.
- [33] OTIENO, L.H., DARJI, N., ISCTRC, Banjul, Gambia (1977).
- [34] LINDER, J., Bull. Res. Council, Israel 8 (1960) 128.
- [35] SCHMITTNER, S.M., MCGHEE, R.B., J. Parasitol. 56 (1970) 684.
- [36] WATKINS, R., J. Invertebr. Pathol. 17 (1971) 67.
- [37] BURGDORFER, W., et al., Acta Trop. 30 (1973) 340.
- [38] KRINKSKY, W.L., BURGDORFER, W., J. Parasitol. 62 (1976) 824.
- [39] REHACEK, J., et al., Zecken Mitt. Abt. Zool. Landesmus Joanneum 33 (1974) 161.
- [40] ZELEDON, R., de MONGE, E., J. Invertebr. Pathol. 8 (1966) 420.
- [41] ADLER, S., THEODOR, O., Ann. Trop. Med. Parasitol. 20 (1926) 109.
- [42] BAILEY, C.H., BROOKS, W.M., J. Invertebr. Pathol. 19 (1972) 342.
- [43] SAFJANOVA, V.M., ALEXSEEV, A.N., Bull. CNRS No. 239 (1979) 153.
- [44] WILLIAMS, P., Bull. Ent. Res. 65 (1976) 615.
- [45] KILLICK KENDRICK, R., et al., Proc. R. Soc. (London), Ser. B 196 (1977) 105.
- [46] MOLYNEUX, D.H., et al., Ann. Trop. Med. Parasitol. 73 (1979).
- [47] RICE, M., et al., Ann. Trop. Med. Parasitol. 67 (1973) 169.
- [48] LIVESEY, J., MOLYNEUX, D.H. (in preparation).
- [49] UCHIDA, S.Z., Angew. Math. Phys. 7 (1956) 403.

DISCUSSION

Max MURRAY: The junctions or points of attachment between parasites and insect gut cells are reminiscent of junctions between mammalian cells, both epithelial and endothelial; the integrity of the latter is Ca^{++} - and Mg^{++} - dependent.

Might it be possible to interfere with points of parasite attachment in the insect host, for example, using EDTA and, as a result, to interfere with the parasite's life cycle?

D.H. MOLYNEUX: I agree that this experimental approach should be tried. There is a problem, however, in choosing the appropriate system. I think the best approach initially would be to use the Crithidia/millipore system or the system using Falcon flasks.

W.J. KLOFT: I am very impressed by the excellent scanning microscope pictures you showed in your oral presentation, which demonstrate the attachment between the *Leishmania* parasites and the gut epithelium cells of the sandflies. We know that rapid multiplication of the parasites can cause the gut to become

blocked, so that regurgitation is the only way of flushing the front parts of the alimentary tract. I was under the impression that this blockage occurs in the foregut, but your pictures seem to demonstrate high multiplication within the midgut. I wonder whether you might be able to give more details about regurgitation from the foregut or the midgut or both as a means of transmitting the parasites.

D.H. MOLYNEUX: There is still a great deal of controversy about the method of transmission of Leishmaniasis. Blockage of the midgut does not seem to occur, although it may be found in the oesophageal valve and in the oesophagus itself. We have observed free unattached parasites in the proboscis, and I believe that it is these forms which infect the mammal. However, any parasite from a sandfly is potentially infective.

J.D. DARGIE (*Scientific Secretary*): What is the evidence that infected tsetse bite more frequently than uninfected flies?

D.H. MOLYNEUX: There is evidence from both Jenni and Elce (personal communication) that infected *Glossina* probe more frequently than uninfected flies. It is simply a result of visual observation of flies which are known to transmit as compared with uninfected ones.

R. GALUN: Does the blocking of the alimentary canal affect the survival of the infected flies?

D.H. MOLYNEUX: Apparently not. There is in fact evidence that infected flies survive longer than uninfected flies.

R. GALUN: Do you get an accumulation of red blood cells around the rosettes?

D.H. MOLYNEUX: We haven't seen this.

EFFICIENCY OF WILD *Glossina pallidipes* Austen AS VECTORS OF PATHOGENIC AFRICAN TRYPANOSOMES

L.H. OTIENO, N. DARJI, J. OLOBO,
P. ONYANGO

International Centre of Insect
Physiology and Ecology (ICIPE),
Nairobi,
Kenya

Abstract

EFFICIENCY OF WILD *Glossina pallidipes* Austen AS VECTORS OF PATHOGENIC AFRICAN TRYPANOSOMES.

Two methods have been used to determine trypanosome infection rates in wild *Glossina pallidipes* in two different localities in Kenya. Trypanosome infection rates obtained by the two methods were comparable, although each method failed to detect a proportion of infections. The number of trypanosomes secreted with saliva depended on the species of trypanosomes. Although there were more flies infected with *T. vivax*, the number of trypanosomes they secreted were consistently fewer than those secreted by flies infected with *T. congolense*.

INTRODUCTION

There had been no published account of longitudinal studies on the prevalence of trypanosomes secreted by wild tsetse until recently when a limited account of what may be happening in the case of *Glossina pallidipes* Austen was given [1]. In this study, it was reported that *G. pallidipes* from Lambwe Valley in Western Kenya infected with *Trypanosoma (Duttonella) vivax* secreted constantly fewer numbers of trypanosomes than similar flies infected with *T. (N.) congolense*. However, the only fly found infected with *T. (T.) brucei* secreted the largest number of trypanosomes whenever it was tested. This work has now been extended to other localities of Kenya where large populations of *G. pallidipes* are infected with pathogenic African trypanosomes.

MATERIALS AND METHODS

Study areas

The tsetse flies used for this work were *Glossina pallidipes* collected from two different localities in Kenya:

- (1) Lambwe Valley game reserve (0°40' S, 34°10' E) situated in the South Nyanza district, Western Kenya.
- (2) Shimba Hills national reserve (4°30' S, 38°45' E) on the Kenya coast near Mombasa.

Trapping methods

In Lambwe Valley where the fly population was high, Langridge and Moloo traps were used. Challier's biconical traps were used in the Shimba Hills.

Examination of flies for trypanosome infections

The flies caught were kept in individual plastic tubes (4.2 cm X 3.2 cm) with nylon gauze at both ends. In this way it was easy to collect salivary secretions from each fly using the batwing membrane technique [2]. The secretions were stained with Giemsa's stain and later examined for the presence and morphology of trypanosomes. When present, the actual number of trypanosomes in the entire secretion were enumerated. It was thus easy to identify, at the same time, the species of trypanosomes secreted. The same flies were later dissected and examined for the presence and location of trypanosomes in the various tissues of the fly.

RESULTS

Salivation and dissection as diagnostic methods

The first round of experiments was carried out in the Lambwe Valley just before the long rainy season. A total of 341 *G. pallidipes* were examined both by salivation and dissection methods. The two methods showed respectively that 38 (11.14%) and 37 (10.85%) flies were infected with trypanosomes. However, the dissection method failed to detect infections in six flies (five *T. vivax* and one *T. congolense*) which had secreted infected saliva. Similarly, the salivation method failed to detect infections in 12 flies which were later shown by dissection to be infected. These infections were all immature. Table 1 summarizes the results of these investigations.

TABLE I. COMPARISON OF SALIVATION AND FLY DISSECTION AS DIAGNOSTIC METHODS FOR DETECTING TSETSE FLIES INFECTED WITH TRYPANOSOMES

The same flies were examined by both methods

Diagnostic method	No. flies examined	No. flies infected			
		<i>T. vivax</i>	<i>T. congolense</i>	<i>T. brucei</i>	unidentified
Salivation	341	27	10	1	-
Dissection	341	25	9	1	2 ^a

^a Gut infections.

TABLE II. TRYPANOSOME INFECTION RATES IN *G. pallidipes* OBTAINED FROM LAMBWE VALLEY AND SHIMBA HILLS

Trypanosome infections were detected both by salivation and dissection

Diagnostic method	Lambwe Valley			Shimba Hills		
	Flies examined	No. infected	Percentage infection	Flies examined	No. infected	Percentage infection
Dissection	49	3	6.1	204	10	4.9
Salivation	403	28	6.9	747	31	4.2
Total	452	31	6.8	951	41	4.3

The second round of investigations was carried out both in the Lambwe Valley and the Shimba Hills during a protracted wet period. These studies differed from the above in that the dissected flies were not the same flies whose salivary secretions were examined for trypanosome infection. Table II summarizes the results obtained. It may be seen from the table that both dissection and salivation methods revealed very comparable results.

Although very few flies (49) were dissected in the Lambwe Valley compared with those which were salivated (403), it is important to note the very similar results obtained, 6.1% and 6.9% respectively. It is important also to note that very similar results were obtained in the Shimba Hills where dissection revealed 4.9% whereas the salivation method detected 4.2% trypanosome infections among the flies examined.

TABLE III. ANALYSIS OF THE ABUNDANCE OF TRYPANOSOMES IN THE SALIVARY SECRETIONS OF *G. pallidipes* INFECTED WITH *T. vivax*, *T. congolense* AND *T. brucei* FROM LAMBWE VALLEY AND SHIMBA HILLS^a

Locality	Lambwe Valley			Shimba Hills		
Trypanosomes	<i>T. vivax</i>	<i>T. congolense</i>	<i>T. brucei</i>	<i>T. vivax</i>	<i>T. congolense</i>	<i>T. brucei</i>
	(12)	(6)	(1)			
Total flies	17	8	-	15	9	-
	(74)	(38)	(8)			
Total salivary drops	28	12	-	25	23	-
	(6.6)	(49.5)	(608.8)			
Arithmetic mean no. of trypanosomes	10.7	48.1	-	15.5	28.6	

^a Figures in parentheses have been taken from earlier studies [1] in the same area.

TABLE IV. AN ASSESSMENT OF THE AGES OF WILD POPULATIONS OF *G. pallidipes* FROM SHIMBA HILLS AND FROM LAMBWE VALLEY
The ages are based on ovarian age determination

	Shimba Hills				Lambwe Valley			
Ovarian age category	0	1-3	4+	Total	0	1-3	4+	Total
Number	22	110	98	230	17	54	3	74
Percentage	9.6	47.8	42.6	-	22.9	72.9	4.0	

Enumeration of trypanosomes secreted with saliva

Both flies from the Lambwe Valley and the Shimba Hills that had been demonstrated to be infected through salivation were kept alive and examined daily (as long as they remained alive) for the presence of trypanosomes in their salivary secretions. When present the trypanosomes were enumerated. A summary of

these experiments is shown in Table III. These results have been compared with earlier [1] observations in the Lambwe Valley. The table shows that in both localities, the arithmetic mean number of *T. vivax* secreted was much lower than that shown for *T. congolense* infections. The higher count of 15.5 *T. vivax* seen in the Shimba Hills compared with 10.7 in the Lambwe Valley may be of interest. At the time of these studies a highly virulent strain of *T. vivax* was isolated in the neighbourhood. *T. brucei* infections were not observed in the two localities.

In order to see whether the prevalence of trypanosome infection rates obtained from the two localities were related to the ages of the flies caught, ovarian ages of a few *G. pallidipes* caught daily were determined according to the method described by Saunders [3]. Table IV shows the ovarian ages of the flies caught. It may be seen from the Table that 22.9% of the flies examined in the Lambwe Valley were nulliparous compared with 9.6% obtained in the Shimba Hills. These figures represent proportions of flies which were unlikely to carry trypanosome infections due to their age. It would appear therefore, that the higher (6.3%) trypanosome infection rates observed in the Lambwe Valley compared with (4.2%) in the Shimba Hills suggest that the incidence of trypanosome infection in the flies was higher in the Lambwe Valley than in the Shimba Hills at the time of the investigation.

DISCUSSION

Burt [4] found that dissection of flies as the sole method of detecting *G. morsitans* infected with *T. rhodesiense* was unreliable, and had to base all his infection rates on results obtained by feeding individual flies on separate clean rats. He therefore tested two methods of examination in parallel [5]: a probe record of a hungry fly, and feeding of the same fly on a susceptible host. He found that each method failed to detect a proportion of infected flies. He found, however, that if a probe method was carried out for a period of about 10 days, every time the fly was hungry a full number of *T. rhodesiense*-infected flies could be detected.

The two methods used in this study to detect trypanosome infections in wild *G. pallidipes* have shown that these methods are very comparable, although each method failed to detect a proportion of infected flies. The superiority of salivation over dissection is that it ensures a simultaneous and accurate identification of trypanosome species. Salivation through a membrane is also more representative of the act of feeding and therefore better than probing directly on to a microscope slide. Mixed *T. vivax* and *T. congolense* are virtually impossible to separate by the dissection method, especially if the infections are massive. The two species are also not easily transmissible to laboratory rodents, and therefore cannot be easily identified through feeding infected flies.

A survey on the literature available has produced very little information as to the number of trypanosomes secreted when an infected tsetse fly feeds. Rodhain and co-workers (quoted by Harley and co-workers [6]), working with *G. morsitans* infected with *T. brucei* and fed through a rat skin membrane, estimated that 1562 metacyclic forms were released during one meal. Fairbairn and Burt [7], however, observed that some 300–450 metacyclic *T. rhodesiense* were needed to initiate infection in man. Otieno and Darji [1] observed that *G. pallidipes* infected with *T. vivax* secreted saliva which contained very few trypanosomes, and some of these flies produced negative saliva for several successive days. They also noted a clear-cut difference between the numbers of trypanosomes exuded by flies infected with *T. vivax* and those infected with *T. congolense*. The present investigations have confirmed these findings, and have further shown that these differences may be trypanosome species specific. The evidence for this is that infected *G. pallidipes* from both the Lambwe Valley and the Shimba Hills localities, which are some 800 km apart, showed similar patterns in trypanosome vectorial capacities.

The low number of *T. vivax* trypanosomes secreted with saliva may be due to their firm attachment on to the smooth chitinous lining of the food canal [8] of the fly; this probably is not the case with *T. congolense*. The present investigations in the Shimba Hills were carried out at a time when there was an outbreak of a highly virulent strain of *T. vivax* in cattle in the coastal area of Kenya. It is interesting to speculate on the relationship between the higher *T. vivax* counts in this area compared with Lambwe and the pathogenicity of this strain on the Kenya coast.

ACKNOWLEDGEMENTS

The ovarian age estimates were determined by M.F.B. Chaudhury and W.F. Snow, both of the ICIPE, Nairobi, Kenya, to whom we are very grateful.

REFERENCES

- [1] OTIENO, L.H., DARJI, N., The abundance of pathogenic African trypanosomes in the salivary secretions of wild *Glossina morsitans pallidipes*, Ann. Trop. Med. Parasitol. (in press).
- [2] YOUDEOWEI, A., A simple technique for observing and collecting the saliva of tsetse flies (Diptera, Glossinidae), Bull. Ent. Res. 65 (1975) 65.
- [3] SAUNDERS, D.S., Age determination for female tsetse flies and the age compositions of samples of *Glossina pallidipes* Aust., *G. palpalis fuscipes* Newst., and *G. brevipalpis* Newst., Bull. Ent. Res. 53 (1962) 579.
- [4] BURTT, E., Incubation of tsetse pupae: increased transmission — rate of *Trypanosoma rhodesiense* in *Glossina morsitans*, Ann. Trop. Med. Parasitol. 40 (1946) 18.

- [5] BURTT, E., Salivation by *Glossina morsitans* on to glass slides: a technique for isolating infected flies, *Ann. Trop. Med. Parasitol.* **40** (1946) 141.
- [6] HARLEY, J.M.B., CUNNINGHAM, M.P. VAN HOEVE, K., The numbers of infective *Trypanosoma rhodesiense* excreted by *Glossina morsitans* during feeding, *Ann. Trop. Med. Parasitol.* **60** (1966) 455.
- [7] FAIRBAIRN, H., BURTT, E., The infectivity to man of a strain of *Trypanosoma rhodesiense* transmitted cyclically by *Glossina morsitans* through sheep and antelope: evidence that man requires a maximum infective dose of metacyclic trypanosomes, *Ann. Trop. Med. Parasitol.* **40** (1946) 270.
- [8] VICKERMAN, K., The mode of attachment of *Trypanosoma vivax* in the proboscis of the tsetse fly *Glossina fuscipes*: an ultrastructural study of the epimastigote stage of the trypanosome, *J. Protozool.* **20** (1973) 394.

DISCUSSION

R. GALUN (*Chairman*): I think the arithmetic mean of trypanosome numbers that you are using is misleading. The total number would be more reliable.

L.H. OTIENO: In an earlier report on this work both the arithmetic mean and the range of the number of trypanosomes counted were given. In the present report the arithmetic means are given to illustrate the close similarity between the numbers of trypanosomes counted in the two localities. These means show that the number of trypanosomes secreted in the saliva is dependent on the species of trypanosome being secreted, but not necessarily on the strain of trypanosome infecting the flies.

D.H. MOLYNEUX: Have you any idea of the levels of infection observed in mammals in the area of your studies?

L.H. OTIENO: Wild game in the Lambwe Valley are reservoirs of *T. brucei*, particularly the bushbuck. However, no information is available on trypanosome infection in the game found in the Shimba Hills.

H.S. HUSSEIN: It seems from the results you have presented that larger concentrations of metacyclic trypanosomes are found in the saliva of the fly when trypanosomes that multiply in the midgut as well as in the salivary glands (*T. brucei*) and the proboscis (*T. congolense*) are involved than where those that multiply in the proboscis only, such as *T. vivax*, are concerned.

L.H. OTIENO: In the case of *T. brucei* I think that the trypanosomes are squeezed out with salivary secretion. However, in the case of *T. vivax* it would appear that the trypanosomes are mechanically scraped off from the tip of the proboscis during the probing process.

P.A. LANGLEY: Can you tell us the reason for the apparently very large difference in age composition of the two fly populations, one in the Lambwe Valley and the other in the Shimba Hills? Was it a genuine difference or was it perhaps a reflection of differences in the behaviour of the flies towards the two capture techniques?

L.H. OTIENO: The differences could have been caused by the climate, although the studies were carried out during the wet season in both places. The trapping methods also differed; Langridge traps were used in the Lambwe Valley and Challier traps in Shimba. These two trapping methods could have been catching flies of different ages.

A.M. VAN DER VLOEDT: When using the ovarian method of physiological age determination, which is fairly accurate, did you observe difference in the time elapsing between the infection blood meal and the appearance of mature forms of trypanosome between the two regions under consideration? Dissection of the individual flies immediately after 'positive' salivation could perhaps give the answer.

L.H. OTIENO: The flies that were examined for the presence of trypanosomes were not the same as those which were used for ovarian age determination, so we had no way of gauging the earliest time at which mature infections appeared in the flies.

D.H. MOLYNEUX: Why haven't you aged your flies past age group 4?

L.H. OTIENO: They were aged from 0 to 7, but we were only interested in the proportion of young flies in the population. Flies of age group 0 were very important to us as they were unlikely to be infected with any trypanosomes.

LEISHMANIASIS VECTOR BEHAVIOUR IN KENYA

M.J. MUTINGA

International Centre of Insect

Physiology and Ecology (ICIPE),

Nairobi,

Kenya

Abstract

LEISHMANIASIS VECTOR BEHAVIOUR IN KENYA.

Leishmaniasis in Kenya exists in two forms: cutaneous and visceral. The vectors of visceral leishmaniasis have been the subject of investigation by various researchers since World War II, when the outbreak of the disease was first noticed. The vectors of cutaneous leishmaniasis were first worked on only a decade ago after the discovery of the disease focus in Mt. Elgon. The vector behaviour of these diseases, namely *Phlebotomus pedifer*, the vector of cutaneous leishmaniasis, and *Phlebotomus martini*, the vector of visceral leishmaniasis, are discussed in detail. *P. pedifer* has been found to breed and bite inside caves, whereas *P. martini* mainly bites inside houses.

INTRODUCTION

Leishmaniasis in Kenya exists in two forms: visceral and cutaneous leishmaniasis. The focus of cutaneous leishmaniasis is so far known to be distinct in altitude and climate from the foci of visceral leishmaniasis. Cutaneous leishmaniasis focus is limited to an altitude of about 6000 ft (1800 m) above sea level while that of visceral leishmaniasis occurs between 3000 ft (900 m) and 4500 ft (1350 m). Climatically the visceral leishmaniasis thrives in areas which are arid or semi-arid, unlike the cutaneous leishmaniasis focus which is wet for the most part of the year.

The role of Phlebotomine sandflies in these foci has indeed been the subject of investigation since the visceral leishmaniasis rose to epidemic level about two decades ago, and as a result of the discovery of cutaneous leishmaniasis focus in the Mt. Elgon area about ten years ago.

CUTANEOUS LEISHMANIASIS

The discovery of the existence of cutaneous leishmaniasis in Mt. Elgon in 1969 led to intensive investigations into the vectors of the disease [1-3]. *Phlebotomus pedifer* turned out to be the only man-biting species in the area, and experiments later incriminated the species as the vector of the disease [3].

TABLE I. BLOOD-MEAL ANALYSIS OF *P. pedifer* FROM MT. ELGON CAVES

Host	Number of blood meals	Percentage
Bovid	663	84.46
Hyrax	65	8.28
Unidentified bovid	26	3.31
Mammal	4	0.51
Man	2	0.25
Sheep/goat	1	0.13
Negative	24	3.06
Total	785	

HABITS

Further studies showed that *Phlebotomus pedifer* is limited to dwelling inside caves and in the entrance of caves [3]. *Phlebotomus pedifer* was observed to have a flight range of not more than five metres from the caves. Investigations into the feeding habits were made through blood-meal analysis. *P. pedifer* was found to feed on various hosts, details being given in Table I.

As is indicated in Table I, most flies fed on bovids. Observations on the various activities in the caves revealed that the caves were frequented by cattle, sheep and goats for salt licks. The flies were observed to bite man, cow, goats and sheep very readily, and therefore the percentages of fed flies proved to be indicative of the availability of the hosts and not an order of host preference inside the caves. In regard to the time of biting, it was observed that the flies bite almost any time of the day provided the host is located in areas of the cave where it is slightly dark as the flies tended to shun brightness or daylight. The breeding of the flies takes place inside the caves on the moist floor where all stages of development were recovered [2].

Control measures against these flies can very easily be instituted and it is possible to eliminate the flies by the use of insecticides. This, however, has not been done because it was more economical to inform the population in the area to keep away from the caves, which are, in any case, a good distance from dwellings.

TABLE II. ANTHROPOPHILIC SANDFLIES, HUMAN-BAITED AND CAPTURED OUTDOORS FEEDING ON MAN AT A TERMITE HILL IN WEST POKOT, KENYA

Species	Total number	Percentage incidence
<i>P. martini</i>	105	94
<i>P. adleri</i>	5	5
<i>P. bedfordi</i>	2	2

TABLE III. INCIDENCE OF VARIOUS SPECIES OF SANDFLIES COLLECTED FROM TERMITE HILLS, SHOWING RELATIVE ABUNDANCE OF VARIOUS SPECIES IN WEST POKOT, KENYA

Species	Total	Percentage
<i>P. martini</i>	1251	22
<i>P. bedfordi</i>	1096	19
<i>P. schwetzi</i>	2143	38
<i>P. africanus</i>	7	—
<i>P. antannatus</i>	1096	19
<i>P. adleri</i>	1	—
<i>P. ingrini</i>	55	1
<i>P. affinis</i>	3	—
<i>P. kirki</i>	20	—
<i>P. clydli</i>	1	—
<i>P. squemipheuris</i>	3	—
Total	5676	

VISCERAL LEISHMANIASIS

The vectors of visceral leishmaniasis are scattered in a much wider range in Kenya than those of cutaneous leishmaniasis. As a result of the outbreak of the disease various authors set out to work on the vectors. Van Someren worked on the

TABLE IV. ANTHROPOPHILIC SANDFLIES, HUMAN-BAITED INDOORS FEEDING ON MAN

Species	Total	Percentage
<i>P. martini</i>	243	98
<i>P. bedfordi</i>	4	2
<i>P. schwetzi</i>	2	1

TABLE V. BLOOD-MEAL ANALYSIS OF VARIOUS SPECIES OF SANDFLIES CAPTURED FROM TERMITE HILLS, WEST POKOT, KENYA

Species	Total	%	Bovine	Man	Reptile	Negative
<i>P. bedfordi</i>	416	66		11	219	196
<i>P. affinis</i>	128	20		4	34	90
<i>P. antennatus</i>	35	6		2	15	18
<i>P. martini</i>	8	1	3		—	5
<i>P. schwetzi</i>	26	4			13	11
<i>P. africanus</i>	20	3			12	8
Total	633		3	17	293	328

vectors in the area north west of Lake Rudolf [4]. Heisch [5] investigated the vectors in the Northern Frontier District, while the foci of Kitui, Meru and Baringo were investigated by Heisch and Guggisberg [6], Heisch, Wijers and Minter [7], Minter [8], Wijers [9], Minter and Wijers [10], Wijers and Minter [11] and Mutinga and Ngoka [11]. These studies indicate that the members of the 'Synphlebotomus complex', *P. martini*, *P. vansomerenae* and *P. celiae*, can be considered at present as vectors of leishmaniasis, *P. martini* being the chief vector of the disease with wider range of distribution than the other two vectors.

BEHAVIOUR OF *P. martini*

Phlebotomus martini has been found resting inside termite hill shafts, rock crevices and animal burrows. Resting sites inside houses were considered until 1977 of little importance by epidemiologists of the disease because it was believed then that the transmission of diseases occurred outside the dwellings. Strong correlation was, however, made between kala-azar patients and proximity of their homes to termite hills [7]. Recent studies [12] have shown that *P. martini* when compared with other species will prefer to bite man. Table II shows results of man-baited experiments in termite hills. These termite hills had been sampled and showed that other species of sandflies were also present in relatively high numbers (Table III).

When the human bait was conducted indoors again *P. martini* was found to bite man almost exclusively (Table IV).

These experiments were repeated for a new focus about 300 miles away at Machakos, Kenya, and the same behaviour of *P. martini* in biting man was found.

Table V shows that even though the *P. martini* sandflies are abundant in termite hills, the fed ones do not rest there.

Table IV shows that *P. martini* bites people indoors. The question remains as to where *P. martini* rests after feeding. Experiments have been set up to try to find this out. Do they rest indoors, and if not where are they resting outdoors? When do they return to the termite hills? These are questions we are trying to answer. The breeding area is another unanswered riddle which is still under careful scrutiny.

For temporary control measures, it is now possible to apply residual insecticides in resting sites, i.e. termite hills and dwellings. This may be a temporary measure if indeed these may turn out to be merely alternate resting sites.

When and if breeding sites are known, it may be possible to attack these also with chemicals or other methods of integrated control measures.

REFERENCES

- [1] MUTINGA, M.J., *Phlebotomus longipes*, a vector of cutaneous leishmaniasis in Kenya, Trans. R. Soc. Trop. Med. Hyg. 65 (1971) 106.
- [2] MUTINGA, M.J., Cutaneous Leishmaniasis and its Transmission Factors, PhD Thesis, University of Nairobi, 1972.
- [3] MUTINGA, M.J., *Phlebotomus* fauna in the cutaneous leishmaniasis focus of Mt. Elgon, East Africa Med. J. 52 (1975) 340.
- [4] ANDERSON, T.F., Kala-azar in East African Forces, East Africa Med. J. 20 (1973) 172.
- [5] HEISCH, R.B., Two years medical work in Northern Frontier District, Kenya Colony, East Africa Med. J. 124 (1974) 3.
- [6] HEISCH, R.B., GUGGISBURG, C.A.W., Notes on sandflies (*Phlebotomus* of Kenya), Ann. Trop. Med. Parasitol. 47 (1953) 44.
- [7] HEISCH, R.B., WIJERS, D.J.B., MINTER D.M., In pursuit of the vector of Kala-azar in Kenya, Br. Med. J. 1 (1962) 1456.

- [8] MINTER, D.M., Studies on the vector of Kala-azar in Kenya: III. Distributional evidence, Ann. Trop. Med. Parasitol. 57 (1963) 19.
- [9] WIJERS, D.J.B., Studies on the vector of Kala-azar in Kenya: II. Epidemiological evidence, Ann. Trop. Med. Parasitol. 57 (1963) 7.
- [10] MINTER, D.M., WIJERS, D.J.B., Studies on the vector of Kala-azar in Kenya: V. Experimental evidence, Ann. Trop. Med. Parasitol. 57 (1963) 24.
- [11] WIJERS, D.J.B., MINTER, D.M., Studies on the vector of Kala-azar in Kenya: I. Entomological evidence, Ann. Trop. Med. Parasitol. 56 (1962) 462.
- [12] MUTINGA, M.J., NGOKA, J.M., Incrimination of the vector of visceral leishmaniasis in Kenya, East Africa Med. J. 55 (1978) 373.

DISCUSSION

D.H. MOLYNEUX: Have you any evidence of *L. donovani* infection from *Arvicanthus* in view of the assertion in your presentation that kala-azar arrived in Kenya from the Sudan?

M.J. MUTINGA: So far many animals have been trapped, including hundreds of rodents, but we have not succeeded in isolating *L. donovani* in rodents. We have, however, isolated it in dogs.

D.H. MOLYNEUX: Have you considered using fluorescent powders for detecting sandflies?

M.J. MUTINGA: Yes, we have considered using them but for the time being we know where the flies rest in houses, so we spray the houses and sort the flies into species. If we find that the flies do not rest inside houses after feeding, we shall have to use fluorescent powder in the termite hills and then follow them from there.

INSECT REPELLENTS; PESTICIDES

(Session 4)

LONG-TERM PROTECTION OF ANIMALS FROM TSETSE BITES THROUGH CONTROLLED-RELEASE REPELLENTS

R. GALUN, M.N. BEN-ELIAHU, D. BEN-TAMAR,
Department of Zoology,
The Hebrew University of Jerusalem,
Jerusalem, Israel

J. SIMKIN
Pennwalt Corporation,
King of Prussia,
Pennsylvania,
United States of America

Abstract

LONG-TERM PROTECTION OF ANIMALS FROM TSETSE BITES THROUGH CONTROLLED-RELEASE REPELLENTS.

Laboratory animals have been given full protection from tsetse bites for a period greater than 10 days by dermal application of some formulations of micro-encapsulated natural pyrethrum. If we take into consideration the two-hours protection conferred by currently used repellents against blood feeders, this is a breakthrough in repellency duration. Moreover, the same formulations are toxic to ticks for more than seven days, permitting us to envisage the possibility of using a single agent against the two major vectors of cattle diseases.

1. INTRODUCTION

Tsetse flies, vectors of trypanosomiasis, and ticks, vectors of several serious livestock diseases, continue to plague the health and economy of great parts of Africa. Different approaches to controlling these vectors have been attempted over the years, including some new chemical control techniques. However, vast areas with favourable climatic and edaphic conditions for cattle production are still infected and cannot be used for raising cattle.

Previous efforts to protect people and domestic animals by repelling the tsetse flies have been disappointing. Attempts to find appropriate repellents were made in the 1940s [1-3], and then discontinued for almost 30 years. Recently, the usefulness of some new repellents against the tsetse has been evaluated. Of nine repellents tested on human skin (including DEET, DMP, ethyl hexanediol,

2,2-n-trimethyl pentanediol), none gave 100% protection for more than two hours [4]. Impregnation of wide-mesh net jackets (ratio, 0.5 g agent per g netting) conferred 100% protection to the wearer for only one day using the most effective agent, di-isopentyl malate [5].

In practice, protection of livestock against tsetse flies and ticks by means of repellents requires a repellency duration of at least one week. An agent giving 100% protection for one week or more could replace chemoprophylactic drugs and also acaricides as a means of protection, and might be the preferred method. To date, the only compound conferring one or two days protection against ticks and tsetse flies is natural pyrethrum [6]. However, pyrethrum decomposes very rapidly because of UV radiation from sunlight, and it also oxidizes readily. In 1975, Galun [7] proposed attempting to increase the residual repellency of pyrethrum using micro-encapsulation as a possible means of reducing evaporation rate, photodegradation and oxidation, and of slowing down absorption of the material into the skin. Moreover, micro-encapsulation was reported to reduce dermal and oral toxic effects on the treated animals [8-10].

The present study reports on the repellency and toxicity of 27 formulations of micro-encapsulated natural pyrethrum, with different capsule walls controlling different release rates of the repellent-pesticide. The micro-encapsulation was carried out by the Pennwalt Corporation. For comparison, non-encapsulated natural pyrethrum and micro-encapsulated synthetic pyrethrum were also tested.

2. MATERIALS AND METHODS

2.1. Preparation of micro-encapsulated formulations

All the micro-encapsulated materials reported here were prepared by Pennwalt Corporation at their Technological Center located at King of Prussia, Pennsylvania. The encapsulations were based upon technology developed and patented by Pennwalt (U.S. Patent 3577515 issued to Pennwalt Corporation). In essence, the test materials were micro-encapsulated by interfacial polymerization which produces a shell or capsule wall around tiny droplets of repellent/insecticide. The capsule walls that were utilized were cross-linked polyamides. An unusual degree of flexibility in preparing capsule walls for specific controlled release rate is inherent in the Pennwalt process. Varying degrees of permeability were effected by using preparative formulations that yielded the desired variation in the wall thickness and the degree of cross-linking of the polymer [8]. Each of these wall characteristics has an independent effect on release rate. All the micro-encapsulated samples reported here were previously screened by Pennwalt and found to possess the intended control release character.

For comparison purposes, a sample of cyclohexane carbamate derivative was encapsulated by the Pennwalt procedure. The active ingredient was suggested and supplied by T.P. McGovern for inclusion in these tests because he found that it showed good repellency against biting flies (USDA, ARS, Beltsville, Md.).

One sample tested was an encapsulated synergized natural pyrethrum in which the capsule wall was made by a different experimental process and consisted of a blend of waxes.

Each sample submitted for tests was rated for release characteristics. Such a rating was based upon empirical data obtained from previous tests. No direct determination of release, by chemical analysis, was performed on the test samples.

Microcapsules ranging in size from 30–50 μm were dispersed in an aqueous medium, into which a thickener was added. Final concentrations of the natural pyrethrum ranged from 0.6%–2.2%. In some formulations, piperonyl butoxide synergist was added in ten-fold concentrations to the pyrethrum, before encapsulation.

2.2. Repellency tests

2.2.1. Application of repellent

Five cubic centimetres of the formulation was applied to semi-shaved bellies of guineapigs and spread evenly over an area of $16 \times 9 \text{ cm}^2$. The same guineapig treatment was used for evaluating repellency and toxicity against both tsetse flies and ticks.

2.2.1.1. Tsetse

The tsetse flies used were *Glossina morsitans*. *Glossina* pupae were made available through the courtesy of the Tsetse Research Laboratory, School of Veterinary Science, Langford, Bristol, United Kingdom. Pupae were kept at 28°C at 70% humidity.

Ten-twelve hatched flies were placed in nylon mesh cages ($14 \times 8 \times 4 \text{ cm}$), and kept at $24^\circ\text{--}25^\circ\text{C}$ in a humid incubator. Repellency tests were carried out with hungry flies, teneral flies, 48 h after emergence, or non-teneral flies, 48 h after the last blood meal. Testing was carried out at 28°C and 70% humidity. The treated guineapig was immobilized on its back, using a specially designed restrainer table, and fly cages placed on the treated bellies on their large surface, and exposed to the repellent for 5 min. Instead of covering the cages during testing as is commonly done, the room was darkened; this change in procedure was done in order to prevent accumulation of noxious pyrethrum vapour in the test cage. Immediately upon removal of the cage, the number of engorged flies was recorded and all the flies transferred to a clean cage. The number of knocked down flies was recorded 1/4 h

TABLE I. REPELLENCY OF PYRETHRUM AND OTHER INSECTICIDES TO TSETSE FLIES

A. Non-synergized natural pyrethrum

Compound designation	Estimated rate of release ^a	Concentration (mg/cm ²)	Repellency (days) ^b	MED (mg/cm ²)
<i>Micro-encapsulated preparations</i>				
4632-97-1	M	0.90	9	0.09
97-2	S	0.90	7	0.052
97-3	S	0.80	7 (9)	0.027
4632-105-1	F	0.50	< 6	0.013
105-2	F	0.52	11	0.026
105-3	F	0.52	8	0.026
105-4	F	0.52	9	0.052
105-5	F	0.42	6	0.026
105-6	F	0.50	8	0.026
105-7	M	0.60	10 (15)	0.014
<i>Non-encapsulated preparations</i>				
4515-118		1.05	6	0.052
118		0.52	6	
118		0.26	4	
118		0.13	4	

later. One-half hour later, the cage was placed on an untreated control animal and additional feeding was noted, an indication of the residual effect of the repellent. Mortality was recorded 24 h following testing. We observed that pyrethrum protected the guineapigs for five to six days, and standardized the testing procedure beginning testing at the sixth day following treatment and testing daily until feeding was observed. Formulations that gave full protection for nine days and more were tested again using flies of different ages, including the most resistant stage, pregnant females [11, 12]. The minimal effective dose (MED), defined as the minimum concentration conferring 90% protection from biting, tested 48 hours after application, was obtained by testing serial dilutions of the formulation.

2.2.1.2. Ticks

The ticks used were adult female *Ornithodoros tholozani* starved for over two years. Testing was carried out under the conditions described above, using the

TABLE I (cont.)

B. Synergized natural pyrethrum

Compound designation	Estimated rate of release ^a	Concentration (mg/cm ²)	Repellency (days) ^b	MED (mg/cm ²)
<i>Micro-encapsulated preparations</i>				
4632-104-1	F	0.55	6	0.013
104-2	F	0.52	< 6	0.027
104-3	F	0.52	7 (9)	0.10
104-4	F	0.52	< 6	0.10
104-5	F	0.52	< 6	0.050
104-6	F	0.56	6	0.10
104-7	F	0.46	6	0.10
4683-42-1	M	0.56	6	0.10
43-1	M	0.42	7 (9)	0.04
43-2	M	0.52	< 5	0.027
45-1	S	0.38	7	0.014
45-2	S	0.38	< 6	0.08
46-1	M	0.35	< 6	0.052
46-2	S	0.46	< 6	0.10
47-1	S	0.52	7	0.052
4659-52	M	0.52	7	0.052
4632-97-5	M	0.35	7	0.027
<i>wax capsule</i>				
<i>Non-encapsulated preparations</i>				
4515-113		0.52	5	0.10

same restraining table for the guineapigs as described but with a special apparatus for testing tick repellency and toxicity harnessed to the guineapig. This consists of a stiff rubber sheet with two holes of 5 cm fitted with brass cylinders with bottom flanges each holding a tightly fitted glass cylinder for holding the ticks, projecting slightly from the bottom, according to the method of Bar Zeev and Gothilf [13]. This apparatus is strapped tightly to the guineapig belly. *Ornithodoros* adults do not climb on glass and are therefore in contact with the treated belly area for the whole period of exposure, 10 minutes for repellency testing, six hours for toxicity testing. Repellency testing was begun two days following application,

TABLE I (cont.)

C. Synthetic insecticides

Compound designation	Estimated rate of release ^a	Concentration (mg/cm ²)	Repellency (days)
<i>Micro-encapsulated preparations</i>			
4683-59-1 bioresmethrin	S	0.52	< 2
4632-97-4 resmethrin	M	1.0	< 2
4632-123-1 permethrin	M	0.20	< 2
4632-123-2 permethrin	M	0.90	< 2
4632-123-3 methyl parathion	M	0.52	< 2
4632-97-6 N ₁ N dipropylcyclohexane carboxamide	M	4.5	< 2
<i>Non-encapsulated preparations</i>			
4683-49 bioresmethrin		0.52	< 2
46-97-7 N ₁ N dipropylcyclohexane carboxamide		0.52	< 2

^a Estimated rate of release: S = Slow release
M = Moderate release
F = Fast release.

^b Numbers in brackets indicate maximal protection period, otherwise minimal protection period is given.

and carried out daily using different batches of ticks, ten ticks per batch, and continued until feeding was observed. The number of attached and of engorged ticks was recorded. Mortality was recorded one week after exposure since delayed mortality seemed to occur. Only formulations conferring seven or more days of protection against tsetse were tested for toxicity to ticks.

RESULTS AND DISCUSSION

Formulations of non-encapsulated natural pyrethrum at a concentration of 0.50 mg/cm² gave guineapigs complete protection from tsetse bites for six days (Table IA, 4515–118). Repellency was not increased by synergizing the pyrethrum, since it conferred only five days protection from tsetse bites (Table IB, 4515–113). Micro-encapsulating natural pyrethrum increased the duration of repellency in some

of the formulations. Fifteen of the 27 formulations did not show increased repellency duration. These had minimal effective doses (MEDs) ranging between 0.013–0.090 mg/cm², a wide range, possibly indicative of a wide range in the rate of release. Twelve of these formulations showed increased repellency duration, with the best formulations (4632–105–7 and 4632–105–2) giving a protection time of 10–15 days. Both of these preparations had low MEDs and were not synergized. Treatments were repeated after eight months storage, and showed the same duration of protection as before, indicating that these micro-encapsulated materials have a long shelf life.

Tsetse flies and ticks are vectors of serious diseases, thus it is necessary to protect people and livestock from the pathogens they carry, rather than merely to decrease irritation caused by their bites. We therefore selected the most severe criterion for repellency, 100% protection, although a preparation giving somewhat less protection in the field would still significantly reduce the damage caused by these pests. In many of the formulations, a high protection level, over 90%, persisted for some days after the first-bite record, extending the period of actual protection beyond that cited in Table I. However, we concentrated on 100% protection within the scope of the present research, and deterioration with time was not followed up. Again, there is good reason to believe that a high level of protection would persist for some time.

A high mortality of flies was observed when repellency tests were carried out during the first five days following treatment. After five days, few flies died, even when the repellent continued to confer full protection. This high mortality necessitated delaying determination of the MED, usually done immediately following drying of the repellent, to 48 hours following application, in order to distinguish between the toxic and repellent effects of pyrethrum. Although the mortality of flies tested after the first five days was lower, the flies were affected by exposure to the repellent and did not feed on untreated animals offered to them about 1/2 h later. A longer recovery period, hours, is apparently needed. More teneral flies died than pregnant ones, whereas repellency appeared to be equally effective on both groups.

Examination of the hair of treated animals under the dissecting microscope showed that the microcapsules were clumped and did not adhere well to the hair. Spreader-sticker adjuvants should improve the residual effect of these formulations.

The synthetic pyrethrins tested did not confer protection from tsetse bites when tested only two days following treatment (Table IC), despite their very high toxicity reported by Hadaway [12]. These include micro-encapsulated remethrin, bioresmethrin, and permethrin as well as non-encapsulated bioresmethrin.

The insecticides, non-encapsulated and micro-encapsulated cyclohexane carbamate and micro-encapsulated methyl-parathion, did not deter biting.

Full protection of cattle from tsetse fly bites is especially important when considered as treatment for slaughter cattle. In Africa, tsetse-free livestock production

TABLE II. REPELLENCY AND TOXICITY OF NATURAL PYRETHRUM AND OTHER INSECTICIDES TO THE TICK *Ornithodoros tholozani*

A. Non-synergized natural pyrethrum

Compound designation	Concentration (mg/cm ²)	Repellency (days)	Mortality (%) (a) ^a	Mortality (%) (b) ^b
<i>Micro-encapsulated preparations</i>				
4632-97-1	0.90	5	30	90
97-2	0.90	3	30	
97-3	0.80	< 2	30	
4632-105-1	0.50	< 2	80	
105-2	0.52	> 2	100	80
105-3	0.52	3	60	60
105-4	0.52	3	70	60
105-5	0.42	< 2	60	
105-6	0.50	< 2	100	30
105-7	0.60	< 2	100	80
<i>Non-encapsulated preparations</i>				
4515-118	0.52	< 2	100	

areas are often located far from the large cities and slaughter animals have to travel for weeks through tsetse-infested areas where they may contact trypanosomiasis. Protection from bites may replace the chemoprophylaxis treatment used under such conditions and which requires more skilful application.

Pyrethrum is also very effective as an acaricide, and if the application of micro-encapsulated pyrethrum can protect cattle from infestations of ticks its use can become much more attractive from the economic point of view. We therefore tested the same formulations for their effects as a tick repellent or toxicant (Table II). Starved adult females of *Ornithodoros tholozani* are repelled for shorter periods than the tsetse flies. However, ticks killed within 24 hours of their attachment to the host do not transmit their pathogens which require several days of activation to be virulent. Unlike *O. tholozani*, hard ticks attach and feed for periods of days, and toxicants can act over a long-term period. We therefore tested the effects of the formulations during a six-hour contact period and found that many of the preparations gave 100% mortality.

TABLE II (cont.)

B. Synergized natural pyrethrum

Compound designation	Concentrations (mg/cm ²)	Repellency (days)	Mortality (%) (a) ^a	Mortality (%) (b) ^b
<i>Micro-encapsulated preparations</i>				
4632-97-5	0.35	< 2	50	
4632-104-1	0.55	3	100	
104-2	0.52	< 2	100	
104-3	0.52	< 2	100	50
104-4	0.52	< 2	80	
104-5	0.52	< 2	80	
104-6	0.56	< 2	90	
104-7	0.46	< 2	80	
4683-42-1	0.56	< 2	0	
43-1	0.42	< 2	100	90
43-2	0.52	< 2	50	
45-1	0.38	< 2	100	100
45-2	0.38	< 2	60	
46-1	0.35	< 2	0	
46-2	0.46	< 2	0	
47-1	0.52	< 2	10	90
4659-52	0.52	< 2	0	100
<i>Non-encapsulated preparations</i>				
4515-113	0.52	2	100	

Ticks have become resistant to most of the available acaricides, therefore frequency of dipping or spraying has been increased to once or twice weekly. The systems presented here may be important in Africa in conferring protection from tick- and tsetse-borne diseases. We therefore believe that the effectiveness of these systems as a means of protecting cattle from these two major pests should be tested in the field in infested areas of Africa.

ACKNOWLEDGEMENTS

We gratefully acknowledge the kind assistance of A.M. Jordan of the Tsetse Research Laboratory, Department of Veterinary Medicine, Langford, Bristol,

TABLE II (cont.)

C. Synthetic insecticides

Compound designation	Concentration (mg/cm ²)	Repellency (days)	Mortality (%) (a) ^a
<i>Micro-encapsulated preparations</i>			
4683-59-1 bioresmethrin	0.52	< 2	100
4632-97-4 resmethrin	1.0	< 2	60
4632-123-1 permethrin	0.2	< 2	90
4632-123-2 permethrin	0.9	< 2	70
4632-97-6 N ₁ N dipropylcyclohexane carboxamide	4.5	< 2	0
<i>Non-encapsulated preparations</i>			
4683-49 bioresmethrin	0.52	< 2	100
4632-97-7 N ₁ N dipropylcyclohexane carboxamide	35.0	3	10

^a 10 min exposure two days after application.^b 6 h exposure seven days after application.

United Kingdom, in forwarding a continuous supply of *Glossina* pupae without which this work could not have been undertaken. The research was supported by the U.S. Agency for International Development by Contract AID/ta-c-1420 with the Pennwalt Corporation.

REFERENCES

- [1] HORNBY, H.E., FRENCH, M.H., Introduction to the study of tsetse repellents in the field of veterinary medicine, *Trans. R. Soc. Trop. Med. Hyg.* 32 (1943) 41.
- [2] FINDLAY, G.M., HARDWICKE, J., PHLEP, A.J., Tsetse fly repellents, *Trans. R. Soc. Trop. Med. Hyg.* 40 (1946) 341.
- [3] HOLDEN, J.R., FINDLAY, G.M., Pyrethrum as a tsetse repellent: human experiments, *Trans. R. Soc. Trop. Med. Hyg.* 38 (1944) 199.
- [4] SCHMIDT, M.L., Relative effectiveness of repellents against *Simulium damnosum* and *Glossina morsitans* in Ethiopia, *J. Med. Entomol.* 14 (1977) 276.
- [5] SHOLDT, L.L., GROTHONS, R.H., SCHRECK, C.E., GOUCK, H.K., Field studies using repellent-treated wide-mesh net jackets against *Glossina morsitans* in Ethiopia, East Africa *Med. J.* 52 (1975) 277.

- [6] GLYNNE JONES, G.D., SYLVESTER, N.K., Pyrethrum as an insect repellent: Part I: Literature reviews, *Pyrethrum Post* 8 (1966) 28.
- [7] GALUN, R., Protection of livestock from tsetse bites by means of repellents, *Pyrethrum Post* 13 (1975) 2.
- [8] LOWELL, J.R., Jr., CULVER, W.H., DeSAVIGNY, C.B., "Effects of wall parameters on the release of active ingredients from microencapsulated insecticides", ACS Symposium Series, No. 53, Controlled Release Pesticides (1977).
- [9] PALMER, J.S., Toxicologic evaluation of a microencapsulated formulation of methyl parathion applied dermally to cattle, *Am. J. Vet. Res.* 39 3 (1978) 429.
- [10] PALMER, J.S., Toxicologic evaluation of microencapsulated formulation of diazinon applied dermally to cattle, *Am. J. Vet. Res.* 39 7 (1978) 1231.
- [11] BURNETT, G.F., The susceptibility of tsetse flies to topical application of insecticides: III. The effects of age and pregnancy on the susceptibility of adults of *Glossina morsitans* Westw., *Bull. Ent. Res.* 53 (1962) 337.
- [12] HADAWAY, A.B., Toxicity of insecticides to tsetse flies, *Bull. World Health Organ.* 46 (1972) 353.
- [13] BAR ZEEV, M., GOTHILF, S., Laboratory evaluation of tick repellents, *J. Med. Entomol.* 110 (1973) 71.

DISCUSSION

W.J. KLOFT: Your results showing the very fast action of several of your new pyrethrum formulations are of particular interest to me. For example, it seems that permethrin acts so quickly that it even prevents certain arthropods which feed on animal hosts from probing and piercing. This is important, because the transmission of pathogens by regurgitation and other pathways could be prevented in this way. Even in aphids — the main vectors of plant diseases — it has been possible to arrest regurgitation. Several recently developed specific aphicides, such as certain carbamates, do in fact prevent aphids from probing and puncturing by their fast action. In this context one should consider the many parallel problems affecting vectors of both animal and plant diseases.

R. GALUN (*Chairman*): Permethrin does not prevent tsetse from biting, but it does prevent mosquitoes, fleas and some species of tick from biting by its fast killing action. It could most probably prevent aphids from probing provided it were permitted to be used on plants.

P. FINELLE: Do your encapsulated pyrethrins have a true repellent action or do they just inhibit piercing? Can they work at a distance or only on contact?

R. GALUN (*Chairman*): Flies do not probe on the treated area but will feed on an adjacent area. Thus, successful protection requires the entire animal to be sprayed.

M.J. MUTINGA: Have you considered the chemical side-effects of repellents on the hosts, or are they not important at this stage?

R. GALUN (*Chairman*): Micro-encapsulation reduces the dermal toxicity to a level that ensures the animal's safety.

D.A. DAME: Recent studies suggest that successful trapping of 1–2% of a tsetse population daily will result in population control and perhaps in elimination. Your studies show that the synthetic pyrethroids are effective toxicants for tsetse even though they may not prevent feeding. It may be that these compounds, if applied to bait animals, would give effective population control provided a sufficient percentage of the natural fly population came to feed on the bait animals.

R. GALUN (*Chairman*): Competition with wild animals as a food source would probably make it difficult to affect enough of the population.

D.A. DAME: Success would definitely depend on the percentage of the natural fly population that could be contacted with the pyrethroid insecticide.

R. GALUN (*Chairman*): Natural pyrethrum will kill most of the flies that land on the treated animal in addition to protecting it from bites. It is therefore preferable to the synthetic pyrethrins.

C.F. CURTIS: I would suggest that it might be better to report your results in terms of time required for decline to 50% repellency (ET_{50}) rather than minimum repellency time. The latter is dependent on sample size and presumably requires relatively large numbers of replicates with each formulation in order for reliable results to be obtained. I suppose that even after 100% repellency has been lost, partial repellency could still be of epidemiological significance.

R. GALUN (*Chairman*): I agree with you, but I'm afraid that if repellents are to be relied upon as a replacement for chemoprophylaxis, one needs 100% protection.

A. HASSAN (*Scientific Secretary*): Do you think that routine use of the micro-encapsulation technique on large animals is likely to lead to any significant residues, e.g. in meat or milk?

R. GALUN: I believe that pyrethrum will be decomposed by the animal and will not accumulate in it.

APPLICATION OF ISOTOPE-LABELLED COMPOUNDS IN THE STUDY OF THE CHEMICAL STABILITY OF PESTICIDES

M. RÖSSELER, D. LUTHER,
H.-C. ABENDROTH, H. KOCH
Central Institute of Isotope and Radiation
Research,
Academy of Sciences of the German
Democratic Republic,
Leipzig,
German Democratic Republic

Abstract

APPLICATION OF ISOTOPE-LABELLED COMPOUNDS IN THE STUDY OF THE CHEMICAL STABILITY OF PESTICIDES.

The user of pesticides requires specific biological modes of action from the corresponding commercial products. Impurities and degradation products may cause uncontrollable toxicological reactions. The evaluation of results obtained in studying subject matters as presented at this symposium is related to some fundamental premises that must be fulfilled if misinterpretations are to be avoided. One such premise is profound knowledge of the chemical stability of the effective substance in question and its formulations under storage conditions as well as under those of analytical sample preparation and detection. The advantages of the application of radioactive-labelled compounds to studies of the following topics are presented by use of the labelled effective substances dimethoate and 1-butyl-amino-cyclohexane-phosphonic acid dibutyl ester: storage stability of the pure effective substance and its formulations; effects of selected impurities, such as technical by-products, moisture or water content, binding or carrier materials, organic solvents, chemical stabilizers and other formulation components on storage properties; temperature dependence of the storage stability; selection of suitable analytical techniques allowing the quantitative determination of the effective substance without interference effects caused by any by-product; reduction of the necessary analytical expense; disclosure of sources of error in the application of usual analytical techniques; improvement of possibilities of an immediate and clearer discrimination between types and amounts of compounds in a chemical system consisting of one pesticide and its degradation or reaction products at the beginning and at the end of an experimental or reaction period. Work on these topics was carried out by means of radiochemical analytical techniques, such as radio thin-layer chromatography (also combined with liquid scintillation counting), radio gas chromatography, autoradiography and isotope dilution analysis. Results are discussed, especially of experiments dealing with dimethoate and its technical by-products.

1. INTRODUCTION

The economy of application of pesticidal effective substances is decisively given by the storage stability of their formulations, by the temperature stability of the latter, and by the stability of the effective substance in question against possible chemical action of individual formulation components and their impurities.

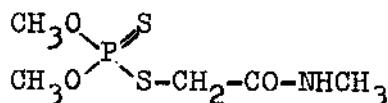
Investigating the chemical stability of pesticidal effective substances in dependence on limiting factors such as degree of purity, composition of the formulation, container material, temperature, and time of storage requires a very great deal of chemical analysis.

The general requirement for the applied analytical procedure, namely quantitative determination of the effective substance without interference effects caused by accompanying bodies, especially in the thermally relatively unstable organophosphorus compounds, encounters considerable difficulties which may often be overcome by application of isotope-labelled compounds.

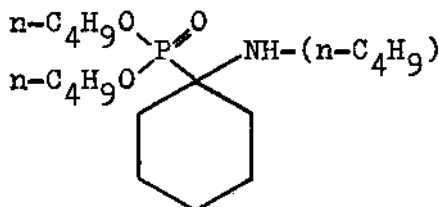
The investigations presented in this study were carried out using the ^{32}P - or ^{35}S -labelled effective substances 0,0-dimethyl-S-[N-methyl-carbamoylmethyl]-phosphorodithioate (I, dimethoate), and 1-butylamino-cyclohexane-phosphonic acid dibutyl ester (II).

Dimethoate (I) is one of the most commonly used organic phosphate insecticides and acaricides with systemic action for use under various conditions.

Its formulations are suitable for control of sucking and biting pests, leaf- and fruitminers, both on field and forage crops, vegetables, fruits, special sub-tropical and tropical cultivations and on livestock.



(I)



(II)

1-Butylamino-cyclohexane phosphonic acid dibutyl ester (II) may be used not only for foliage destruction in the potato, desiccation in rape, leguminous plants and plants of special cultivations grown for seed, but also for defoliation in cotton and tree nursery fruit trees. Furthermore, it has been applied for weed control in some cultivated plants.

2. METHODS

For subsequent applications, given as examples, the following general operational method will be valid:

- (a) Investigation of the storage stability of the pure effective substance and its formulations.
- (b) Study of the effect of selected impurities on storage stability.
- (c) Selection of suitable carrier materials for dust formulation and wettable powders.
- (d) Selection of suitable solvents for liquid formulations.
- (e) Investigation of selected organic compounds as chemical stabilizers.

The purified radioactive-labelled effective substance of known specific activity is added to the formulation preparations in homogeneous distribution. Samples are taken at fixed time intervals with subsequent chromatographic separation of sample aliquots. The character and quantity of chemical conversions that have taken place are determined by measuring the activity distribution in the chromatograms obtained.

The use of radioactive-labelled compounds permits the chemist to determine their content by detection of the radioactivity. The most important procedure for the determination of individual compounds in a given mixture consists in its separation.

Chromatographic techniques are convenient for separation through their high resolving power and reliability. In our laboratory we have employed thin-layer chromatography as well as gas-liquid chromatography to resolve organo-phosphorus compounds.

Table I presents a survey of the advantages and disadvantages of the various techniques.

2.1. Thin-layer radiochromatography

In our separation experiments 20 × 20 cm glass chromatoplates were used. The adsorbent consists of a thin uniform film (0.25 to 0.75 mm) of alumina or silica gel. After the samples had been applied, the plates were developed. Special

TABLE I. COMPARISON OF APPLIED ANALYTICAL METHODS

	Conditions and detector	Limit of detectability of ^{32}P -labelled compounds ^a	Advantages	Disadvantages
Thin-layer radiochromatography	2π -methane gas-flow proportional counter (strip scanning)	~ 50 Bq ($\sim 7 \times 10^{-9}$ g of dimethoate)	No thermal decomposition	No direct mass detection
	Liquid-scintillation counting (elution analysis)	~ 2 Bq ($\sim 3 \times 10^{-9}$ g of dimethoate)		Lower resolving power than in gas-liquid chromatography Only one separation per hour
Gas-liquid radiochromatography	Gas-flow proportional counter (measuring volume 8 cm^3 ; velocity of carrier gas $30\text{ cm}^3/\text{min}$; residence time: 0.267 min)	~ 150 Bq ($\sim 2 \times 10^{-7}$ g of dimethoate)	Simultaneous determination of mass and activity High resolving power Low expenditure of time for separation (10 min per analysis)	Unsuitable for thermally unstable compounds

^a Specific activity of dimethoate (^{32}P): 7.4×10^8 Bq/g; limit of detectability equal background counting rate.

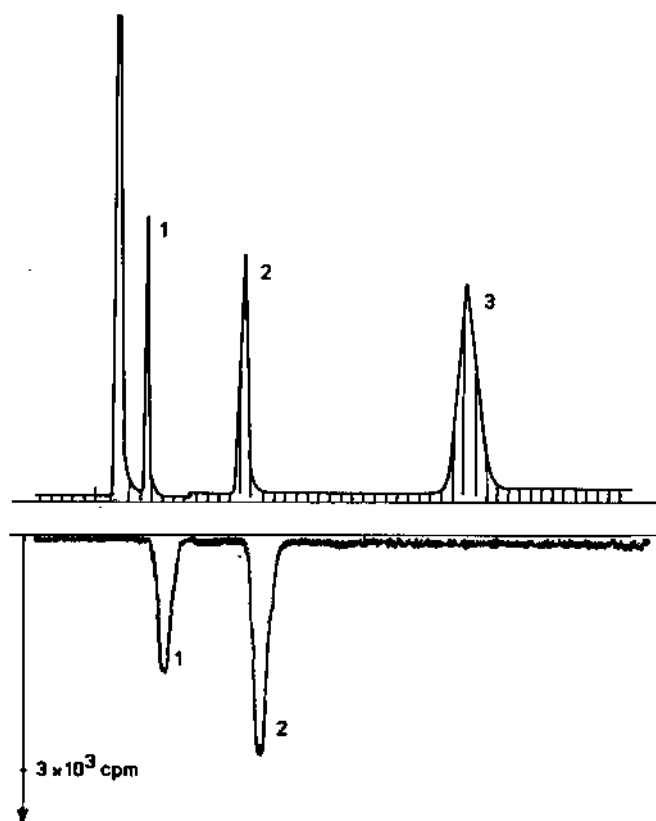


FIG.1. Gas liquid radiochromatogram of an analysis sample:

- 1 – *O,O,S-trimethyl phosphorodithioate (^{32}P)*
- 2 – *O,O-dimethyl-S-(O-methylcarboxymethyl)-phosphorodithioate (^{32}P)*
- 3 – *dimethoate (non-labelled).*

procedures can be found in books and reviews giving details of thin-layer chromatography. For visualization of the individual organophosphorus components the plate is sprayed with an aqueous solution of palladium dichloride.

Radiometric detections are carried out by elution analysis as well as by strip scanning. In elution analysis an area of adsorbent is scraped from the plate, and the sample is recovered by extraction and detected sensitively by liquid scintillation counting. Strip scanning denotes a method in which the plate is moved under a radiation detector, when the radiochromatogram obtained directly shows the distribution of radioactivity on the chromatoplate.

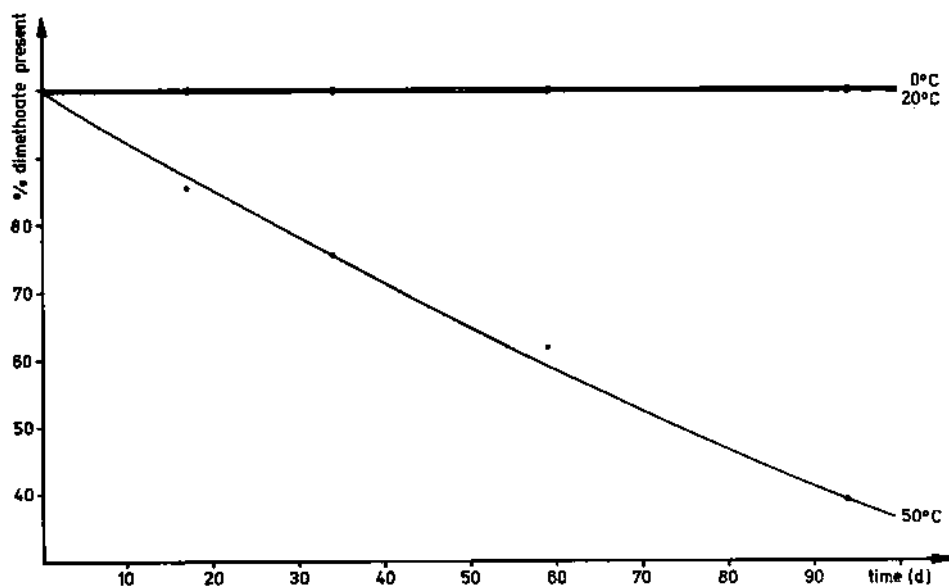


FIG.2. Storage stability of thin-layer chromatographically purified dimethoate at various temperatures.

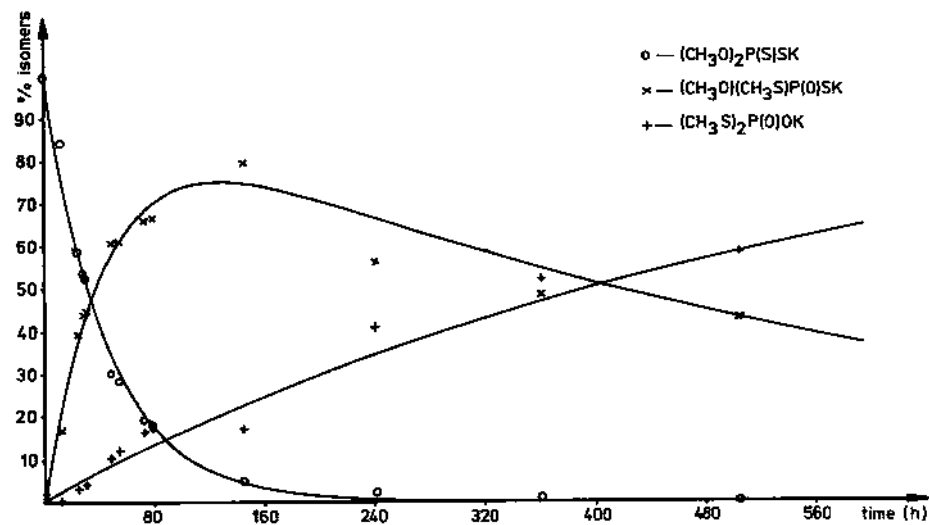


FIG.3. Isomerization of *O,O*-dimethyl phosphorodithioic acid (potassium salt) in aqueous solution at $T = 60^{\circ}\text{C}$.

2.2. Gas-liquid radiochromatography

This method separates volatile compounds. To prevent thermic and catalytic decomposition of organophosphorus compounds it is necessary to use glass columns and a glass flash heater also. The packing of the column consists of Gas-Chrom-Q (80–100 mesh) coated by 11% DC 200 + 0.01% Versamid 900.

The main advantage of the gas-liquid radiochromatography procedure is the possibility of determining the mass and the activity of compounds simultaneously by using a continuous flow system. After the compounds have left the column the stream is split: then the mass is measured by a hydrogen-flame ionization detector and radioactivity is detected in a proportional counter tube developed in our laboratory. A chromatogram is shown in Fig. 1.

3. RESULTS AND DISCUSSION

3.1. Investigation of the storage stability of the pure effective substance and its formulations

First, an exact estimation of the effect of variable factors of the stability of an effective substance will be obtained after particulars of the storage stability of the pure compound are available.

Results obtained at various temperatures with the thin-layer chromatographically purified effective substance dimethoate are given as an example. As shown in Fig. 2, the pure compound remains completely unaffected after a three-month storage at 0° and 20°C, whereas it shows a high degree of decomposition at 50°C.

Provided the several decomposition or rearrangement products can be separated from each other, it is possible to obtain additional information on the kinetics of these conversion processes. Thus, the operational method presented for stability studies is generally suitable also for technological investigations, and studies of pesticide metabolism.

Isomerization of O,O-dimethyl-phosphorodithioic acid (as potassium salt) is given as an example for a re-arrangement reaction. The compound serves as starting material in the technical synthesis of dimethoate.

The isomerization proceeds as follows:

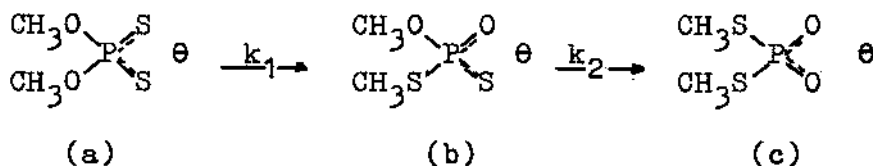


TABLE II. EFFECT OF WATER CONTENT ON STORAGE STABILITY OF DIMETHOATE CRYSTALS AT $T = 23 \pm 1^\circ\text{C}$

Water content (%)	Dimethoate (%) present after a storage time of			
	30 d	65 d	146 d	237 d
0.1	99.5	97.2	95.1	97.2
0.4	100.3	98.7	97.7	96.7
0.8	99.9	98.4	98.7	97.8
1.4	98.7	100.8	99.4	99.7
2.2	100.2	101.1	97.5	98.8

TABLE III. SELECTION OF SUITABLE CARRIER MATERIALS FOR DIMETHOATE DUST FORMULATIONS AND WETTABLE POWDERS AT $T = 22^{\circ}\text{C}$

Type of formulation	Carrier	pH	Storage time (d)	Dimethoate present (%)
Dust	Amorphite	3	19	96.0
			111	91.8
	Slate powder	7-8	18	95.6
			110	89.7
	Electrical filter ash	11	20	1.0
Wettable powder	Amorphite	3	27	97.0
			112	94.9
	Diatomite	8	27	94.0
			112	91.0
	Betonite	10	27	76.4
			112	41.0

Distribution of the isomers as a function of the storage time of the solution determined by measurement of the ^{32}P -activity distribution among the three isomers (a, b, c) is shown in Fig.3.

3.2. Study of the effect of selected impurities on storage stability

Investigation of the effect of a fixed water content on the storage stability of dimethoate crystals at $T = 23^{\circ} \pm 1^{\circ}\text{C}$ is given as an example of investigations for the type mentioned. Table II summarizes the results obtained using ^{35}S -labelled dimethoate. As shown, dimethoate crystals are of remarkably high stability under given experimental conditions.

In all cases lowering of concentration, present after a storage time of eight months, amounts to less than 3.5%.

Taking into consideration the usual spread of analysis values obtained by measurements of activity distribution, there is no significant difference among samples of variable water content in the selected concentration range with regard to stability.

TABLE IV. STORAGE STABILITY OF 1-BUTYLAMINO-CYCLOHEXANE PHOSPHONIC ACID DIBUTYL ESTER FORMULATIONS AT $T = 35^{\circ}\text{C}$ DEPENDING ON THE SOLVENT USED

Solvent	Storage time (d)	$C_0(\%)^a$	$C(\%)^b$
Cyclohexane	68	85.1	81.4
Lubricating oil	68	77.1	100.5
Diesel oil	68	71.9	102.5
n-Butyl alcohol	100	71.7	55.9
Cyclohexanone	100	72.3	80.4
n-Butyl acetate	98	74.0	69.3
Without (e.g. technical effective substances only)	139	87.7	89.2

^a C_0 = content (%) of the effective substance in the formulation at starting point t_0 .

^b C = effective substance (%) present after given storage time.

3.3. Selection of suitable carrier materials for dust formulations and wettable powders

Marked dependence of decomposition of the effective substance on the pH-value of the formulation in question resulted from aging experiments at room temperature ($T = 22^{\circ}\text{C}$) using dust formulations and wettable powders for the effective substance dimethoate.

Carrier materials, the aqueous suspended matter of which shows pH-values in the alkaline range, gave rise to a comparatively fast dimethoate decomposition. In contrast to this, carriers with pH-values ≤ 7 are suitable as formulation components (Table III).

3.4. Selection of suitable solvents for liquid formulations

Tests of organic solvents as liquid formulation medium in the preparation of formulations of the effective substance 1-butylamino-cyclohexane phosphonic acid dibutyl ester are given as examples.

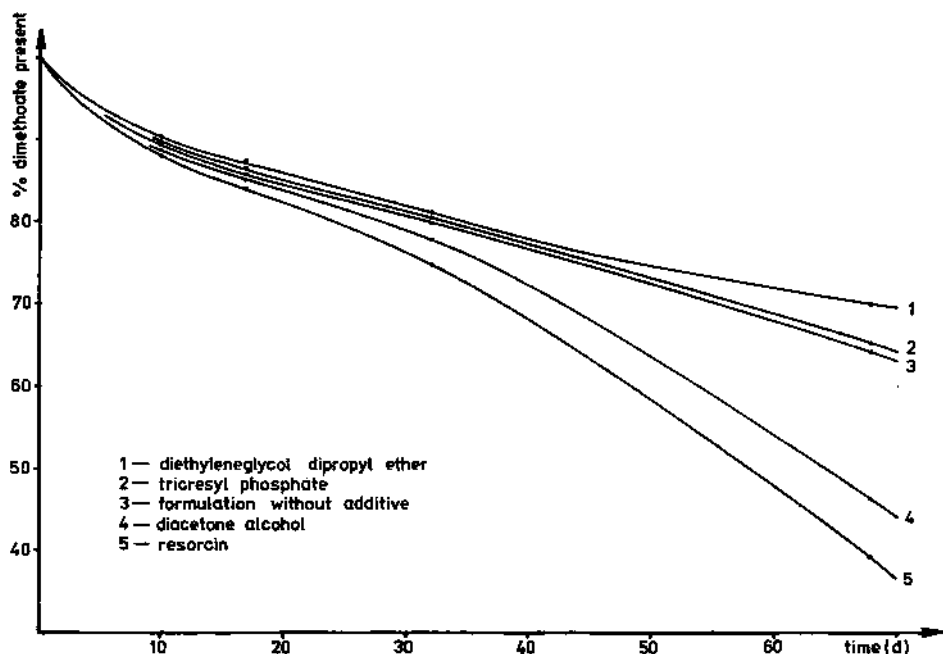


FIG.4. Selection of organic compounds as chemical stabilizers for a liquid trial formulation of dimethoate at $T = 50 \pm 2^\circ\text{C}$.

Among the solvents listed in Table IV only lubricating and diesel oils are suitable for formulation purposes.

On the other hand, a well-defined negative modulation of the stability of the effective substance, related to the storage stability of the undiluted technical compound, is caused by the other solvents.

3.5. Examination of selected organic compounds as chemical stabilizers

A test for use in dimethoate formulations has been carried out for a series of organic compounds, from which a stabilizing effect would have been expected according to the literature.

As shown by the results listed in Fig.4, only diethylene glycol dipropyl ether yields a low stabilizing effect among the compounds tested.

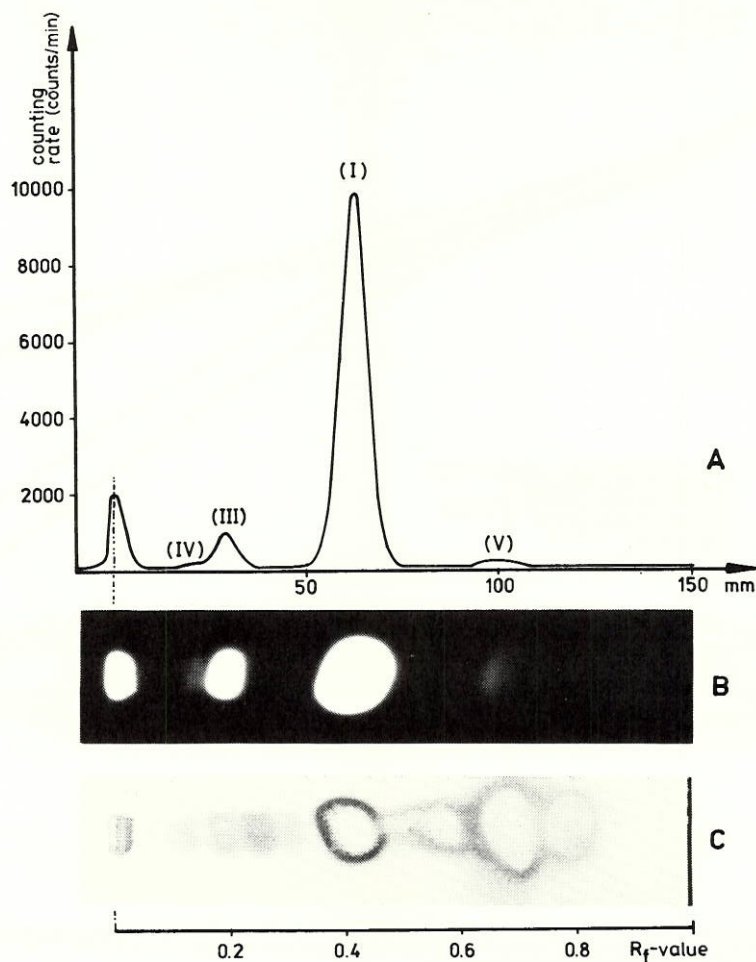


FIG. 5. Dimethoate formulation with 10% diacetone alcohol after a storage time of 17 days at $T = 50 \pm 2^\circ\text{C}$:

A — ^{32}P -activity distribution

B — radioautograph

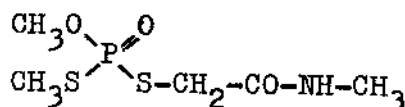
C — thin-layer chromatogram.

Triethyl- and tricresyl phosphates behave indifferently, whereas diacetone alcohol and resorcinol hasten the decomposition.

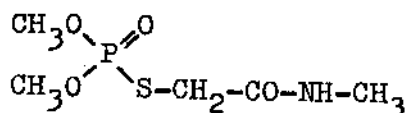
Figure 5 exhibits ^{32}P -activity distribution (A) in a test solution with diacetone alcohol, taken after 17 days, together with the corresponding radio-autograph (B) and the figure (C) of the developed thin-layer chromatogram.

(A) and (B) markedly signify that dimethoate has mainly been converted into hydrophilic decomposition products (starting spot) and the S-methyl isomer

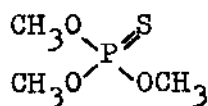
of dimethoate (III). O,O-dimethyl-S-[N-methylcarbamoylmethyl]-phosphorothiolate (IV), the oxygen analogue of dimethoate, and O,O,O-trimethyl phosphorothioate (V), are formed in low concentrations only.



(III)



(IV)



(V)

The results demonstrate that the use of radioactively labelled compounds allows highly sensitive determination of individually labelled effective substances with relatively small effort.

Thus, a survey may be obtained on the behaviour of the effective substances investigated under variable conditions. The information will be of interest also in the application of such compounds in environmental protection.

DISCUSSION

A. HASSAN (*Scientific Secretary*): You indicate in Table III that the stability of dimethoate depends on the type of carrier material. Since these materials have different pH levels, I would have thought that degradation was directly correlated with the pH rather than with the carrier type.

M. RÖSSELER: Yes, indeed, I fully agree with you. From data we have obtained when testing carrier materials for dust formulations and wettable powders, we have to conclude that the pH value of the formulation is responsible for the degree of degradation in a given time. The pH value of the formulation itself, however, depends on the type of carrier material used in the test.

BIOTRANSFORMATION OF LINDANE IN MAMMALS AND ITS SIGNIFICANCE FOR SAFETY ASSESSMENT OF THE PESTICIDE

U.V. GOPALASWAMY, M.P. PHONDKE, A.S. AIYAR
 Biochemistry and Food Technology Division,
 Bhabha Atomic Research Centre,
 Trombay,
 Bombay,
 India

Abstract

BIOTRANSFORMATION OF LINDANE IN MAMMALS AND ITS SIGNIFICANCE FOR SAFETY ASSESSMENT OF THE PESTICIDE.

Lindane accounts for about 50% of the total pesticide usage in India for agriculture and other uses. In view of its relative chemical stability and lipid solubility, it is known to enter the food chains. Investigations have, therefore, been undertaken on the fate and metabolism of lindane in rats. Many of the earlier studies on mammalian transformation of organochlorine pesticides have been carried out with intact animals, and it is difficult to rule out the possibility of some of the metabolic products identified in urine, faeces and tissue arising as a result of microbial activity. In the present studies, an attempt has been made to identify the metabolic capability of rat liver enzymes for the transformation of lindane. Following the incubation of liver slices with U-¹⁴C-lindane in the presence of a broad spectrum antibiotic to prevent microbial growth, radioactivity was found to be associated with hexachlorobenzene, penta and trichlorophenols. Although dehydrochlorination reactions of the pesticide, as a detoxification mechanism in mammalian organism, have been reported earlier, its direct aromatization to hexachlorobenzene is a novel pathway. Another interesting observation emerging from these studies relates to the liberation of labelled CO₂, during the incubation of the pesticide with liver slices. This would indicate degradation of the carbon ring of pesticide either as a cyclohexane derivative or following its aromatization; although such cleavage of the ring of lindane is known to occur in micro-organisms, this is the first time evidence for a similar possibility with a mammalian organism has been obtained. The conversion of lindane to hexachlorobenzene in the rat may have implications for the safety of this pesticide from the genetic view point since several studies have reported that hexachlorobenzene is carcinogenic in experimental animals. In view of the reported correlation between carcinogenicity and mutagenicity, both lindane and hexachlorobenzene have been tested for possible mutagenicity using the *Salmonella typhimurium* test system; the results have been equivocal with variable response being observed in different experiments.

1. INTRODUCTION

The use of organochlorine pesticides constitutes an important component of the (a) efforts to control agricultural pests, and (b) public health measures to

TABLE I. USE OF BENZENE HEXACHLORIDE FOR AGRICULTURAL AND PUBLIC HEALTH PURPOSES IN INDIA

Pesticide	Total consumption ($\times 10^{-6}$ kg)	
	1976-77 ^a	1977-78 ^a
Benzene hexachloride	24.59	28.64
Total	51.92	58.98
% of total	47.36	48.56

^a 12 months.

eliminate insect vectors of diseases, in most areas of the developing world. In particular, benzene hexachloride (BHC) is being used in increasing quantities because of its low cost, ease of manufacture, broad spectrum of biological activity and ready availability, notwithstanding the fact that for ecological reasons its use in industrialized countries has dropped sharply. It has been established that it is lindane, the gamma isomer of the compound, which has the insecticidal property, but in practice technical BHC, consisting of a mixture of several isomers, is most often used, because of the advantage of the lower cost of production. The extent of consumption of BHC in relation to total pesticide usage in India is indicated in Table I.

The ability of the organochlorine pesticides to enter the food chain in significant quantities, as a result of their relatively high stability, lipid solubility and propensity for bio-accumulation, poses a possible health hazard to man, the proper evaluation of which requires experimental studies in other mammalian organisms. Several investigations have been carried out on the metabolism and toxicity of BHC, as well as of lindane, in a variety of experimental animals, and the subject has been periodically reviewed [1, 2].

On the basis of the nature of urinary metabolites isolated from rats fed BHC, Grover and Sims [3] proposed a pathway involving mainly dehydrochlorination reactions yielding chlorinated aromatic compounds. This postulated scheme of detoxification of the pesticide has formed the basis of several subsequent studies to characterize the metabolites. Whereas there is general agreement that a series of chlorinated phenols and benzenes are the ultimate products which are excreted either in the free form or as conjugated water-soluble compounds, opinions on the nature of the early transformation(s) of lindane are divergent. In agreement with the earlier proposal [3], and based on their own extensive investigations,

Engst and co-workers [4] have suggested that γ -pentachlorocyclohexene (PCCHE) is the first intermediate arising from lindane by dehydrochlorination, and that it has a central position in mammalian degradation of lindane. On the other hand, Chadwick and his collaborators consider that PCCHE may not be on the main degradative pathway [5]; a novel dehydrogenation reaction of lindane, identified by them and yielding γ -hexachlorocyclohexene (HCCHE) may represent an important step in the mammalian transformation [6].

An aspect of particular importance relates to the possible chronic toxicological effects of lindane, because of its widespread use, persistence in the environment and consequent build-up in tissues. The recent awareness of the presence of genetic toxicants in the environment, resulting in such subtle effects as mutagenicity, carcinogenicity and teratogenicity with prolonged periods of latency, makes it imperative that the safety of the pesticide, as also its known metabolites, be adequately assessed. The carcinogenicity of lindane has remained controversial [7-11] and points to the urgent need for additional information.

In considering the toxicity of lindane in mammalian organisms, note must be taken that several dietary factors have been reported to influence the metabolism and excretion of lindane, thereby causing significant changes in the steady-state levels in tissues [13-15]. Such findings may have practical significance, since in general the more rapidly a compound is eliminated from the body, the less toxic it is likely to be.

The present study relates to some of our observations on the foregoing aspects of lindane metabolism that may have a bearing on the toxicity assessment of the pesticide.

2. MATERIALS AND METHODS

2.1. Chemicals

Analytical standards of γ -hexachlorocyclohexane (lindane), pentachlorophenol (PCP) and some other metabolites were obtained from the Environmental Protection Agency, Research Triangle Park, North Carolina, United States of America. Hexachlorobenzene (OAS grade) was purchased from BDH Chemicals, Poole, United Kingdom. Glucose-6-phosphate and NADP were products of Sigma Chemical Co., St. Louis, Missouri, United States of America, and 3,4-benz (α) pyrene of Calbiochem, San Diego, California, United States of America. Aroclor 1254 was obtained through the courtesy of Monsanto Chemical Co., St. Louis, Missouri, United States of America. $U-^{14}C$ -lindane (sp. act. 46 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, United Kingdom. The antibiotic 'Keflin' was a product of Eli Lilly and Co., Indianapolis, Indiana, United States of America. All other chemicals and solvents were of analytical reagent grade.

2.2. Animals

For metabolic transformation studies using liver slices, young adult male Wistar rats maintained on a nutritionally adequate laboratory stock diet and weighing 100–150 g were employed; some of the rats were administered a single dose of Aroclor 1254 (50 mg/kg body wt), by stomach tube, 48 hours before sacrifice, for induction of microsomal enzymes. Swiss albino female weanling mice were used in studies to assess the influence of nutritional status on excretion of the pesticide.

2.3. In vitro metabolic transformation of lindane

The rats were decapitated, thoroughly bled and the livers excised. After quick chilling in cold Krebs-Ringer phosphate buffer, pH 7.4, the livers were cut into thin uniform slices of less than 0.5 mm thickness with a Stadie-Riggs tissue slicer. The liver slices (1.0g), suspended in 4 ml of Krebs-Ringer phosphate buffer containing 1 μ Ci of U- ^{14} C-lindane and 10 mg of a cephalosporin antibiotic, 'Keflin', were incubated for 60 min at 37°C in Warburg flasks. The central well in the flasks contained 0.2 ml of 20% KOH, with a fluted filter paper dipped into it. The metabolic transformations were terminated by the addition of 1.0 ml 2N HCl to the incubation mixture.

2.4. Isolation of metabolites and counting of radioactivity

The KOH in the central well was quantitatively transferred together with the filter paper, by repeated washings, into a test tube. The CO₂ absorbed was precipitated as BaCO₃ [16] and the radioactivity counted in a liquid scintillation spectrometer, after suspension in toluene containing 4% Cab-O-Sil. The incubation mixture was transferred to a test tube containing 1g NaCl. The incubation flask was rinsed with 1.0 ml of 2N HCl, followed by 15 ml of benzene which was added to the incubation mixture. The tube was shaken vigorously on a Vortex mixer for 1 min, centrifuged for 3–5 min at 3000 rev/min and the benzene layer pipetted off. The extraction was repeated once more and the combined extracts were dried under vacuum. The metabolites were separated by thin layer chromatography, first with benzene as solvent and then with chloroform as solvent. The spots corresponding to various standards were separately scraped off the plates, eluted and evaporated to dryness. The radioactive samples of hexachlorobenzene and pentachlorophenol were diluted with the respective cold compound and repeatedly crystallized from cyclohexane : hexane (1:1) and carbon tetrachloride respectively. The radioactivity was measured in a liquid scintillation spectrometer.

2.5. Excretion studies

For these experiments, mice which had been maintained for a year on a laboratory stock diet were used. Two of the mice were from an ad libitum fed group, whereas the other two were from a group whose feed intake had been restricted to approximately half that consumed by the other group. All the mice were individually caged in metabolism cages and were orally administered $U\text{-}^{14}\text{C}$ -lindane (1 μCi per animal, diluted with unlabelled lindane 3 mg/100 g body weight) dissolved in 0.5 ml of groundnut oil. The urine and faeces of each animal were collected daily over a seven-day period and preserved. An aliquot of the dried and powdered faeces from each animal was suspended in toluene containing 4% Cab-O-Sil for liquid scintillation counting. Radioactivity in urine samples was counted by taking 0.5 ml of the sample in toluene : Triton X 100 (2 : 1 vol./vol.) for liquid scintillation counting. In separate studies, reported elsewhere [17], the urinary metabolites were fractionated into neutral metabolites, polar metabolites, free chlorophenols and conjugated products.

2.6. Mutagenicity testing

The mutagenicity of lindane and hexachlorobenzene was examined using the microbial/liver microsomal test system of Ames [18]. The compounds were dissolved in acetone (500 $\mu\text{g}/\text{ml}$) and were added at different concentrations to the molten top agar, along with 0.1 ml ($\sim 10^7$ cells) of the tester strain of *Salmonella typhimurium* TA 98, and 0.4 ml of the S_9 reaction-mix containing the microsomes and cofactors. The microsomal fraction was obtained from rats which had been administered Aroclor 1254 (500 mg/kg body wt) 96 hours earlier. The animals were fasted for 24 hours before they were killed. Livers were homogenized in 0.15M KCl (1 g $\equiv$ 3 ml), centrifuged at 9000 g for 10 min, and used as the source of microsomal enzymes. The S_9 fraction contained (per ml) 0.4 ml of the microsomal fraction, 0.2 ml of 0.2M Na_2HPO_4 , 0.02 ml of 0.4M MgCl_2 , 0.02 ml of 1.65M KCl, 1.52 mg glucose-6-phosphate and 3.05 mg NADP. 3, 4 Benz (α) pyrene was used as a positive control and acetone, which was used as the solvent as a control. The plates were incubated at 37°C for 48 hours and the number of revertant colonies counted.

3. RESULTS AND DISCUSSION

The large number of studies that have been carried out earlier on the identification of metabolites, especially in the urine, following the oral administration of lindane in the whole animal, have been helpful in elucidating the overall detoxification of the pesticide. Such efforts do not, however, yield

TABLE II. INCORPORATION OF RADIOACTIVITY FROM U-¹⁴C-LINDANE INTO METABOLITES BY RAT LIVER SLICES

Metabolite	Radioactivity incorporated (dis/min)	
	Control	Aroclor administered
Hexachlorobenzene	35 010 ± 296	80 070 ± 416
Pentachlorophenol	3 010 ± 194	7 650 ± 211
Carbon dioxide	1 120 ± 152	2 660 ± 145

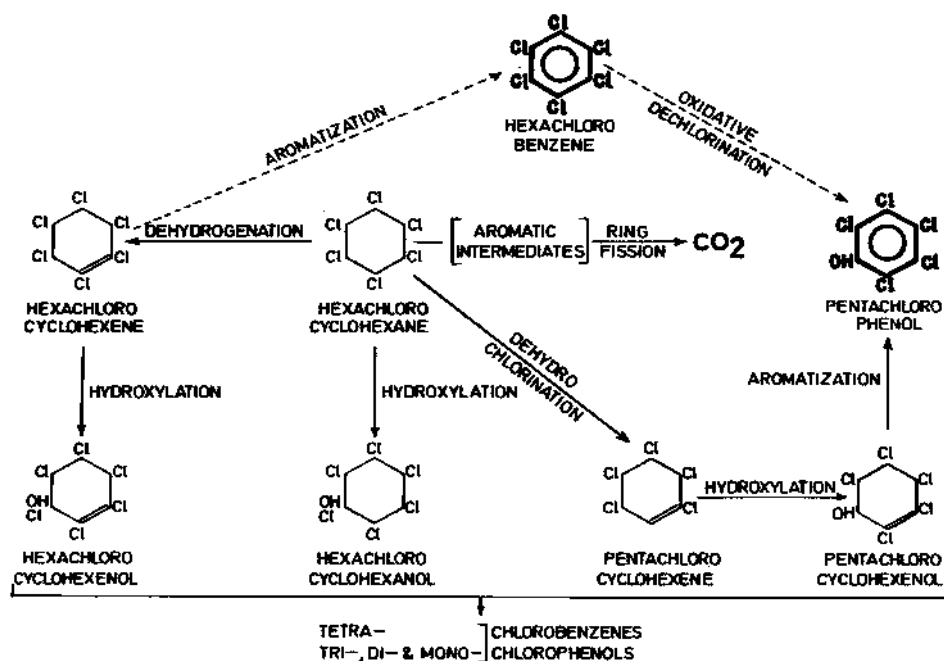


FIG.1. Mammalian biotransformation of lindane. The metabolites identified in the present studies are shown in bold; the broken lines represent pathways that have been proposed for the first time.

unequivocal data on the mammalian capability for specific metabolic transformations, as they do not delineate the influence of the intestinal micro-organisms. The possibility also exists that the characterization of urinary metabolites, comprising the ultimate water-soluble excretory products, may not generate comprehensive information on the metabolic pathway, since the tissues may contain metabolites that are either very poorly or not at all degraded and may, therefore, tend to accumulate. In the present studies, the metabolism of ^{14}C -labelled lindane has been followed in liver slices and the various known metabolites separated by published procedures. However, in the data presented here, attention has been focused on those metabolites whose existence has not been reported so far in animal tissues or where the evidence for their formation is still fragmentary.

The data on the incorporation of radioactivity from lindane into hexachlorobenzene (HCB), pentachlorophenol (PCP) and carbon dioxide are summarized in Table II. The formation of HCB from lindane would represent an aromatization process involving sequential removal of six hydrogen atoms, but with all the chlorine atoms being retained in the molecule. This is not a novel biological reaction, as HCB has been identified as a metabolite of lindane in both micro-organisms and plants [2, 19–23]. The presence of this compound has not, however, been established in animal tissues, although its possible existence has been referred to in one investigation [24]. In our experiments, after the preliminary chromatographic separation, HCB has been purified by repeated crystallization and efforts are currently underway for a more rigorous assessment of the radio-purity of the metabolite. Both lindane and HCB have been shown to be converted into a large number of common metabolites in animals [2, 25–31]. In the light of the present finding, it is tempting to speculate that at least some of these metabolites of lindane may arise via HCB (Fig. 1). It is also interesting to note that both lindane and HCB exhibit a similar degree of induction of liver microsomal enzymes [32]. It is likely that the formation of HCB may involve the partially dehydrogenated metabolite, HCCHE, as an intermediate. The formation of HCCHE, has been shown to be catalysed by microsomal enzymes [6], and it has been proposed that it may take place by the oxenoid mechanism [33]. Since the labelling of HCB in the present study is enhanced by prior treatment of the animal with Aroclor, the mediation of microsomal metabolism in this conversion can be inferred. The dehydrogenation of lindane to HCB has a parallel in the conversion of several hydro-aromatic precursors to benzenoid derivatives [34–40], and may be a more widespread reaction mechanism in mammalian organisms than is generally believed.

The incorporation of radioactivity from ^{14}C -lindane into PCP, as observed in the present study, is in conformity with its reported presence as a metabolite of lindane in rats [41] and rabbits [42]. Although it has been proposed that PCP may arise from PCCCHE by one of two pathways [41], there is a distinct possibility that it may be formed from HCB by oxidative dechlorination. This suggestion

TABLE III. MUTAGENICITY OF LINDANE AND HEXACHLOROBENZENE IN *Salmonella typhimurium* TA 98

Compound	Concentration ($\mu\text{g}/\text{plate}$)	Number of revertants ^a (per plate)	
		-S ₉	+S ₉
Benzpyrene	1	15	40
	5	23	90
<i>Expt. 1</i>			
Lindane	50	9	155
	100	8	182
HCB	50	6	179
	100	4	185
<i>Expt. 2</i>			
Lindane	50	12	153
	100	8	170
HCB	50	16	175
	100	9	208
<i>Expt. 3</i>			
Lindane	50	11	25
	100	10	21
HCB	50	-	26
	100	6	28

^a Spontaneous revertants: 11–14 per plate.

derives support from the reported formation of PCP from HCB in animals [26–28, 30, 31].

Perhaps the most striking finding in the present study relates to the degradation of the lindane molecule to yield CO₂. The capability for the total breakdown of the aromatic ring of lindane has generally been attributed only to micro-organisms [43], although there is a single report of the breakdown of the pesticide to CO₂ in the housefly [44]. Very recently, the formation of labelled CO₂ from ¹⁴C-labelled lindane in the rat has been reported [15]. It is not possible to conclude, however, whether the formation of CO₂ represents a mammalian transformation

TABLE IV. INFLUENCE OF NUTRITIONAL STATUS OF MICE ON EXCRETION OF RADIOACTIVITY FROM ORALLY ADMINISTERED U-¹⁴C-LINDANE

Nutritional status	Radioactivity excreted			
	Urine		Faeces	
	dis/min ($\times 10^{-5}$)	% Dose	dis/min ($\times 10^{-5}$)	% Dose
Fed ad libitum	4.13	18.48	0.69	3.12
Restricted intake	8.28	37.30	1.08	4.90

or is due to metabolism by the intestinal microflora. Since the experiment reported in this study was conducted in the presence of a wide-spectrum antibiotic that would preclude microbial activity, the formation of CO₂ appears to be mediated by liver enzymes. The enhanced formation of CO₂ in the Aroclor-treated rats would again point to the microsomal nature of this breakdown. It may be mentioned in this context, that in addition to lindane [15], the ring fission of DDT in vivo in the rat to yield CO₂ has also been reported [45]; the latter study indicates that quantitatively this pathway may be of equal importance to renal excretion of the pesticide [45]. It is not clear at present whether the mechanism of this ring opening is similar to those in the aromatic amino acids, tyrosine and tryptophan.

The mutagenicity studies on lindane and HCB have provided equivocal results, as indicated in Table III. Whereas a distinctly positive mutagenic response was observed at times with both the compounds, the response was found to be variable in different experiments. In all cases, where a mutagenic response was noted, activation by rat liver microsomal fraction was found to be necessary. The reasons for the inconsistencies in the findings in different experiments are not clear, but even if the positive effects are to be accepted as not being an artefact, both the compounds possess only a weak mutagenic potential. Additional studies using the Ames test [18], as well as the host-mediated assay [46], are currently in progress. It has been reported by other investigators that lindane does not cause gene mutations [47-49], and is also negative in the dominant lethal test [47]. It is of interest to note, however, that both HCB and PCP which could arise from lindane in the mammalian organism [present studies, 24, 41, 42], may be potential genetic toxicants. The ability of PCP to induce gene conversion in *Saccharomyces cerevisiae* and chromosomal aberrations in human lymphocyte cultures has been

documented [47]. HCB is known to be transferred across the placenta in the rabbit [50] and rat [51], and has also been shown to be teratogenic in rats [52]. A life-time feeding study in the hamster has also indicated the carcinogenicity of this compound [53]. The ability of lindane to induce cancer in experimental animals has remained a controversial issue, with divergent findings from various laboratories [7–12]. Even on theoretical considerations, the compound is rather suspect, since it can give rise to chlorinated benzenes which are metabolized via arene oxide intermediates; the latter possess carcinogenic potential because of their ability to interact with nucleic acids. If they possess a low potential for causing cancer in man, it is unlikely that epidemiological studies will reveal the fact, especially since vast segments of the population are exposed to the pesticide. There is an urgent need for a critical evaluation of the safety of lindane by animal experimentation.

Several factors, many of them related to diet, are known to influence the rates of detoxification, excretion and accumulation of pesticides. These may have implications for the safety of the pesticide, since a slower detoxification may signify a greater risk. In the present studies, carried out with mice, it is observed that mice which had been maintained for a prolonged period on a restricted diet, showed a more rapid excretion and less retention of an orally administered dose of U-¹⁴C-lindane (Table IV). Among other dietary factors which have been reported to have a significant effect on lindane metabolism and excretion in the rat are nature and quantity of dietary lipid [13], the presence of inducers of microsomal enzymes in the diet [14] and dietary fibre [15]. A likely explanation for the present finding could be provided on the basis of a two-compartmental model of pharmacodynamics of lipophilic xenobiotics, consisting of a central metabolic compartment (e.g. liver, kidney and blood), which is in contact with a second otherwise isolated and metabolically inert storage compartment (e.g. adipose tissue). The differences in pesticide retention in relation to nutritional status could result from changes in the size of the body stores of fat. Alternatively, the differences in the rate of excretion may be related to qualitative and/or quantitative changes in the binding capacity of serum and tissue proteins for the pesticide. Although the protein-bound and the free forms of the pesticide may exist in a dynamic equilibrium, the former, which is in the form of a high molecular weight complex, is of limited availability for metabolic transformations as well as for transport across membranes, e.g. glomerular filtration. Food deprivation has also been reported to enhance the rate of excretion of dieldrin in rats [54], and the mobilization of DDT from the tissues into plasma in cockerels [55]. These observations may have implications for man, in as much as the recommendations on acceptable daily intakes are based on toxicological studies in animals, which may not have taken into consideration the possible influence of the nutritional status on the induction of toxic effects.

REFERENCES

- [1] MATSUMURA, F., *Toxicology of Insecticides*, Plenum Press, New York and London (1976).
- [2] ENGST, R., MACHOLZ, R.M., KUJAWA, M., *Residue Reviews* **68** (1977) 59.
- [3] GROVER, P.L., SIMS, P., *Biochem. J.* **96** (1965) 521.
- [4] ENGST, R., MACHOLZ, R.M., KUJAWA, M., *Nahrung* **20** (1976) 83.
- [5] FREAL, J.J., CHADWICK, R.W., *J. Agric. Food Chem.* **21** (1973) 424.
- [6] CHADWICK, R.W., CHUANG, L.T., WILLIAMS, K., *Pestic. Biochem. Physiol.* **5** (1975) 575.
- [7] FITZHUGH, O.G., NELSON, A.A., FRAWLEY, J.P., *J. Pharm. Expl. Therap.* **100** (1950) 59.
- [8] DE BARROS, S.B., SALIBA, A.M., *Toxicology* **10** (1978) 271.
- [9] GOTO, M., HATTORI, M., MIYAGAWA, T., *Chemosphere* **1** (1972) 279.
- [10] WEISSE, I., HERBST, M., *Toxicology* **7** (1977) 233.
- [11] THORPE, E., WALKER, A.J.T., *Food Cosmet. Toxicol.* **11** (1973) 433.
- [12] ITO, N., NAGASAKI, H., AOE, H., SUGIHARA, S., MIYATA, Y., ARAI, M., SHIRAI, T., *J. Natl. Cancer Inst.* **54** (1975) 801.
- [13] CHADWICK, R.W., SIMMONS, W.S., BRYDEN, C.C., CHUANG, L.T., KEY, L.M., CHADWICK, C.J., *Toxicol. Appl. Pharmacol.* **39** (1977) 391.
- [14] CHADWICK, R.W., CHADWICK, C.J., FREAL, J.J., BRYDEN, C.C., *Xenobiotica* **7** (1977) 235.
- [15] CHADWICK, R.W., COPELAND, M.F., CHADWICK, C.J., *Food Cosmet. Toxicol.* **16** (1978) 217.
- [16] AIYAR, A.S., FATTERPAKER, P., SREENIVASAN, A., *Biochem. J.* **90** (1964) 558.
- [17] GOPALASWAMY, U.V., AIYAR, A.S., *Nuclear Techniques in Studies of Metabolism, Effects and Degradation of Pesticides*, Dept. of Atomic Energy, Government of India (in press).
- [18] AMES, B.N., *Mutat. Res.* **31** (1975) 1.
- [19] ENGST, R., MACHOLZ, R.M., KUJAWA, M., *Nahrung* **18** (1974) 737.
- [20] KOHLI, J., JONES, D., SAFE, S., KORTE, F., *J. Environ. Sci.* **B 11** (1976) 23.
- [21] KOHLI, J., WEISGERBER, I., KLEIN, W., *Pestic. Biochem. Physiol.* **6** (1976) 97.
- [22] KOHLI, J., JONES, D., SAFE, S., *Can. J. Biochem.* **54** (1976) 203.
- [23] STEINWANDTER, H., *Chemosphere* **6** (1976) 119.
- [24] SEIDLER, H., MACHOLZ, R.M., HARTIG, M., KUJAWA, M., ENGST, R., *Nahrung* **19** (1975) 473.
- [25] PARKE, D.V., WILLIAMS, R.T., *Biochem. J.* **74** (1960).
- [26] MEHENDALE, H.M., FIELDS, M., MATHEWS, H.B., *J. Agric. Food Chem.* **23** (1975) 261.
- [27] ENGST, R., MACHOLZ, R.M., KUJAWA, M., *Nahrung* **19** (1975) 603.
- [28] LUI, H., SWEENEY, D., *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **51** (1975) 225.
- [29] ROZMAN, K., MUELLER, W., IATROPOULOS, M., COULSON, F., KORTE, F., *Chemosphere* **5** (1975) 289.
- [30] ENGST, R., MACHOLZ, R.M., KUJAWA, M., *Bull. Environ. Contam. Toxicol.* **16** (1976) 248.
- [31] KOSS, G., KORENSKY, W., STEINBACH, K., *Arch. Toxicol.* **35** (1976) 107.
- [32] DEN TONKELLAR, D.M., VAN ESCH, G.J., *Toxicology* **2** (1974) 371.
- [33] HAMILTON, G.A., *Molecular Mechanisms of Oxygen Activation* (HAYAISHI, O., Ed.), Academic Press, New York (1974) 405.

- [34] BERNHARD, K., CALFISCH-WEILL, H., *Helv. Chim. Acta* **28** (1945) 1697.
- [35] BEER, C.T., DICKENS, F., PEARSON, J., *Biochem. J.* **48** (1951) 222.
- [36] BERNHARD, K., VUILLEUMIER, J.P., BRUBACHER, G., *Helv. Chim. Acta* **38** (1955) 1438.
- [37] MITOMA, C., POSNER, H.S., LEONARD, F., *Biochim. Biophys. Acta* **27** (1958) 158.
- [38] AYENGAR, P.K., HAYAISHI, O., NAKAJIMA, M., TOMIDA, I., *Biochim. Biophys. Acta* **33** (1959) 111.
- [39] BALDWIN, B.C., ROBINSON, D., WILLIAMS, R.T., *Biochem. J.* **76** (1960) 600.
- [40] AIYAR, A.S., GOPALASWAMY, U.V., SREENIVASAN, A., *Biochem. Biophys. Res. Commun.* **45** (1971) 893.
- [41] ENGST, R., MACHOLZ, R.M., KUJAWA, M., LEWERENZ, H.J., PLASS, R., *J. Environ. Sci. B* **11** (1976) 95.
- [42] KARAPALLY, J.C., SAHA, J.G., LEE, Y.W., *J. Agric. Food Chem.* **21** (1973) 811.
- [43] MATSUMURA, F., BENEZET, H.J., PATIL, K.C., *J. Pesticide Sci.* **1** (1976) 3.
- [44] BRIDGES, R.G., *Nature (London)* **184** (1959) 1337.
- [45] ABOU-DONIA, M.B., MENZEL, D.B., *Experientia* **32** (1976) 500.
- [46] AIYAR, A.S., SUBBA RAO, V., *Mutat. Res.* **48** (1977) 17.
- [47] FAHRIG, R., *IARC Scientific Publication* **10** (1974) 161.
- [48] SHIRASU, Y., MORIYA, M., KATO, K., FURUHASHI, A., KADA, T., *Mutat. Res.* **40** (1976) 19.
- [49] SHAHIN, M.M., VON BORSTEL, R.C., *Mutat. Res.* **48** (1977) 173.
- [50] VILLENEUVE, D.C., PANOPIO, L.G., GRANT, D.L., *Environ. Biochem. Physiol.* **4** (1974) 112.
- [51] VILLENEUVE, D.C., HIERLIHY, S.L., *Bull. Environ. Contam. Toxicol.* **13** (1975) 489.
- [52] KHERA, K.S., *Food Cosmet. Toxicol.* **12** (1974) 471.
- [53] CABRAL, J.R.P., SHUBIK, P., MOLLNER, T., RAITANO, F., *Nature (London)* **269** (1977) 510.
- [54] HEATH, D.F., VANDEKAR, M., *Br. J. Ind. Med.* **21** (1964) 269.
- [55] ECOBICHON, D.J., SASCHENBRECKER, *Toxicol. Appl. Pharmacol.* **15** (1960) 420.

DISCUSSION

M. RÖSELER: According to Section 2.4 of your paper, the $^{14}\text{CO}_2$ activity was counted in a liquid scintillation spectrometer after absorption of the gas and precipitation as $\text{Ba}^{14}\text{CO}_3$, and after suspension in toluene containing 4% Cab-O-Sil. I should think this method of detection would be relatively insensitive because of the high self-absorption of the precipitate. Did you try to absorb CO_2 in an amine in order to prevent precipitation followed by detection in a liquid phase?

A.S. AIYAR: We have on occasion compared the method of counting the CO_2 as precipitated carbonate with a technique where it is absorbed in 'Hyamine'. Although the counting efficiency is lower in the first method, we deliberately used it because lindane is known to be volatilized during incubation, and precipitation of the CO_2 as BaCO_3 enables thorough washing with organic solvents to remove any lindane that may have condensed in the central well. When the CO_2

is absorbed in 'Hyamine', there is no possibility of eliminating the condensed lindane.

M. RÖSSELER: For studying the mechanism of lindane degradation, especially that of consecutive reactions, I recommend the use of labelled precursors and the kinetic isotope method. Have you tried this?

A.S. AIYAR: We propose to study the pathways in greater detail and to quantify the transformations. At present, our data are essentially qualitative but sufficient to warrant the conclusions reached.

C. MELLA LIZAMA: Why did you use such a large dose of lindane in mice?

A.S. AIYAR: In the mouse studies, which were carried out to assess the possible influence of nutritional status on the metabolism of lindane, the animals were given $U-^{14}C$ -lindane (negligible mass) diluted with cold lindane (3 mg/100 g body weight). This is not a large dose of the pesticide.

C. RAZAFINDRAKOTO: Have you done experiments on the biotransformation of organophosphorus insecticides?

A.S. AIYAR: No, we have not. The organophosphorus pesticides are known to be metabolized rapidly by animal organisms, and a large amount of work has been done on several such compounds by various investigators.

R. GALUN (*Chairman*): What are the restrictions imposed on the use of lindane in different parts of the world?

A.S. AIYAR: I am afraid I cannot give you very precise information. A large number of developing countries continue to use lindane as technical benzene hexachloride (BHC) without any restrictions, as it is one of the most economical broad-spectrum insecticides available. In a few of these countries limits have been laid down on the maximum permissible levels of residue in food. In the United States of America lindane is considered more acceptable than DDT; there is a ban on the use of the latter, but the use of lindane is permitted for certain specific applications, e.g. in forestry operations. I believe that many of the other industrialized countries also have a similarly cautious approach to the use of this pesticide.

M.E. LEEGWATER-VAN DER LINDEN: Do the figures given in Table I indicating the amount of lindane used over the past years relate to single years or does the period 1976-77 relate to two years?

A.S. AIYAR: All the figures for consumption of lindane (BHC) relate to single years. For accounting and statistical purposes, the year begins on 1 April and ends on the following 31 March.

A. HASSAN (*Scientific Secretary*): I am a bit sceptical about the production of $^{14}CO_2$ in vitro and not in vivo. Would you care to comment on this?

A.S. AIYAR: I did not say that $^{14}CO_2$ evolution from lindane has not been observed in vivo. We have not studied this, but there is one report indicating that it does happen. I merely wanted to point out that the in vivo study does not conclusively establish the enzymatic capacity of the animal to produce such a

reaction; since some micro-organisms are known to be capable of carrying out this transformation, the possibility of a contribution of intestinal microflora cannot be ruled out. In our in vitro system, a broad-spectrum antibiotic was included to ensure the absence of microbial metabolism.

**STERILE INSECT TECHNIQUE
AND STERILITY INDUCTION**

(Session 5)

LA LUTTE CONTRE *Glossina palpalis gambiensis* Vanderplank PAR LACHERS DE MALES IRRADIES EN HAUTE-VOLTA

Etude de paramètres opérationnels

D. CUISANCE, H. POLITZAR, M. CLAIR, E. SELLIN,
Y. TAZE, G. BOURDOISEAU, J. FEVRIER
Centre de recherches sur les trypanosomoses animales,
Bobo-Dioulasso,
Haute-Volta

Abstract-Résumé

PROGRAMME FOR THE CONTROL OF *Glossina palpalis gambiensis* Vanderplank BY THE STERILE-MALE TECHNIQUE IN UPPER VOLTA: A STUDY OF OPERATIONAL PARAMETERS.

The Institute for Stock-Breeding and Veterinary Medicine in Tropical Countries (Institut d'élevage et de médecine vétérinaire des pays tropicaux - IEMVT, France), in association with the German Society for Technical Co-operation (Deutsche Gesellschaft für technische Zusammenarbeit - GTZ, Federal Republic of Germany), has been experimenting for four years in Upper Volta on genetic control of *G.p. gambiensis*, a vector of trypanosomiasis in both man and animals, by the release of sterile males. A mass rearing facility has been set up for this glossina, the first of its kind in Africa; at present it has 45 000 females which produce 250 000 males for irradiation (11 000 rad) annually. This required a parallel facility for rearing 1000 host animals (400 rabbits and 600 guinea pigs). The releases are made twice a week on five experimental sectors isolated by barriers, which represent 32 km of forest galleries inhabited by *G.p. gambiensis* and *G. tachinoides* populations of medium density. The whole of this study zone is compared with a control covert 5.5 km in length. In each covert the influence of different parameters was examined: sterile-to-wild male ratio, spacing of release points, combination with insecticide treatment or not, and variation over time of the numbers of males released. First observations indicate that the sterile-male technique is efficient, and analysis of different parameters should make it possible to choose the optimum conditions for applying it. However, if the method is to be used on a larger scale, industrial-type rearing facilities will be required; yet the possibilities are limited by the difficulty of maintaining large numbers of host animals in tropical regions. Artificial feeding on membranes might offer one solution to the problem, but it must be shown to be acceptable and operationally viable in local conditions.

LA LUTTE CONTRE *Glossina palpalis gambiensis* Vanderplank PAR LACHERS DE MALES IRRADIES EN HAUTE-VOLTA - ETUDE DE PARAMETRES OPERATIONNELS.

L'Institut d'élevage et de médecine vétérinaire des pays tropicaux (IEMVT, France), auquel s'est associée la Deutsche Gesellschaft für technische Zusammenarbeit (GTZ, République fédérale d'Allemagne), expérimente depuis quatre ans sur le terrain en Haute-Volta la lutte génétique par lâchers de mâles stériles contre *G.p. gambiensis*, vecteur des trypanosomiasis chez l'homme et le bétail. Pour la première fois en Afrique un élevage massif de cette glossine a été créé. Il comprend actuellement 45 000 femelles produisant annuellement 250 000 mâles irradiés (11 000 rad). Il a impliqué la mise en place d'un élevage parallèle de 1000 hôtes

nourriciers (400 lapins et 600 cobayes). Les lâchers ont lieu deux fois par semaine sur cinq tronçons expérimentaux isolés par des barrières et représentant 32 km de galeries forestières où vivent des populations de densités moyennes de *G.p. gambiensis* et de *G. tachinoides*. L'ensemble de la zone d'étude est comparé à un gîte témoin long de 5,5 km. Dans chacun des gîtes, l'action de différents paramètres a été examinée: rapport mâles stériles/mâles sauvages, espacement des points de lâchers, association ou non avec un traitement insecticide, modulation du nombre de mâles lâchés dans le temps. Les premières observations montrent l'efficacité de la méthode du mâle stérile. L'étude de différents paramètres permet de dégager les conditions d'emploi de cette méthode génétique. Toutefois, l'utilisation de la méthode à plus grande échelle implique des élevages de glossines de type industriel, élevages limités par les difficultés de maintenir de gros effectifs nourriciers en régions tropicales. L'alimentation artificielle sur membrane pourrait apporter une solution à ce problème, mais doit tout d'abord se révéler acceptable et opérationnelle dans les conditions locales.

1. INTRODUCTION

Les traitements insecticides contre les glossines ont fait ces dernières années de grands progrès en matière d'efficacité, de sélectivité et d'amélioration des techniques d'application. Toutefois, les nouveaux projets de développement de l'élevage en Afrique et d'intensification des activités agricoles impliquent une action contre les glossines [1] dans les zones de savane humide et les régions préforestières ou forestières. Or, les insecticides n'y donnent pas toujours les résultats escomptés, du fait du climat (saison des pluies longues), des conditions microclimatiques au niveau du sol (pas ou peu de phénomène d'inversion), de la taille et la densité de la végétation, de l'importance du réseau hydrographique (toxicité pour la faune aquatique). Les recherches actuelles sont donc entreprises pour mettre au point de nouveaux moyens de lutte visant à s'associer aux méthodes classiques pour les renforcer ou les compléter.

La lutte génétique constitue l'une de ces directions de recherche à laquelle contribue le Centre de Recherche sur les trypanosomoses animales de Bobo-Dioulasso où est expérimentée depuis quatre ans la lutte par lâchers de mâles stériles contre *Glossina palpalis gambiensis* [2], vecteur important des trypanosomoses de l'homme et du bétail en Afrique occidentale.

Cette technique fait appel à un élevage de masse de l'insecte qui nécessite lui-même un élevage d'animaux nourriciers. Une méthodologie assez complexe a permis de faire varier sur le terrain des paramètres visant à savoir où, quand, comment, combien de mâles irradiés doivent être lâchés pour rendre le plus efficace possible cette technique de lutte.

Quelques résultats sont présentés ici et mettent en évidence les principes de base de l'application de la méthode.

2. ELEVAGE DES ANIMAUX NOURRICIERS

2.1. Le lapin

Le lapin a d'abord été choisi comme hôte nourricier. Commencé en 1974, cet élevage est passé de 181 individus en 1975 à 287 en 1976, 388 en 1977 et 449 en 1978. La moitié sert effectivement à l'alimentation des glossines, le reste étant représenté par les reproducteurs et les jeunes. Cet élevage est relativement difficile en région tropicale pour trois raisons principales:

- les problèmes pathologiques sont accentués sous les climats chauds (pneumopathies en particulier),
- les problèmes de reproduction, vraisemblablement liés à une hygrométrie basse durant de nombreux mois, sont importants,
- l'alimentation est difficile à assurer durant la saison sèche.

Les cages contenant les glossines sont placées sur les oreilles des lapins, immobilisés dans des appareils à contention. Chaque lapin «travaille» un jour par semaine, nourrissant ainsi environ 1000 glossines.

2.2. Le cobaye

Devant les difficultés d'élevage du lapin, une colonie de cobayes a été créée en janvier 1977 avec environ 150 individus. Fin 1977, l'effectif s'élevait à 470 individus et, fin 1978, il atteignait 636 individus, dont la moitié environ utilisés comme hôtes nourriciers. Aucune prophylaxie sanitaire et médicale n'est appliquée vis-à-vis de ces animaux rustiques et bien adaptés à la chaleur. Seule une mycose cutanée a nécessité quelques mesures sanitaires.

Les cages contenant les glossines sont placées entre les flancs des cobayes qui nourrissent chacun environ 250 glossines une fois par semaine.

Le lapin est un animal de choix pour l'alimentation des glossines en raison des manipulations réduites qu'il nécessite par rapport au cobaye. En contre-partie, il exige une surveillance sanitaire plus complexe que ce dernier et des locaux plus importants.

La comparaison coût-bénéfice de ces deux types d'élevage est en cours d'évaluation. Il semble que le bilan des avantages et des inconvénients s'équilibre et que les deux espèces rendent à prix égal les mêmes services.

Une troisième possibilité d'alimentation des glossines est actuellement offerte par l'utilisation de membrane artificielle recouvrant des plaques contenant du sang défibriné. Elle est en cours d'étude dans les conditions du Centre de recherche sur les trypanosomoses animales (CRTA) de Bobo-Dioulasso.

TABLEAU I. DONNEES SUR L'ELEVAGE DE *G. p. gambiensis* DANS LES DEUX INSECTARIUMS (CRTA)

		Nombre moyen de femelles/jour	Production pupes	Pourcentage éclosion	Mortalité (en %) du nombre moyen par jour	Nombre de mâles produits
Insectarium I	1976	20 469	383 095	85,34	1,55	56 095
	1977	31 705	659 648	85,96	1,58	162 496
	1978	30 717	685 440	80,09	1,91	185 586
Insectarium II	1977	3 648	57 977	86,14	1,54	9 438
	1978	9 354	203 063	85,86	2,23	45 662
Total mâles produits						459 277

3. ELEVAGE DE *Glossina palpalis gambiensis*

Les glossines ne sont élevées au laboratoire que depuis peu (une quinzaine d'années) et en Europe principalement. Les deux élevages africains les plus importants numériquement sont actuellement réalisés, l'un à Tanga en Tanzanie (*G.m. morsitans*) [3], l'autre à Bobo-Dioulasso en Haute-Volta (*G.p. gambiensis*) [4].

Celui de Haute-Volta a commencé en mars 1975 à partir de 5000 pupes venant de Maisons-Alfort (Dr. Itard). En mai 1976, la colonie a atteint 30 000 femelles reproductrices. Depuis, elle a oscillé entre 25 000 et 50 000 femelles réparties en deux insectariums indépendants, l'un utilisant le lapin et l'autre le cobaye. Les conditions thermo-hygrométriques sont impérativement de $25^{\circ}\text{C} \pm 1$ et $85\% \pm 5$ (humidité relative) et sont obtenues par un système simple associant des climatiseurs et des humidificateurs à pulvérisation d'eau (type *Defensor*), faciles à remplacer ou à réparer.

Onze personnes travaillent pour l'alimentation de tout l'effectif des glossines, soit environ quatre personnes pour 15 000 glossines.

Les résultats obtenus sont résumés dans le tableau I.

Cet élevage a permis d'obtenir depuis sa création plus de 450 000 mâles en vue des lâchers, la production annuelle se situant à plus de 230 000 mâles en 1978.

Il a permis également de mettre en évidence les problèmes soulevés par un élevage de ce type en Afrique:

- une grande variation de température et d'humidité entre le jour et la nuit, entre la saison sèche et la saison des pluies, d'où un travail important des machines,
- manque de fiabilité de l'approvisionnement en eau et en électricité,
- sensibilisation du personnel à un travail où chaque faute est lourde de conséquences.

4. STERILISATION DES MALES DE *G.p. gambiensis*

Les glossines sont triées après un refroidissement à 4°C qui permet leur sexage. Les mâles sont alors séparés des femelles et nourris au plus tôt une heure après l'anesthésie par le froid.

La stérilisation se fait par introduction de lots de 500 mâles dans un irradiateur au césium 137. Ils sont soumis à une dose de 11 000 rad, préalablement déterminée au laboratoire et permettant d'obtenir à la fois un bon taux de stérilité (95%) et de conserver le pouvoir inséminant [5]. Ces mâles sont ensuite transportés sur les lieux de lâchers dans des caissons humidifiés (cadre métallique entouré de toile de jute mouillée).

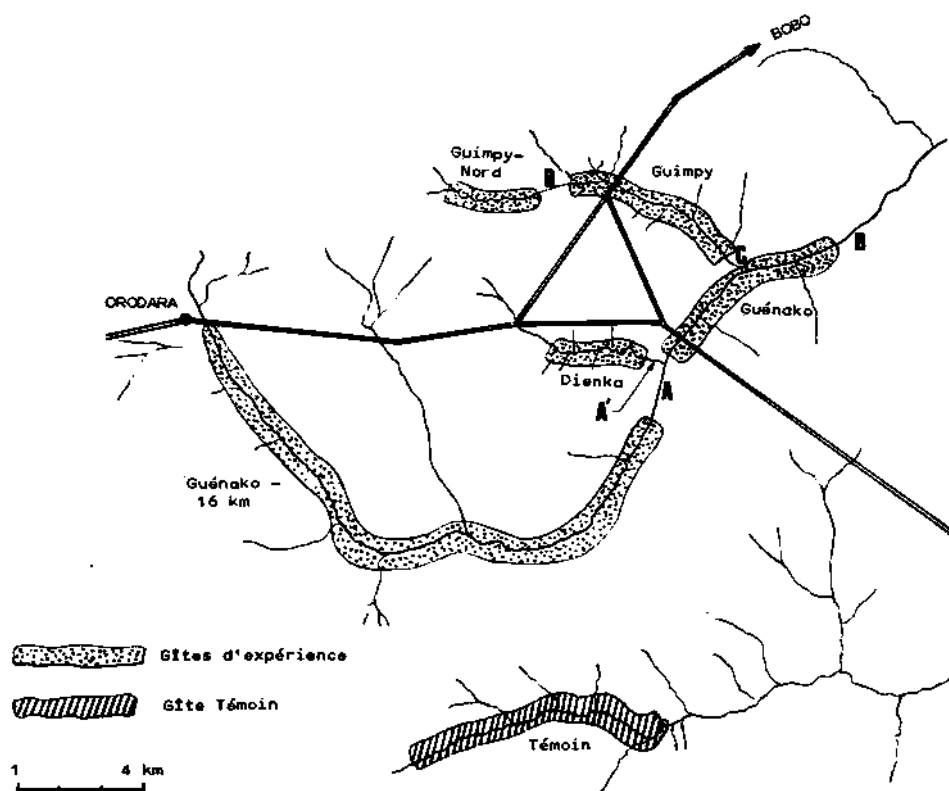


FIG.1. Zone d'expérimentation de la méthode du mâle stérile contre *Glossina palpalis gambiensis* (Haute-Volta).

5. ETUDE DES PARAMETRES OPERATIONNELS SUR LE TERRAIN

5.1. Zone expérimentale (fig.1)

Elle a été choisie à 70 km de Bobo-Dioulasso sur trois rivières qui constituent les vraies sources de la Volta Noire: Guénako, Guimpy, Dienkoa. Celles-ci sont bordées de galeries forestières homogènes, continues, larges de 20 m en moyenne, couvrant en grande partie le lit mineur très encaissé de ces cours d'eau permanents. Deux espèces de glossines: *G.p. gambiensis* (95%), *G. tachinoides* (5%) y sont présentés.

Cinq tronçons expérimentaux et un témoin sont délimités ainsi:

- gîte Guénako: 5,5 km (en aval de Kourinion)
- gîte Guimpy: 5,5 km (affluent de la Guénako)

- gîte Guénako: 16 km (entre Orodara et Kourinion)
- gîte Guimpy-Nord: 2,8 km (en amont de la Guimpy)
- gîte Dienka: 2,2 km (affluent de la Guénako)
- gîte Dienkoa (= gîte témoin) : 5,5 km.

L'ensemble de ces gîtes est isolé, soit naturellement, soit dans la plupart des cas par des barrières d'éclaircissement total (A, A', B, C, D) renforcées par des traitements insecticides et par un piégeage continu (pièges biconiques) [6]. Les plus longues barrières mesurent 1,300 km. Quarante kilomètres de pistes et 12 ponts ont été créés pour pouvoir effectuer les lâchers et les sondages réguliers par marquage-recapture, permettant de suivre la dynamique de la population dans chaque gîte d'expérience.

5.2. Résultats

Dans un premier temps, on a cherché à mettre en évidence l'efficacité des mâles stériles en se plaçant dans des conditions optimales. Cette efficacité étant confirmée, on a fait varier différents paramètres pour essayer de dégager les conditions d'application de la méthode.

5.2.1. Gîte témoin

Avant de décrire les faits d'observation sur les gîtes où sont effectués les lâchers, il convient d'observer l'évolution de la population du gîte témoin, celui-ci ne subissant que des contrôles réguliers sans autre intervention (fig.2).

On constate depuis trois ans que la densité des populations sauvages de *G.p. gambiensis* baisse naturellement de février jusqu'à mai où elle atteint son minimum, puis s'élève jusqu'en saison des pluies et en début de saison sèche pour décroître à nouveau. Les variations de pluviométrie modulent plus ou moins ce cycle qui apparaît très voisin d'une année à l'autre.

5.2.2. Lâchers réduits, continus, rapprochés faisant suite à un piégeage continu: gîte Guénako et Guimpy

Avant les lâchers, un piégeage continu de 35 jours (pièges biconiques posés tous les 200 mètres), effectué uniquement sur le gîte Guimpy a permis d'abaisser de 50% la population de *G.p. gambiensis* en affectant toutes les classes d'âge. On a lâché ensuite les mâles stériles 2 fois par semaine puis 1 fois par semaine, tous les 200 mètres au long de ces rivières; les quantités ont été en moyenne de 570 mâles par semaine sur Guénako, 700 mâles sur Guimpy pendant 8 mois d'expérience.

Ces quantités sont inférieures à celles prévues initialement et qui devaient permettre d'atteindre des rapports mâles stériles/mâles sauvages de, respectivement,

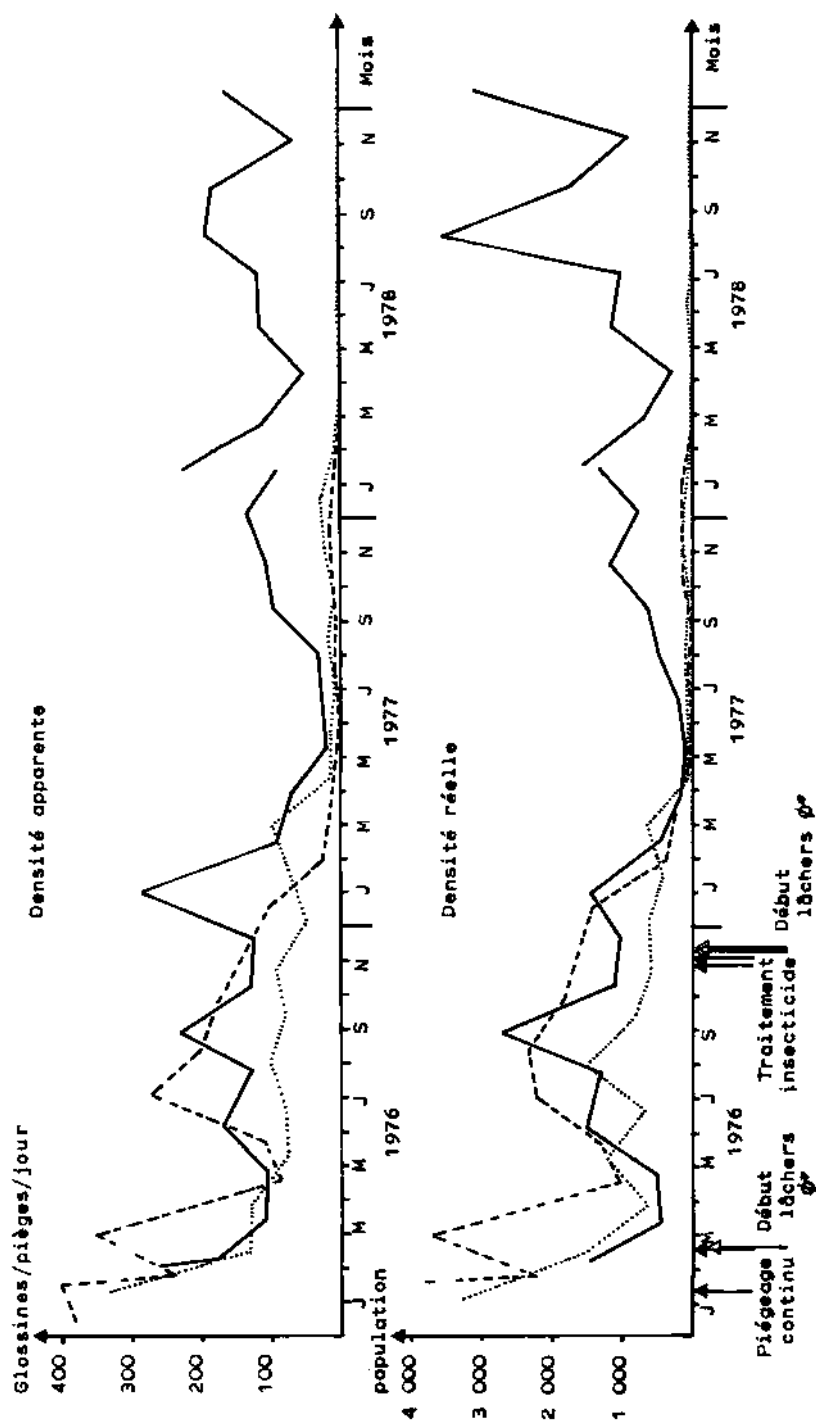


FIG. 2. Evolution de la densité de *G.p. gambiensis*. — Guénako; ---- témoin; Guimpy.

1/1 et 7/1. Un accident dans l'élevage, réduisant les quantités de mâles disponibles, n'a en effet permis d'atteindre que des taux moyens de 0,7/1 et 2,5/1. Dans ces conditions, aucun effet n'a été observé sur la population sauvage du gîte Guénako, et seulement un effet réduit sur le gîte Guimpy dont la densité n'a crû que modérément pendant la saison des pluies par rapport à celle du gîte témoin (fig.2).

5.2.3. Lâchers massifs, continus, rapprochés, effectués 10 à 14 jours après un traitement insecticide non rémanent: gîte Guénako et Guimpy

Dans ces mêmes gîtes, dont la densité de glossines a été peu modifiée, on a pratiqué un traitement insecticide non rémanent (Thiodan avec Swingfog). Dans chaque gîte les lâchers de mâles irradiés ont commencé 10 jours et 14 jours après ce traitement. Les lâchers sont massifs et continus en vue d'obtenir respectivement un rapport ϕ/δ de 3/1 et 7/1 (ϕ : mâles stériles; δ : mâles sauvages). Ils sont rapprochés dans l'espace (200 m) et dans le temps (2 fois par semaine). On a lâché ainsi en moyenne 1400 ϕ par semaine sur Guénako et 1300 ϕ sur Guimpy, aboutissant à un taux moyen ϕ/δ de 9,3/1 et 9,8/1 permettant de qualifier ces lâchers «d'inondatifs».

La densité de *G.p. gambiensis* a baissé constamment par rapport à celle de *G. tachinoides* et un écart très net s'est précisé entre le gîte témoin et les deux gîtes receveurs de mâles stériles. Les densités ont baissé de 70% au bout de 12 mois et, 16 mois après les premiers lâchers, elles sont devenues nulles sur ces deux gîtes (fig.2). Il est vraisemblable que ce niveau aurait été atteint plus tôt si l'étanchéité des barrières avait été totale et si les délais entre le traitement insecticide non rémanent et le début des lâchers avaient été plus réduits. Le traitement insecticide abaisse légèrement la densité de population mais laisse surtout se constituer une population jeune, notamment de femelles vierges à partir des pupes déposées avant les pulvérisations. D'où l'intérêt de procéder aux lâchers de mâles stériles immédiatement après le traitement chimique.

5.2.4. Lâchers massifs, continus, mais espacés, effectués 48 heures après un traitement insecticide non rémanent: gîte Guénako 16 km

Dans les précédentes observations, les lâchers se faisaient tous les 200 mètres. Un espacement supérieur permettrait de simplifier la technique d'application de la méthode du mâle stérile et d'en diminuer le coût. Il convenait donc d'apprécier l'espacement géographique optimal entre les points de lâchers, permettant aux mâles stériles d'être représentés au mieux auprès des femelles sauvages tout au long du gîte.

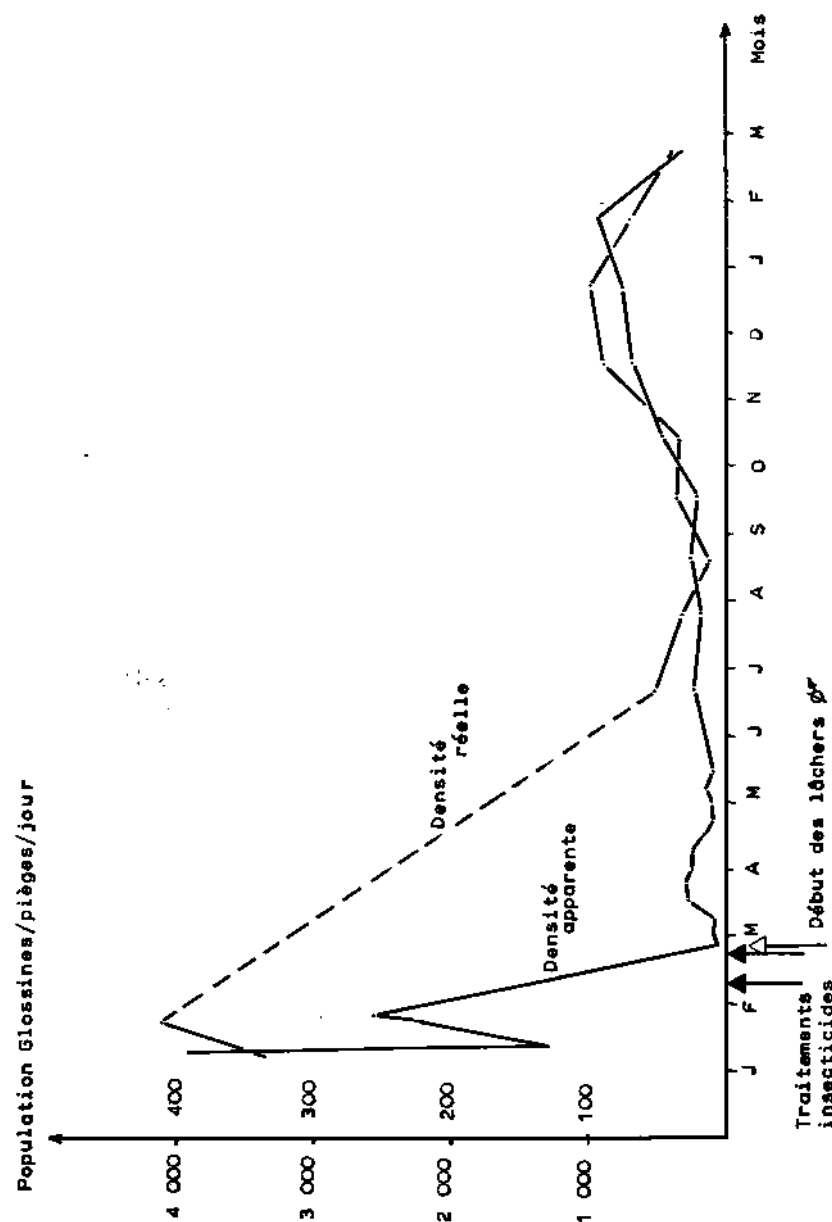


FIG. 3. Evolution de la densité apparente et de la densité réelle de *G. p. gambiensis* sur le gîte Guénako-16 km.

Deux épandages U.L.V. par hélicoptère de dècamèthrine (0,2 g/ha) ont été appliqués à 15 jours d'intervalle [7], en vue de ramener la densité sauvage à un niveau compatible avec les quantités de mâles stériles disponibles pour obtenir un rapport ϕ/δ de 7/1. Les lâchers ont immédiatement suivi et ont été ininterrompus, selon un rythme de 2 lâchers par semaine. Durant 1 mois et demi tous les lâchers se font en un point unique au milieu du gîte (km 8), puis durant 1 mois, ils se font tous en un autre point unique (km 4), ce changement ayant pour but de savoir si la dispersion est liée au point de lâcher. Les captures, faites toutes les 48 heures, donnent lieu à un nouveau marquage des individus récoltés.

Il apparaît que les mâles stériles lâchés au km 8 ou au km 4 se dispersent de façon identique aussi bien en amont qu'en aval.

Le rapport ϕ/δ obtenu a atteint une valeur $\geq 7/1$ sur une grande partie du gîte à l'exception de 3 zones.

Toutefois, la courbe montre une bonne représentation des mâles stériles dans un rayon de 2 km autour du point de lâcher (analyse statistique en cours).

De ce fait, depuis juin 1978, les lâchers ont été espacés de 2 km sur tout le gîte et ont donné les résultats suivants:

- Le rapport 7/1 souhaité est obtenu sur une plus grande partie de la galerie forestière, à l'exception de la région proche d'Orodara qui constitue un biotope particulier (plantations). Cet espacement des points de lâcher tous les 2 km répond donc bien à une représentation satisfaisante des mâles stériles dans le gîte.
- La densité de *G.p. gambiensis* a fortement chuté après les deux épandages d'insecticide et est restée à un bas niveau pendant 7 mois, en particulier pendant la saison des pluies où normalement les densités croissent (fig.3). L'impact des mâles stériles a été certain, mais pas aussi intense qu'il aurait dû l'être du fait des premiers lâchers qui ont été volontairement très espacés (1 point sur 16 km) et n'ont pas assuré une présence uniforme des mâles irradiés.

5.2.5. *Lâchers massifs, continus, rapprochés, sans traitement insecticide préalable: gîte Guimpy-Nord*

Dans les protocoles précédents, on a fait appel à un traitement préalable du gîte avec un insecticide non rémanent qui entraînait une baisse de densité et un remaniement de la pyramide des âges au bénéfice des jeunes individus.

Dans ce nouvel essai, on a procédé à des lâchers dans une population naturelle non modifiée: 650 mâles irradiés ont été lâchés chaque semaine (en 2 fois), tous les 200 mètres de galerie, permettant d'atteindre un rapport moyen de 8,4 $\phi/1\delta$ (prévu: 7/1). Ils ont commencé en saison des pluies.

La densité (fig.4) a baissé de façon constante durant la saison sèche suivante, puis a crû à nouveau, mais très faiblement en saison des pluies. Après 17 mois de lâchers, le niveau de population est actuellement très bas, mais l'éradication n'est pas encore totale.

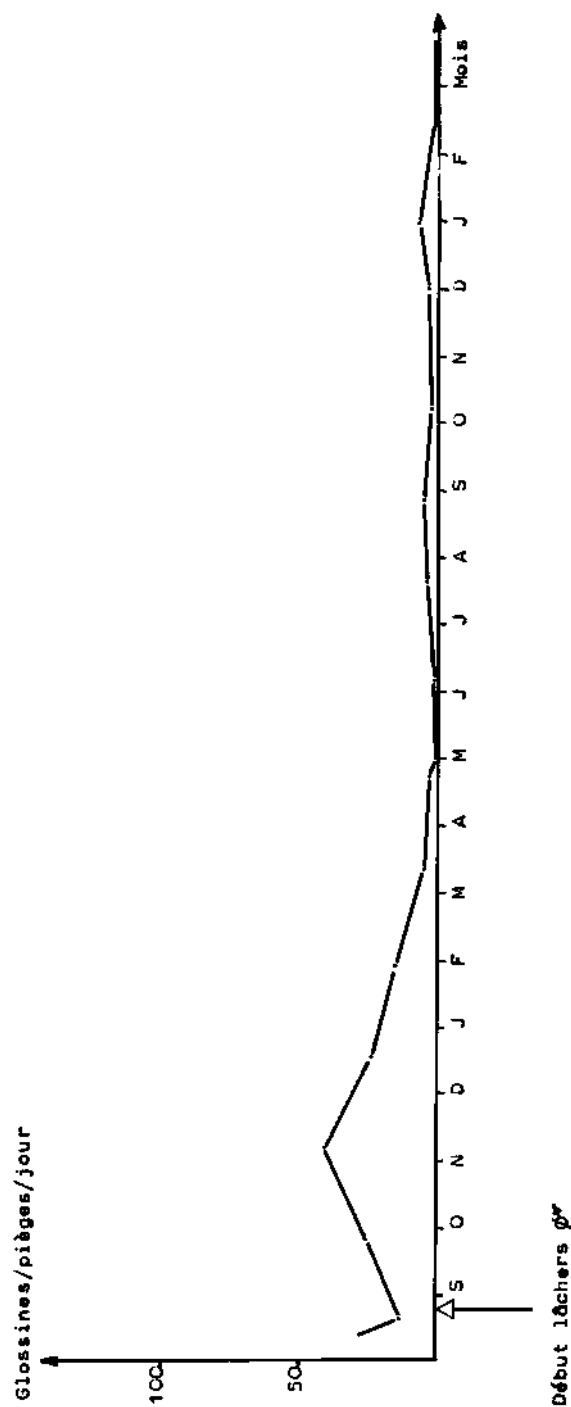


FIG. 4. Evolution de la densité apparente de *G. p. gambiensis* sur le gîte Guimpy-Nord.

Il semble que l'impact des mâles stériles soit certain, mais que leur effet soit plus lent à apparaître. Il convient d'attendre les prochains mois pour pouvoir tirer les conclusions définitives.

5.2.6. Lâchers modulés, continus, espacés, effectués 48 heures après un traitement insecticide non rémanent: gîte Dienka

Dans tous les essais précédents, les quantités de mâles stériles lâchés sont demeurées constantes, sans adaptation à l'évolution ultérieure de la densité de population sauvage. Il convient donc d'éviter une surconsommation inutile de mâles stériles et de lâcher des quantités nécessaires mais suffisantes pour obtenir l'efficacité désirée. Après le traitement insecticide non rémanent, les mâles ont été lâchés de façon continue, en des points distants de 1 km, mais en adaptant les quantités à lâcher à la densité de population, mesurée après chaque sondage mensuel, de sorte que le rapport ϕ/δ soit toujours compris entre 7/1 et 10/1. Les lâchers sont continus depuis 10 mois et ont commencé à la fin de la saison sèche.

La densité de la population sauvage (fig. 5) a décroît régulièrement, même en saison des pluies. Cette évolution semble se confirmer avec la saison sèche et sera à suivre particulièrement à l'approche de la nouvelle saison des pluies.

6. CONCLUSION

L'objectif de ces 3 années d'expérimentation était de soumettre à l'épreuve de la pratique sur le terrain la méthode du mâle stérile contre une glossine de galerie forestière.

Les travaux ont mis en évidence l'efficacité de cette méthode, dont les conditions d'application répondent aux points suivants:

- le début des lâchers se situera avec profit en pleine saison sèche, moment de baisse naturelle pour l'espèce visée dans cette zone climatique,
- les mâles stériles doivent se trouver dominants par rapport aux mâles sauvages, dans des proportions d'au moins 10/1 dès le début des opérations et de façon continue,
- la diffusion observée des mâles stériles permet d'espérer un espacement des points de lâchers d'au moins 1 km (analyse statistique en cours),
- des études annexes ayant montré un taux de «longévité 50%» de 9 jours [8], un lâcher hebdomadaire devrait être suffisant,
- l'opportunité d'un traitement insecticide non rémanent appliqué préalablement aux lâchers ne pourra être fermement établie qu'à la fin des observations en cours, mais tout laisse présager que l'intégration des deux méthodes est profitable et nécessaire,

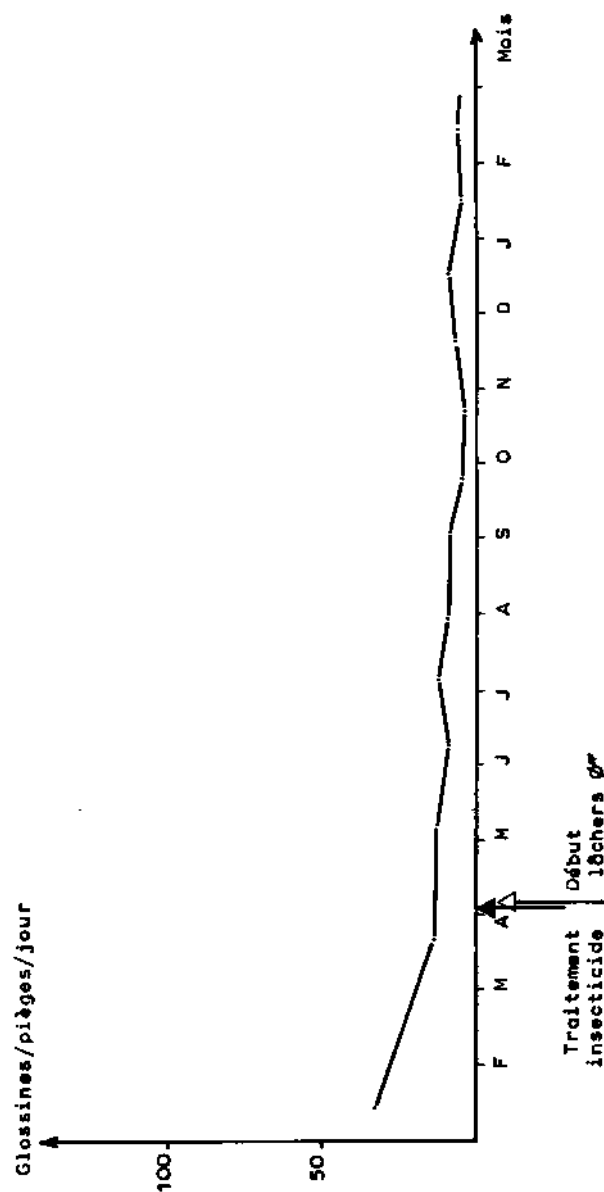


FIG. 5. Evolution de la densité apparente de *G.p. gambiensis* sur le gîte Dienka.

- la méthode du mâle stérile ne peut être dirigée que contre des populations moyennes ou faibles de glossines dans l'état actuel de la production en masse de cet insecte,
- son application à grande échelle est dépendante des progrès qui permettront de réaliser des élevages vraiment industriels.

Les résultats obtenus d'après ces expériences, dont une partie est en cours, montrent que la méthode génétique est efficace [9] aux conditions énumérées ci-dessus, puisque sur 32 km en expérimentation, 11 km qui avaient subi en priorité les lâchers de mâles irradiés sont actuellement libres de *G.p. gambiensis* et que sur le reste, les courbes évolutives de densité sont décroissantes.

L'élevage massif des glossines sur animaux de laboratoire ne peut répondre à la demande d'opérations de plus grande envergure. L'élevage sur membrane artificielle, qui a fait ses preuves en Europe, est en cours d'expérimentation en Haute-Volta; de sa réussite dépendra en grande partie l'avenir de la méthode du mâle stérile sur le terrain.

REFERENCES

- [1] POLITZAR, H., CUISANCE, D., «Les glossines, facteur limitant de la production bovine en Afrique. Essai de lutte biologique», IVth World Conf. on Animal Production, 20-26 août 1978, Buenos-Aires, Argentine.
- [2] CLAIR, M., CUISANCE, D., POLITZAR, H., SELLIN, E., TAZE, Y., Synthèse des recherches effectuées en 1975-1976 et 1977 sur la lutte génétique contre *Glossina palpalis gambiensis* Vanderplank 1949 par lâchers de mâles irradiés, Centre de recherches sur les trypanosomiasés animales (1978), 43 pages avec illustrations.
- [3] DAME, D.A., WILLIAMSON, D.L., "Status of sterile insect release method (SIRM) with *Glossina morsitans morsitans*", Ibid.
- [4] SELLIN, E., POLITZAR, H., CUISANCE, D., CLAIR, M., L'élevage de *Glossina palpalis gambiensis* Vanderplank 1949 (Diptera, Muscidae) à Bobo-Dioulasso, Haute-Volta, Revue Elev. Méd. vét. Pays trop. 30 1 (1977) 41.
- [5] TAZE, Y., CUISANCE, D., POLITZAR, H., CLAIR, M., SELLIN, E., Essais de détermination de la dose optimale d'irradiation des mâles de *Glossina palpalis gambiensis* Vanderplank 1949 en vue de la lutte biologique par lâchers de mâles stériles dans la région de Bobo-Dioulasso (Haute-Volta), Revue Elev. Méd. vét. Pays trop. 30 3 (1977) 269.
- [6] CHALLIER, A., LAVEISSIERE, C., Un nouveau piège pour la capture des glossines (*Glossina: Diptera-Muscidae*), description et essais sur le terrain", Cah. ORSTOM, sér. Ent. Méd. Parasit. 11 4 (1973) 251-262.
- [7] VAN DER VLOEDT, A.M.V., BALDRY, D.A.T., POLITZAR, H., KULZER, H., CUISANCE, D., The suppression of a riverine population of *Glossina palpalis gambiensis* by the aerial application of OMS-1998 (K-Othrin, NRDC 161), prior to the release of sterile males, ICIPE, Workshop on the Epidemiology of trypanosomiasis, Nairobi, 2-7 avril 1979.

- [8] POLITZAR, H., CUISANCE, D., LAFAYE, A., CLAIR, M., TAZE, Y., SELLIN, E., Expérimentation sur le terrain de la lutte génétique par lâchers de mâles stériles: longévité et dispersion des mâles irradiés de *Glossina palpalis gambiensis* (Haute-Volta), sous presse.
- [9] CUISANCE, D., POLITZAR, H., CLAIR, M., TAZE, Y., SELLIN, E., «Opération de lâchers de mâles stériles de *G.p. gambiensis* à Bobo-Dioulasso (Haute-Volta): efficacité de la lutte autocide», Joint FAO/IAEA Research Coordination Meeting on the Use of the Sterile Insect Technique for Tsetse fly Eradication or Control, Anvers, Belgique, 18–22 septembre 1978.

DISCUSSION

D.J. ROGERS: How did you translate your apparent density estimates into real estimates and what are the units of area for the latter?

D. CUISANCE: The apparent density is expressed as the number of tsetse flies trapped over the total length of the site during one day of trapping. The actual density is expressed by a simple Lincoln index for the whole site.

D.J. ROGERS: What percentage of your field-marked wild flies did you recapture during your Lincoln index work?

D. CUISANCE: The percentage was 10–15% of wild flies and 10% of sterile males.

F.M.J. EVENS: You recommend (Section 6 of your paper) beginning releases of sterile males early in the dry season when the population is at its lowest. Would it not be better to begin treatment early in the rainy season at the moment of peak eclosion?

D. CUISANCE: The ideal moment to begin releases is just before the maximum drop in the wild population, in other words in February. At this time the number of pupae in the soil is small and the adult population is concentrated over a small surface area.

P. FINELLE: The last figure you showed in your oral presentation seemed to show that the insecticide treatment was hardly effective at all.

D. CUISANCE: The effect of the treatment with insecticide was not shown in the figure as it was of very short duration (24–28 hours) and because the treatment was non-persistent. In fact, the figure should have shown the drop in density produced.

A.M. VAN DER VLOEDT: With regard to the effectiveness of decamethrin, I would just like to say that a single aerosol treatment has reduced a population of non-teneral flies by at least 90–98%, males proving to be more susceptible. The important point is, however, that all the older flies (i.e. those that could have been infected) were killed.

P. FINELLE: Have you any idea how many sterile males need to be released in order to compensate for re-invasions?

D. CUISANCE: The number of sterile males needed depends on the risk of re-invasion. In our case it would appear that 400 sterile males released each week in every cleared site are enough.

M. WEISS: In view of the relatively bad survival rate of your sterile males, would it not have been better to carry out the irradiation procedure in a saturated nitrogen atmosphere?

D. CUISANCE: We have not tried irradiating insects in a nitrogen atmosphere, but this technique might contribute to greater longevity.

A.M. VAN DER VLOEDT: The results achieved by the IEMVT team indicate that the released sterile males survived long enough to be effective, in that mating and insemination occurred. If survival were increased so that the sterile males lived as long as or longer than natural flies, there might be a risk of released flies becoming infected and consequently of transmitting trypanosomes.

D. CUISANCE: I quite agree with you.

P.A. LANGLEY: You mentioned that it is important to conserve numbers of released sterile flies in view of their high production costs. In fact, from the theoretical point of view, ratios of nine or ten sterile flies to one wild fly are high.

D. CUISANCE: These ratios do seem high, but the results show that they are necessary in order for the technique to be successful.

P.A. LANGLEY: Have you ever released marked, unirradiated males in order to see whether the reduced survival is due to laboratory rearing methods rather than to irradiation?

D. CUISANCE: We have not released unirradiated reared flies because we did not want to introduce fertile tsetse in sites where eradication was being attempted.

P.A. LANGLEY: Have you compared the survival of flies released as puparia with that of flies released as adults?

D. CUISANCE: No, we have not.

P.A. LANGLEY: I ask because it is of great economic importance for the lives of flies released in a control programme to be extended.

D. CUISANCE: We doubt whether it is so important to extend the lives of the sterile males, because after six matings, which would probably take about ten days, their inseminating capacity is in any case reduced.

H. POLITZAR: I might just add that adults were released (after their first meal in the laboratory) because of the presence of human sleeping sickness: we did not want to risk incurring criticism for increasing the risk of transmission.

H. WETZEL: Release in the adult stage does not preclude irradiation at the very late puparial stage, whereby only the male puparia are irradiated. Once the males emerge they can be fed in the laboratory as often as you wish and released afterwards.

D. CUISANCE: This is a good idea which should be tried out.

F.M.J. EVENS: I gathered that the survival time of the sterile males was nine days, which is very short. Do you know what the survival time of wild males in the biotope is?

D. CUISANCE: It is very difficult to estimate since there is no precise method of age determination for males; one can rely to some extent on observation of wing wear, but this is an approximate method.

R. GALUN: I wish to congratulate you on your success in eradicating tsetse flies from 11 linear kilometres. This success was badly needed in order to get support for continuing the SIT with tsetse flies.

D. CUISANCE: Thank you for your kind words; the whole team at Bobo-Dioulasso is responsible for this success. This week we have eradicated *G.p. gambiensis* over three further kilometres, which brings the length of tsetse-free gallery forest to 14 kilometres.

INTEGRATION OF STERILE INSECTS AND PESTICIDES FOR THE CONTROL OF THE TSETSE FLY

*Glossina morsitans morsitans**

D.A. DAME[†], D.L. WILLIAMSON, P.E. COBB,
D.B. GATES, P.V. WARNER, A.G. MTUYA,
H. BAUMGARTNER

Division of Livestock,
Tanzanian Ministry of Agriculture
and
Agricultural Research, Science
and Education Administration,
United States Department of Agriculture
and
FAO, Tsetse Research Project,
Tanga, Tanzania

Abstract

INTEGRATION OF STERILE INSECTS AND PESTICIDES FOR THE CONTROL OF
THE TSETSE FLY *Glossina morsitans morsitans*.

The effectiveness of integrating conventional insecticidal control with the release of sterile insects was tested in Tanzania with laboratory-reared *Glossina morsitans morsitans*. Two aerial applications of endosulfan suppressed the adults of an initial population of about 260 000 *morsitans* and 120 000 *Glossina pallidipes* by more than 95% on each application. Irradiated *morsitans* pupae were then distributed twice weekly in numbers depending on the varying density of the natural population within a 200-square kilometre test plot, averaging about 135 sterile males per square kilometre per month. After six months the check population of *pallidipes* had returned to normal density (0% control), but *morsitans* was only at 10% of its normal level (90% control). Although further reduction of *morsitans* was prevented by immigration of flies, 85–90% control was maintained throughout the remaining seven months of releases. The sterile males were highly competitive, preventing population increase at a ratio of about 1 sterile male:1 indigenous male; eradication would probably have been achieved if migrating fertile flies had not penetrated the perimeter. The sterile males were supplied from a self-sustaining colony of about 60 000 flies of Tanzanian origin maintained in insectaries at Tanga. The female flies produced 5–6 pupae each with a mean weight of 29 mg; these

* This paper reports the results of research only. Mention of a pesticide in this paper does not constitute a recommendation for use by the U.S. Department of Agriculture or the Tsetse Research Project, nor does it imply registration under FIFRA as amended.

[†] Insects Affecting Man and Animals Research Laboratory, Agricultural Research, Science and Education Administration, USDA, Gainesville, Florida 32604, United States of America.

45 000 females produced sufficient progeny for about 27 000 sterile males to be liberated monthly. For each 100 000 pupae produced, 31 800 viable males were released at a cost of US \$220 per thousand. Half of this cost was for host animal maintenance; approximately 580 locally procured or bred goats and 130 rabbits of imported origin were maintained indoors. Tanga strain flies reared in vitro at the International Atomic Energy Agency in Vienna in a 10 000-fly, back-up colony also performed normally when tested independently in the field.

Glossina morsitans morsitans is a major deterrent to economical livestock production throughout much of the tsetse-infested area of Africa because of its efficiency as a vector of animal trypanosomiasis. Of the 22 known species of tsetse flies, *morsitans* is unique in that it is distributed over most of the entire range of the genus *Glossina*. Because of the wide-spread, economic impact of *morsitans*, we selected it as a test species for research on the use of the sterile insect technique for the control of vectors of trypanosomiasis.

The phase of the research that we will report here was conducted under the auspices of and with joint funding by the United States Agency for International Development (AID) and the Republic of Tanzania. The Agricultural Research arm of the Science and Education Administration of the United States Department of Agriculture cooperated with the Division of Livestock of the Tanzania Ministry of Agriculture to create the facilities, provide the staff and conduct the research.

1. THEORETICAL CONSIDERATIONS

Under most circumstances, the sterile insect release method for tsetse fly control must be supplemented with other forms of control to achieve the most economical results. As the first table (I) reveals, a desirable ratio of sterile flies to indigenous flies at the beginning of a control program would be 3:1. However, the release of 3 sterile males for each native male into a dense population of *morsitans* might require 6 000 or more flies per square kilometer per month if the natural population were not first reduced by other means. Releases of such magnitude are clearly neither feasible nor practical for tsetse flies. However, sterile insects are likely to be most efficient when the density of the natural population is low. Therefore, limited insecticidal applications should provide an acceptable, cost-efficient method of reducing the indigenous tsetse population to a manageable level, i.e. one low enough so the sterile insects available from a rearing facility would be expected to cope.

Once the indigenous population has been suppressed and releases have been conducted for a generation, about 3 months for the purpose of this discussion, the numbers of released flies can be diminished without reducing the ratio of sterile to native males. This occurs because of the decline in the numbers of newly emerging adult flies caused by the sterility induced into the population during the first three months. In fact, as is shown in the table, even though the number released is reduced after three months, the effective ratio of sterile males to indigenous males can actually be expected to increase steadily. After a year or so of this stress the indigenous population should have been eliminated.

The next table (II) shows that it should be possible to utilize the release capacity of a rearing facility to progressively attack an infested area that is adequately isolated but is too large to be dealt with in a single year. In this situation, a new sector of the fly belt could be treated with released flies each quarter. This would optimize the use of insects by assuring a steady demand for the product. This model is flexible and is applicable to linear populations of flies, e.g. the riverine species such as *Glossina palpalis* and *Glossina tachinoides*, as well as for dispersed populations.

This then is the principle: reduce the target population to levels commensurate with the release capability and then use sterile insects to achieve eradication. The theoretical uses and benefits of this approach for tsetse fly control have been dealt with in detail in Volume 10 of IAEA's Atomic Energy Review [1]. We shall not repeat that information except to say that the technique could reduce insecticide usage 60% or more. The resulting reduction in environmental impact due to pesticide residues could be highly significant.

2. STERILE INSECT PRODUCTION

Techniques for colonizing and producing large surpluses of tsetse flies were developed relatively recently in England by Nash and Jordan [2] with *Glossina austeni* pupae imported from Zanzibar. In 1967 these scientists successfully colonized *morsitans*, and in 1969 when this colonized strain was evaluated in its original natural environment in the Zambezi River valley, it was found to disperse and survive normally [3]. The *in vivo* rearing system [4] perfected by these scientists was adopted for use in our program at Tanga, and we have been fortunate to have Dr. Jordan as an active consultant on rearing and fly production techniques.

TABLE 1. THEORETICAL TREND OF A LOW-DENSITY POPULATION OF TSETSE FLIES (AVERAGE 39 MALES AND 39 FEMALES PER SQUARE km) SUBJECTED TO STERILE MALE RELEASES AT THE RATES AND PERIODS INDICATED (AFTER KNIPLING [9], WHO BASED HIS CALCULATIONS ON 100 MALES AND 100 FEMALES PER SQUARE MILE)

Period (months)	Natural density at beginning of each period (males plus females)	No. of sterile males released each period	Estimated average ratio of sterile to fertile males	Assumed natural density at the end of each period
1-3	77	348 (116/month)	3:1	29
4-6	29	174 (58/month)	4:1	8
7-9	8	87 (29/month)	7:1	2
10-12	2	43 (14/month)	16.5:1	<1

Large-scale rearing of *morsitans* has involved the development and adaptation of systems for housing and managing large numbers of goats and rabbits under tropical conditions. At the termination of the release program 583 goats and 132 rabbits were being maintained. A breeding program was an important factor in the establishment of healthy herds. Initially, goats were purchased on the open market or obtained from government stations in Tanzania; rabbits were imported. The suitability of individual animals as hosts for tsetse flies was assessed, and unsuitable animals were rested from feeding or discarded. New goats were quarantined and treated for trypanosomiasis and helminth infections before being used for fly feeding. Since the animals were maintained indoors under quarantine-type conditions and are not routinely exposed to parasites and disease, they were likely to lose acquired immunity and tolerance to pathogenic organisms. Therefore, the key to successful use of goats and rabbits as hosts proved to be the development of optimal nutritional and animal husbandry practices appropriate to laboratory-housed animals. For example, the provision of spacious, well-ventilated housing was an important factor in disease control, particularly respiratory infections. Daily veterinary surveillance of the entire herd was required, and ill, parasitized or unthrifty animals were either treated under quarantine or returned to pasture for treatment and further observation. Also difficulties were encountered with shortages of animal foods so insofar as possible forage crops were produced on the station grounds to supplement local supplies. Detailed records of animal maintenance show that approximately half the cost of fly production could be attributed to maintenance of the animal host.

During the release program, the fly colony comprised approximately 45 000 females and 15 000 males housed in three separate and self-supporting insectaries especially designed for tsetse flies and built by project staff. Each insectary included separate fly maintenance, handling and feeding rooms in which the climate and photoperiod were strictly controlled. Daily the newly emerged flies were immobilized by exposure to 4°C and then collected, sexed and placed in mating cages. After mating the females were retained in cages until 81 days old; males were used for mating on no more than 6 occasions. They were given an opportunity to feed every day, including Sundays, on hosts that were rested for 2 days between use to prevent skin reactions. Precautions were taken to prevent insecticidal contamination of the colonies, e.g. all fly room staff were required to change their clothes and wash before beginning work. Also care was taken to ensure that animals used for fly feeding were free of substances such as sulphonamides and antibiotics, that can cause sterility of the insect. Animal

TABLE II. ESTIMATED NUMBER OF STERILE TSETSE FLY MALES NEEDED TO ERADICATE A LOW DENSITY POPULATION IN A NEW UNIT OF 1 000 SQUARE km EACH QUARTER

Release months	Number of males released per month in the indicated 1 000 square km block					Total no. released
	Block					
	A	B	C	D	E	
1-3	116 000					116 000
4-6	58 000	116 000				174 000
7-9	29 000	58 000	116 000			203 000
10-12	14 500	29 000	58 000	116 000		217 500
13-15 ^a		14 500	29 000	58 000	116 000	217 500

^a Process can be moved into new blocks as long as is required.

foods were routinely assayed for pesticides to prevent fly losses by exposure to insecticides in or on the host.

The Tanga colony was initiated with flies that emerged from pupae collected in northeastern Tanzania near Tanga. The fecundity of this strain of *morsitans* is somewhat lower than that of the widely used Rhodesian strain. Nevertheless, excellent fly breeding was achieved throughout the project. During their lifetime females produced a mean of 5-6 pupae with a mean weight of 29 mg. Daily collections of pupae were kept separately and when 52% had eclosed the remainder were sterilized. About 28% of the males were used for stud purposes. From every 100 000 pupae produced some 31 810 viable sterilized males were released.

3. PUPAL STERILIZATION AND RELEASE

The excess mature pupae that were to be used for releases were partially separated by sex by using a differential timing system related to pupal development, so most of the released insects were males [6]. They were irradiated in a nitrogen gas atmosphere, which suppressed the emergence of flies during irradiation and reduced radiation damage in the nonreproductive tissues of the fly [5]. Sterilization was achieved by exposure to 11.8 kr of gamma radiation from a Cesium-137 irradiator. After irradiation the pupae were dispensed into packets for release, transported under refrigeration (10°C) by road to the field, and delivered manually to the appropriate release sites in the experimental plot. There they were placed in sand containing a fluorescent powder. Within a few minutes all the flies emerged more-or-less synchronously [7], were automatically marked with the powder as they crawled up through the sand, and then dispersed within an hour or so.

4. FIELD TRIALS

Earlier field studies conducted in the Rhodesia phase of this program in 1967-69 with chemosterilized *morsitans* revealed sterile insects released as adults were only 20% effective, but those released in the pupal stage were equally competitive with males in the natural population [8]. An island population of *morsitans* was eradicated with the less competitive adult male by releasing 5 times as many males as should have been required. In another release effort 98% control was achieved using the more efficient pupal release system; 100% control would probably have been achieved if outside events had not prematurely terminated that program.

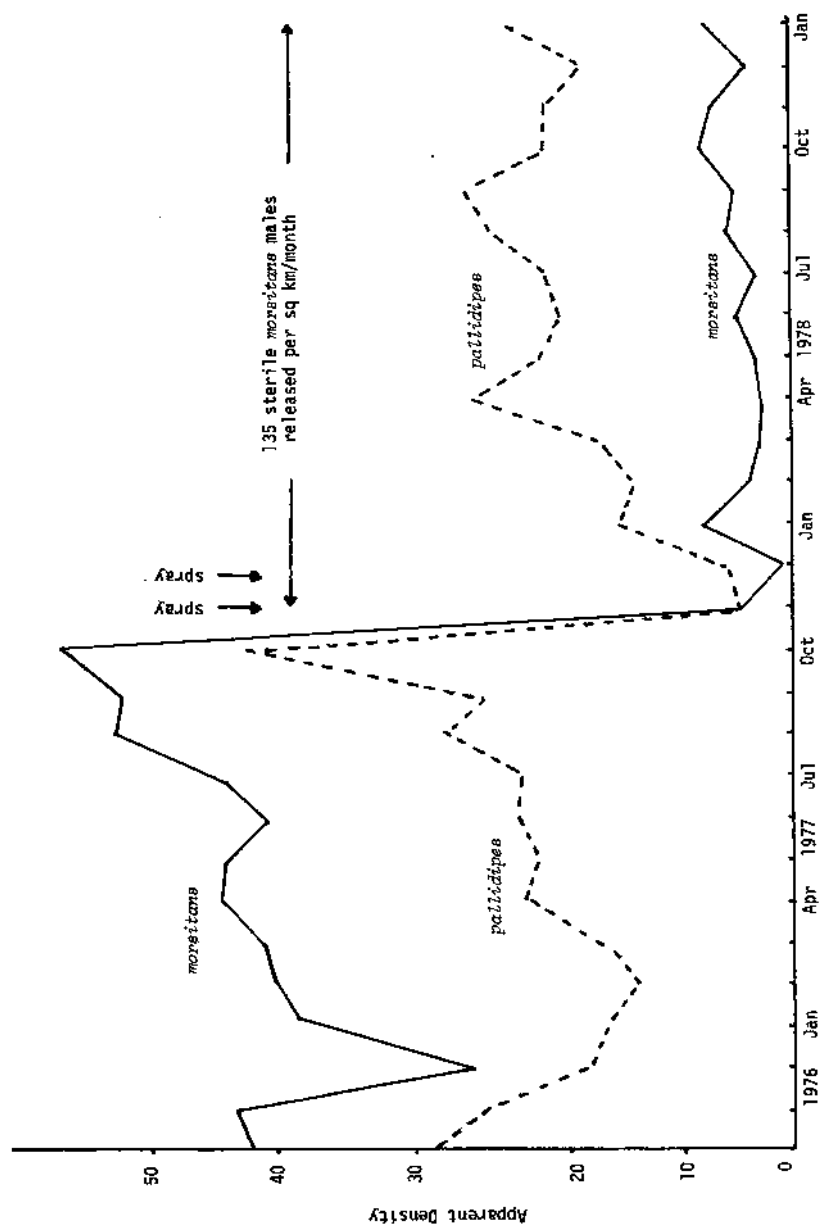


FIG. 1. Tsetse fly population trends at Mkwaja Ranch, 1976-79.

After achieving proficiency in fly rearing at Tanga, we began field studies to determine the capability of the laboratory-reared flies for population control. A field site of ca. 200 sq km was selected on Amboni Estates Ltd's Mkwaja Ranch, located 95 km south of Tanga. Initially, the site was semi-isolated by a 1-km clearing around 70% of the border, and considerable work was done to complete the barrier. During the release program the barrier was to be reinforced along the outside perimeter with applications of residual DDT on tree trunks to kill tsetse that came to rest before attempting to cross the barrier.

First, detailed studies of the fly population were conducted during 1976-77 by teams traversing 200 km of flyrounds weekly in the experimental and control areas. The distribution and dynamics of the population were then determined with the assistance of computer program analyses. Two major vector species were abundant in the test plot, *morsitans* (ca. 1300/sq km) and *pallidipes* (ca. 600/sq km); localized minor populations of *Glossina brevipalpis* were also detected.

The second step was to suppress the population of tsetse flies in the experimental site by 2 aerial sprays of endosulfan applied at 20 g/ha. These were applied in November 1977 with a 28-day interval between sprays. Each application of this nonresidual aerosol gave virtually complete control of the adult tsetse. The subsequent population consisted of females that emerged from pupae in the soil and were the target of the sterile male releases.

Sterile pupae were placed at 120 release sites in a pattern closely matching the distribution of the indigenous fly population. Each release site was visited once each 14 days. The releases were made twice weekly and provided a monthly average rate of 135 sterile males per square km/month.

The level of sterility in the target *morsitans* population was assessed during the early part of the release period when female flies were relatively abundant. During this period infertility in the native *morsitans* females increased about five-fold. Later, when females became difficult to detect because of the population reduction achieved, it became impractical to monitor induced sterility in this manner.

Over the first six months the *morsitans* population fell to ca. 10% of its normal density as a result of the impact of the sterile males (Figure 1). In contrast, during that

same period the *pallidipes* population, which had been exposed to the insecticide but was not under stress by sterile males, returned to its normal prespray density.

In spite of further releases at the same intensity for seven more months, the *morsitans* population stabilized at a level representing a reduction of between 85-90%, indicating that immigration of fertile flies from outside the plot might be influencing the level of control. This was confirmed by making extensive studies with marked flies that were released outside the cleared barrier and recaptured inside the experimental plot. The movement observed was sufficient to balance the effect of the sterile male releases at ca. 90% reduction. This fly immigration was the consequence, at least in part, of the inability to maintain an effective deposit of DDT on the outer edge of the barrier because of the extremely heavy rains experienced during this phase of the research program. During the year the rainfall continued through the normal dry periods and broke a 22-year record.

The released males effectively held the indigenous population at 85-95% control despite at an average ratio of only 1 sterile male for each indigenous male. The competitiveness of the sterile males was revealed to be extremely high. Had the barrier been completely effective, it is likely that complete eradication would have been achieved.

5. DISCUSSION

The program discussed here was a research effort undertaken to determine whether high quality sterile male tsetse flies could be produced in Africa in sufficient quantities to support a sterile male release program. The results demonstrate that this has been achieved. The flies produced *in vivo* were relatively expensive, 22 cents each. However, during the course of the trials, funding was provided to IAEA to help in developing *in vitro* methods of rearing *morsitans*. As a result, IAEA was able to perfect a technique for rearing *morsitans* on preserved horse blood by using artificial membranes. In fact, for 18 months a reserve colony of 10 000 Tanga flies was maintained *in vitro* at the IAEA Seibersdorf Laboratory outside Vienna. When it was field-tested in Tanzania in the latter part of 1978 it was found to be normal. This availability of acceptable *in vitro* techniques of rearing should reduce the cost of fly production considerably because it eliminates the need to maintain the flies on live host animals, which now accounts for half of the production costs at Tanga.

The use of sterile tsetse flies in future programs in combination with pesticides or other control methods can now be realistically anticipated. However, further fine-tuning is needed to increase cost-effectiveness. For example, the development of acceptable aerial release methods would allow easier dispersal throughout the target zones and would help assure adequate release coverage.

Also in areas with multiple vectors of trypanosomes, it may be necessary to release more than one species to eliminate transmission. Although this will certainly increase the cost of the control operation relative to the release of a single species, such increases may be acceptable when compared with the rising cost of conventional control methods. The cost effectiveness of each program to be considered will have to be weighed separately, and full consideration must be given to isolation requirements and to the environmental and social impact and to land use implications.

The sterile insect technique requires time and expertise to develop. However, where the demand for trypanosomiasis control is high and where the infested areas are sufficiently large, the economic return from the initial investment to develop the technique will be accompanied by the reduced environmental hazards resulting from the integrated use of complementary control technologies.

REFERENCES

- [1] JOINT FAO/IAEA DIVISION OF ATOMIC ENERGY IN FOOD AND AGRICULTURE, Application of sterility principle for tsetse fly suppression: Review of a panel, At. Energy Rev. 10 1 (1972) 101.
- [2] NASH, T.A.M., JORDAN, A.M., BOYLE, J.A., A promising method for rearing *Glossina austeni* Newst. on a small scale, based on the use of rabbits' ears for feeding, Trans. R. Soc. Trop. Med. Hyg. 60 (1966) 183.
- [3] DAME, D.A., BIRKENMEYER, D.R., NASH, T.A.M., JORDAN, A.M., The dispersal and survival of laboratory-bred and native *Glossina morsitans morsitans* Westw. (Diptera, Glossinidae) in the field, Bull. Ent. Res. 65 (1975) 453.
- [4] NASH, T.A.M., JORDAN, A.M., "Methods for rearing and maintaining *Glossina* in the laboratory", The African Trypanosomiases, George Allen and Unwin Ltd., London (1970) 441.
- [5] LANGLEY, P.A., CURTIS, C.F., BRADY, J., The viability, fertility and behaviour of tsetse flies (*Glossina morsitans*) sterilized by irradiation under various conditions, Entomol. Exp. Appl. 17 (1974) 97.
- [6] BIRKENMEYER, D.R., DAME, D.A., Storage and sexual separation of *Glossina morsitans morsitans* Westwood puparia, Ann. Trop. Med. Parasitol. 69 3 (1975) 399.

- [7] CURTIS, C.F., LANGLEY, P.A., Use of nitrogen and chilling in the production of radiation-induced sterility in the tsetse fly *Glossina morsitans*, Entomol. Exp. Appl. 15 (1972) 360.
- [8] DAME, D.A., SCHMIDT, C.H., The sterile male technique against tsetse flies, *Glossina* spp., Bull. Ent. Soc. Am. 16 1 (1970) 24.
- [9] KNIPLING, E.F., The Potential Role of the Sterility Principle for Insect Population Control with Special Reference to combining this Method with Conventional Methods, U.S. Dept. Agriculture, Rep. ARS 33-98 (1964).

DISCUSSION

J.L. GRINGORTEN: I understand from the section on 'Pupal sterilization and release' that you attribute the synchronized emergence of the adult flies to both the cold and nitrogen treatments. Do you in fact need both: is the nitrogen necessary for synchronization?

D.A. DAME: Nitrogen both protects the fly from radiation damage and inhibits emergence; cold also inhibits emergence. Radiation, on the other hand, stimulates emergence. We need cold to prevent premature emergence outside the irradiator and nitrogen for the irradiation treatment.

J.L. GRINGORTEN: What would be the time-spread of emergence without the treatments?

D.A. DAME: Without these treatments, irradiation would have given synchronous and immediate emergence. If, after most of the females had eclosed, the pupae had been left at ambient temperatures, emergence would have been spread over two-three days. However, the pupae were chilled to prevent this.

H. WETZEL: Spraying with insecticide was repeated after a 28-day interval. What was the reason for this interval being chosen?

D.A. DAME: In order to get maximum cost-efficiency we used a 28-day interspray interval with the expectation that about 90% of the pupae underground would emerge in that period and that we would eliminate a high proportion of these females before their first larviposition; the sterile males would then have only to confront the remaining 10% and those emerging from the relatively few pupae that were deposited during the interspray period (incidentally, we did release sterile males during the interspray period in order to influence population density further). However, because of very heavy rainfall the pupal period was extended, and it seems that perhaps only about 60% of the pupae emerged as adults before the second spray, leaving 40% to emerge after the second spray. This made it difficult to achieve the 3:1 ratio expected and in fact the maximum ratio obtained was about 1.5 sterile males to each indigenous male. Nevertheless, the data now indicate that this ratio would have caused the population to decline had immigration from outside the experimental plot not continually revitalized it.

P.A. LANGLEY: You released marked flies on the control side of the barrier and recaptured them in the experimental area, thus illustrating that the barrier was not 100% effective. This was done rather late in the release programme. Have you any reason to believe that the barrier was 100% effective at any time during the experiment?

D.A. DAME: Before the release programme began the barrier was tested with released marked flies and some crossed it. It was expected that the application of DDT around the perimeter would reduce immigration significantly — that is, to a level that would not present a challenge to the experimental design. Since environmental conditions prevented the DDT applications being completed in good time and in an effective manner, I doubt whether the barrier was 100% effective at any time during the experiment.

C.F. CURTIS: Did you consider releasing sterile males beyond the barrier zone to try to ensure that the immigrant females were sterile when they arrived, i.e. to create a 'biological barrier zone'?

D.A. DAME: The population of flies outside the barrier was very dense along much of the barrier perimeter and in its surroundings. The release of sterile males without other suppression measures would have had only a negligible effect unless unreasonably large numbers could be released. Thus, no attempt was made to create a 'biological barrier zone'.

M. FRIED: Were there parts of the treatment area in which the wild population dropped effectively to zero?

D.A. DAME: The data have not been fully analysed, but indications are that the main infiltration was on the west and south-west perimeter of the 200 km² plot, and that complete elimination may have been achieved in the eastern and central portions of the plot. We hope the data will enable us to clarify this point.

Max MURRAY: Did the release of sterile flies have any effect on the prevalence of trypanosomes in the cattle population on the ranch — as gauged for example from the number of treatments required?

D.A. DAME: This is quite difficult to answer. The cattle were under prophylactic and curative treatment with isometamidium and berenil, and just before the aerial applications our samples showed about 45% incidence of trypanosomiasis in the cattle. I believe that the sample was taken just before the routine curative dose was to be applied, i.e. just at the end of the prophylactic cycle. Later the incidence fell to less than 20% and at the end of the release programme it was about 35%. By this time the *pallidipes* population was back to normal, and there was a residual population (10%) of indigenous *morsitans* and an equal number of sterile released flies. It is impossible to interpret this information in terms of the effect of the released flies. However, there is published evidence that sterile flies transmit trypanosomes without difficulty. In an eradication programme such transmission would cease when the programme ended, and would inevitably be less than transmission before the treatment because of

the reduction in the natural fly population before the release of flies. The schedule of treatments for protection against trypanosomes was not under our control.

R.E. PURNELL: Is there any way of altering the sex ratio of laboratory-reared flies?

D.A. DAME: Tsetse flies have a very low reproductive rate, so genetic manipulation and selection of the insects for desirable characteristics are quite difficult, although not impossible. It should be possible to alter the sex ratio by means of one or more techniques, but it may not be practical to do so.

D.A. LINDQUIST (*Chairman*): What data are available on the longevity of laboratory-reared, sterilized male *G. morsitans* released in Tanzania – that is, how long does it take for 50% of them to die (Lt_{50})?

D.A. DAME: Although the field data have not yet been fully analysed, the laboratory studies show that these males survive as well as unirradiated males. In the field the initial observations indicate a mean survival of 35–40 days ($Lt_{50} \cong 35$) compared with a roughly estimated mean survival for indigenous flies of about 30–50 days ($Lt_{50} \cong 35$).

GENERAL DISCUSSION

on the FAO Animal Health Division field research on tsetse flies

M.J. MUTINGA: Does the FAO operate training centres in Africa for speakers of French and English.

J.G. LEROUX: The FAO project in Zambia entitled *Training and Applied Research in the Dry Savannah Zone* has a major training component aimed at middle-level field control personnel from English-speaking countries. It is based at Mazabuka and is expected to become operative during 1979.

In addition, training courses aimed at the leadership cadres are organized approximately every two years. During the first quarter of 1979 two such courses of about 3½ months' duration were completed in collaboration with France and the United Kingdom. A seminar of three weeks' duration was conducted during February and March 1979 in Bobo-Dioulasso, Upper Volta, with bilateral assistance and in collaboration with the World Health Organization (WHO) and the Organization for African Unity (OAU). This seminar was concerned principally with tsetse control.

R.C. PINHÃO: There seems to be scanty information about the distribution of tsetse flies in Mozambique; this seems to apply to the northern part of the country, where military operations have been going on for several years. Have you any recent information about the progress of the invasion of *G. morsitans* south of the Limpopo River, which was described some years ago?

J.G. LEROUX: The northern two thirds of Mozambique is the area on which there is little information. No reliable information is available regarding the *G. morsitans* advance and the present status of the control campaign of the early 1960s because the Massagena area has been politically sensitive for quite some time. The suspected *G. morsitans* advance around Muabsa in the east was investigated during 1978. The conclusion reached was that the infestation is highly unlikely to proceed further south because of adverse ecological conditions.

USE OF THE STERILE MALE TECHNIQUE AS AN ADJUNCT TO INSECTICIDAL AND PHYSICAL METHODS FOR STABLE FLY CONTROL ON THE ISLAND OF ST. CROIX, U.S.V.I.

R.S. PATTERSON, G.C. LABRECQUE*,
D.F. WILLIAMS

Insects affecting Man and Animals Research Laboratory,
Science and Education Administration,
Agricultural Research,
USDA,
Gainesville,
Florida,
United States of America

Abstract

USE OF THE STERILE MALE TECHNIQUE AS AN ADJUNCT TO INSECTICIDAL
AND PHYSICAL METHODS FOR STABLE FLY CONTROL ON THE ISLAND OF
ST. CROIX, U.S.V.I.

A three-year feasibility study was conducted on the island of St. Croix, U.S.V.I., on the use of the sterile male technique as an adjunct to insecticidal and physical measures to control the stable fly, *Stomoxys calcitrans* L. The native stable fly population cycled in relation to moisture; it was the highest during the rainy season in the autumn (1.9×10^6) then gradually declined to a low point (0.7×10^5) during the summer in the dry season. The releases were carried out with an indigenous strain of flies reared on the island. The adult flies (24–48-h old) were exposed to 2 kR of gamma irradiation in a cobalt source. This treatment plus marking reduced the competitiveness by about 15%. The sterile flies were released in lots of 4000 at prearranged points throughout the island. Approximately 1×10^5 sterile males/day for 18 months were released at 2 km intervals over the 218 km² island. This reduced the native population by about 50%. On selected farms where insecticide or sterile males could not be released, the fly pupal parasite, *Spalangia endius* Walker, was released. Sanitation and cleanup of breeding habitats were recommended to all farmers, but were practised by very few. When all control techniques were in practice, the native stable fly population was reduced by 99.9%. A few fertile flies were found during the last six months of this experiment. These came from isolated breeding sites, flies immigrating from other islands about 40 km away or were introduced with imported livestock and/or pets. During this period no larval breeding was observed indicating that immigration was probably the main cause of the fertile flies on the island.

* Present address: Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture,
International Atomic Energy Agency, Vienna.

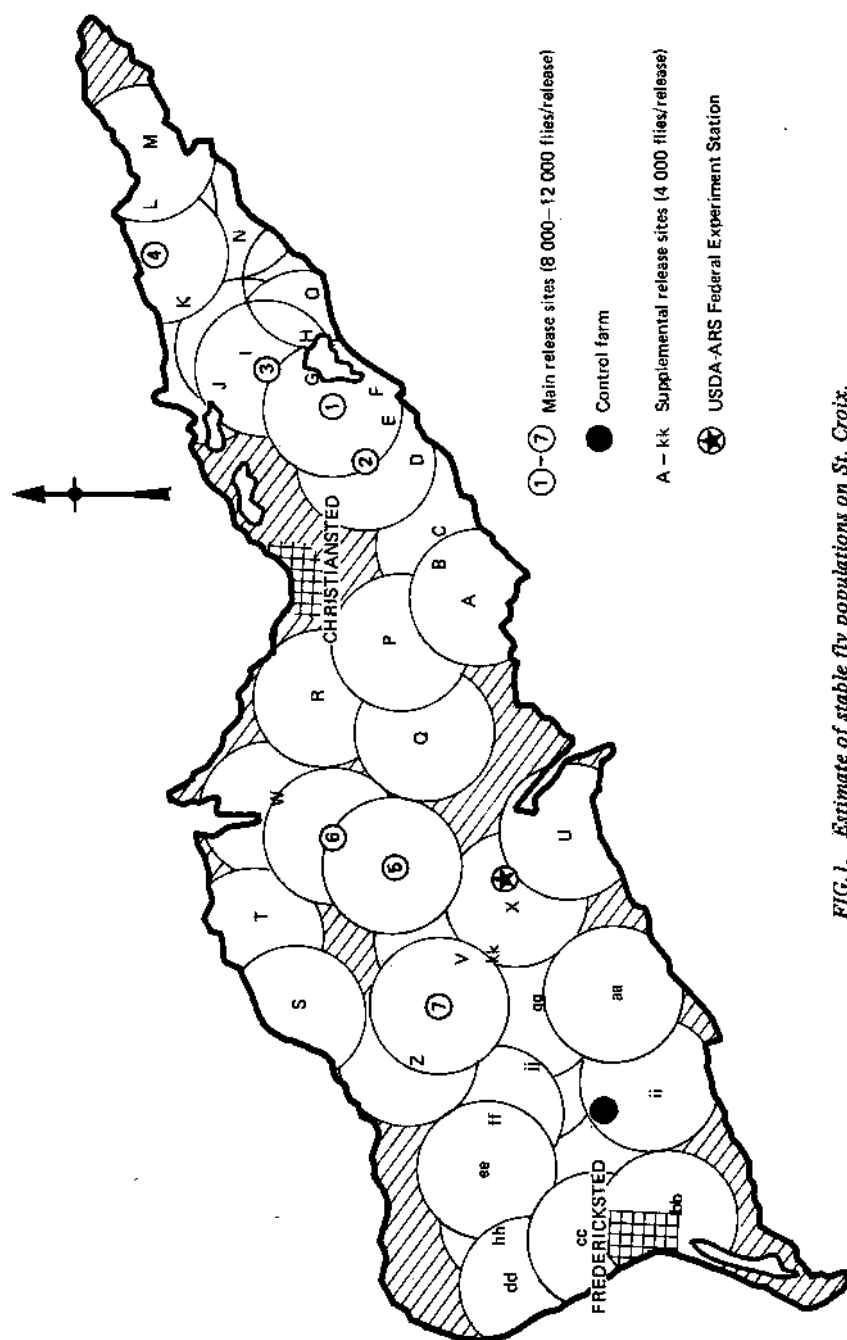


FIG.1. Estimate of stable fly populations on St. Croix.

The feasibility of controlling the stable fly *Stomoxys calcitrans* (L.) by sterile male releases (SMR) was first demonstrated by LaBrecque and co-workers [1] at a small dairy in central Florida. Although the released flies were impaired by the sterilization, they were able to compete sufficiently in the wild to cause a high degree of sterility in the native females followed by a population suppression. Unfortunately, the farm was not isolated and wild flies continued to infiltrate into the release area. Consequently, the length of control once the releases were terminated was relatively short.

In order to demonstrate the practicality of the sterile male technique, it would be necessary to try it in an isolated area free of immigrating flies. The island of St. Croix was selected for this study because of its isolation, stable fly population and available facilities.

St. Croix is located in the Caribbean Sea about 60° 45' W longitude and 17° 45' N latitude, approximately 65 km southeast of Puerto Rico, the nearest island known to produce large numbers of stable flies. The climate is semi-tropical with an annual range in temperature of 21° to 28°C. There is a wet season (Sep.—Feb.) with periods of excessive rainfall and a dry season (Mar.—Aug.) when severe droughts are frequently experienced. The island's 218 km² can be divided into three distinct zones: a semi-arid grassland on the eastern end, a fertile central plain, formerly planted in sugar-cane, and a mountainous rain forest in the northwestern area. St. Croix supports about 1000 dairy and 8000 beef cattle, about 1000 wild and domestic horses plus numerous goats, sheep and swine. A survey of the area revealed over 100 large stable fly breeding sites mainly where livestock are maintained, and about 1000 backyard pens with five or less domestic animals. This creates situations capable of breeding numerous stable flies especially during the wet season. There is little farming on the island. Sorghum is being grown on about 200 to 300 ha in the central valley. Most of the tillable land lies fallow.

Population densities of the wild stable flies were determined by counting adult flies feeding or resting on cattle. Studies by LaBrecque and co-workers [2] in Florida indicated that each fly observed on an animal represented about 55 in the environment. A similar study on St. Croix produced almost identical results. Therefore these counts were made at weekly intervals throughout the study on ten animals selected at random at the seven principal dairies and feed lots. Since we know the approximate number of animals on the island we could estimate the daily size of the fly population based on the average number of flies per animal. A generation time was about three weeks as this was the elapsed time from egg to sexually mature adult in the laboratory at an average 25°C, the average mean temperature for St. Croix. Thus, from an average of 210 counts per generation a quantitative index of the fly population could be determined. From the data obtained (Fig.1), fly populations on the island average 7.4×10^5 for the wet season with a range of 3.0×10^5 to 1.9×10^6 , and

TABLE I. AVERAGE NUMBER ($\times 1000$) OF FLIES TO BE RELEASED DAILY AT INDICATED STERILE TO FERTILE (S:F) RATIOS ASSUMING A DAILY EMERGENCE OF 25%

Season	RI ^a	S:F Ratio			
		0.5:1	1:1	2:1	5:1
Wet	1.6	119	238	475 ^b	1188 ^c
Dry	0.9	31	63	125	313

^a RI = rate of increase.

^b Feasible for 1/2 of the island only.

^c Not feasible with existing rearing capacity.

TABLE II. NUMBER OF GENERATIONS TO ACHIEVE 0 POPULATION WHEN SELECTED AT THE FOLLOWING STERILE (S) TO FERTILE (F) MALE RATIOS

Season ^a	Population ($\times 1000$)	RI	Ratio — S:F			
			0.5:1	1:1	2:1	5:1
W	950	1.6	—	> 50	22	11
D	250	0.9	25	16	11	7

^a W = wet season, D = dry season.

during the dry season populations average 2.0×10^5 with a range of 0.7×10^5 to 3.5×10^5 .

Based on the data obtained, the rate of increase (RI) per generation seldom exceeded 2X during the wet season although once it was 6.3X, and the maximum observed during the dry season was 1.2X. The daily emergence rates based on adult emergence as well as on the daily loss rates (DLR) derived from marked-release-recapture studies in static populations indicated that the daily emergence population makes up about 25% of the total adult population.

We now had some idea of the size of the wild stable fly population and thus had to determine the number of sterile males needed for suppression. Table I shows the average number of flies required to be released daily to cause a favourable sterile : fertile ratio at different seasons. Table II gives the number of generations

TABLE III. COST OF REARING ONE MILLION ADULT STABLE FLIES WEEKLY DURING 1976 ON ST. CROIX (EXCLUDING HOUSING AND INITIAL EQUIPMENT SUCH AS RACKS AND LARVAL TRAPS)

Item	Number or amount used	Total cost (US\$)	Cost/wk (US\$)
Racks for cages and trays	30	600	
Cages (adult)	125	625	
Trays (larval)	300	2400	
Covers (larval trays)	300	150	
Misc. containers	50	150	
Pupal washer	1	80	
Pupal driers	4	100	
Wheat bran	142 kg/wk		17.00
Sugar-cane bagasse	57 kg/wk		6.00
Cotton	6 kg/wk		21.00
Blood	40 litres/wk		5.25
Sodium citrate			1.20
Electricity			25.00
Salaries (2 Biol. Aides at US\$2.80/h, 40 h/wk + OT)			224.00
Total			299.45

needed at various sterile : fertile ratios to achieve complete eradication in the wet and dry seasons on St. Croix. The daily production from the St. Croix colony, a strain established from local wild flies, was about 250 000–300 000 pupae per day with existing facilities and staff. Of this number of pupae about 70 000 were needed daily to maintain the colony. The remaining flies were irradiated and later released in the field. Both males and females were released following exposure of 24- to 48-h-old adults to 2 kR of gamma rays (cobalt-60). This dosage yielded 100% sterility in the females and $98 \pm 2\%$ sterility in males. Following irradiation these adults were vigorous and highly competitive with the native strain. Studies by Williams and co-workers [3] indicated only a 5% reduction caused by the irradiation.

The immature flies were reared on a diet of wheat bran and sugar cane bagasse plus water. Each larval tray was seeded with about 60 000 eggs with a subsequent yield of 21 444 pupae per tray. Adult eclosion averaged more than 90% thus rearing efficiency was about 30%. The rearing techniques were

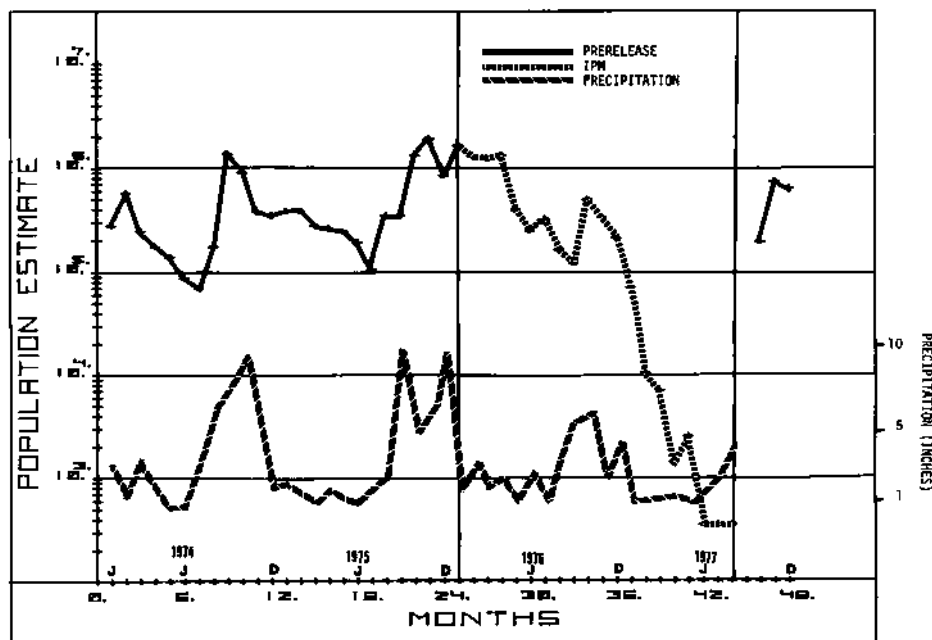


FIG. 2. Release sites of irradiated stable flies on St. Croix, U.S.V.I., 24 August 1976.

modified for St. Croix from that described by Bailey and co-workers [4]. The cost of rearing the flies is given in Table III. Strict security was maintained throughout the study since rearing was performed within the test area, and escaped flies could be detrimental to our goal of total population suppression.

All the flies that were sterilized and released on St. Croix were marked with fluorescent dusts. The dust increased the DLR of the flies by about 10% [3], but we felt marking was essential since we could rapidly derive the field ratio of sterile to normal males, assay only wild females (unmarked) for sterility, and monitor the irradiated females for fertility. The ratio of sterilized to normal flies in the total population could also be calculated based on the ratio found in animal-baited traps [5] located throughout the island. Based on Williams' calculation from laboratory and field cage studies, the marked sterile flies were approximately 15% less competitive than normal flies. In the field the sterilized flies exhibited less competitiveness than observed in the field cages. This level of competitiveness was probably caused by the flies being released in areas where they had to migrate long distances to obtain a blood meal or to mix with the wild fly population rather than a true loss of competitiveness caused by any physical damage to the flies.

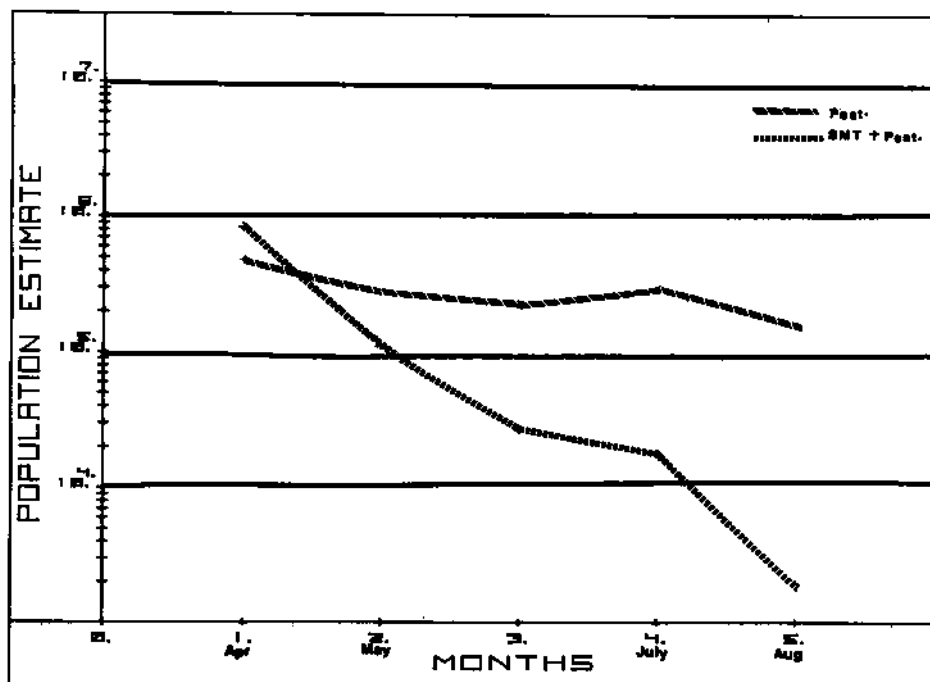


FIG.3. Estimate of stable fly populations on St. Croix (using pesticides versus pesticides and sterile male technique).

The sterilized flies were released at 2 km intervals throughout the island (Fig.2) except in the densely populated areas such as the towns, villages, housing areas and employment areas. Because of inaccessibility, due to lack of roads, releases were not conducted in areas of the rain forest and a salt marsh on the uninhabited eastern end of the island. The sterile flies were released in lots of 4000 from disposable paper bags from a vehicle at 2 km intervals throughout the island except in the densely populated areas. At the major dairies and feed lots, 12 000 sterile flies were released near the animal holding pens. The released flies had a tendency to orient towards the large animals and did not disperse evenly throughout the island. Aerial releases might have given a more even distribution.

When the study was initiated, only one farm was growing and feeding sorghum. Each subsequent year the number of acres planted in sorghum increased with a corresponding increase in the breeding sites and number of wild stable flies. This was because sorghum mixed with animal wastes is an excellent breeding medium for stable flies.

Production of flies for release could not be increased because of the small staff and limited facilities. Therefore we decided to reduce the wild population by treating the major larval habitats created by sorghum feeding with methoxychlor. The 1% spray acted mainly as an adulticide to the newly emerging adults and ovipositing females. The major larval sites were treated routinely starting about three months before the systematic releases of sterile flies. These treatments caused an estimated 50–60% overall reduction in the fly populations in the areas. This estimate is based on the fact that although the methoxychlor sprays were used at the major breeding sites throughout the island, the sterile insects were released on only the eastern half (about 58 km²) for a four-month period. Therefore the effect of the chemical alone and that of the chemical and the sterile insect releases (Fig.3) could be calculated. The data clearly indicate that the spraying checked all population growth, but that other control methods such as the release of sterile insects were needed to cause a downward trend in the population.

Since the use of methoxychlor sprays or release of sterile flies was denied at some breeding sites, other control measures were used. Although cultural control is the preferred method of fly population reductions, few farmers co-operated. Some were persuaded to feed silage from troughs rather than from the ground, and in one instance where one farmer refused the use of pesticides, he accumulated all waste material at a central point at regular intervals thereby reducing fly breeding. The parasite *Spalangia endius* Walker was also used in a caged layer operation of about 20 000 chickens to control house flies and stable flies. We released about 2.5×10^5 parasites weekly for approximately eight months, and eliminated both the house fly and stable fly problem. The releases were carried out in the manner described by Morgan and co-workers [6]. During the last six months of the study, an additional control measure was introduced. An attractant toxicant trap system as described by Meifert and co-workers [7] was used at several farms to eliminate the few remaining flies. Unfortunately we did not have sufficient traps to cover all isolated and urban breeding sites where releases could not be conducted because of concentrated human populations.

The effectiveness of this integrated pest management programme for stable fly control was evaluated by two methods:

- (1) An estimate of the total population based on the number observed feeding on the animals corrected for the sterile to normal ratio observed in the field.
- (2) The degree of sterility in the wild females.

At weekly intervals, 'animal counts' were made and heifer-baited traps set at ten farms. Consequently, the number of flies observed on an animal could

be amended to derive only the wild flies feeding and the total wild stable fly population estimated. Since all released flies were marked, the ratio of released to wild could be determined.

The degree of sterility in the wild population was assayed by capturing wild females in the heifer trap in a 4-hour period. To collect the flies, the animal was removed and the cage vacuumed using a 115 V AC vacuum cleaner modified with a plastic collecting tube. Where electric power was not available, a 12 V vacuum unit using the vehicle electrical system was used. The flies were transferred to a holding cage following collection and returned to the laboratory, chilled, counted and separated by sex. Unmarked females were placed in individual plastic tubes for oviposition.

The tubes had an open base and a screened top and were fitted into holes in a piece of wood. The bottom of each hole was fitted with a piece of cotton covered with black cloth which served as an oviposition site. The cotton was kept moist by resting the blocks of wood in metal trays containing about 16 mm of water. The flies were fed daily by placing a small piece of cotton saturated with citrated bovine blood on the screened top. When oviposition occurred the cloth was removed and placed on a small wet sponge in a petri dish, held for 74 hours and the eggs checked for hatch under a microscope.

As stated previously, the integrated system was started on the eastern half of the island (58 km²) in March 1976. Sterility steadily increased in the wild population, as shown in Fig.4, until August. At that time the fly population was at such a low level that releases on the western half of the island (about 83 km²) were initiated using only enough sterile flies on the eastern half to maintain population suppression. However, because of a change in the milling of the bran used in the larval diet there was a severe curtailment of fly production and subsequent releases. We terminated regular releases on the western half of the island and tried to maintain releases on the eastern half. Still sterility declined to 70% in November with a corresponding increase in the wild population, as shown in Fig.4. Once colony fly production returned to normal, sufficient flies were again available for release, and sterility on the eastern side rose again to 99% followed by a steady decline in the wild population. Throughout this period sterility on the western half of the island remained at the same level and the population did not decrease until sterile insects were again released in sufficient numbers. Larvicide treatments were used throughout this period which helped to curtail the rapid buildup of the population.

By January all systems of the IPM program were functional and, as shown in Fig.1, the wild fly population rapidly declined to an estimated low level of only about 350 wild flies on the 218 km² island. Sterility likewise rose and maintained itself, for the most part, at about 99+%. However, since the wild fly population was very low, frequently weeks elapsed before a wild female could be captured for sterility assessment. If it was fertile, it could not be determined

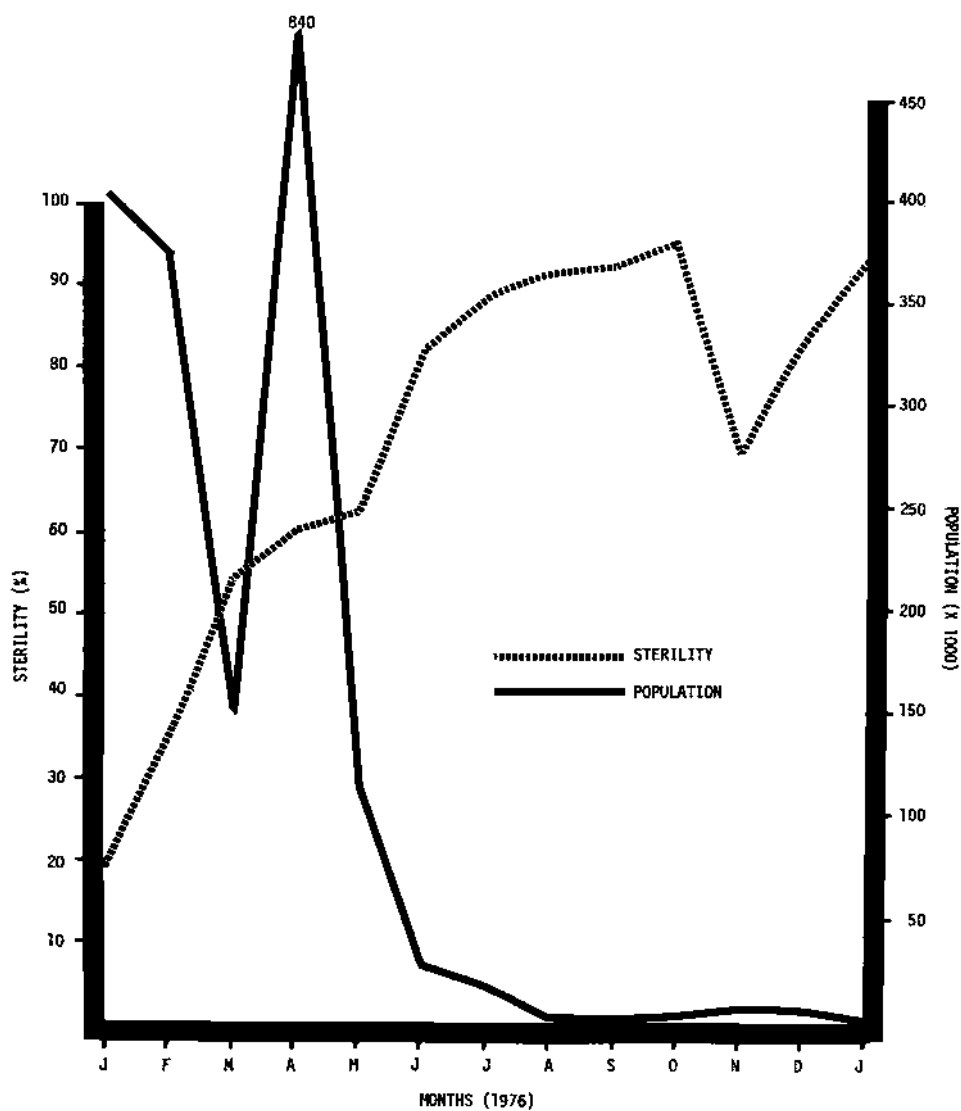


FIG. 4. Sterility and population of wild stable flies from east end of St. Croix using IPM with sterile males.

whether it came from some small isolated breeding area, was indigenous to St. Croix, or was introduced with livestock imported from the other islands.

Although we tried for three months, we could never entirely eliminate the wild stable flies. It is speculated that the flies could have been eliminated if (1) we could have established a quarantine and inspected all planes and boats coming to St. Croix to spray for flies; (2) if we could have dispersed the sterile flies better or even had more manpower to put out a greater number of the attractant toxicant panels; or (3) if closer inspection and treatment of urban areas could have been accomplished where domestic animals were quartered. However, unless a strict quarantine is maintained, they will quickly re-establish themselves on the island. The importation of flies along with animals from planes and boats, plus native flies breeding at small isolated spots in the urban areas of the towns and villages, created a foci for the rapid spread of wild fly population once the control programme was suspended. The study was terminated at the start of the rainy season when survival and rates of increase were at their maximum. During the three-generation period following the termination of releases the population increased from 350 to 210 000: an average of 8–9X per generation. Since 6X was the highest rate of increase recorded in either Florida or St. Croix, 9X is considered high even under optimal survival conditions.

This study proves that an IPM programme using sterile insects could successfully control stable flies over a large area. Although it is possible to obtain total population control by the release of sterile males alone, the use of integrated control techniques is a more practical approach. A newly instituted sorghum-feeding programme increased the number of flies on the island so that the planned number of released flies was insufficient to cause population control. Source reduction, insecticide treatments and management procedures overcame this problem as well as actual and potential complaints from excessive numbers of released flies. The release of a pupal parasite at one special problem site, and the use of an attractant-toxicant device, demonstrated two additional technologies that can be incorporated into an IPM programme.

REFERENCES

- [1] LABRECQUE, G.C., MEIFERT, D.W., RYE, J., Jr., Experimental control of stable flies, *Stomoxys calcitrans* (Diptera: Muscidae) by releases of chemosterilized adults, Can. Entomol. **104** (1972) 885.
- [2] LABRECQUE, G.C., BAILEY, D.L., MEIFERT, D.W., WEIDHAAS, D.E., Density estimates and daily mortality rate evaluation of stable fly (*Stomoxys calcitrans*) (Diptera: Muscidae) populations in field cages, Can. Entomol. **107** (1975) 597.
- [3] WILLIAMS, D.F., LABRECQUE, G.C., PATTERSON, R.S., Effect of gamma rays and/or fluorescent pigments on sterility and survival of the stable fly, Fla. Entomol. **60** (1977) 297.

- [4] BAILEY, D.L., WHITFIELD, T.L., LABRECQUE, G.C., Laboratory biology and techniques for mass producing the stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), J. Med. Entomol. 12 (1975) 189.
- [5] WILLIAMS, D.F., SKOV, O., PATTERSON, R.S., Two traps for collecting live stable flies *Stomoxys calcitrans* (L.) in the field, Mosq. News 37 (1977) 404.
- [6] MORGAN, P.B., PATTERSON, R.S., LABRECQUE, G.C., WEIDHAAS, D.E., BENTON, A., Suppression of a field population of house flies with *Spalangia endius*, Science 189 (1975) 388.
- [7] MEIFERT, D.W., PATTERSON, R.S., WHITFIELD, T., LABRECQUE, G.C., WEIDHAAS, D.E., Unique attractant-toxicant system to control stable fly populations, J. Econ. Entomol. 71 (1978) 290.

DISCUSSION

R. GALUN: In view of the fact that the bite of *Stomoxys* is painful, did you get co-operation from the farmers with your sterile insect releases?

R.S. PATTERSON: We had excellent co-operation from the farmers, as they were interested in successful fly control. We did get some complaints when we released flies in or near urban areas. The only complaint we had from farmers was about the release of a large number of sterile flies next to the herds, causing the animals to run away. For this reason we made numerous releases at two-hour intervals of only 4000 flies at each point and did not release flies next to the herds.

C.F. CURTIS: Do you think that *Spalangia* is a more acceptable control agent for a biting fly than sterile males? Have you assessed the economics of using *Spalangia* for large-scale control?

R.S. PATTERSON: *Spalangia* wasps are a very acceptable agent for stable fly control, especially for the suppression of *S. calcitrans* which produces large numbers of pupae in a relatively small area. One problem with *Spalangia* wasps is that they attack all muscoid flies. Therefore one has to consider all species of fly pupae in the area where the releases of parasites are to take place. The cost of rearing *Spalangia* wasps is about US\$ 500 per million and each wasp normally stings between two and four fly pupae in its lifetime in the field. So some idea of the total fly population is needed if one is to do an efficient parasite release. We have considered the economic implications of using *Spalangia* for large-scale control in which the aim is to control whole complexes of muscoid flies breeding in an area such as poultry farms. The cost of parasite releases compares favourably with that of chemical control for equal fly suppression.

L.H. OTIENO: Do you know of any cases of cattle dying from *Stomoxys* bites? I ask this because there are reports from Zanzibar that cattle have died in this way.

R.S. PATTERSON: We had no cases of cattle dying from *Stomoxys* bites on St. Croix during this three-year study. I have just returned from two months on the island of Zanzibar where I observed three animals which the local

farmers claimed had died from *Stomoxys* bites. I am not a veterinarian but I doubt whether the animals died directly as a result of *Stomoxys* bites. These and other animals in the same herd were under tremendous stress and it is estimated that 1500 to 2000 flies were feeding on each animal every day plus large numbers of mosquitoes at night. These weakened animals were therefore very susceptible and any infection might have been lethal for them.

D.H. MOLYNEUX: Have you any figures on the productivity of cattle before and after your control programme?

R.S. PATTERSON: No, we asked the farmers but they noticed no difference in productivity; they found simply that the animals were easier to handle. In the United States of America research workers have estimated that there must be at least 20 flies per animal to cause a reduction in weight gain or in milk production. Throughout most of the study on St. Croix, the number of flies per animal was less than 20.

D.H. MOLYNEUX: Were the figures you presented showing *Stomoxys* reduction with pesticides alone and with pesticides and SIT together comparable in terms of the amount of pesticides used and the time of application?

R.S. PATTERSON: The figures presented are comparable. The treatments with pesticide were performed at the same time and with the same chemical and dosage rate. On half the island the pesticide alone was used and on the other half the pesticide was used with SIT. We did this for two reasons: the first was to determine the influence of pesticide on the total population when only a part of the total larval breeding area was treated, and the second reason was that we did not have enough sterile flies to release over the entire island at that time.

H. POLITZAR: Are released flies marked and, if so, how?

R.S. PATTERSON: All the released sterile flies were marked with fluorescent dust. The dust was placed in the air stream from a small fan and was filtered through an enclosed chamber which contained the flies in their release cages and/or bags. The dusting reduced the vigour of the flies by about 10% as estimated by large-size field cage studies. The marking on the flies was not visible to the naked eye but could easily be seen under UV light.

ISOTOPE APPLICATIONS; PHYSIOLOGY

(Session 6)

USE OF ISOTOPES AS MODEL SUBSTANCES TO ELUCIDATE THE MODE OF TRANSMISSION OF PATHOGENS TO ANIMALS AND PLANTS BY ARTHROPOD VECTORS

W.J. KLOFT, H.L. CROMROY*, E.S. KLOFT

Institut für Angewandte Zoologie

der Universität Bonn,

Bonn,

Federal Republic of Germany

Abstract

USE OF ISOTOPES AS MODEL SUBSTANCES TO ELUCIDATE THE MODE OF TRANSMISSION OF PATHOGENS TO ANIMALS AND PLANTS BY ARTHROPOD VECTORS.

Experimental work was begun with J.F. Butler and L.A. DuBose on the biting flies *Stomoxys calcitrans* (L.) and *Haematobia irritans* (L.), which are important ectoparasites of livestock. Using radioisotopes as model substances, several possible mechanisms for the transmission of pathogenic materials to animals could be worked out: (1) mechanical transmission from contamination of the mouthparts; (2) transfer through regurgitation; (3) transfer via saliva; (4) transfer through the alimentary tract. On the basis of experiences gained over a long period in applying radioisotopes to a variety of different nutritional types of insects we could work out a generalized scheme for the paths of uptake and excretion of radioisotopes through the organism of insects. First, experiments were carried out to apply this model to arthropods which are blood feeders, such as the stable fly and hornfly, tsetse flies, sheep keds (*Melophagus ovinus* L.), mosquitoes (*Culex* sp., *Aedes* sp.), *Triatoma infestans* Klug (Hemiptera, Triatomidae), and the tick *Ornithodoros moubata* Murray. On the basis of earlier experiences and new experiments with aphids we could discover with tracer techniques very similar principles and possibilities for the transfer of pathogens also in the plant-sucking aphids (vectors of virus diseases in plants) as in blood-feeding arthropods. In particular the phenomenon of regurgitation which could be found in biting flies, appears in the literature and in our experience in ticks as well as in *Triatomidae* and tsetse flies (in the last two only under stress conditions) and could even be found in aphids. Thus aphids and certain biting arthropods can immediately transfer pathogens after having fed on an infected host plant or host animal, if migrating to another host. To find this out and to specify the different possibilities was mainly possible through the use of radioisotopes as model substances. The results with aphids are presented with special discussion of the ingestion-egestion hypothesis of noncirculative virus transmission, described by Harris in 1977.

* Permanent address: Department of Entomology and Nematology, University of Florida, Gainesville, Florida, United States of America.

1. INTRODUCTION

This Symposium has been organized by the Joint FAO/IAEA Division of the United Nations. Many years of personal experience and the work of several authors have shown the excellent possibilities offered by tracing the relationships between an ectoparasitic arthropod and its relationships to either plant or animal hosts. We found out that radioisotopes can be used as model substances to elucidate the mode of transmission of pathogens by arthropod vectors to animals and plants. The authors are grateful to Mr. G.L. LaBrecque for giving us permission to include in this paper certain review material since only comparison of earlier and new results on plant as well as animal hosts allows a better understanding of the host parasite relationships.

2. EXPERIMENTS WITH APHIDS AS IMPORTANT VECTORS OF VIRUS DISEASES

Plant-sucking insects such as aphids, coccids (e.g. mealybugs), leafhoppers and planthoppers (psyllids and membracids), psyllids, whiteflies, and thrips belong together with mites and nematodes to the most important animal vectors of plant diseases. Among these virus diseases together with fungal and bacterial diseases play the principal role. Our present knowledge has been covered in two recent handbooks [1, 2]. In a paper [3] presented during the IAEA-FAO symposium in 1967 the advantages given by the use of radioisotopes in the investigation of inter-relationships between aphids and host plants was pointed out. Figure 2 in this earlier paper illustrates in a schematical manner various possibilities and methods. These methods, further progress and different applications in the field of aphid research are described [4] in the above-mentioned handbook [1]. The use of artificial holodric diets has been intensified and these techniques could be applied to more aphid species. An up-to-date list of the composition of artificial diets, species and number of reared generations is given by Kunkel [5].

2.1. Special arrangement of mouthparts and salivary gland system in Aphididae

Several studies have been made on the mouth parts, the function of the stylets, the functional anatomy of the alimentary tract and the salivary gland system [6-11]. Kunkel [12] has also studied very intensively the morphology and the histology of the salivary glands of Aphidina belonging to different families. After careful preparation of live specimens the unfixed glands (principal gland, accessory gland, salivary duct, accessory duct) have been sketched in way which allows a comparison between the families Adelgidae, Phylloxeridae, Pemphigidae, Anoeciidae, Chaitophoridae, Lachnidae, Callaphididae, Pterocommatidae and

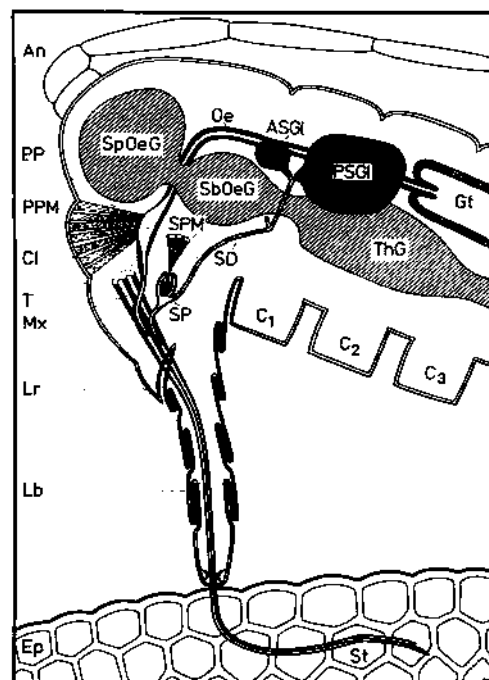


FIG.1. Schematic drawing showing the arrangement of mouthparts (stylets), foregut (oesophagus) and the salivary glands in the head and anterior thoracic segments of an aphid. The salivary glands and the stylets (Mx) are marked in black.

ASGI = Accessory salivary gland; An = Antenna; C_{1...3} = Coxae 1-3; Cl = Clypeus; Ep = Epidermis (of the plant); Gt = Midgut; Lb = Labium; Lr = Labrum; Mx = Maxilla; Oe = Oesophagus; PP = Pharyngeal pump; PPM = Pharyngeal pump muscle; PSGI = Principal salivary gland; SbOeG = Sub-oesophageal ganglion; SpOeG = Supra-oesophageal ganglion; SD = Salivary duct; SP = Salivary pump; SPM = Salivary pump muscle; St = Stylets; T = Epipharyngeal organ (taste organ); ThG = Thoracic ganglion complex.

Aphididae. Special attention was given to the cell structure in the principal salivary glands of cecidogenous (= gall-forming) Coccinea and Aphidina. On the basis of the very detailed studies of Weber [6, 7], Sorin [8] and our own experience we present in this study a schematical drawing (Fig. 1) that shows and explains didactically the arrangement of the mouthparts (stylets), food channel, salivary glands with salivary duct, and the foregut (oesophagus). Thus can easily be understood the functional anatomy of this system and the co-operation of its different parts. However, for more detailed information about steering and controlling of the stylets see Weber [6, 7].

2.2. Individual beginning of the uptake of food

To analyse the acquisition and transmission times especially for non-circulative virus, it is important to know exactly the time at which food uptake begins. As we pointed out [3, 4], neither measurements nor the sensitive autoradiographic techniques detected radioactivity inside aphids after probing-puncturing (i.e. piercing the epidermis and one or two layers beyond) on radioactive (labelled with ^{32}P) host plants. 'The measurements showed that the insects take up food only after stinging when they reach the phloem; the activity then suddenly increases' [3]. Schnorbach [13] in our laboratory has tried to work out the individual beginning of food uptake within groups of *Aphis craccivora* Koch. Although aphids are transferred in groups on labelled host plants and settle almost at the same time, there is considerable variation in the individual beginning of food uptake. To analyse exactly the course of time of the whole process, between piercing, reaching the phloem and beginning individually to take up food, the above-mentioned [13] measurements of the length of stylets which are not covered by the labium were combined with histological sections through plant tissue and tracer techniques. However, the amounts of the food taken up at the very beginning are so small that they could not be detected by hitherto used methods. To overcome these problems food has been offered either as plants or diets labelled with inorganic ^{32}P as phosphate. The radioactive aphids immersed in plain water were checked in the tritium channel of a Packard Tri-carb liquid scintillation spectrometer. The radioactivity of the specimens could be detected through the Cerenkov effect.¹ The big advantages of this method are the very low background oscillating only over a very small range. Since the relatively high energy of the beta particles of ^{32}P penetrates practically without any absorption the very thin cuticle of aphids [15], the efficiency is rather high. For some time several authors believed that electronic systems would give more exact results for monitoring the feeding of aphids [16, with further references]. However the results obtained by using the Cerenkov effect seem to show that the real beginning of food uptake cannot be exactly described with the help of electronic monitoring. It seems that contact of the stylet tips with the phloem content were interpreted as uptake of food. But in spite of the aphids reaching the phloem within a relatively short time, the real ingestion of phloem sap can be delayed. In principle our former result [17] could be confirmed: Aphididae do not begin earlier with food uptake than seven to ten minutes after piercing, this time seeming to correspond with the time required to reach the phloem.

¹ The Cerenkov effect was used, as far as we know for the first time in entomology in 1972 [14]. The senior author suggested to his student Tennessen a method to follow the course of food uptake (with ^{32}P -labelled food) and excretion of live (first instar) aquatic larvae of dragonflies.

On a diet 100% of *A. craccivora* begin ingestion within 50–60 min, on labelled plants 100% of this species need 300 min (stem of *Vicia fabae*) or 240 min (leaves). These latest results [13] also confirm our previous investigations with *Myzus ascalonicus* Donc. [18]. The food uptake in *A. craccivora* is at first retarded (possibly taste proofing, see Fig. 1, T = epipharyngeal taste organ) but then increases constantly [13].

2.3. The problem of regurgitation by aphids

As we have shown [19, 20], regurgitation of material from the foregut plays an important role in the social feeding behaviour of termites and especially social Hymenoptera. Studying these feeding relationships with radioisotopes has been very successful as we have shown since 1956 in a series of papers; a summary of methods and results was presented in 1977 [20]. Observations of the membrane feeding behaviour of aphids [21] indicate that the pharyngeal pump (PP in Fig. 1) can function in either direction. We began tracer experiments to further elucidate the question of regurgitation, the previous results of our group (Esser, Safe, unpubl., see also Ref. [22]) are briefly summarized here. Experiments were carried out with the aphid *Aulacorthum circumflexum* (Buckt.) reared on *Vicia faba* L., and fed on labelled *V. faba* sprouts, standing in a nutritive solution containing THO (specific activity 0.2 mCi/ml). The aphids were removed from the plant and allowed to 'clean' (decontaminate) their mouthparts by probing on nonradioactive plants [4, 17, 20]. The aphids were transferred to leaves of nonradioactive plants where they probed for 0.5–15 min. Leaf discs obtained by the use of a cork borer were burned in a Packard Autooxidizer, Model No. 504. We found that during probing times ranging from 1–3 min a relatively large amount of ^3H was transferred to the leaves. The results are given in Table I.

2.4. Discussion of the results for aphids as virus vectors with special attention to the ingestion-egestion hypothesis [23]

Plant-sucking aphids, among these especially phloem-feeding Aphididae, belong to the most important vectors of virus diseases. Here we apply and discuss our results only with respect to the nonpersistent, noncirculative virus transmission. The two major hypotheses of noncirculative virus transmission, stylet-borne and ingestion-egestion, are discussed in detail by Harris [23]. Aphids can acquire virus during probes as brief as 5 s, however, uninterrupted probes of 15–60 s are optimal for successful acquisition of virus. Acquisition and inoculation thresholds ranged between 4–5 s only. These short times have been found by several authors [23], and strongly support the stylet-borne transmission hypothesis. However, the idea of transmission by regurgitation has been discussed, in addition to Harris and

TABLE I. DEPOSITION OF RADIOACTIVE MATERIAL (VIA SALIVATION AND/OR REGURGITATION) BY ADULT APHIDS, *Aulacorthum circumflexum* (Buckt.) (Adapted from Ref. [4])

Aphid I			Aphid II			Aphid III		
Probing time (min)	Activity in the leaf (counts/min above background)	Amount of secreted material ($\times 10^{-3} \mu\text{l}$)	Probing time (min)	Activity in the leaf (counts/min above background)	Amount of secreted material ($\times 10^{-3} \mu\text{l}$)	Probing time (min)	Activity in the leaf (counts/min above background)	Amount of secreted material ($\times 10^{-3} \mu\text{l}$)
0.5	0.0		1	0.0		1	88.3	2.71
1	0.0		2	86.6	4.6	2	74.8	2.3
2	12.5	1.47	3	0.0		3	32.8	1.01
2	0.0		5	17.7	0.94	3	79.6	2.45
2.5	0.0		5	3.9	0.21	3	1.1	0.03
8	0.0		5	41.2	2.19	5	88.7	2.73
15	17.5	2.06	7	6.1	0.32	11	10.7	0.39
			10	0.0		13	58.1	1.79
			13	0.7	0.04	15	14.9	0.46
			15	41.6	2.21			

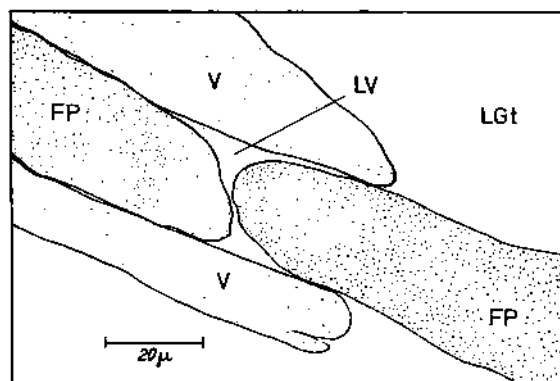


FIG.2. Valve between foregut and midgut (*Valvula cardiaca*) with food particles (FP). The situation has been observed on a live *Megoura viciae* Buckt. through the midgut epithelium, using a phase contrast microscope.

V = rim of the valve, extending into the lumen (= interior) of the midgut (LGt); LV = lumen of the valve. Adapted from a schematic drawing of Ehrhardt [24], which was based on a phase contrast microphotogram.

Bath [21] by other authors. Ehrhardt [24] excluded the possibility of regurgitation of midgut content due to the construction of the valvula cardiaca (Fig. 2). He argues that this valve between the oesophagus and midgut could be passed by ingested food only in one direction. Garrett's [25] radioisotope and virus transfer experiments suggest that aphids can also egest material from the foregut during brief probes into plants. Large proportions of the tracer (in his experiments ^{32}P) that the aphids carried, and sometimes all of it, could be transferred by regurgitation. Garrett points out that 'most of the transferred tracer had certainly not been assimilated with saliva. In these experiments, the aphids did not excrete and regurgitation seems unlikely'. This author did not note that since 1960 [17, 18, see summaries in 15, 3, 4, 19] we have pointed out that a time factor is involved, which enables a distinction to be made between excretion via saliva (necessary time for ^{32}P in the case of peroral uptake: 3–4 h) and other mechanisms. We must always realize that there exist in Rhynchota in general and in aphids especially two totally separated capillary tubes in the stylets. The larger food channel opens into the oesophagus whereas the saliva is injected by the salivary pump (PP, Fig. 1) through the salivary duct (SD). This duct passes through the tip of the hypopharynx and leads into the lumen of the salivary channel within the stylets. The tip of the hypopharynx fits in an effective way into the salivary channel with perfect neatness thus preventing any mixing of the content of both capillary tubes (Fig. 1). We do not know the fate of ^3H in the aphid. But a rapid exchange with all liquids, tissues and organic matter can be expected. Therefore it might be more difficult to

differentiate between salivation and regurgitation than with ^{32}P . But even in the case of rapid exchange between H and ^3H (tritium), a time of only 1–5 min is certainly too short for resorption from the midgut, transport via haemolymph to the salivary glands and resecretion with the saliva. Nevertheless the relatively high activities deposited in several instances (Table I) seem to demonstrate the occurrence of regurgitation. This holds especially true if we take the value of Lamb and co-workers [26] where they calculate the viscous phase of saliva as $3.7 \times 10^{-6} \mu\text{l}$ for an average salivary sheet, and compare it with our calculated values which were nearly 500 times higher.

In Table I the amount of secreted material was calculated by assuming that aphids and ingested material have isospecific activities and that the average weight of an aphid is 0.6 mg.

On the basis of his own experience [21], Harris [23] gives a very interesting 'ingestion-egestion-hypothesis'. He states: 'The function of egestion . . . is not always apparent, but it often serves to clear clogged food and salivary sheath canals. Could it not serve the same function during phloem feeding? Injured sieve tubes are quickly plugged by massive depositions'. Actually cylinders can also be formed out of the liquid food (phloem content) which might block even the valve between the oesophagus and midgut (Fig. 2). Ehrhardt described this as early as 1963 [24], and we reproduce here in this context his figure, which is not well known. As he reports, the formed food particles dilute in the posterior part of the midgut sac. We know about the virus-inactivating role of aphid saliva: as Harris [23] pointed out, the tip of the stylets is not always ensheathed by saliva, it moves backwards and forwards, and often penetrates deeper. Material can be observed to move backwards and forwards in gentle pulsations thus preventing contact and inactivation effects by the salivary sheath. So neither ingested nor – in case of regurgitation – egested virus particles will be inactivated, which is important for virus transmissions through regurgitation. One more advantage of regurgitation might be flushing of clogged food and the cylinders mentioned above (Fig. 2).

3. EXPERIMENTS WITH ECTOPARASITIC ARTHROPODS FEEDING ON ANIMAL HOSTS

This symposium is devoted to vectors of animal diseases, nevertheless it is important to compare the situation in plant-sucking arthropods.

We have carried out experiments with different blood-feeding arthropods such as biting flies, *Stomoxys calcitrans* (L.), *Haematobia irritans* (L.), tsetse flies, sheep keds (*Melophagus ovinus* L.), *Triatoma infestans* Klug. Experiments have also been started with mosquitoes (*Culex*, *Aedes*) and *Ornithodoros*, but these must be continued. As described in Refs [27] and [28], the flies were fixed on an insect pin with glue in an upright normal horizontal position. Legs and wings

were glued together in order to avoid any surface contamination from mouthparts or faeces which might result from movement of the wings and legs. Flies were fed a ^{32}P -labelled 10% sucrose solution through a glass capillary attached by a teflon tube to a Hamilton Microliter® measured dosage syringe, so that known amounts of fluid (2–3 μl) could be offered. Tsetse flies, *Melophagus* and ticks were fed through artificial membranes such as Parafilm®, silicone membrane [29] or Baudruche membrane. Mosquitoes were fed with 7.5% glucose (labelled with ^{32}P as phosphate), the glucose was soaked in filter paper strips, and slightly rolled in a teflon tube. The teflon tube containing the filter paper strip was placed in a hole in a cork. This cork closed an Erlenmeyer flask serving as container for the mosquitoes. Only a small tip of the soaked paper was drawn out thus preventing external contamination of the mosquitoes sitting on the tube while feeding. If necessary adenine nucleotides (ATP) were added as phagostimulants according to Galun (review paper [30]).

3.1. Transfer from external contamination of the mouthparts

We had fed also hornflies and stable flies with (^{32}P)-labelled defibrinated blood from capillaries. We checked subsequently what amounts of external radioactive contaminations of the mouthparts were transferred during insertion into unlabelled agar blocks or blood capillaries. Transfer of rather high amounts (here expressed as counts/min above background) could be detected especially after the first 'decontamination' (1–5 min) in the unlabelled material. But also after the second 'decontamination' (feeding for 2–8 min) radioactivity could still be detected. The third feeding on an unlabelled source showed no more transfer. Similar results were obtained with stable flies (details are given in Ref. [28]). The transfer of ^{32}P , as a model for any pathophorous materials, has not yet been checked for the other bloodfeeders we worked with. But we can assume in general that this mechanism is important for the transfer of pathogens.

3.2. Transfer by regurgitation

Biting flies and other bloodfeeding arthropods having fed on radioactive blood were transferred to nonradioactive matter such as agar blocks or capillaries with sucrose solution. Sometimes stable flies in particular transferred rather high amounts (in *Stomoxys calcitrans* up to one tenth of the totally ingested radioactive blood). These quantities could only be explained by the occurrence of regurgitation. Actually this phenomenon is well known from several Diptera as regurgitative feeding. Confirmation was obtained from the observation that sometimes the agar block or the sucrose capillaries became pink in colour, erythrocytes could even be seen with a microscope. In many similar experiments with hornflies no regurgitation at all was visible.

We have also been interested in whether the phenomenon of regurgitation occurs in tsetse flies. A student, Giannetti [31], has transferred fully engorged tsetse flies (*Glossina m. morsitans* Westwood and *G.p. palpalis* (Robineau-Desvoidy)) immediately after feeding on to agar plates in order to find out whether regurgitation occurs. Subsequent examinations under the microscope showed no positive results. If stress factors are applied to the flies, such as pressing gently the completely filled abdomen, clipping off the wings, etc., the flies regurgitated immediately large amounts of the blood they had just imbibed. To determine the amount of regurgitated blood, the flies had been fed with ^{32}P -labelled blood through membranes. Also in these experiments the flies regurgitated only after slight pressure on the abdomen. The amount of regurgitated blood ranged between one tenth to three tenths of the effectively imbibed blood volume. It can be stated at present that regurgitation in tsetse flies occurs only under the following preconditions:

- (a) Feeding must have just finished so that the crop is still filled.
- (b) The fly has to be under stress.

The importance of regurgitation of pathogenic agents probably is minor compared with stable flies and ticks (see subsequent notes). Tsetse flies normally feed on one single host animal till the midgut and crop are filled. Interruption of feeding and changing to another host animal on to which could be regurgitated pathogenic agents might not be usual. Nevertheless, this possibility should be taken into consideration in the case of biting on mammals feeding or resting in herds or flocks. Stress factors are easily caused by defence movements (such as a swishing tail) of the infested animals.

Melophagus ovinus L. was checked with similar techniques. Baudruche membranes and silicone membranes [29] were used to supply defibrinated blood (from cattle). To the ^{32}P -labelled blood ATP was added as a phagostimulant. In many experiments no regurgitation could be found. When the small flies were slightly pressed (as with the tsetse flies) blood was ejected only through the rectum.

Triatoma infestans Klug were fed blood labelled with ^{32}P through various membranes (household wrapping foil, silicone membrane, Parafilm, Baudruche membrane). The membranes were removed before the insects were fully engorged. Subsequently a microvial filled with melted agar (35°C) was used to clean (decontaminate) the stylets. External radioactivity, washed from the stylets, could be detected in the melted agar. To check for the possible release of any material on an unlabelled food source to which the *T. infestans* had been transferred immediately after that procedure, the *T. infestans* were placed on a membrane covering a microvial filled with water at 35°C . After piercing through the membrane we observed the release of substances that appeared in the water as streaks, and when strong mechanical pressure was applied to the abdomen, as could happen during defence movements of the human or animal host, red

substances were released. These showed radioactivity and red blood cells could be proven.

We also carried out experiments with ticks to ascertain the occurrence of this phenomenon in these ectoparasites. We obtained adult unfed *Ornithodoros moubata* Murray which were fed through silicone membranes [29] of 0.25 mm thickness with radioactive-labelled (^{32}P) blood. The ticks accepted this device; however, after only 45-min feeding time (Argasidae are so-called 'rapid feeders') they were transferred to nonradioactive blood, covered with the same type of membrane. After another 45-min feeding time they were removed and subsequently the blood was checked for radioactivity using the Cerenkov effect mentioned above. It was shown that 1.7% of the engorged radioactive blood, corresponding to $\approx 0.864 \mu\text{l}$, could be found. Since any external contamination was carefully avoided we must assume regurgitation. Intensive attention has been paid to an oral secretion [32, 33] under the assumption that this secretion might contain the causative agent for paralysis. However, regurgitation is not mentioned in addition to oval secretions (saliva itself and cement) of the salivary glands [22, 30, 33]. Two statements in Gregson's work [32] should be mentioned (his monography of 1973 [34] was not available to us): 'Once a tick has reached a peak in its secretion, further substantial secretions cannot be induced, even several hours later, without renewed feeding on a host'. While discussing his experiments on injections of collected oral secretions to different vertebrates (lamb, mice, frog) he states: 'Not one of these experiments showed any indication of a paralysing agent'. It should be considered whether both facts could indicate regurgitation. The first statement could be explained by diminution of filling pressure due to regurgitation of uptaken surplus. The lack of any paralysing agent could be understood by assuming this phenomenon too as regurgitated blood should not induce toxic effects.

3.3. Transfer via saliva

We have discussed above the problem of salivation in ticks in the context of oral secretions. As mentioned in Section 2.4 a time factor is involved to differentiate between regurgitation and salivation of perorally ingested ^{32}P . This tracer requires a certain time for reabsorption in the midgut, transport via haemolymph, movement into the salivary glands and resecretion with the saliva in connection with a further food uptake [28]. The earliest recovery of radioactive saliva from agar blocks (into which the insects could probe) was in hornflies 1 h 57 min after feeding at a temperature of 25° – 26.5°C . Stable fly females resecreted radioactive saliva after 6 h (5 cases) and after 8 h 10 min (15 cases). In one case radioactive resecretion was detected 3 h 21 min after food uptake. Since stable flies regurgitated, we always had to ascertain by optical methods (red blood cells) whether we had the radioactivity from regurgitation or salivation.

In *Musca domestica* L. (20°–23°C) and *Lucilia cuprina* (Wiedemann) (23°–30°C), 6–8 h were necessary for resecretion of perorally ingested ^{32}P via saliva [35]. But further research with actual disease agents would be needed to show whether this is an important transmission pathway. Work on the transmission of a bacterial infection (*Pseudomonas aeruginosa*) has been done in tsetse flies [36, 37] and to discriminate between salivation and eventual regurgitation the factor of time must be investigated.

4. DISCUSSION

One more way of transfer would be via defecation after passage through the alimentary tract. Several cases are well known, and faeces can be incorporated easily by scraping. Regurgitation is, in addition to the plant-feeders mentioned (Aphididae) and blood-feeders, of importance in other cases: Phlebotomine sandflies transmit Leishmaniasis by regurgitation, and pestfleas can transfer *Pasteurella pestis* in a similar way. The pathophorous organisms have such a strong rate of multiplication that they block the foregut, and even the midgut [38], of these ectoparasitic insects. The only way to clear the alimentary tract is often the regurgitation of masses of these organisms [39]. We must expect that regurgitation especially occurs in much more arthropods than is hitherto known. There is no doubt that radioisotopes can serve as excellent model substances to elucidate regurgitation and all other modes of transmission of pathogens by arthropod vectors to animals and plants.

ACKNOWLEDGEMENTS

Thanks are due to the Deutsche Forschungsgemeinschaft for providing one of us (H.L. Cromroy) with a grant to work as visiting professor at the University of Bonn during May–November 1978. The Federal German Ministry of Scientific Research and Technology (BMFT) established during 1966–78 a very well-equipped radioisotope laboratory and financed further expenses for radioisotopes, laboratory equipment, etc. Very helpful in laboratory experiments and discussion of different problems have been W.J. Esser, M. Giannetti, G. Hesse, H. Kunkel, A.I. Safe and H.-J. Schnorbach. Special thanks are given to E.M. Wolfram, who worked out Fig. 1. W. Maier, Institut für Medizinische Parasitologie, University of Bonn, kindly helped with the first mosquito experiments which will be continued with him. W. Stendel, Bayer AG, Wuppertal, kindly provided us with ticks (*Ornithodoros moubata* Murray).

REFERENCES

- [1] HARRIS, K.F., MARAMOROSCH, K. (Eds), *Aphids as Virus Vectors*, Academic Press, London (1977).
- [2] MARAMOROSCH, K., KOPROWSKI, H. (Eds), *Methods in Virology*, Vol. 6, Academic Press, London (1977).
- [3] KLOFT, W., EHRHARDT, P., KUNKEL, H., "Radioisotopes in the investigation of interrelationships between aphids and hostplants", *Isotopes and Radiation in Entomology* (Proc. Symp. Vienna, 1967), IAEA, Vienna (1968) 23.
- [4] KLOFT, W.J., "Radioisotopes in aphid research", *Aphids as Virus Vectors* (HARRIS, K.F., MARAMOROSCH, K., Eds), Academic Press, New York-San Francisco-London (1977) 291.
- [5] KUNKEL, H., "Membrane feeding systems in aphid research", *Aphids as Virus Vectors* (HARRIS, K.F., MARAMOROSCH, K., Eds), Academic Press, New York-San Francisco-London (1977) 311.
- [6] WEBER, H., Skelett, Muskulatur und Darm der schwarzen Blattlaus *Aphis fabae* Scop. (mit besonderer Berücksichtigung der Funktion der Mundwerkzeuge und des Darmes), Mitt. Inst. Pflanzenkr. Bonn, Stuttgart (1928).
- [7] WEBER, H., *Biologie der Hemipteren*, Biol. Studienbuecher, Berlin (1930).
- [8] SORIN, M., The mouth parts of *Aphis craccivora* Koch and the penetration of stylets into host plants, *Jpn. J. Appl. Entomol. Zool.* 5 (1961) 217.
- [9] EHRHARDT, P., Einfluss von Ernährungsfaktoren auf die Entwicklung von saugenden Insekten unter besonderer Berücksichtigung von Symbionten, *Z. Parasitenkd.* 31 (1968) 38.
- [10] PONSEN, M.B., "Anatomy of an aphid vector: *Myzus persicae*", *Aphids as Virus Vectors* (HARRIS, K.F., MARAMOROSCH, K., Eds), Academic Press, New York-San Francisco-London (1977) 63.
- [11] FORBES, A.R., "The mouthparts and feeding mechanism of aphids", *Aphids as Virus Vectors* (HARRIS, K.F., MARAMOROSCH, K., Eds), Academic Press, New York-San Francisco-London (1977) 83.
- [12] KUNKEL, H., Ernährungsphysiologische Beziehungen der Stenorrhynchen zur Wirtspflanze unter besonderer Berücksichtigung der Coccinea and Aphidina, Inaugural Dissertation, University of Bonn (1966).
- [13] SCHNORBACH, H.-J., Individueller Beginn der Nahrungsaufnahme innerhalb einer Blattlausgruppe auf Wirtspflanze und Kuenstlicher Diaet, Diplomarbeit, University of Bonn (in preparation).
- [14] TENNESSEN, K.J., KLOFT, W.J., Liquid scintillation counting of living first instar larvae of *Tetragoneuria cynosura* (Say, 1839) labelled with ^{32}P to follow food uptake and excretion, *Odonatologica* 1 4 (1972) 233.
- [15] KLOFT, W., EHRHARDT, P., "Studies on the assimilation and excretion of labelled phosphate in aphids", *Radioisotopes and Radiation in Entomology* (Proc. Symp. Bombay, 1960), IAEA, Vienna (1962) 181.
- [16] LEONARD, S.H., HOLBROOK, F.R., Minimum acquisition and transmission times for potato leaf roll virus by the green peach aphid, *Ann. Entomol. Soc. Am.* 71 4 (1978) 493.
- [17] KLOFT, W., Wechselwirkungen zwischen pflanzensaugenden Insekten und den von ihnen besogenen Pflanzengeweben, *Z. Angew. Entomol.* 45 4 (1960) 337.
- [18] KLOFT, W., KUNKEL, H., "Einblicke in die Speichelausscheidung bei *Myzus ascalonicus* Donc. mit Hilfe der Tracermethodik", *Proc. XI. Int. Congr. Entomol.*, Vienna 1960 1 (1961) 746.

- [19] KLOFT, W.J., KLOFT, E.S., Untersuchungen ueber Aufnahme und Ausscheidung von radioaktiven Tracern durch den Organismus von Insekten als Grundlage fuer radio-oekologische Forschungen in der Entomologie, Mitt. D. Ges. Entomol. 1 1 (1978) 6.
- [20] KLOFT, W.J., Part V. Applied Part, Laboratory Training Manual on the Use of Isotopes and Radiation in Entomology, 2nd Edn, Technical Reports Series No. 61, IAEA, Vienna (1977) 141.
- [21] HARRIS, K.F., BATH, J.E., Regurgitation by *Myzus persicae* during membrane feeding: its likely function in transmission of nonpersistent plant viruses, Ann. Entomol. Soc. Am. 66 (1973) 793.
- [22] ESSER, W.J., "Untersuchungen an einem Chinolin-Carbamat-Aphizid unter besonderer Beruecksichtigung seines Verhaltens in den Wirtspflanzen, seiner spezifischen Wirkungsweise auf Aphiden und der Ursachen der selektiven Toxizität, Inaugural Dissertation, Bonn (1979).
- [23] HARRIS, K.F., "An ingestion-egestion hypothesis of noncirculative virus transmission", Aphids as Virus Vectors (HARRIS, K.F., MARAMOROSCH, K., Eds), Academic Press, New York-San Francisco-London (1977) 165.
- [24] EHRHARDT, P., Untersuchungen ueber Bau und Funktion des Verdauungstraktes von *Megoura viciae* Buckt. (Aphidae, Homoptera) unter besonderer Berücksichtigung der Nahrungsaufnahme und der Honigtauabgabe, Z. Morphol. Oekol. Tiere 52 (1963) 597.
- [25] GARRETT, R.G., "Non-persistent Aphid-borne viruses", Viruses and Invertebrates (GIBBS, A.J., Ed.), North Holland & American Elsevier, New York (1973) 476.
- [26] LAMB, K.P., EHRHARDT, P., MOERICKE, V., Labelling of aphid saliva with Rubidium-86, Nature (London) 214 5088 (1967) 602.
- [27] KLOFT, W.J., BUTLER, J.F., KLOFT, E.S., Radioisotopes as model substances for the mode of transmission of pathophorous agents by insects to plants and animals, J. Plant Diseases and Protection 83 1/2/3 (1976) 80.
- [28] BUTLER, J.F., KLOFT, W.J., DuBOSE, L.A., KLOFT, E.S., Recontamination of food after feeding a ³²P food source to biting muscids, J. Med. Entomol. 13 4/5 (1977) 567.
- [29] BAUER, B., WETZEL, H., A new membrane for feeding *Glossina morsitans* Westw. (Diptera, Glossinidae), Bull. Entomol. Res. 65 (1976) 563.
- [30] GALUN, R., "The physiology of hematophagous insect/animal host relationship", Proc. XV. Int. Congr. Entomol., Washington, D.C. (1976) 257.
- [31] GIANNETTI, M., Physiologische Untersuchungen an Tsetse Fliegen (*Glossina m. morsitans* Westwood und *Glossina p. palpalis* (Robineau-Desvoidy)) nach Peroraler Applikation von ³²P (unpubl. exp. Staatsexamensarbeit, University of Bonn).
- [32] GREGSON, J.D., Experiments on oral secretions of the Rocky Mountain Wood Tick, *Dermacentor andersoni* Stiles (Acarina: Ixodidae), Can. Entomol. 89 1 (1957) 1.
- [33] O'HAGAN, J.E., "Chemical exchanges between the mouth parts of ticks and their hosts", Proc. XV. Int. Congr. Entomol., Washington, D.C. (1976) 266.
- [34] GREGSON, J.D., Tick Paralysis: an Appraisal of Natural and Experimental Data, Can. Dep. Agric., Monogr. No. 9 (1973).
- [35] KLOFT, W.J., BUNGARD, K., Erfassung der Resekretion eines peroral aufgenommenen Tracers mit dem Speichel bei Fliegen, Z. Angew. Zool. 62 (1975) 83.
- [36] BAUER, B., "Bacterial infection in *Glossina morsitans* Westw., fed through membranes", Proc. 3rd Int. Congr. Parasitology, Munich 2 (1974) 943.
- [37] BAUER, B., WETZEL, H., "Effect of bacteria on tsetse flies fed through membranes" Sterility Principle for Insect Control 1974 (Proc. Symp. Innsbruck, 1974), IAEA, Vienna (1975) 495.
- [38] MOLYNEUX, D.H., "Patterns of development of trypanosomes and related parasites in insect hosts", these Proceedings, IAEA-SM-240/20.
- [39] GEIGY, R., HERBIG, A., Erreger und Überträger tropischer Krankheiten, Verlag f. Recht u. Ges. AG, Basle (1955) 472.

MIDGUT ABSORPTION OF PROTEINS BY TSETSE FLIES, *Glossina morsitans morsitans* Westwood (Diptera: Glossinidae)

G. NOGGE, M. GIANNETTI
Institut für Angewandte Zoologie
der Universität Bonn,
Bonn,
Federal Republic of Germany

Abstract

MIDGUT ABSORPTION OF PROTEINS BY TSETSE FLIES, *Glossina morsitans morsitans* Westwood (Diptera: Glossinidae).

Experiments have been conducted to determine how far tsetse flies are able to absorb undigested proteins from the midgut and to transport into the haemolymph. Comparison of the protein pattern of blood serum and haemolymph as revealed by electrophoresis shows no congruency except for the albumin fraction, which is about 10% of the total protein contents. The peritrophic membrane of tsetse flies allows the passage of FITC-conjugated dextrans up to but not greater than a molecular weight of approximately 45 000. It can be concluded therefore that complete serum globulins cannot pass through the peritrophic membrane, but fragments of globulins and albumin might do so. The presence of serum proteins in the haemolymph of tsetse flies after oral administration was investigated by immunological techniques. With the help of immunoelectrophoresis as well as Ouchter-Lony tests, albumin, Fab-, and Fc-fragments were found to be present in the haemolymph. Tsetse flies usually fed on bovine blood replace bovine albumin present in their haemolymph by human albumin when fed on human blood. Quantitative determinations with the aid of rocket immunoelectrophoresis after Laurell revealed the occurrence of human albumin in relation to the size and number of blood meals. When fed continuously on human blood, the amount of human albumin rises following eight to ten blood meals after which the achieved level is maintained. Human albumin disappears from the haemolymph and is replaced by bovine albumin when the flies are fed again with bovine blood. On the basis of these results the amount of albumin absorbed from a single blood meal lies in the range of 0.01 to 0.035% of its albumin contents.

During a research project on the significance of endosymbionts for tsetse flies, the symbiotic micro-organisms have been eliminated by oral application of specific antibodies [1]. In the course of these investigations whole tsetse flies, after removal of the midgut mycetome and symbionts after immuno-absorption of adherent mycetome proteins, were used as antigen for immunization of rabbits. Tsetse flies were allowed to take blood directly from the ear of the sensitized

rabbits. Flies were then prepared for indirect immune fluorescent technique (IIFT). Flies which had been fed on a rabbit immunized with whole fly without symbionts revealed fluorescence in the midgut epithelium and various other tissues, whereas flies which had been fed on a rabbit immunized with symbionts showed a positive IIFT-reaction in the mycetome but in no other tissue.

These investigations brought up the question of midgut absorption of proteins. As antibodies are immunoglobulins, that means proteins, it should be expected that they are digested by proteolytic enzymes as soon as they reach the midgut. The first argument against this is that proteolytic enzymes according to Gooding [2] are not present in the anterior part of the midgut of tsetse flies, where the mycetome is situated. Secondly it has been confirmed for a number of insects that meal proteins may enter midgut cells, haemolymph and certain other tissues without being broken down, e.g. in the cattle grub, *Hypoderma bovis* (De Geer) [3]. More recently Schleim and co-workers were able to show that antibodies, e.g. against muscles or nerves of *Sarcophaga falcitata* Pand., fed to the flies, are specifically attached to the tissues that had served as antigens [4].

Experiments are currently in progress to find out how far serum proteins are protected from digestive enzymes and can then be absorbed and transported into the haemolymph of tsetse flies.

Passage of proteins firstly is limited by the permeability of the peritrophic membrane. It has been proved that the peritrophic membrane of tsetse flies allows the passage of FITC-conjugated dextrans up to a molecular weight of approximately 45 000 (Peters & Nogge, unpubl.). From this result it may be concluded that complete antibodies, e.g. IgG (mol.wt 150 000), may not pass through the peritrophic membrane, but fragments of IgG, e.g. Fab-fragment, and albumin might do so.

Comparison of the protein pattern of blood serum and haemolymph of tsetse flies as revealed by cellulose-acetate as well as polyacrylamide electrophoresis shows a striking congruency for albumin but no other fraction. This fraction is about 10% of the total protein contents of the haemolymph. To prove whether this and possibly other protein fractions are identical with serum proteins, immunoelectrophoresis and immunodiffusion tests (Ouchterlony) were conducted. By immunoelectrophoresis only albumin was detectable, which was expected from the permeability of the peritrophic membrane.

In Ouchterlony tests haemolymph was allowed to diffuse against the following antibodies: anti-IgG (complete), anti-Fab-, anti-Fc-fragment and anti-albumin. In agreement with the result of the immunoelectrophoresis there was no complete IgG in the haemolymph, whereas Fab- and Fc-fragments as well as albumin were present. The strongest response was achieved in the albumin test [5]. This result is very interesting in view of recent findings of Langley and co-workers [6] and Takken (personal communication), who were able to show that the presence of albumin in the diet is of the greatest importance for tsetse fly reproduction.

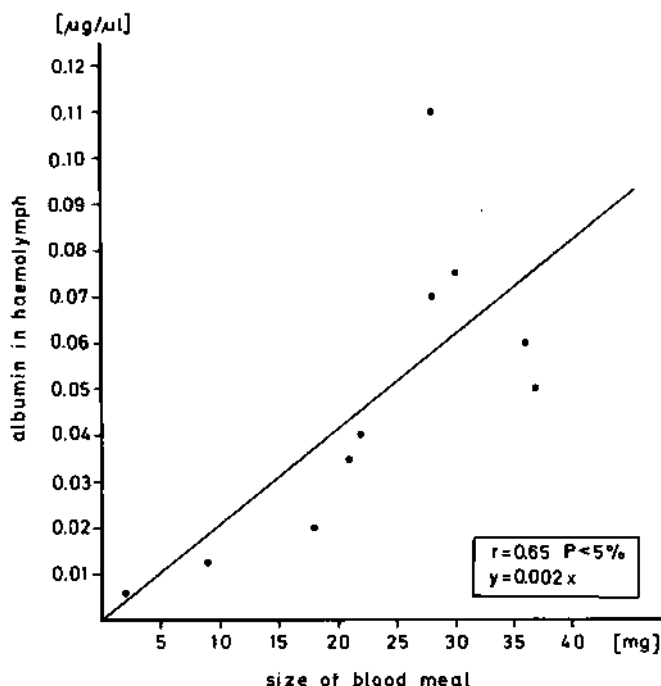


FIG.1. Human albumin found in the haemolymph of tsetse flies 24 hours after feeding on human blood in relation to the size of the blood meal.

The amount of albumin absorbed from the gut into the haemolymph was determined by help of rocket immunoelectrophoresis after Laurell. Tsetse flies usually fed on bovine blood replace bovine albumin in their haemolymph by human albumin when fed on human blood. There is a linear correlation between absorption of albumin and the size of blood meals (Fig.1). After an average blood meal of 20 mg, 0.04 μg of human albumin can be observed following eight to ten blood meals after which the achieved level is maintained (Fig.2).

Human albumin disappears again from the haemolymph and is replaced by bovine albumin when the flies are fed again with bovine blood. From these data the daily turnover of albumin can be estimated to be about 0.01 $\mu\text{g}/\mu\text{l}$ haemolymph, which is 5.8% of the total.

On the basis of these results, the amount of albumin absorbed from a single blood meal can finally be calculated. An average blood meal of 20 mg contains 880 μg albumin. On the premise that the haemolymph of *Glossina morsitans* has a volume of 8 μl , the total uptake of albumin per blood meal ranges from 0.08 μg to 0.32 μg , which means 0.01% to 0.035% of the albumin contents of the blood meal.

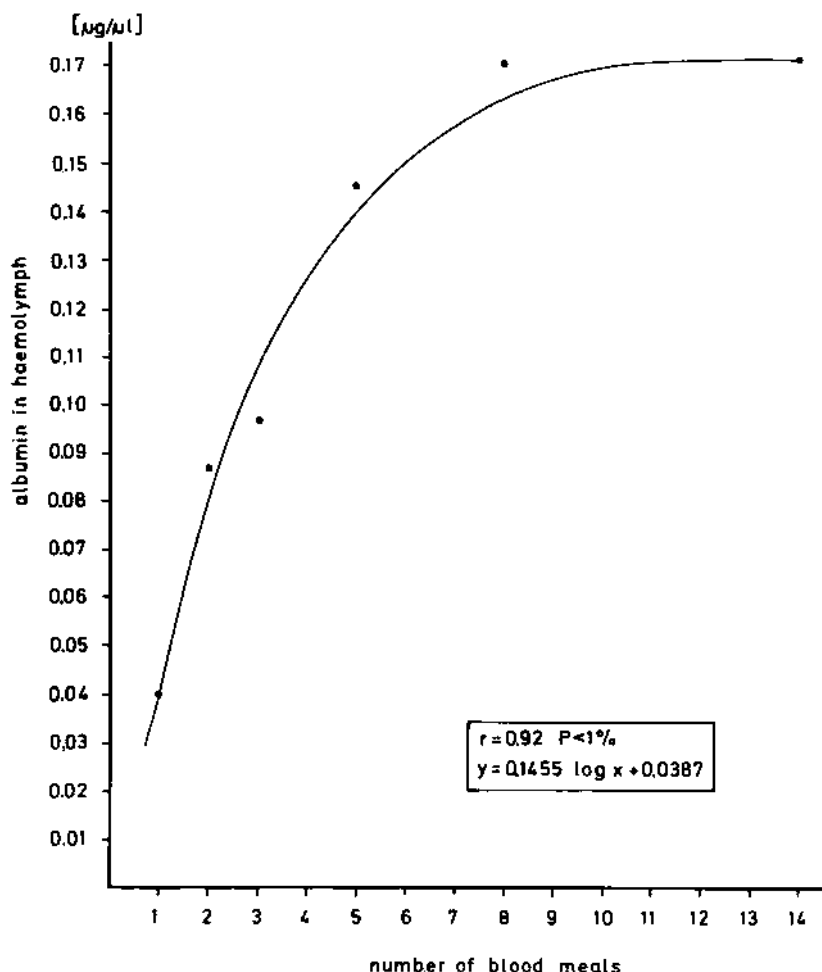


FIG.2. Increase of human albumin in the haemolymph of tsetse flies when fed continuously on human blood.

It has been stated already that albumin plays an important role in tsetse fly reproduction. The reason for this is a matter of speculation. The absorption of proteins, which do not have to be broken down to amino acids, means a saving in energy, which may be of high importance for an insect which depends totally on an energy-poor diet such as blood. Experiments in progress will show whether or not albumin absorbed into the haemolymph will be transferred further to other organs, e.g. fat body or ovary. Alternatively it may have a carrier function similar to the function of albumin in vertebrate blood.

It is interesting to note that the albumin corresponding fraction in the haemolymph can be synthesized also by the flies themselves, e.g. when fed on an albumin-free diet.

The synthesis, however, is obviously possible only at the cost of other pathways of synthesis, resulting in the production of smaller offspring.

REFERENCES

- [1] NOGGE, G., Aposymbiotic tsetse flies, *Glossina morsitans morsitans*, obtained by feeding on rabbits immunized specifically with symbionts, *J. Insect Physiol.* **24** (1978) 299.
- [2] GOODING, R.H., Digestive processes of haematophagous insects: XIII. Evidence of *Glossina morsitans morsitans* Westwood (*Diptera: Glossinidae*), *Can. J. Zool.* **55** (1977) 1557.
- [3] NOGGE, G., Resorption von Serumproteinen in die Haemolymph bei Larven von *Hypoderma bovis* (De Geer) (*Diptera, Hypodermatidae*), *Experientia* **27** (1971) 524.
- [4] SCHLEIN, Y., SPIRA, D.T., JACOBSON, R.L., The passage of serum immunoglobulins through the gut of *Sarcophaga falcipalata* Pand., *Ann. Trop. Med. Parasitol.* **70** (1976) 227.
- [5] NOGGE, G., GIANNETTI, M., Midgut absorption of undigested albumin and other proteins by tsetse, *Glossina morsitans* (*Diptera: Glossinidae*), *J. Med. Entomol.* **16** 2 (1979).
- [6] LANGLEY, P.A., PIMLEY, R.W., MEWS, A.R., FLOOD, M.E.T., Effect of diet composition on feeding digestion, and reproduction in *Glossina morsitans*, *J. Insect Physiol.* **24** (1978) 233.

DISCUSSION

L.H. OTIENO: We have recently seen that, in *G. morsitans* fed on a rabbit immunized with a strain of *T. brucei*, agglutinating antibodies against that particular strain of trypanosomes could be found in the haemolymph of the flies that were fed. Since antibodies against trypanosomes are usually of high molecular weights, I would like to know how these antibodies cross into the flies' haemolymph.

G. NOGGE: As I pointed out, IgG is not able to pass through the peritrophic membrane and therefore cannot be absorbed into the haemolymph. The same is of course true for IgM, which is an even bigger molecule. But fragments, e.g. fats which have antibody characteristics and activities, are able to pass through. In any case your observation is very interesting and supports my own findings.

H. WETZEL: As a result of information received from you earlier, we supplemented an inadequate diet with albumin and observed an increase in fertility (reproduction) and a higher mean weight of puparia compared with the controls. However, the improved diet and the added albumin did not result in a further increase in fly performance.

R. GALUN: Since less than 1% of the protein passes through the gut wall unchanged it probably means very little from the point of view of energy saving. I would ascribe this phenomenon to an imperfection in the digestive tract.

G. NOGGE: The importance of the absorption of undigested proteins is still a matter for speculation. You may be right in saying that the amount of protein absorbed is negligible from the point of view of energy saving. On the other hand, the importance of albumin in the diet for the reproduction of tsetse flies, in my view, would tend to suggest that an imperfection is not the cause.

P.A. LANGLEY: Would you care to speculate through which part of the gut wall the albumin molecules pass?

G. NOGGE: We don't know yet, but we hope to find out in the near future by means of the indirect immune fluorescent technique.

J. VERCOE: What effect does the removal of the symbionts in the digestive tract of the fly have on its digestion, metabolism and reproductive performance?

G. NOGGE: If the symbionts are eliminated, for example by specific antibodies which do not produce side-effects on the flies, survival of the flies remains normal, but their reproduction decreases until it reaches zero. Our investigations on the significance of the endosymbionts show clearly that they normally provide their hosts with substances such as the B-vitamins in which vertebrate blood is deficient. We have no evidence, however, which would show that the symbionts are directly involved in blood digestion.

C. MELLA LIZAMA: As you have shown, albumin can pass unchanged into the haemolymph and influence the reproductive behaviour of the fly. Can you comment on the possible effect of hormones that are normally carried by the prealbumin fraction, such as thyroxine?

G. NOGGE: If the importance of albumins for tsetse flies lies in their carrier function, it may be that they transport certain substances from the gut into the haemolymph. But since it is known from the investigations by Nash and others at Bristol that hormones of vertebrates do not have any influence on tsetse flies, it seems unlikely that hormones attached to albumin or prealbumin play a significant role.

USE OF GAMMA-IRRADIATED BLOOD FOR FEEDING TSETSE FLIES

B. BAUER, K.H. IWANNEK, H.J. HAMANN
Gesellschaft für Strahlen- und Umweltforschung
mbH München,
Institut für Strahlenbotanik,
Hanover

G. ADAMSKY
Technische Universität Hannover,
Institut für Biophysik,
Hanover,
Federal Republic of Germany

Abstract

USE OF GAMMA-IRRADIATED BLOOD FOR FEEDING TSETSE FLIES.

Freshly collected pig blood was sterilized by ^{60}Co gamma irradiation. A dose of 100 krad proved to be effective against *Pseudomonas aeruginosa* at concentrations of at least $4.7 \times 10^5/\text{ml}$. *Glossina morsitans morsitans* (Westw.) were maintained on this blood by feeding them five days per week on silicone membrane for nine months. Their performance was compared with a control fed untreated blood. In spite of the slightly faster haemolysis of the irradiated blood, the results from the experimental group compared favourably with the control. This was especially true for the percentage of daily mortality. The irradiation seems to offer a promising approach for the establishment of mass rearing plants in Africa based on in vitro feeding provided the blood from donor animals is suitable for tsetse flies.

INTRODUCTION

The mass maintenance of tsetse flies on an in vitro feeding technique depends on the use of blood that is not contaminated with pathogenic bacteria. Up to now there has been no safe blood collection technique which can be recommended for a mass rearing plant in Africa. Even with the well developed methods of Mews and co-workers [1], "... low levels of bacterial contamination are occasionally detected". Further, the possibility of overlooking latent bacterial contaminations cannot be entirely excluded. This put emphasis on the need for decontamination methods after blood collection. The procedure used should be highly effective against candidate pathogens, but should not impair the suitability of the blood for the in vitro maintenance of the flies. These requirements limit the number of possible decontamination methods. Several antibiotics proved to be effective against the bacteria in the blood but had negative effects on the fecundity of the treated females [2 - 5].

The irradiation of food with ionizing radiation in order to obtain a certain degree of preservation has been well known for a number of years. This study deals with the prophylactic gamma-ray sterilization of freshly collected pig blood. A colony of *Glossina morsitans morsitans* (Westw.) is maintained on this blood, and its performance is measured in terms of survival, fecundity, offspring size and emergence.

MATERIALS AND METHODS

The pig blood was collected in a slaughter house. After defibrination by agitating with glass beads 10–12 ml portions of the blood were filled into glass vials. For bacteriological examination blood was plated on nutrient agar and incubated for at least 24 h at 37°C.

One part of the blood was irradiated with gamma rays irrespective of its bacteriological state. The irradiations were performed in a gamma cell (AEG-Pantatron) containing two ^{60}Co -sources of $2.9 \times 10^3 \text{ Ci}$ ($1.073 \times 10^4 \text{ Bq}$) each.

To guarantee simultaneous irradiation of the greatest possible number of samples with the same dose, the blood portions were arranged in the irradiation chamber in a curve with a constant dose rate of $(0.52 \pm 0.07) \times 10^4 \text{ rad/min}$.

The dose rate was determined by the modified Fricke dosimeter consisting of 0.001M FeSO_4 , 0.8N H_2SO_4 , oxygen saturated, and 0.001M NaCl [6]. This dosimeter is one of the most proved chemical systems for the quantitative determination of radiation exposures. Its principle is that in sulphuric aqueous solutions the ferrous ions are oxidized by the radiation to ferric ions. This reaction is dependent on dose but largely not influenced by concentrations, radiation energy and dose rate. The yield of ferric ions, which is the measure of exposed dose, was determined by an absorption spectrophotometer (Zeiss PMQ II).

In the main part of the experiment each blood sample was exposed to a total dose of 100 krad. Only during one month of colony maintenance the blood was treated with 300 krad. After irradiation the blood was again bacteriologically investigated and finally stored at 4°C.

Glossina morsitans morsitans (Westw.) were maintained exclusively on this blood by feeding them five days per week on silicone membrane for nine months. To eliminate possible free radicals in the treated blood the flies were fed with a minimum time lag of 16 h.

The control flies were offered untreated blood that did not exhibit bacterial contamination after its incubation.

The membrane system used for feeding consisted of a silicone base covered with a silicone membrane described elsewhere [7, 8].

In an additional test, blood that was bacteriologically clean was infested with *Pseudomonas aeruginosa*, which is known to be extremely pathogenic to *G. morsitans* [9]. Different stages of dilutions from 1.2×10^4 to 4.7×10^5 /ml were exposed with doses ranging from 25 through 100 krad. Checks were made to find out the viability of bacteria in the irradiated blood. Suspensions irradiated with 100 krad after infection were offered to male *G. morsitans* which were maintained for 21 days.

RESULTS AND DISCUSSION

In the preliminary bacteriological experiment it was found that a dose of 100 krad was highly effective against *P. aeruginosa* inoculated into the blood. After irradiation no viable bacteria could be detected in the suspensions although parts of them had been incubated for at least 48 h at 37°C in nutrient broth or in blood agar. Males which were offered contaminated blood after its irradiation had no increased mortality compared with controls over a test period of 21 days. A dose of 75 krad still proved to be effective at the bacterial concentrations given above. The dose of 50 krad reduced the number of bacteria to 700/0.1 ml.

The feeding experiment with flies fed exclusively on irradiated blood began on 1 August 1978. The colony parameters compared with the experimental and the control group over nine and seven months respectively are presented in Fig.1.

The fecundity expressed as viable pupae/total number of females per month ranged from 1.0 to 2.3 for either group without significant difference. With an increase in the dose from 100 to 300 krad in November there was a distinct drop in the fecundity of the flies fed irradiated blood. The simultaneous decrease in the fecundity for both groups in December was obviously caused by a reduction in feeding as a consequence of several holidays.

The mean pupal weights of the control were about 1.4 – 3.8 mg heavier than those of the experimental group. However, there was a trend recognizable towards an attenuation of these differences. During the month when blood irradiated with 300 krad was fed a negative effect is again observable. A similar drop in the mean weights of the control is probably due to bacterial infection.

The percentage of daily mortality for the experimental group seldom exceeded 0.75% during the period of observation. An increase in the dose did not affect the longevity of the experimental flies. Compared with this the mortality for the control flies repeatedly exceeded 1.0%.

The emergence rate (i.e., the number of viable adults hatching from 100 pupae) exhibits no significant difference between both groups.

The slight degradation of all the parameters during the last three months could be the result of late effects following the feeding of highly irradiated blood in November. Further work is necessary to elucidate the negative influence of an increased dose on the reproductive performance of *G. morsitans*.

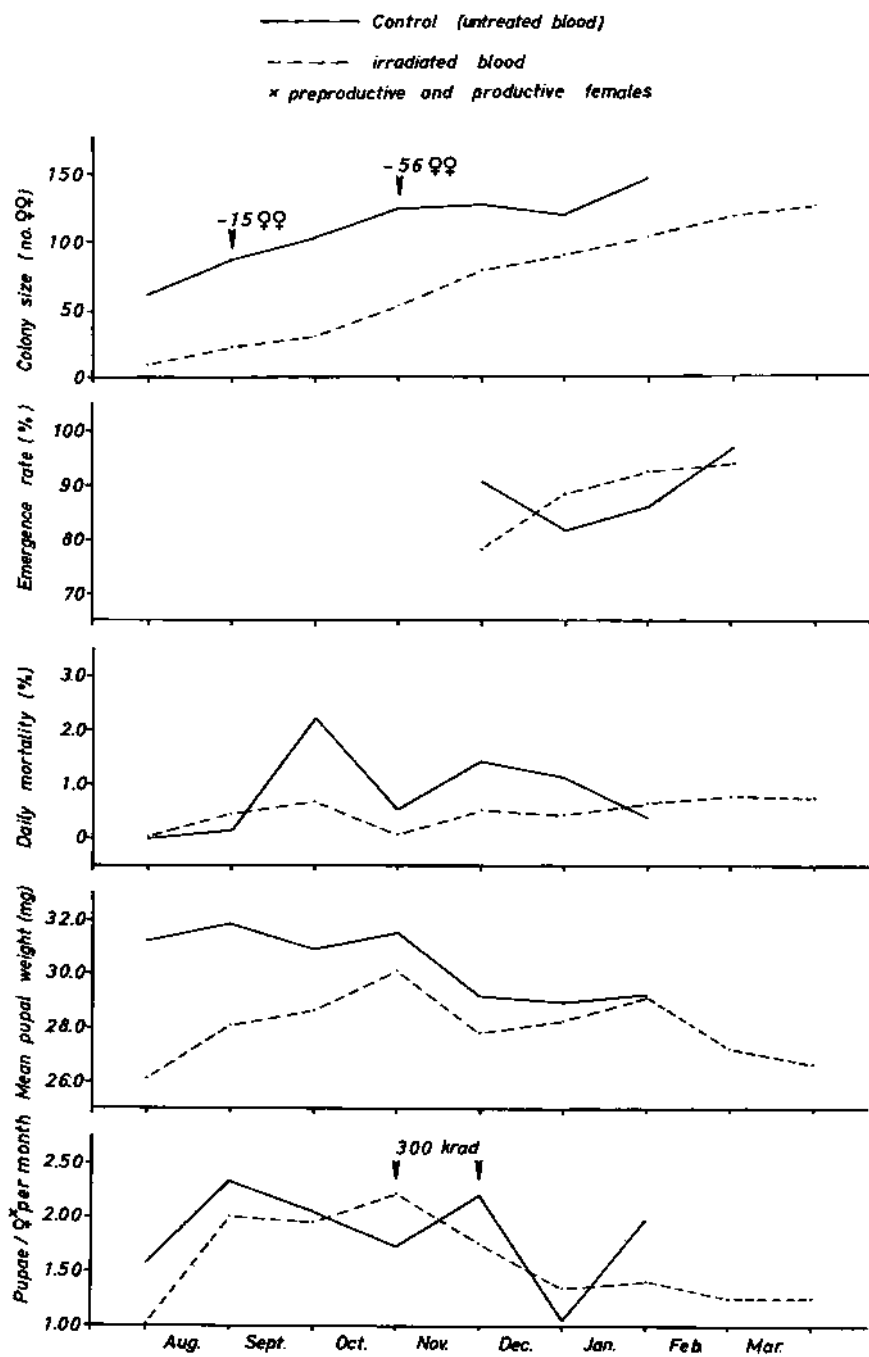


FIG.1. Monthly records of the in vitro colony.

Nevertheless, our data allow the conclusion that, in spite of the reduced feeding regime and some suboptimal mating conditions, the maintenance of *G. morsitans* on gamma-irradiated pig blood proved to be feasible.

In view of the radiation effects on blood this result is not self evident. It was established for many years that ionizing radiation induces considerable damage in blood exposed in vitro to doses such as were applied in our experiment. The most significant visible effect is haemolysis in the erythrocytes. Already after 20 h, erythrocytes irradiated with 100 krad lose 25% of their haemoglobin [10]. The haemolysis was found to depend on the concentration of the cells and the composition of the extracellular fluid during irradiation. It is introduced and accompanied by a number of biochemical changes in the erythrocytes: perturbation of the membrane permeability; destruction of membrane sulphhydryl groups; loss of potassium and accumulation of sodium in the erythrocytes [11]; decrease of intracellular ATP and glutathione content; activation of hexokinase and of glucose-6-phosphate dehydrogenase [12]. All the reactions result in a diminished survival of the cells. In spite of these more or less drastic effects, the blood irradiated with 100 krad was obviously accepted by *G. morsitans* without observable injury to the colony performance.

If this result can be established with blood from African donor animals the irradiation seems to offer a promising approach for the establishment of a mass rearing plant in Africa. The advantages of the method for an in vitro feeding regime are:

- (1) The feeding is independent of the bacterial contamination of the donor blood.
- (2) The application of antibiotics is unnecessary.
- (3) The laboratory procedures are simplified because glass ware and blood containers need not be sterilized before irradiation.
- (4) According to other authors, the irradiation leads to an attenuation of trypanosomes which is characterized by a loss of infectivity [13 - 17]. For *Trypanosoma rhodesiense* the doses to prevent mortality in mice and to immunize cattle were reported to be 20 and 70 krad respectively. Thus the treatment of blood with 100 krad is likely to attenuate the trypanosomes to such a degree that the parasites lose their infectivity for tsetse flies. Therefore, the possibility of using blood from African donor animals would offer an acceptable access for the introduction of in vitro feeding techniques in tsetse-infested countries, provided the blood has been irradiated before it is used.

SUMMARY

The use of gamma-irradiated blood for feeding *G. morsitans* was proved to be feasible. A dose of 100 krad was effective against *P. aeruginosa* inoculated

into the blood at a concentration of 4.7×10^5 /ml. The result from an experimental group fed exclusively on irradiated blood compared favourably with a control group. This was especially true for the percentage of daily mortality. The irradiation allows the *in vitro* feeding of tsetse flies irrespective of the contamination of the blood with bacteria or, probably, trypanosomes. The requirements of sterility in the feeding regime are drastically reduced. Thus far, the irradiation seems to offer a promising approach for the establishment of mass rearing plants in Africa based on *in vitro* feeding, provided the blood from donor animals is suitable.

REFERENCES

- [1] MEWS, A.R., LANGLEY, P.A., PIMLEY, R.W., FLOOD, M.E.T., Large-scale rearing of tsetse flies in the absence of a living host, *Bull. Entomol. Res.* **67** (1977) 119.
- [2] NOBRE, G., DOS SANTOS, A.M.T., "The use of antibiotics in artificial breeding of *Glossina morsitans* - a bacteriological study", *Proc. Int. Symp. Tsetse Fly Breeding under Laboratory Conditions and its Practical Application*, Lisbon (1970) 335.
- [3] AZEVEDO, J.F. DE, DOS SANTOS, A.M.T., DA COSTA PINHÃO, R., "New data on the feeding membrane and blood meals type of *Glossina morsitans*", *Proc. Int. Symp. Tsetse Fly Breeding under Laboratory Conditions and its Practical Application*, Lisbon (1970) 199.
- [4] OLIVEIRA, J.C., NOBRE, G., Bacteriological and mycological study of a laboratory-bred *Glossina* colony", *Proc. Int. Symp. Tsetse Fly Breeding under Laboratory Conditions and its Practical Application*, Lisbon (1970) 341.
- [5] WETZEL, H.W., BAUER, B., "Influence of antibiotics on *Glossina morsitans* Westwood", *Sterility Principle for Insect Control 1974 (Proc. Symp. Innsbruck, 1974)*, IAEA, Vienna (1975) 501.
- [6] FRICKE, H., HART, E.J., "Chemical dosimetry", *Radiation Dosimetry II* (ATTIX, F.H., ROESCH, W.C., Eds), Academic Press, New York, London (1966) 167.
- [7] BAUER, B., AIGNER, H., *In vitro* maintenance of *Glossina palpalis palpalis* (Robineau-Desvoidy) (Diptera: Glossinidae), *Bull. Entomol. Res.* **68** (1978) 393.
- [8] BAUER, B., IWANNEK, K.H., HAMANN, H.J., ADAMSKY, G., The maintenance of *Glossina morsitans morsitans* (Westwood) (Diptera: Glossinidae) on gamma-irradiated pig blood: Preliminary results, *Entomol. Exp. Appl.* (submitted to).
- [9] BAUER, B., WETZEL, H.W., "Effect of bacteria on tsetse flies fed through membranes", *Sterility Principle for Insect Control 1974 (Proc. Symp. Innsbruck, 1974)*, IAEA, Vienna (1975) 495.
- [10] MYERS, D.K., BIDE, R.W., Biochemical effects of X-irradiation on erythrocytes, *Radiat. Res.* **27** (1966) 250.
- [11] KOLLMANN, G., SHAPIRO, B., MARTIN, B., The mechanism of radiation hemolysis in human erythrocytes, *Radiat. Res.* **37** (1969) 551.
- [12] ARKY, I., SZASZ, I., GARDOS, G., SZELENYI, J.G., BREUER, J.H., VARTERESZ, V., HOLLAN, S.R., Biochemical changes in blood induced by *in vitro* X-irradiation, *Haematologia* (Budapest) **3** (1969) 51.
- [13] HALBERSTAEDTER, L., The effect of X-rays on trypanosomes, *Br. J. Radiol.* **11** (1938) 267.

- [14] SANDERS, A., WALLACE, F.G., Immunization of rats with irradiated *Trypanosoma lewisi*, Exp. Parasitol. 18 (1966) 301.
- [15] DUXBURY, R.E., SADUN, E.H., Resistance produced in mice and rats by inoculation with irradiated *Trypanosoma rhodesiense*, J. Parasitol. 55 (1969) 859.
- [16] DUXBURY, R.E., SADUN, E.H., ANDERSON, J.S., Experimental infections with African trypanosomes: II. Immunization of mice and monkeys with a gamma irradiated recently isolated human strain of *Trypanosoma rhodesiense*, Am. J. Trop. Med. Hyg. 21 (1973) 885.
- [17] WELLDE, B.T., DUXBURY, R.E., SADUN, E.H., LANGBEHN, H.R., LÖTZSCH, R., DEINDL, G., WARUI, G., Experimental infections with African trypanosomes: IV. Immunization of cattle with gamma-irradiated *Trypanosoma rhodesiense*, Exp. Parasitol. 34 (1973) 62.

DISCUSSION

T.G.T. JAENSON: How long can the irradiated blood be stored before it should no longer be used for feeding?

H.J. HAMANN: We didn't store the irradiated blood longer than seven days. For this length of time the blood can certainly be used for in vitro feeding of *G. morsitans*.

T.G.T. JAENSON: Do you consider gamma-irradiated or freeze-dried blood more appropriate for membrane feeding under tropical conditions?

H.J. HAMANN: So far we haven't compared the suitability of gamma-irradiated blood with that of freeze-dried blood for membrane feeding of tsetse flies. However, it is well known that freeze drying normally does not kill bacteria, so I doubt whether freeze drying can be used for blood from African donor animals, which is surely more often contaminated than that of European animals.

H. WETZEL: The success of membrane feeding of tsetse flies depends to a very large extent on the technique used. Our experience using membrane feeding over several years to rear large numbers of *G. palpalis* and *G. morsitans* is (1) that the danger of bacterial infection is overestimated and is normally associated with poor technique, (2) that freeze drying of blood is an excellent way of storing it and has the advantage that all bacteria, even those arising from experimental contamination, are killed and cannot grow after reconstitution of the freeze-dried blood (whereas the use of freeze drying for preserving bacteria, on the other hand, requires specific additives to protect them), and (3) that the use of haemolysed blood does not on its own impair fly performance.

H.J. HAMANN: Your statement that the danger of bacterial infection is overestimated is surprising, since in your own laboratory as well as at Langford and in my laboratory a lot of work has been done to find out the best way of avoiding contamination. I agree that bacterial infections are the result of poor technique, but nevertheless the problem exists in cases where you cannot be sure that the blood to be used is not contaminated. This is especially true of African donor animals.

Secondly, I have serious doubts about whether freeze drying generally kills bacteria. This may be true for your aseptic technique and under your special test conditions, but it is a familiar fact that some species of bacteria normally survive freeze drying. This could place limitations on the value of your method where African conditions of hygiene prevail and where blood from African donor animals is used.

G. NOGGE: Is it possible to irradiate quantities larger than 10 – 12 ml, particularly under African conditions?

H.J. HAMANN: Yes, but we use such small portions because of our irradiation conditions and feeding needs. The size of the quantity irradiated is limited by the degree of homogeneity of the radiation field. The dose deviation within the irradiated portion should not be greater than about 20%, because the sterilizing effect in the bacteria as well as the damaging effect on the blood are dependent on dose.

W.J. KLOFT: Aren't there any radiation effects (such as radiolysis and breakdown, especially of proteins) from such high radiation doses? You mentioned haemolysis, which might not affect tsetse flies; but I am thinking, in particular, about radiochemical effects, mainly on the proteins.

H.J. HAMANN: The literature available to me did not consider radiation effects in the blood other than those I mentioned in my presentation. We are continuing our studies and plan to investigate different blood parameters experimentally. However, if there are additional radiation effects, they obviously do not have a negative effect on the colony performance.

P. FINELLE (*Scientific Secretary*): Your trials seem to prove that irradiation can eliminate bacteria in the blood. However, where trypanosomes are concerned, the data are purely hypothetical. It is very important that this point be cleared up before the technique is considered for practical use under African conditions.

H.J. HAMANN: So far we haven't investigated the effect of gamma radiation on trypanosomes in the blood experimentally, and in this sense our conclusions are hypothetical. On the other hand, it has been demonstrated by other workers that *T. rhodesiense*, *T. gambiense* and *T. lewisi* lose their infectivity after exposure to ionizing radiation (X- and gamma rays) in doses higher than 20 krad (see the references given in my paper).

D. DAME: You mentioned in your oral presentation that free radicals are formed during the irradiation process. Of what type are they and how did you eliminate them from the irradiated blood?

H.J. HAMANN: We have to expect these free radicals, which are normally induced in aqueous solutions by radiation. The radicals recombine in about one hour. The blood was not used for feeding until at least 16 hours had passed.

ROLE OF ADULT FAT BODY AND MILK GLAND IN LARVAL NOURISHMENT OF *Glossina morsitans*

P.A. LANGLEY

Tsetse Research Laboratory,
University of Bristol,
School of Veterinary Science,
Langford, Bristol,
United Kingdom

E. BURSELL

Department of Zoology,
University of Rhodesia,
Mount Pleasant, Salisbury,
Rhodesia

Abstract

ROLE OF ADULT FAT BODY AND MILK GLAND IN LARVAL NOURISHMENT OF *Glossina morsitans*.

The *Glossina* larva is nourished entirely in utero by 'milk' composed of equal parts lipid and protein or protein-derivatives, produced by the adult female accessory gland or milk gland. A series of experiments in which activities of the female fat body and milk gland were studied separately, showed that during early pregnancy fat body synthesized and stored triglyceride and, to a lesser extent, protein, utilizing either ^{14}C leucine or ^{14}C palmitate in the process. Late in the pregnancy cycle, synthetic activity of the fat body was reduced whereas that of the milk gland increased, both lipid and protein synthesis being conspicuous at this time. There was apparently a switch in mid-pregnancy at which time the milk gland became the dominant organ for synthesis of nutrient substances. Results support the hypothesis that the adult fat body provides the major store, derived from blood meals ingested during early pregnancy, from which the milk gland obtains the lipid component of the milk. The gland itself synthesizes the bulk of the protein components from digested blood meals ingested during the latter half of pregnancy. Control of the processes identified, and their cyclical nature, suggests a neuroendocrine involvement. Identification of this involvement, and the underlying control mechanisms for hormone synthesis and degradation, may well lead to more specific methods of vector control acting through disruption of larval nutrition.

INTRODUCTION

The adult female *Glossina* nourishes its growing larva in utero with a secretion from its highly modified accessory gland. This secretion, known as larval milk, is composed of 30% solids and 70% water, the solid matter comprising approximately

equal proportions of lipid and protein [1]. Complex regulatory mechanisms obviously exist to co-ordinate the supply of this milk with the cyclical reproductive events which terminate in the birth of a fully grown third instar larva at regular intervals of nine or ten days (at 25°C) [2].

Evidence is available that the milk is synthesized from lipids stored during early pregnancy, coupled with proteins acquired from blood meals ingested during later stages of the pregnancy cycle [3]. There is, apparently, no extensive store for protein within the adult female body and the uterine gland synthesizes proteins *de novo* from amino acids [4]. Clearly a knowledge of the timing of transfer of nutrients between maternal tissues and from mother to offspring would provide a basis for understanding the regulatory mechanisms involved. Interference with nutrient synthesis for milk production at the molecular level might well provide new avenues for control of these vectors of African trypanosomiasis.

The present work describes the involvement of the maternal fat body and uterine gland in supplying nutrients for milk synthesis.

MATERIALS AND METHODS

Adult female *Glossina morsitans morsitans* were taken from the Langford routine colony maintained at 25°C and fed on a diet of defibrinated pig blood [5]. Females were synchronized on their second reproductive cycle as previously described [6], and pregnancy was assessed in terms of the developmental stage of the uterine content as well as the time lapse since the previous larviposition.

In vivo techniques

Radioactive substrates were injected into females either early (days 2 to 3) or late (days 6 to 8) in pregnancy. Using U-¹⁴C leucine, flies were injected 24 h after feeding and sacrificed 0.5 h later, whereas in the case of U-¹⁴C palmitate, flies were sacrificed 1 h after injection. This provided sufficient incorporation of radioactivity into the adult fat body and uterine gland without incurring significant losses through transfer to the growing larva. Hence, the distribution of substrate between lipid and protein fractions of the fat body and uterine gland could be evaluated at different stages of pregnancy.

The transfer of radioactivity from fat body to uterine gland was monitored at 24-h intervals throughout pregnancy following a single injection of U-¹⁴C leucine during early pregnancy.

Quantitative separation of fat body and uterine gland was achieved by dissection in distilled water following fixation of the whole abdominal content in Formal Calcium for 1 h and washing in several changes of Ringer [7] to remove extracellular radioactivity. The fixative preserved both the lipid and protein

TABLE I. DISTRIBUTION OF RADIOACTIVITY IN LIPID AND PROTEIN EXTRACTS OF ABDOMINAL FAT BODY AND UTERINE GLAND, OF FEMALE *Glossina morsitans* IN EARLY OR LATE PREGNANCY, 1 h AFTER INJECTION OF THE LIVING FLY WITH U-¹⁴C LEUCINE OR U-¹⁴C PALMITATE. Means and standard errors are given, with *n* as the number of flies on which estimates are based: F.B. = fat body; U.G. = uterine gland

Pregnancy stage	n	Disintegrations (min ⁻¹)				% Distribution			
		Lipid		Protein		Lipid		Protein	
		F.B.	U.G.	F.B.	U.G.	F.B.	U.G.	F.B.	U.G.
<i>Leucine</i>									
Early	4	6373 ±1554	802 ±293	1447 ±696	362 ±147	71	9	16	4
Late	4	1047 ±185	2134 ±395	309 ± 91	1705 ±163	20	41	6	33
<i>Palmitate</i>									
Early	4	2892 ±658	1230 ±161	668 ± 61	426 ± 22	55	24	13	8
Late	4	1042 ±161	4854 ±1201	376 ± 28	933 ±255	14	67	5	13

content of the tissues and radioactive losses during dissection were negligible. Separated tissues were dried under vacuum at 70°C in small glass tubes, lipids being then extracted with 300 µl chloroform: methanol (2:1), and residues were hydrolysed with 300 µl 1N NaOH at 100°C for 10 min before neutralization with 17 µl glacial acetic acid. Aliquots of extracts were removed for thin layer chromatography of lipids on silica gel plates, and after development in hexane: di-ethyl ether: acetic acid (80:20:1) lipids were made visible by exposure to iodine vapour. Triglyceride spots were marked and the plates exposed to air at 70°C for 30 min to remove iodine. The spots were then scraped off and transferred to a scintillation vial for counting. The radioactivity of all extracted materials was measured by addition of 5 ml NE250 scintillant (Nuclear Enterprises), and samples were counted in an LKB Ultrabeta 1210 scintillation spectrometer, using the channels ratio method to correct for quenching effects.

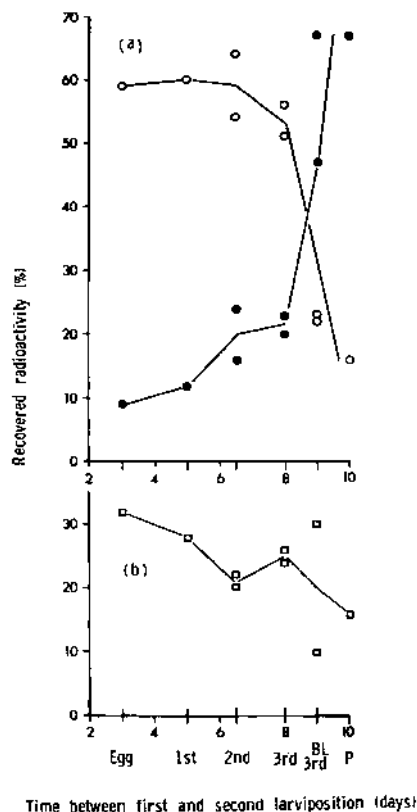
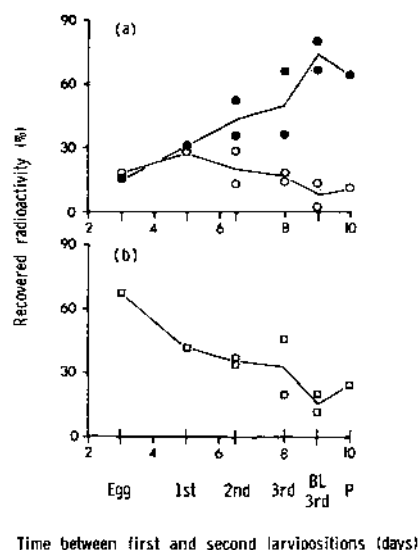


FIG. 1. Distribution of radioactivity between lipid extracts of (a) abdominal fat body and uterine gland and (b) remaining carcass, at successive 24 h intervals in *Glossina morsitans* females following a single injection of $U-^{14}C$ leucine in early pregnancy. Results expressed as percentage of radioactivity recovered from total lipid extract of each fly: $\circ$ — $\circ$, abdominal fat body; $\bullet$ — $\bullet$, uterine gland plus larva; $\square$ — $\square$, carcass. Pregnancy state is indicated by uterine content and given on the abscissa; 1st, 2nd, 3rd = larval instars; BL = Blacklobed; P = post-parturient.

In vitro techniques

Small numbers of fat cells and short lengths of uterine gland were carefully dissected from living tissues in Ringer [7] (but without organic acids and plus 1% bovine serum albumin). Tissues were then incubated in 10 μ l of Ringer containing 10% serum albumen and the radioactive substrate as specified. After 1.5 to 4 h they were washed to remove excess substrate and dried in a small glass vial before extracting with 100 μ l chloroform:methanol (2:1) for chromatographic separation as above.



FIG'2. Distribution of radioactivity between hydrolysate extracts of (a) abdominal fat body and uterine gland, and (b) remaining carcass at successive 24 h intervals in *Glossina morsitans* females following a single injection of U-¹⁴C leucine in early pregnancy. Results expressed as percentage of radioactivity recovered from total hydrolysate extract of each fly: ○---○, abdominal fat body; ●---●, uterine gland plus larva; □---□, carcass. Symbols as in Fig.1.

TABLE II. IN VITRO SYNTHESIS OF TRIGLYCERIDE BY FAT BODY AND UTERINE GLAND FROM ADULT FEMALE *Glossina morsitans*

Means and standard errors are given, with *n* as the number of determinations on which estimates are based

Substrate (conc.)	Stage of pregnancy	<i>n</i>	Fat body (p moles fly ⁻¹ h ⁻¹)	Uterine gland
Leucine (27 μM)	Early	11	877 ± 108	10 ± 3
	Late	7	109 ± 21	18 ± 8
Palmitate (7 μM)	Early	6	70 ± 11	46 ± 12
	Late	7	76 ± 10	35 ± 10

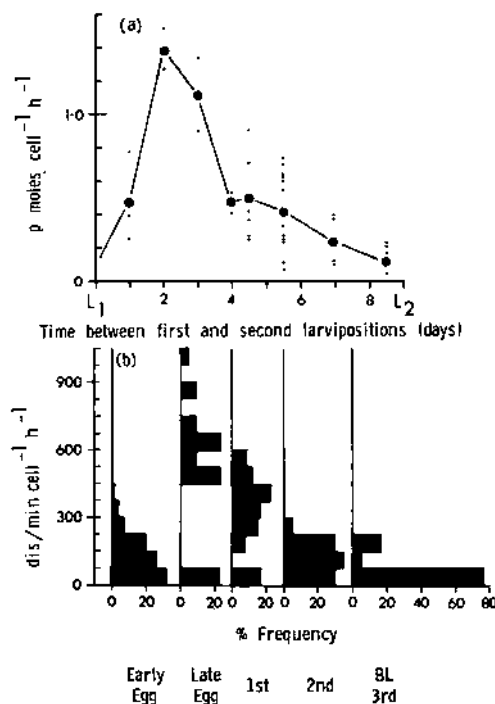


FIG. 3. The *in vitro* incorporation of radioactivity into fat body lipids at different stages of pregnancy with U-¹⁴C leucine as substrate. (a) Incorporation into triglyceride following incubation of about 200 cells for approximately 2 h. Individual estimates (●) are shown and a line has been drawn to join average values (○). Leucine concentration was 0.53 mM with a specific activity of 20 $\mu\text{Ci mmol}^{-1}$. (b) Frequency distributions of the radioactivity of single cells incubated for 4 h in 0.14 mM leucine at a specific activity of 340 $\mu\text{Ci mmol}^{-1}$. Pregnancy state is indicated by uterine content: symbols as in Fig. 1.

To estimate the radioactivity of single fat cells, washed fat body was fixed for 1 h in Formal Calcium, washed in Ringer and then dissected in distilled water. Individual cells were dried on fragments of a glass cover slip at 70°C and transferred to a scintillation vial containing 5 ml scintillation fluid for counting.

RESULTS

Injection of U-¹⁴C leucine or U-¹⁴C palmitate into the haemolymph of adult female *Glossina morsitans* resulted in extensive incorporation of label into fat body lipid provided injections were made in early pregnancy. During late pregnancy

such injections led to less extensive incorporation. Conversely, uterine gland lipids became more heavily labelled if injections were made during late rather than early pregnancy (Table I). A similar change in the activity of these two tissues was seen with respect to protein synthesis, although incorporation into protein never achieved more than about one third of the maximum levels measured in lipid.

To determine the relative roles of fat body and uterine gland in synthesizing the lipid and protein components of larval milk, both tissues were assayed separately at daily intervals following a single injection of U-¹⁴C leucine early in pregnancy. Results (Fig.1) demonstrated a massive transfer of lipid material (mainly triglyceride) from the fat body to the uterine gland at the time of appearance of the third instar larva within the maternal uterus, which marks the beginning of the most rapid growth phase of the larva [6]. A similar but less dramatic increase in labelled protein within the uterine gland did not arise at the expense of fat body protein, but was largely derived from other and unspecified body proteins (Fig.2).

A study of the synthetic capability of fat body and uterine gland *in vitro* was undertaken to explore further the role of these two tissues in larval milk synthesis. To a large extent the capability of isolated fat cells and of small portions of uterine gland to incorporate U-¹⁴C leucine and U-¹⁴C palmitate mirrored the *in vivo* situation (Table II). In addition, it was found that triglyceride synthesis within fat cells depended not only upon the activity of individual cells but also upon the number of cells that were active at any one time (Fig.3).

DISCUSSION

In analysing the process of milk synthesis in the adult female *Glossina*, we are dealing with a dynamic system which does not involve a switch from one specific activity to another. Results have shown that both fat body and uterine gland possess an inherent capacity to synthesize lipid and protein material throughout pregnancy. However, it is clear from the results presented that during early pregnancy a major activity of the fat body is the synthesis of triglyceride. It has also been shown that at the time of maximum nutrient demand by the third instar larva, triglyceride is transferred from the fat body to the uterine gland. As pregnancy proceeds, the ability of the uterine gland to synthesize protein increases but that of the fat body does not. It is thought that the last large blood meal ingested by the fly in each pregnancy cycle (on the 6th or 7th day) probably supplies the bulk of the protein component of the milk directly from the amino-acid products of its digestion, and that this is coupled in the uterine gland with lipids derived from the triglyceride store laid down in the fat body during the earlier part of pregnancy. Such a system is economic in terms of protein metabolism, does not require the presence of an extensive, alternative protein store in

the insect, and permits the growing larva to fill the abdominal space left by the diminishing reserves of the fat body and the blood meal.

The observation that synthetic capability of the fat body depends not only on variations in activity of individual cells but also on fluctuations in the number of cells active at any one time, provides further evidence of the complexity of the system. It suggests strongly that fat body activity is regulated through a mosaic of cells, perhaps having different thresholds of response to changing titres of a humoral agent. The fact that extirpated cells continue to synthesize triglyceride in a medium which lacks humoral components of the haemolymph indicates that competent cells require only an initial stimulus, following which their preprogrammed activity continues for some time.

It therefore remains to be seen whether an untimely release of fat body triglyceride can be achieved in *Glossina* under the influence of an adipokinetic hormone such as exists in locusts [8]. However, techniques developed for the present work should at least provide a basis for exploring the regulatory mechanisms involved in larval nutrition, and open prospects for attempts to control these insects by interference with this vital process at the molecular level.

REFERENCES

- [1] CMELIK, S.H.W., et al., Comp. Biochem. Physiol. **29** (1969) 447.
- [2] TOBE, S.C., LANGLEY, P.A., Annu. Rev. Entomol. **23** (1978) 283.
- [3] LANGLEY, P.A., PIMLEY, R.W., J. Insect Physiol. **25** (1979) 193.
- [4] TOBE, S.C., et al., Tissue Cell **5** (1973) 633.
- [5] MEWS, A.R., et al., Bull. Entomol. Res. **67** (1977) 119.
- [6] LANGLEY, P.A., PIMLEY, R.W., Bull. Entomol. Res. **65** (1975) 129.
- [7] GEE, J.D., J. Exp. Biol. **63** (1975) 391.
- [8] MAYER, R.J., CANDY, D.J., J. Insect Physiol. **15** (1969) 611.

DISCUSSION

D.J. ROGERS: Could you speculate on the function of the 25% of cells that are apparently inactive during the pregnancy cycle? Is it possible that they are involved in some other activity, such as flight?

P.A. LANGLEY: We may well be dealing with more than one metabolic pool of cells, some of which are releasing lipids whereas others are synthesizing them. Some such system would be necessary to ensure that other metabolic activities involving consumption of lipids could continue uninterrupted during pregnancy.

D.J. ROGERS: Could there be competition between these two processes (pregnancy and flight activity) for the limited reserves of the fat body?

P.A. LANGLEY: I do not like the term 'competition', because the nature of the phenomenon is entirely unknown. However, it is likely that at times of nutritional stress the survival of the species is ensured by disruption of reproductive processes rather than of metabolic processes which are vital for survival of the individual adult fly.

A.M. VAN DER VLOEDT: It might be worth while extending your ^{14}C leucine labelling studies to females that have been inseminated by sterilized males. We know that after fertilization of the egg embryogenesis stops in such females very soon and, consequently, no larval stage follows. The milk gland system does not then need to synthesize proteins as in the normal situation. On the other hand, such females build up enormous amounts of fat body. Do you consider that those females are in some sort of physiological imbalance?

P.A. LANGLEY: I do not think there is any question of an imbalance. The female fly mated to a sterile male will probably be in a physiological state similar to that of a virgin female in respect of its fat body function. Virgin females synthesize triglyceride to the same extent as pregnant flies, but this level then remains constant in the virgin and the uterine gland shows little sign of growth. I agree that it would be useful to measure the rate of turnover of triglyceride in virgins and in females mated to sterile males. This should enable us to determine the rate of utilization of lipids for processes other than those associated with reproduction.

C.F. CURTIS (*Chairman*): I would like to add that there is evidence from both *G. palpalis* and *G. austeni* that females mated to partially sterilized males produce pupae of abnormal weight (above normal in *G. palpalis* and below normal in *G. austeni*). In *G. austeni*, reproduction in such partially sterile females terminates abnormally early. These are not genetic effects but physiological ones and it seems likely that they arise from the abnormal accumulation of fat in females which have had repeated pregnancy failures. These effects are of some significance when the overall impact of releases of incompletely sterilized males are assessed.

P.A. LANGLEY: This observation may also be of significance for predictions of the causal relationships between fat body and uterine gland activity in the fully fertile fly.

H. WETZEL: It has been shown previously that nutrition taken up by the female is converted into fat during early pregnancy and to protein in the later part. Feeding of the fly is important at every stage of egg or larval development, so a particular large meal in the middle (or near the end) of the pregnancy cycle might not be of great importance.

P.A. LANGLEY: Obviously, all the blood meals ingested by the fly are important, but I would say that to deprive a fly of a blood meal in early pregnancy may not be so deleterious as to deprive it in later pregnancy. All I am suggesting is

that the bulk of the protein component of the larval milk is supplied by meals ingested in late pregnancy. We relate this to the developmental stage of the larva in utero rather than to the chronological age of the fly.

R. GALUN: Your hypothesis about the different responses of fat body cells and milk gland cells to hormonal stimulation is based on their *in vitro* responses. Is it possible that the medium composition requirements are different and therefore that what you observed is merely an artifact?

P.A. LANGLEY: The medium was deliberately kept as simple as possible in order to evaluate rates of triglyceride synthesis among isolated fat cells in the presence of a single labelled amino acid. Portions of uterine gland were tested in the same way. Of course, the requirements of medium composition are unknown, and ideally we should use the insect's own haemolymph. However, since the tissues behave differently when incubated in the same medium *in vitro* it seems likely that they may respond differently when functioning *in vivo* in a medium which is common to each, namely the haemolymph itself.

**ECOLOGY AND BEHAVIOUR
OF TSETSE FLIES**

(Session 7)

1. The first part of the paper is devoted to the study of the properties of the function $f(x)$ defined by the equation

$$f(x) = \int_0^x \frac{1}{1+t^2} dt$$

for $x \in \mathbb{R}$. It is shown that $f(x)$ is an odd function and that it satisfies the inequality

$$f(x) \leq \frac{1}{2} \ln \frac{x+1}{x-1} \quad \text{for } x > 1.$$

2. The second part of the paper is devoted to the study of the function $g(x)$ defined by the equation

$$g(x) = \int_0^x \frac{1}{1+t^4} dt$$

for $x \in \mathbb{R}$. It is shown that $g(x)$ is an even function and that it satisfies the inequality

$$g(x) \leq \frac{1}{2} \ln \frac{x+1}{x-1} \quad \text{for } x > 1.$$

3. The third part of the paper is devoted to the study of the function $h(x)$ defined by the equation

$$h(x) = \int_0^x \frac{1}{1+t^6} dt$$

for $x \in \mathbb{R}$. It is shown that $h(x)$ is an even function and that it satisfies the inequality

$$h(x) \leq \frac{1}{2} \ln \frac{x+1}{x-1} \quad \text{for } x > 1.$$

CONSTRUCTION DETAILS OF THE CHALLIER/LAVEISSIERE BICONICAL TRAP

L. RYAN, D.H. MOLYNEUX

Department of Biology,
University of Salford,
Salford,
United Kingdom

Abstract

CONSTRUCTION DETAILS OF THE CHALLIER/LAVEISSIERE BICONICAL TRAP.

Detailed constructional information on the biconical trap, first described by Challier and Laveissière for the attraction and capture of tsetse flies, is supplied. Some of the advantages of this trap compared with other biting fly traps are briefly discussed.

INTRODUCTION

The Challier/Laveissière biconical trap (Fig.1) is widely used in West Africa in ecological studies and control operations [1-4] to capture *Glossina tachinoides*, *G. palpalis gambiensis* and *G. fuscipes quanzensis*. In East Africa it is being evaluated against *G. morsitans morsitans* and *G. pallidipes* (Challier in Kenya, personal communications), and it has also been used successfully against these two species in Rhodesia with host odours incorporated (E. Bursell, personal communication, 1978). Interest in this type of trap is increasing since it is collapsible, easily carried and quickly assembled, requirements not met by most other tsetse traps [5, 6]. The value of this trap has already been proven in several field programmes.

However, in view of the absence of construction details in the original description given by Challier and Laveissière [1], its wider use has been restricted. Moreover, lack of standardization in design and constructions may in future make it difficult to compare results from different workers. In view of these difficulties and because of the experience gained in making 60 biconical traps at the Department of Biology, University of Salford, details of the construction of the trap are presented with a pattern and instructions for its manufacture.

MATERIALS AND EQUIPMENT

Roman numbers in parentheses refer to commercial films listed at the end of the paper.

- (1) 2 m² of blue unfinished cotton drill (i) is dyed with non-water-soluble Phthalogen Blue IF3GM (ii), a precursor which forms copper thalocyanine pigment in the fibre. Other more robust and readily available blue materials are being field tested.
- (2) 2 m² of black cotton, cambric cloth (iii) or any lightweight unfinished black material would suffice, such as a polyester fabric.
- (3) 2 m² of grey, PVC-coated glass fibre netting (iv) — quality T 685 with 7.0 × 5.5 threads per centimetre.
- (4) Four 50-cm spring-loaded, interconnected, seam-welded aluminium tubes (O.D. 2.25 cm; I.D. 2.1 cm). These are constructed to form a collapsible tube, the axis of length 1.8 m (v).
- (5) Hollow plastic tubing (O.D. 1.7 cm; I.D. 1.35 cm) to form a circle of diameter 0.9 m. This may be coiled to a diameter of 30 cm for packing, the cut ends being joined by a 5-cm cylinder of 1.3-cm-thick wood.
- (6) The apex and geigy cages (Fig.2) are made of 3.15-mm diameter, copper-coated mild steel, spot welded and plastic coated.
- (7) Cloth sleeves for the apex and geigy cages are of black terylene netting (vi) (number 6480), commonly used to cover tsetse-rearing cages.
- (8) Thread for sewing is black polyester (vii).
- (9) A good sewing machine, fitted with a number 14 needle (viii).

Description of the trap

The general construction of the trap is that of two cones joined at their bases with the points at either end of a vertical axis. The whole consists of four independent parts (Fig.2).

Part 1

(See Fig. 2.) The axis (F) is a hollow tube of aluminium 1.8 m long manufactured as a tent pole (v) with outside diameter 2.25 cm. This consists of four interconnecting sections, three of which are spring-loaded with the fourth remaining unattached to these sections. The lower end of the loose section (K) is sharpened so that the whole cylinder may be driven into the ground leaving a length of 8 cm visible (H). This section may be shortened to a length suiting the

ground. The other three interconnected sections fit on to this. The lower portion of the axis should be covered in a non-setting adhesive to prevent access to predators. A suitable formulation is Öcetak A10 (ix).

Key to abbreviations in text
(all measurements are in centimetres unless otherwise stated)

A — inner, black screens	H — 8 cm, glue-covered length of K
a — apex base	K — separate section of the axis (F)
B — lower blue cone	L — leg struts of apex
b — rectangular base	M — middle washer of apex
C — upper, collecting cone	Q — swivel axis for suspension
c — apical supports for geigy cage	R — ropes suspending trap
D — apex	S — plastic hoop and sleeve
E — geigy cage	T — non-return opening
F — axis	W — positions of spot welding
G — rectangular openings	Z — spacer block

Part 2

The apical frame (D) is a metal frame covered with netting (vi). This is constructed entirely of 3.15-mm copper-coated mild steel and when complete is plastic coated to reduce snagging of the net. It consists (Figs 3 and 4) of a circle of iron (a), 13 cm in diameter on which is mounted a pyramid of rectangular base (b), 9.2 cm, and 13 cm high. The small opening at the top, 1 × 1.5 cm, provides a passage to the geigy cage (E) which rests on two supports (C) situated 7 cm from the top of the pyramid. The circular base (a) has four horizontal spokes running to the centre through a washer (O.D. 2 cm; I.D. 1 cm) then vertically downwards for 18 cm. These spokes fit into the top of the axis.

Part 3

The body of the trap (Fig.2) consists of two cloth cones joined at their bases (diameter 90 cm) along the edge of the circumference with a second seam 5 cm in from the edge. This forms a sleeve (S) into which fits a circle (2.73 m circumference, 0.88 m diameter) of hollow plastic. This plastic tube holds the trap open and is superior to metal being lighter and easily coiled into a small space for transport.

Text cont. on p. 351.



FIG.1. *The Challier/Laveissière biconical trap.*

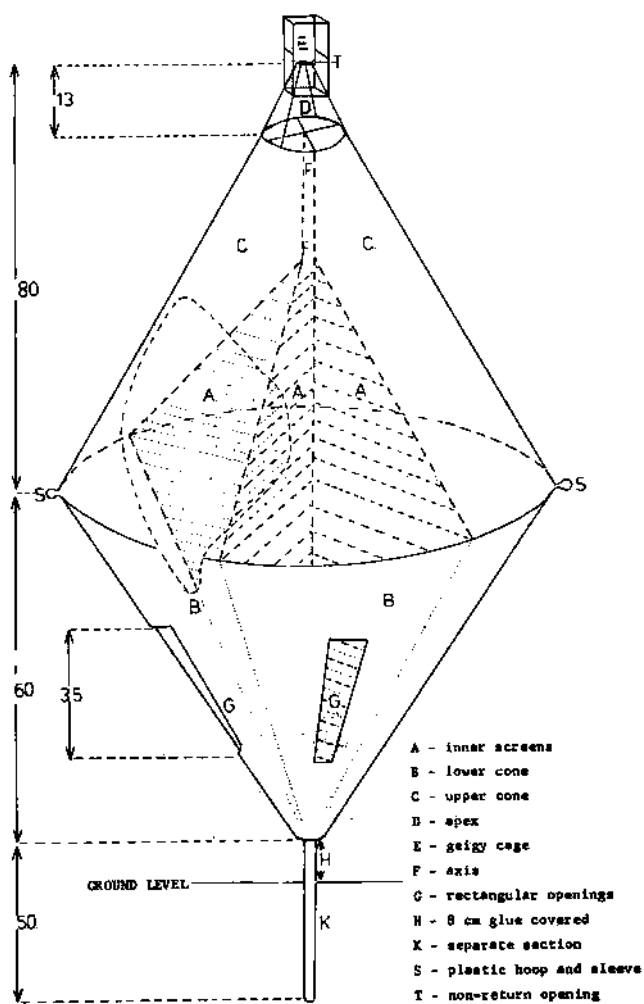


FIG.2. Diagram of complete trap.

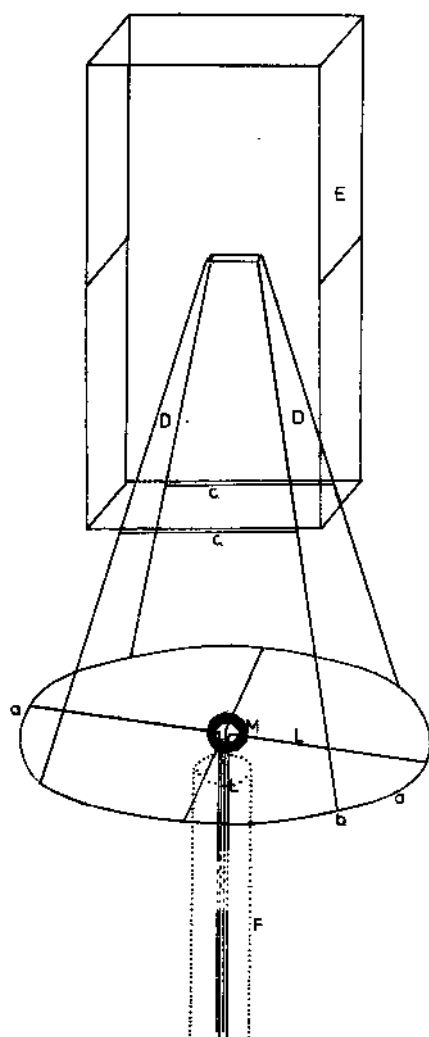


FIG.3. Structure of the geigy cage and apex frame. See Fig.2 for details.

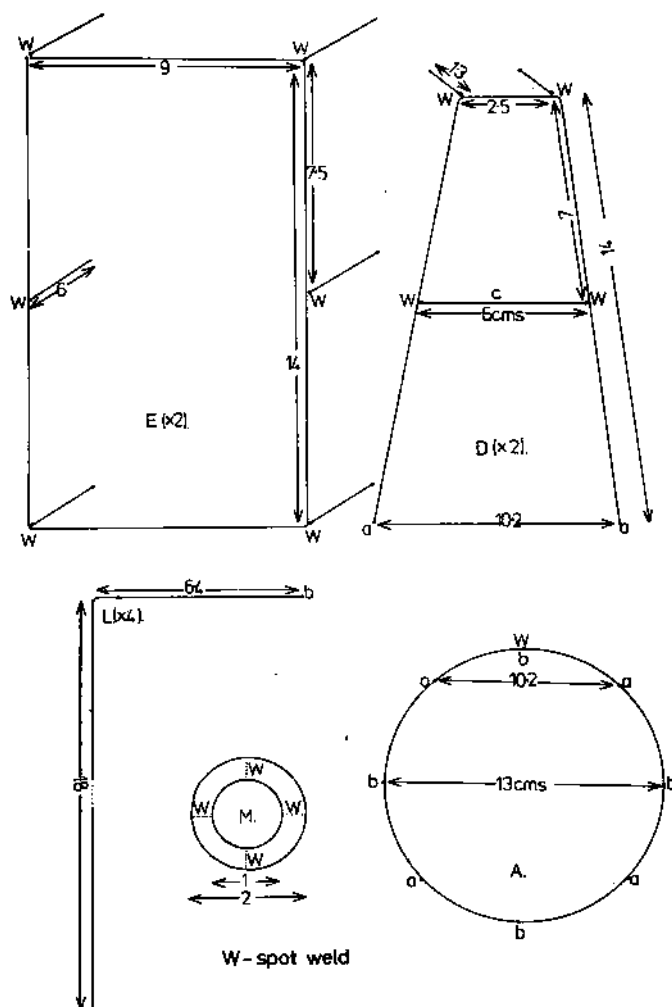


FIG.4. Metal construction of the apex and geigy cage showing position of spot weld.

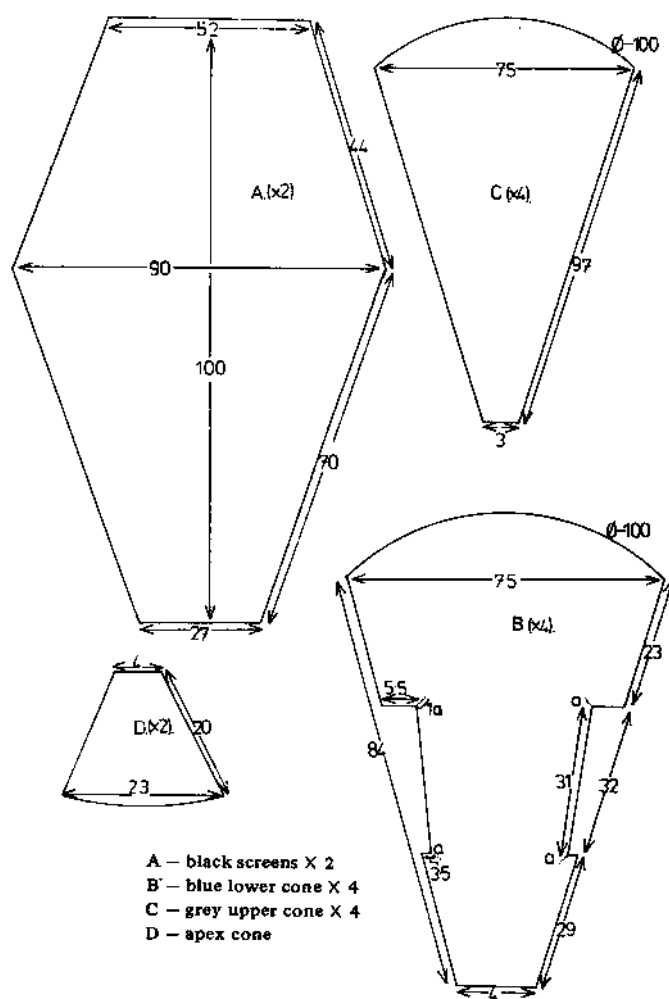


FIG. 5. Pattern for a biconical trap.

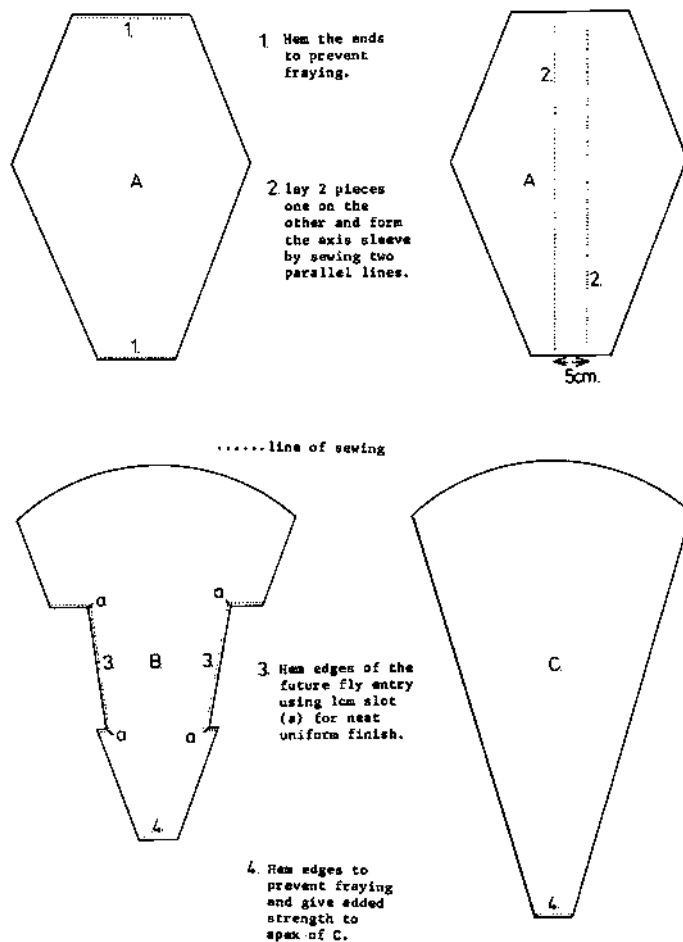


FIG.6. Preliminaries to assembly.

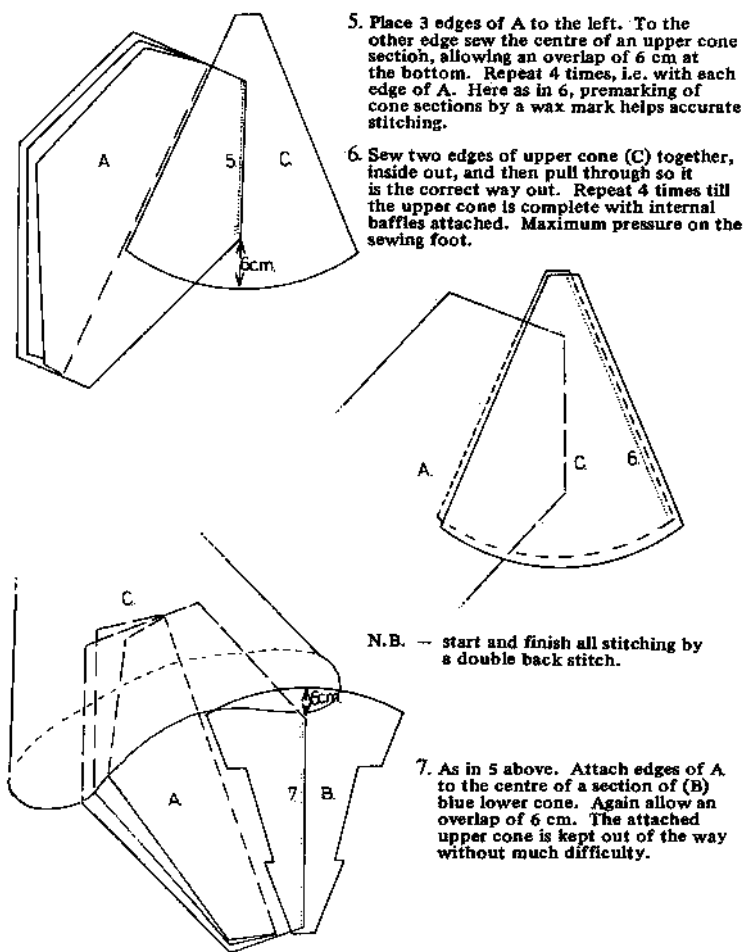


FIG. 7. Trap assembly.

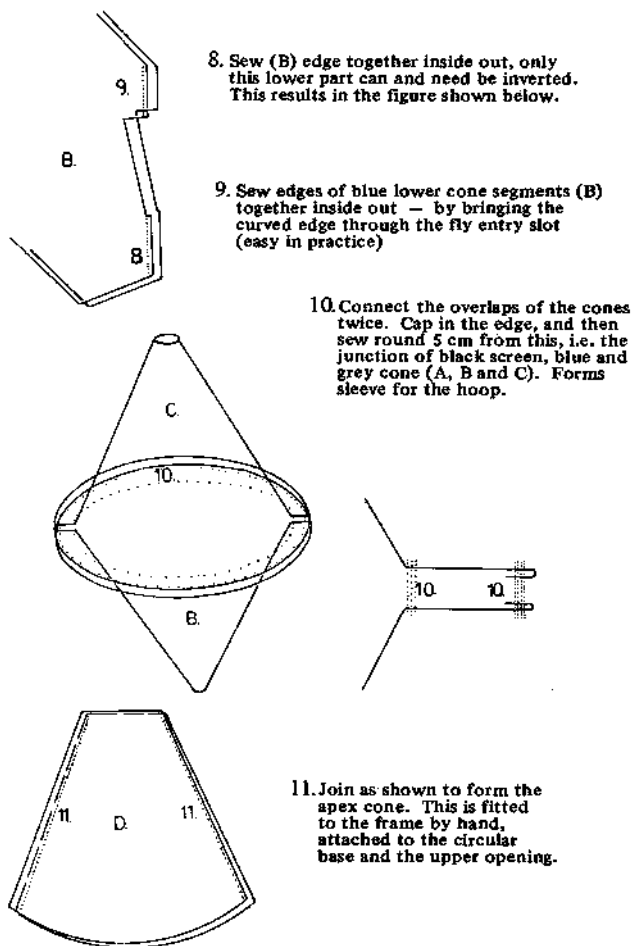


FIG.8. Trap assembly (continued).

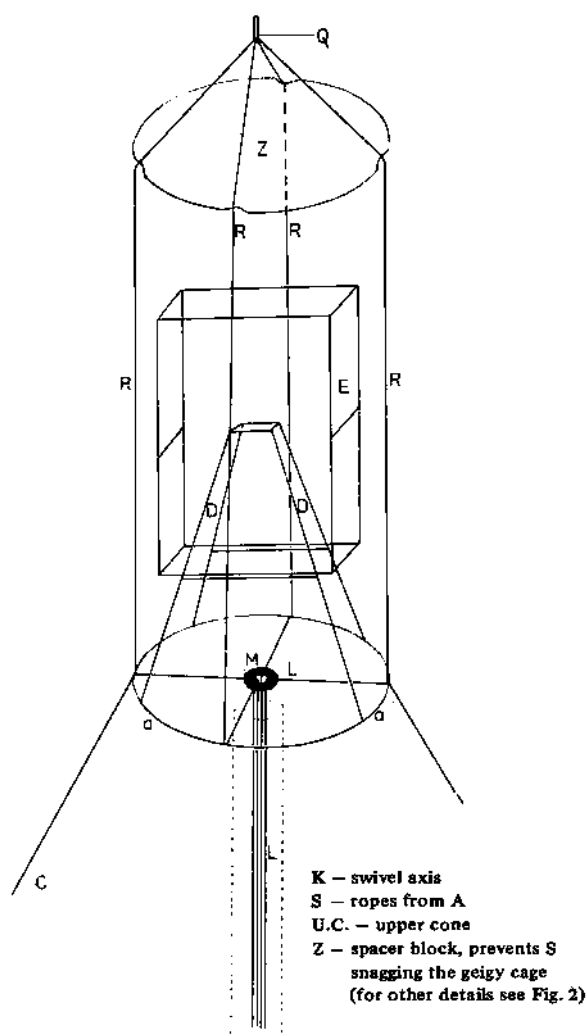


FIG.9. Means of suspending the biconical trap.

The upper cone (80 cm high) is made of robust netting, either white or grey, of a type that is UV stabilized and will not fray when torn. The apex of this cone is formed by the apex frame netting, and to allow for this the upper cone (C) finishes 10 cm before its true apex as a circular opening, diameter 8 cm. It is quicker and easier to make the upper cone (C) as a full cone and fit it directly to the apex frame (D). However, damage is likely to occur where the geigy cage (E) rests on apical supports (C) and too much repair work would make this area dark. Also, if one fits the upper cone to the apex opening (T) collapsibility is restricted. The lower cone (60 cm high) is made of cotton or polyester and cotton: best results are obtained using cloth dyed blue with a copper thalocyanine dye (Challier, personal communications). Four equally spaced openings with tapering rectangular shape (G) around the cone (large base 11 cm, small base 6 cm, length 35 cm) give entry to the fly via the lower cone and thus into the body of the trap. Internally, these screens (A) divide the body into four equal sections. These screens of black polyester/cotton are attached to each other centrally and to the cones by their opposite edges. A cylindrical slot is formed centrally by these screens to accommodate the aluminium axis (F). The pattern is shown in Fig.5 and Figs 6–8 show in annotated form how the trap is sewn together. The pattern (Fig.5) allows minimum cloth wastage and mass production using modern cutting techniques by a professional cutter (x). All sewing may be done on a domestic machine in one or two hours.

Part 4

The geigy cage (E), as shown in Figs 2–4, 9, rests on the apex (c). If the geigy cage is made cylindrical of 8 cm diameter with four struts, it is easier to make and is more stable on the cylindrical apical support (c). The geigy sleeve fits over the apex cone and is held by an elastic band or velcro (a). It must be removed every four hours to prevent excessive wind fray.

DISCUSSION

The weight of the trap is 1.66 kg (cloth 800 g, axis 380 g, plastic tube 224 g, apex and geigy 240 g, + 16 g). However, this weight can be improved by using lighter fabrics. The trap packs into a parcel of $15 \times 10^3 \text{ cm}^3$ and may be assembled ready for use or packed for storage by one person in under five minutes. When being moved in the field the pole is extracted and the cloth cones folded to the centre and carried under the arm. This design (Fig.2) has proven benefits over other traps specifically designed to catch tsetse, as it is lightweight, easily made and erected, and cheap and compact when packed [1]. This trap needs to be evaluated with regard to tabanids and other biting flies. It is an improvement on

both the Manitoba trap [7, 8], because it needs only one support pole, not a tripod, and the Canopy trap [9, 10], which requires guy ropes.

The trap described above differs from the original [1] in having a collapsible central axis (F), with a cylindrical, unpointed stake (K), and a plastic horizontal axis (S) which will coil. The apex (D) and geigy cage (E) are plastic coated to reduce snagging of the material. The upper cone is made of grey, PVC-coated netting, which is very robust and transparent.

The conical trap may also be suspended from a branch or tripod allowing more swaying movement (Fig.9) which may increase trap catches [11]. The materials for the trap and labour may be costed at about US \$40 but this could be considerably reduced by improvising, i.e. a coat-hanger or a lampshade may form the apex figure and geigy cage, a sapling or a scrap metal rod the central axis, water piping the horizontal axis and any available material and curtain net as the trap body.

Traps can be supplied ready to use if a reasonably large number are required; inquiries should be made to the authors.

ACKNOWLEDGEMENTS

We wish to thank B. Braneld at U.M.I.S.T. for help and advice and supply of cloth and dye. Several firms have been very understanding and undertaken very short runs. We are grateful to the Ministry of Overseas Development for financial support (Scheme No. R3425).

REFERENCES

- [1] CHALLIER, A., LAVEISSIERE, C., Cah. ORSTOM, Ser. Ent. Met. Parasitol. 11 (4) (1973) 251.
- [2] CHALLIER, A., ORSTOM Mem. 64 (1976) 294.
- [3] FREZIL, J.L., CARNEVALE, P., Cah. ORSTOM, Ser. Ent. Med. Parasitol. 14 (3) (1976) 225.
- [4] MOLYNEUX, D.H., BALDRY, D.A.T., de RAADT, P., LEE, C.W., HAMON, J., Ann. Soc. Belge Med. Trop. 58 (1978) 185.
- [5] CHALLIER, A., 'Trapping technology', Tsetse - the Future for Biological Methods in Integrated Control (MARSHALL - LAIRD, Ed.), IDRC 077e (1977) 109.
- [6] HARGROVE, J., Ecol. Entomol. 2 (1977) 123.
- [7] THORESTEINSON, A.H., BRACKEN, G.K., HANEC, W., Entomol. Exp. Appl. 8 (1965) 189.
- [8] THOMPSON, P.H., Ann. Entomol. Soc. Am. 62 (1) (1969) 50.
- [9] ROBERTS, R.H., Mosq. News 36 (4) (1976) 530.
- [10] CATTS, E.P., Mosq. News 30 (1970) 472.
- [11] LANGRIDGE, W.P., I.S.C.T.R.C., 14th Meeting, Dakar, OAU/STRC Publ. No. 109, Nairobi, (1975) 277.

MANUFACTURERS' ADDRESSES

- (i) A. Brunnschweiler (U.K.) Ltd, Newton Bank Print Works, Hyde, Cheshire, England.
- (ii) Bayer (U.K.) Ltd.
- (iii) J. Walton's, P.O. Box No. 1, Hollingworth, via Hyde, Glossop, Derbyshire, SK14 8NS, England.
- (iv) Fothergill and Harvey Ltd, Summit, Littleborough, Lancashire, OL1 9QP, England.
- (v) Blacks Outdoor Centre Ltd, 200 Deansgate, Manchester, England.
- (vi) J.C. Small and Tidmus Ltd, Perry Road, Sherwood, Nottingham, NG5 3AF, England.
- (vii) J.P. Coats (U.K.) Ltd, 12 Seedhill Road, Paisley, PA1 1JT, Scotland.
- (viii) Singer Co. (U.K.) Ltd, 15 Stanley Street, Manchester, England.
- (ix) Öecos Ltd, 130 High Street, Kimpton, Hertfordshire, England.
- (x) S. & G. Cutting Ltd, Peel Trading Estate, Silk Street, Salford 3, England.

DISCUSSION

L.H. OTIENO: It would be a good idea to consider incorporating some sort of gadget into your new trapping device that would scare off wild game, e.g. elephants and baboons, since these animals interfere a lot with traps.

D.H. MOLYNEUX: It is extremely difficult to construct a trap which cannot be interfered with.

TSETSE MOVEMENT IN WIND FIELDS

*Possible epidemiological and
entomological implications for
trypanosomiasis and its control**

D.H. MOLYNEUX

Department of Biology,
University of Salford,
Salford,
United Kingdom

D.A.T. BALDRY

UNDP/FAO Tsetse Research and
Training Project,
UNDP Regional Office for
SE Africa,
Lusaka, Zambia

C. FAIRHURST

Department of Biology,
University of Salford,
Salford,
United Kingdom

Abstract

TSETSE MOVEMENT IN WIND FIELDS: POSSIBLE EPIDEMIOLOGICAL AND ENTOMOLOGICAL IMPLICATIONS FOR TRYPANOSOMIASIS AND ITS CONTROL.

Evidence is presented that tsetse flies (*Glossina*) can be dispersed by wind. This dispersal in West Africa is suggested to be along a south-west north-east axis. The implications of wind dispersal of *Glossina* for chemical and genetic control operations are discussed. Field experiments necessary to test this hypothesis are recommended. A study of human trypanosomiasis foci in West Africa has revealed that foci are orientated in roughly parallel lines in a south-west north-east direction. This directionality was significant when compared with seven other compass points. It is proposed that foci could be populated by infected flies dispersed from the south west, where denser populations exist, on the prevailing winds in the late dry/early rainy season. The significance of these ideas in relation to the epidemiology of *Trypanosoma brucei rhodesiense* in Ethiopia and *T. evansi* are discussed.

* The abstract only is printed here since the paper has been published in *Acta Tropica* 36 (1979) 53.

DISCUSSION

W.J. KLOFT: Your observations on long-distance wind-borne migration of tsetse flies were of special interest to me. We did some work in 1977 on the introduction of the Old World screw-worm *Chrysomya bezziana* (Bezzi's blowfly) into the Arabian Gulf States (W.J. & E.S. Kloft, unpubl.). These countries bring vessels each containing 2000 live sheep from Australia and thereby introduce this livestock pest. We have been able to prove this from many examinations of infested sheep; the development of the fly larvae introduced showed that infestation had occurred during the journey. The Australian authorities claim that Australia is free of the pest, and we agree with them. The vessels pass a certain distance from infected areas (Indonesia, India) and may well act as giant traps attracting the flies, perhaps even over long distances. These comments illustrate the fact that much more attention should be devoted to long-distance migration caused by meteorological factors such as anabatic winds.

D.J. ROGERS: A few years ago I reviewed many published examples of the advances of tsetse fly belts throughout Africa (J. Anim. Ecol. 46 (1977) 309). The average daily rate of fly movement was quite small (approximately 252 m per day) compared with the sort of distances mentioned by Mr. Molyneux. Thus it seems that the majority of any tsetse fly population migrates or disperses at a rather slow rate, although I agree that even a small portion migrating vast distances may be epidemiologically very important.

What becomes rather curious in the light of Mr. Molyneux' evidence is the rather stable pan-African distribution of many species. Fly belts expand and contract at relatively slow rates over periods of several decades (e.g. *G. morsitans* in Zambia). Thus I wonder what is the long-term fate of those individuals that migrate large distances beyond the species' local distribution. Certainly, migration rates of many insects are continuously being revised upwards.

D.H. MOLYNEUX: I would agree with these observations. It is perhaps only a part of the population which is dispersed by wind. I also believe that, at the limits of their natural distribution or beyond them, these flies may not be capable of establishing permanent populations. However, as you suggest, they may still be epidemiologically very important.

H. WETZEL: Do you believe that changes taking place in the flies' habitat may force them to migrate further than they would have done had the biotope remained unchanged?

D.H. MOLYNEUX: I don't think that with our present knowledge this question can be answered.

D. CUISANCE: I do not believe that the slight modifications of the biotope which occur in our forest galleries have induced the tsetse to disperse over large distances. We know that a fraction of the wild population naturally tends to

move over quite large distances, since tsetse have passed from one gallery to another without the first having been modified at all.

K.H. POLITZAR: In our work about 500 000 flies have been marked altogether; given the numbers released and what we know about the flies' longevity, we estimate that about 5000 sterile males have usually been present in the area at the same time. Every time surveys have been carried out outside the project area, one or several sterile males have been found. This indicates that some movement both along rivers and across river systems separated by savannah occurs.

D.A. DAME: In eradication programmes such as those mounted against *Simulium* in West Africa and *Cochliomyia* in the south-western United States of America and Mexico, fly migration has turned out to be much more extensive than previously thought. Therefore, where *Glossina* is concerned, very high priority should be given to studies of the extent of fly migration, not only in areas with special meteorological conditions but also in those where large-area eradication programmes are contemplated.

D.H. MOLYNEUX: In the Tanga programme, was migration influenced by wind?

D.A. DAME: At Mkwaja Ranch the immigration of tsetse from outside the test plot seems to have been associated more with animal and human activities than with meteorological conditions.

R. GALUN: *G. morsitans* is known to be active only 10–15 minutes per day. Is there any information on other *Glossina* species?

D.H. MOLYNEUX: I cannot tell you whether any similar information is available for other species. However, riverine flies disperse away from the gallery forest when humidity rises at the end of the dry season and move to the top of the canopy at night. Both these activities would indicate that they are susceptible to wind movement.

DYNAMICS AND BEHAVIOUR OF TSETSE POPULATIONS

D.J. ROGERS

Hope Department of Entomology,
University Museum,
Oxford,
United Kingdom

Abstract

DYNAMICS AND BEHAVIOUR OF TSETSE POPULATIONS.

A scientific rather than empirical approach to the pest problem requires that we understand the natural limits to an animal's distribution and abundance before we attempt to manipulate them. Distribution and abundance are defined in terms of natural fertility levels and natural levels of abiotic, density-independent mortalities. These birth and death processes interact with the biotic, density-dependent mortalities, which must operate at some time during the animal's life-cycle if an equilibrium is to be achieved. The aims and effects of applying pest control measures can all be described within the context of these birth and death processes. Theoretical, analytical and experimental approaches have been used to provide information on the natural levels of fertility, and abiotic and biotic mortalities for tsetse flies. The resultant predictions of the bioclimatic limits of one species, *Glossina morsitans*, correspond well with the present known pan-African distribution of the species, and may explain the greater ease with which tsetse are controlled at the edge of their distribution, e.g. Northern Nigeria and southern Africa, compared with the more central areas where bioclimatic conditions are more favourable, e.g. Zambia. Further analytical and experimental work requires quantification of the biases of the various sampling methods for tsetse, so that relative population estimates can be turned into absolute estimates. The feeding cycle for male tsetse and the pregnancy cycle for females, derived from fat and haematin reserves of field-caught flies, are useful frameworks for investigating the field behaviour of tsetse and hence the sampling biases of different capture methods.

INTRODUCTION

Pest control is an attempt to limit an animal's distribution by reducing its abundance, ultimately to zero. Most examples of successful partial control have generally occurred near the natural limits of the species' distribution - in the case of the tsetse fly these are in Northern Nigeria [1] and southern Africa [2]. Within the central area of the species' distribution the same control measures are not as successful for various reasons.

A scientific rather than empirical approach to the pest problem requires that we understand the natural limits to an animal's distribution and abundance before we attempt to manipulate them. This is emphasized by the fact that present empirical methods of pest control are often not as effective as we might expect on the basis of laboratory

experiments. Two quite different explanations are proposed for this failure:

1) A failure of technology: clearly many organisational and technical skills are required to carry out a successful spray programme. If the insecticide ends up in the wrong place at the wrong time, or is emitted in an incorrect droplet size, an obvious explanation for any failure to achieve the desired population reduction is available.

2) A failure to understand the natural ecology of the target species: species are in a state of dynamic balance, and equilibrium densities are maintained over a wide range of changes in natural and applied mortalities. This is the ecological reason why even a perfectly run spray programme may produce the undesirable 'side effect' of increasing population size.

Since either explanation alone can account for a failure to achieve population reduction we tend to find that some scientists are pre-disposed (by their training and experience) to accept the first explanation, and others to accept the second, whilst the truth probably lies somewhere in between. This paper examines how the second approach can contribute to more rational population management.

POPULATION PROCESSES

Natural populations experience birth and death processes that are in exact balance at the equilibrium density. The only way in which such an exact balance can be maintained through time is for at least one of these processes to be directly related to density. For convenience in the following discussion we shall assume that this balance is maintained by constant fertility and density-related mortality, although constant mortality and density-related fertility would have the same effect. Similarly, for convenience we shall ignore population movement which has the same effect on local population density changes as fertility (immigration) or mortality (emigration), although this simplifying assumption may not be acceptable on a larger scale [3].

Density-dependent processes are always mediated by biotic factors and generally involve competition, parasitism and predation. The severity of each of these factors is usually reduced at low population density and so allows the population to increase again, and vice versa. Superimposed on these biotic factors are those caused by weather, which, because they operate independently of density, have a disturbing effect on it and are usually called abiotic.

With these two types of processes in mind we can now delimit both an animal's distribution and its abundance. At the edge of a species' distribution density will be low and density-dependent, biotic processes virtually absent. The population will only exist if, on an annual basis, the total fertility just exceeds the total abiotic mortality, which is density independent. Thus the distribution can be defined as those places in which:

$$\sum_{n=1}^{\infty} \log f_n \geq \sum_{n=1}^{\infty} d.i.m._n$$

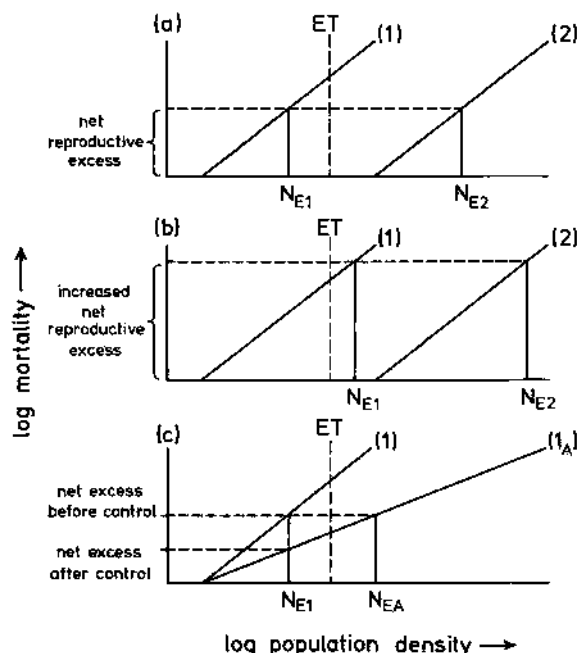


FIG.1. A graphical representation of the basic population processes.

- (a) The interaction of the net reproductive excess with two different density-dependent mortalities (1) and (2) to give two different equilibrium densities (N_{E1} and N_{E2}). ET = arbitrary economic threshold.
- (b) The effect of increased net reproductive excess in a more favourable environment.
- (c) The effect of applying a pesticide that reduces the strength of the density-dependent mortality from (1) and (1_A), resulting in a higher equilibrium density (N_{EA}). The log net reproductive excess must be reduced to half its previous level in order to maintain the status quo (i.e. equilibrium density at (N_{E1})).

where f is the rate of increase and d.i.m. is the abiotic mortality (on a log. scale) for each month, n , of the year.

An animal's abundance is some function of the difference between these two quantities, i.e.

$$\text{function} \left(\sum \log f_n - \sum \text{d.i.m.}_n \right)$$

with symbols as before. This expression represents the annual net reproductive excess. Just how it fixes equilibrium density is determined by its interaction with the density-dependent mortality that must operate sometime in the life-cycle. This is shown in Fig. 1a where two density-related mortalities are shown with different intercepts on the x-axis. The same net reproductive excess interacts with the two mortalities to give two quite different equilibria (N_{E1} and N_{E2}) (where the equilibrium is achieved when fertility = mortality). This figure

shows how the threshold level of a density-related response may be crucial in determining an insect's pest status. In the one case the insect's equilibrium density is above an arbitrary economic threshold (ET) and hence the insect is a pest; in the other case it is not.

Let us look again at the net reproductive excess. In an environment in which the density-independent mortality is less severe, the net excess is greater and Fig. 1b shows how both populations are now above the ET. In this case the relative importance of the density-dependent processes vis à vis density independent processes is increased.

If we revert to the first case, with the lower net excess, we may find that many pest control measures reduce the strength of the density-dependent relationship (e.g. pesticides killing off causative agents such as parasites and predators), with the result shown in Fig. 1c. Because the density-dependent mortality is lower, the balance between it and fertility is achieved at a higher equilibrium density (N_{EA}). Usually, of course, control measures also increase the effective density-independent mortality operating on the population, and therefore reduce the net reproductive excess. But in the present example they would have to reduce the logarithmic net excess to about one half its previous value (i.e. reduce the net excess to its square root value) just to maintain the status quo. In order to reduce the population they would have to reduce the net excess still further, a quite considerable reduction.

Graphs such as those in Fig. 1 can explain all of the known effects of applying pesticides to natural environments. The terms 'secondary pest' or 'side effects' of pesticides are simply euphemisms applied by those who do not understand the natural workings of complete ecosystems rather than of single species in them. Using such graphs we can prepare a list of what we need to know if we are to make pest control more scientific and less empirical than heretofore. This list includes as a minimum:

- 1) Natural fertility rates throughout the year;
- 2) Natural levels of abiotic mortalities;
- 3) Natural levels and degrees of density-related mortalities and their causes;
- 4) The likely effects on 1) and 3) of any proposed control measures, be it pesticides or sterile male, whose aim is effectively to increase 2).

TSETSE POPULATION DYNAMICS

A variety of approaches has been used to provide information on each of the first three of the above list for tsetse flies:

- 1) Natural fertility rates - a theoretical approach.

The temperature dependencies of the tsetse's interlarval and developmental periods given by Glasgow [4] allow us to construct a simple model of tsetse population growth that fixes the maximum rates of increase (Fig. 2). No apologies are given for the purely theoretical

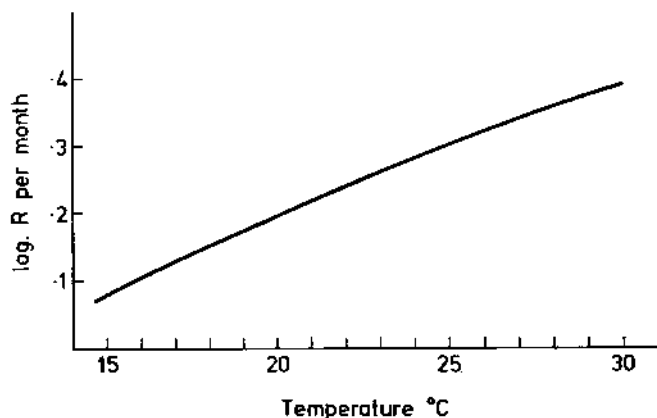


FIG.2. Temperature dependence of the rate of increase of a tsetse population in the absence of any mortality.

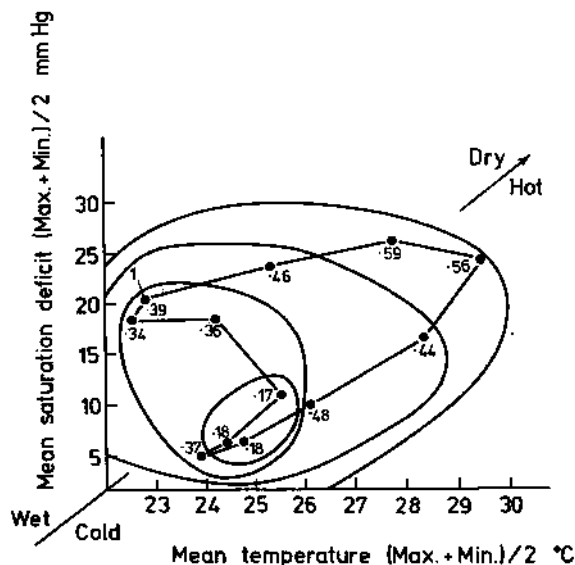


FIG.3. The irregular polyhedron is the annual climogram for Bauchi, Northern Nigeria (1 = January and the other months follow round in a clockwise sequence). To each month has been assigned the value of the density-independent mortality for *G.m. submorsitans* and contours have been drawn to enclose months of approximately equal mortality. The environmental optimum for the subspecies is within the smallest contour.

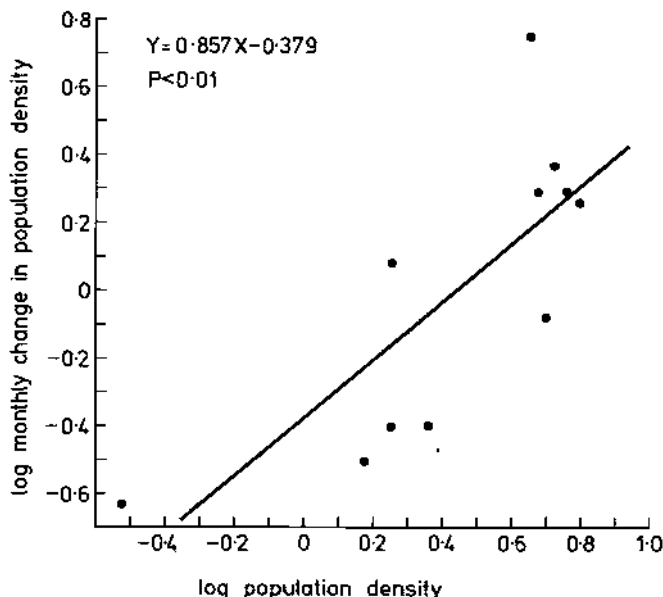


FIG.4. Relationship between log monthly change in population size and log population density for female *G.m. submorsitans* in Northern Nigeria; e.g. change from May to June in eleven consecutive years (1964-74). $Y = 0.857X - 0.379$, $p < 0.01$.

attack on this much studied problem - ovarian ageing only determines a population's actual potential, not its maximum potential in which we are presently interested.

2) Natural abiotic mortality rates - an analytical approach.

An analytical method to appear later this year [5] allows us to estimate levels of natural weather-induced mortalities for tsetse on the basis of fly-round data. Although such a method of sampling an haematophagous insect is prone to severe and hitherto unquantified sampling biases (but see [6], [7], and [8]), the results (only some of which we can look at here) can help us to understand the problems of tsetse control in different parts of Africa. In Fig. 3 the irregular polyhedron represents the annual climogram for the Sudan vegetation zone of Northern Nigeria. Against each month's point has been written the estimated density-independent mortality for that month. The smooth curves are estimated mortality contours, enclosing months of approximately equal mortality. The environmental optimum is within the smallest contour and mortality increases at varying rates as conditions depart from this optimum: We now have such mortality contours for two sub-species of *Glossina morsitans* and for *G. palpalis palpalis*.

For *G. morsitans* we can combine estimates of 1) and 2) to predict the possible bioclimatic limits of the species. When this was done [5]

it was found that 94% (n=33) of sites within the present known pan-African distribution of the species [9] fall within these predicted limits, whereas 50% (n=58) of sites an average of less than 200 km away from the present distribution fall outside them. This provides corroborative support for both the theoretical and analytical approaches.

3) Natural biotic mortality rates - an experimental approach.

Laboratory-produced puparia of *G. fuscipes* buried under fairly natural conditions in the field at Lugala, Uganda, showed a significant density-dependent mortality over 2-week periods. The major cause of this mortality seemed to be predation by ants [10]. Although such semi-natural experiments can be criticised in a number of respects, they clearly demonstrate that abundant natural predators do take a larger proportion of high density rather than low density puparial populations. Little work has been done on quantifying predation of the adult tsetse, but this too may be density related [10].

An alternative approach for investigating biotic mortalities is the analytical one. Fly-round records can be examined to find the relationship between the logarithmic change in population size and population density. One example is shown in Fig. 4. The slope of the relationship is high, indicating great sensitivity of the mortality to changing density and therefore great resistance to population change brought about by any external agency. If an externally applied mortality simply reduces density within the range of operation of this natural density-dependent mortality, then the latter relaxes its severity to compensate for that change. Thus insecticides that kill off, say, 90% of a population of insects in a cage in the laboratory will not reduce populations in the field by 90% however perfect the application technique may be. Similar considerations must be applied to sterile male programmes.

4) The interaction of control measure and natural mortalities.

By drawing together these three facets of tsetse population dynamics, we can predict that the nearer the tsetse is to its abiotic optimum, the more important will be density-dependent mortalities and therefore the more difficult will be control measures. This is tentatively borne out by the two study areas that gave us the bioclimatic limits for *G. morsitans*. Northern Nigeria is very near to the predicted natural limits and hence insecticidal control is relatively easy. Zambia is much closer to the identified optimum for its local sub-species and here the same levels of insecticidal control as are used in Nigeria are totally ineffective. Dose rates have to be increased quite significantly to achieve the same end result.

THE IMPORTANCE OF A GOOD SAMPLING PROGRAMME

We can do little towards gaining the sort of information we require for analytical and experimental methods without an efficient sampling programme. We should not be so concerned that fly-round capture methods are biased; very few trapping techniques provide random samples [11]. What we should be doing is trying to establish in what way and to what extent traditional and new trapping methods exhibit such biases, and how they change on a seasonal basis. This will enable us to turn relative estimates of population size into absolute estimates.

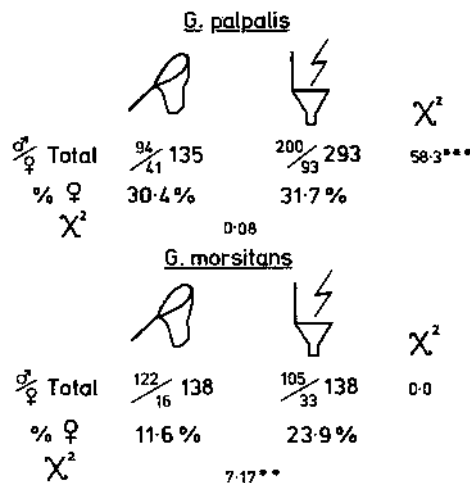


FIG. 5. Results of simultaneous comparisons of hand-net and electric back-pack trapping of *G. palpalis* in Nigeria and *G. morsitans* in Zambia.

It is of course difficult or impossible to establish the bias of any capture method (e.g. hand nets) without reference to another method of less or different bias. Here I believe electric trapping methods [12], [13] can be immensely useful. Fig. 5 shows the results of simultaneous comparisons of hand-net and electric back-pack trapping of *G. morsitans* in Zambia and *G. palpalis* in Nigeria. For *G. morsitans* the two methods return the same total catch of flies, but the electric method catches a significantly higher female percentage. For *G. palpalis* the sex ratio remains the same, but the electric trap catches, on average, more than twice as many flies. These results indicate that the catch of any particular capture method is some function of both fly behaviour and fly abundance and/or persistence around the bait.

The sex ratio alone is not particularly meaningful for assessing the sampling bias of capture methods as it denotes the relative ability of capture devices to catch both male and female tsetse, either one of which or both may vary to give a different percentage female catch. Currently we are investigating the underlying mechanisms of sampling biases, in terms of the field behaviour of flies in relation to capture devices [7], [8]. By relating fat and haematin reserves of field-caught flies to a relative or absolute time scale, we can establish graphs which represent the feeding cycle in the case of males and the pregnancy cycle in the case of females. Associated with these repeated cycles are changes in the field behaviour of flies, and the varying capture rate of flies by different devices during the cycles is proving to be very useful in establishing quantitative estimates of sampling biases.

Thus through a closer look at old and new capture methods we hope to be able to measure changes in absolute tsetse population density.

Such information can then provide a sound basis for theoretical, analytical and experimental attacks on the problem of natural population regulation and the effects on it of proposed control measures.

CONCLUSIONS

Finally there is one aspect of all this work which is often neglected. Projects succeed or fail on the basis of the people involved. All the equipment in Africa is useless if applied incorrectly and with little forethought of the scientific problems involved. We do not need people who know all the answers, we need people who know how to ask the right questions.

REFERENCES

- [1] DAVIES, H., J. Appl. Ecol. 8 (1971) 563.
- [2] TOIT, R. du, KLUGE, E.B., FIEDLER, G.D.H., "The eradication of *G. pallidipes* from Zululand by chemical means" (Proc. 5th meeting Int. Sci. Comm. Tryp. Res., Comm. Tech. Coop. S. Sahara, Pretoria, 1954), Publ. No. 206, 141 (1954).
- [3] TAYLOR, L.R., TAYLOR, R.A.J., Nature (London) 265 (1977) 415.
- [4] GLASGOW, J.P., The Distribution and Abundance of Tsetse, Pergamon, Oxford (1963).
- [5] ROGERS, D.J., J. Anim. Ecol. (in press).
- [6] ROGERS, D.J., RANDOLPH, S.E., Bull. Entomol. Res. 68 (1978) 283.
- [7] ROGERS, D.J., RANDOLPH, S.E., Bull. Entomol. Res. 68 (1978) 639.
- [8] RANDOLPH, S.E., ROGERS, D.J., Bull. Entomol. Res. 68 (1978) 655.
- [9] FORD, J., KATONDO, K.M., The Distribution of Tsetse Flies (*Glossina*) in Africa 1973, Organisation of African Unity, Scientific and Technical Research Commission, Cook, Hammond & Kell Ltd, London (1977).
- [10] ROGERS, D.J., Ecology of *Glossina*: Natural regulation and movement of tsetse fly populations, Revue d'élevage et de médecine vétérinaire des pays tropicaux (Supplement: Les moyens de lutte contre les trypanosomes et leur vecteurs; Actes du Colloque, Paris, March 1974) (1975) 35.
- [11] SOUTHWOOD, T.R.E., Ecological Methods with Particular Reference to the Study of Insect Populations, Chapman Hall, London (1978).
- [12] VALE, G.A., Bull. Entomol. Res. 64 (1974) 199.
- [13] ROGERS, D.J., SMITH, D.T., Bull. Entomol. Res. 67 (1977) 153.

DISCUSSION

G. HASIBEDER: Great Britain is one of the countries in which important biomathematical research has been done in recent years, and there are famous biomathematicians working in Oxford. Do you work together with biomathematicians?

D.J. ROGERS: Yes, where necessary, although at the moment I believe that biologists should always try to do their own statistics and build their own population models. Models are only as good as the field information on which they are based.

G. HASIBEDER: Do you know of any theoretical mathematical models which are used in pest management?

D.J. ROGERS: These are very many, the most useful coming from the group at Selwood Park (Imperial College) in the United Kingdom. For useful references see Conway's paper in *Theoretical Ecology* (R.M. May, Ed., Blackwell Scientific Publications).

G. HASIBEDER: Do you think that common mathematical models will be developed in the next few years, which will be possible to apply to several different insects simply by inserting different parameter values?

D.J. ROGERS: Each species is unique in many respects. Nevertheless the first three processes I mentioned in the paper must apply to all species, including our own. Thus ecologists can suggest an overall approach, a methodology, but not, I think, a basic model in which we just change parameter values.

L.H. OTIENO (*Chairman*): Early this year there was a lot of rain in Kenya, and the Lambwe valley (West Kenya), the home of *G. pallidipes*, was completely flooded. At this time hardly any flies were being caught by Langridge traps, but soon after the rains (1–2 months) there was an influx of young *G. pallidipes*. Have you any idea how these flies survived the rough weather?

D.J. ROGERS: One should try to establish whether they survived the conditions you mention, or simply escaped them elsewhere and migrated into your experimental area later. This could depend on how close the nearest flooded area suitable for puparial survival was.

FIELD STUDIES WITH IRRADIATED
Glossina morsitans morsitans
 Westwood (Diptera: Glossinidae)
FROM A COLONY MAINTAINED ON
A MEMBRANE FEEDING REGIMEN*

M. WEISS, W. TAKKEN
 Joint FAO/IAEA Division of
 Atomic Energy in Food and Agriculture,
 International Atomic Energy Agency,
 Vienna

Abstract

FIELD STUDIES WITH IRRADIATED *Glossina morsitans morsitans* Westwood (Diptera: Glossinidae) FROM A COLONY MAINTAINED ON A MEMBRANE FEEDING REGIMEN.

In a field study, carried out at Mkwaja Ranch, Tanzania, the behavioural pattern of membrane-reared *Glossina morsitans morsitans* Westwood males and females (Seibersdorf flies) was compared with that of animal-host reared flies (Tanga flies). The flies were irradiated in the late pupal stage in a saturated nitrogen atmosphere. A total of 4167 Seibersdorf and 4065 Tanga males and females were hand-marked with non-toxic oil paint and released in daily batches over a 25-day period. Flies were recaptured chiefly by applying the fly-round method. Recapture began two days after the first release and ended 30 days after the last release. The recapture rates were 5.66% for Seibersdorf and 4.50% for Tanga flies. Within the catches, only 57% as many Tanga females were recaptured compared with Seibersdorf females, but the Tanga females had a higher insemination rate. The age distribution of recaptured flies of both groups was almost identical (males compared with males, females with females). Dispersal rates of the two populations from the release site were also equal. This indicated that there was no difference in the longevity and dispersal behaviour between membrane- and animal-host-reared *G. m. morsitans*.

DISCUSSION

T.G.T. JAENSON: The insemination in the recaptured Seibersdorf females reached 100% at a slower rate than in the Tanga females. The numbers recaptured and dissected may be small — but if the difference is a real one, do you think that it is due to a slower development of sexual maturation in the Seibersdorf females? And is it maybe in some way related to the higher proportion of Seibersdorf females recaptured compared with the Tanga females?

* The abstract only is printed here since the paper is to be published in the Bulletin of Entomological Research.

M. WEISS: It does not seem probable to me that the process of sexual maturation takes significantly longer in one of the two strains than in the other. If there was such a difference, it would also have been observed in the laboratory. There may be relationship between the insemination rates and the availabilities of Seibersdorf and Tanga females. However, it is difficult to be sure about this.

H. WETZEL: I should think that you would tend to overestimate the difference in insemination time in view of the fact that the number of females caught was so small.

M. WEISS: It is true that the data are based on a relatively small number of recaptured females. We do not overestimate the difference in time, however, as insemination in both strains turns out to be a function of female age. We want to make it clear that there is a trend which indicates such a difference, even though we cannot evaluate this finding in terms of female behaviour.

A.M. VAN DER VLOEDT: During your dissections for evaluating the insemination rate among recaptured females, did you observe different pregnancy stages in utero? Assuming that some released females were inseminated by sterile males from one of the release batches, one would expect some females to have failed to develop a normal pregnancy between 15 and 20 days following release. If you did find different stages, this would provide direct evidence that the released males are effective mates.

Your satisfactory insemination rate in the released females is interesting. This proves that the irradiation of female pupae does not influence the adult female's receptivity or willingness to mate.

M. WEISS: We did not observe any pregnancy stage in the recaptured females. It is clear that since the females were sterile they could not have developed a pregnancy. Some pregnant females were found among the catches, but these were wild females which were not taken into account.

FEEDING AND REARING BEHAVIOUR IN TSETSE FLIES

L.H. OTIENO, Y. YOUDEOWEI*

International Centre of Insect
Physiology and Ecology (ICIPE),
Nairobi,
Kenya

Abstract

FEEDING AND REARING BEHAVIOUR IN TSETSE FLIES.

Batwing membrane was used to study salivation and feeding behaviour of tsetse flies. Probing and salivation were observed to be stimulated by tarsal contact with the membrane. Salivation and feeding responses varied from day to day with characteristic alternating high and low responses. The feeding process was invariably accompanied by a resting period. Attempts to rear *G. morsitans* artificially through the use of batwing membrane showed that the flies needed an initial adjustment period to in vitro maintenance.

INTRODUCTION

The transmission of human and animal trypanosomiasis is dependent upon a great number of biological and ecological factors involving the vertebrate hosts, the tsetse fly vector and the causative agent of the disease, the trypanosome organism. Among these factors, feeding behaviour is one of the crucial components in effecting trypanosome transmission. The feeding behaviour of tsetse flies in particular has therefore attracted the attention of workers since the early part of this century when Rodhain and co-workers (1912), quoted by Yorke and Blacklock [1], showed that tsetse could suck up citrated blood through a membrane from a freshly removed mouse skin. They [1] later made use of the technique to show that *Glossina palpalis* could feed avidly through rat's skin on fresh defibrinated blood and also suspensions of washed red blood cells in normal saline. They considered that the ingredient in the blood which was so attractive to the flies was the haemoglobin, although commercial preparations of this compound hardly induced any engorgement. They also found that the tsetse will feed through other membranes such as sheep bladder, peritoneal tissues and membrane composed of a thin rubber sheet, although only one fly fed successfully on the latter.

* Research associate from the University of Ibadan, Nigeria.

Since these pioneering efforts at artificial feeding of *Glossina*, a need has arisen in recent years to explore in greater detail the feeding behaviour of tsetse, in an attempt to mass rear the fly for experimental work and also for possible use of the sterile insect technique. The in vitro feeding technique which involves the use of suitable membranes has recently received added impetus [2–4] in the search for methods for mass rearing. Rice and co-workers [5] observed the powerful attractiveness of bat's wing membrane to tsetse, and ascribed it to its rough surface, dark colour, internal texture, chemical composition and thickness. Youdeowei [6] later developed a method for routine examination of tsetse salivation behaviour using the batwing membrane.

In this communication, a brief survey of feeding behaviour, mainly through salivation of some species of *Glossina*, is described. A note on the possible attempt to rear *G. morsitans* through feeding on batwing membrane is also described.

MATERIALS AND METHODS

Tsetse flies

G. pallidipes were collected from the Lambwe Valley, South Nyanza, Western Kenya.

G. morsitans and *G. austeni* were obtained from the International Centre of Insect Physiology and Ecology (ICIPE) insectary.

G. brevipalpis were collected from Kibwezi forest, Machakos district, Kenya.

Bats

Rousettus aegyptiacus and *Hipposideros commersoni* were collected from the Similani caves, near Mombasa, on the Kenya coast.

Otomops martiensseni were collected from the Suswa Hills in the Rift Valley, central Kenya.

Salivation of tsetse flies

Flies were kept singly in plastic tubes measuring 4.2 cm X 3.2 cm, covered at both ends by nylon gauze. The salivary secretions were obtained from individual flies by allowing them to probe through a membrane of a batwing using the method described by Youdeowei [6].

Rearing of *G. morsitans* in the laboratory

The general methods used in our laboratory for the maintenance of *G. morsitans* follow closely the method described by Mews and co-workers [7] with minor modifications. The flies are kept in a room maintained at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and about 60–80% relative humidity. The room is fitted with a tungsten switch which regulates light intensity on a 12/12-hour cycle.

Two-day-old females that have recently been fed are mated with males seven or more days old that have not been fed within the last six hours. Twenty-four males are placed in each cage of 20 females. Two days later all males are removed. The mated females are then separated into groups of ten and kept in polyvinyl chloride (PVC) cages. A total of six such cages containing mated females are kept in a 30 cm X 40 cm tray.

The larvae deposited are kept in the breeding box. Ten days before the date of emergence, the pupae are placed in an emergence box. Usually mated females are kept and recorded up to the ninth A.G.P. after which they are discarded. The flies are fed daily on rabbit ears.

RESULTS

Salivation process

The process of probing and salivation was viewed under a binocular stereoscope. It was observed that when the tarsi of the fly made contact with the membrane, the haustellum shot out from its normal resting position between the palps, and the labellum was used to cut the membrane with a rasping action. A small quantity of saliva was then released, probably to moisten the membrane. After a few seconds of probing attempts, the membrane was pierced and the haustellum projected through the membrane. Once the haustellum had pierced the membrane there was a copious flow of saliva on the tip of the haustellum which was deposited on the surface of the membrane. This process was the same for all flies tested. Flies responded equally well to the various membranes used (membranes from different bats).

Salivation in flies fed daily

Twenty mated female *G. morsitans* were isolated in single tubes and tested daily for salivation. After salivation they were fed on rabbit ears. The number of flies salivating and feeding was recorded. The results are presented in Fig. 1. From this it may be seen that when flies were fed daily, the pattern of salivation showed peaks followed by troughs. This indicated that the tendency to salivate

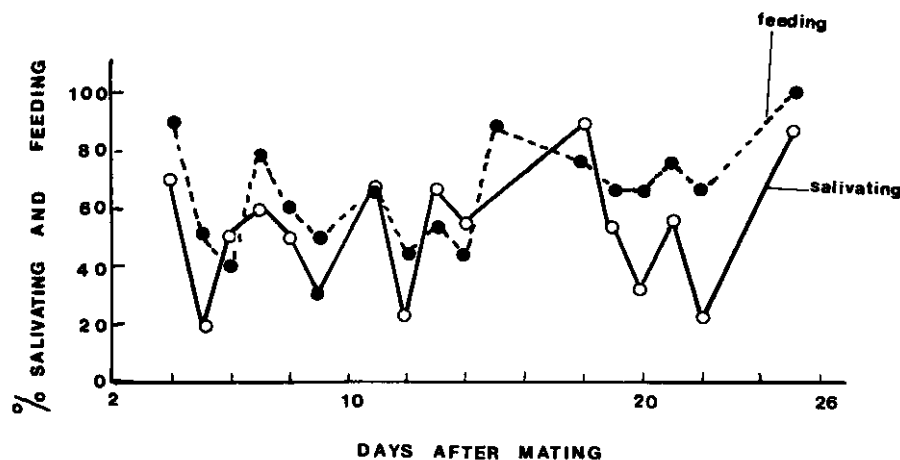


FIG. 1. Daily salivation and feeding patterns in female *G. morsitans*.

varied from day to day. A high degree of salivation was followed by a low degree of salivation. The feeding pattern followed the same trend as the salivation.

Feeding process in three species of *Glossina*

An electronic device described by Margalit and co-workers [8] was used to study the feeding process in *G. morsitans*, *G. austeni* and *G. pallidipes*. In this apparatus, when the fly probes and makes contact with the mouse skin, an electrical circuit is completed and variations in the current flow are recorded on a strip chart recorder. Using this device it was possible to show that three phases of feeding process occurred:

- (1) Probing, piercing and initial salivation (PPS).
- (2) Feeding (F).
- (3) Rest (R).

In most instances the sequence was repeated but in other instances it was carried out once only when the fly was observed to be fully engorged with blood. It was, however, possible to measure the time taken for the various phases of the feeding process. Table I illustrates typical records of the duration of these phases in three species of tsetse flies when rest periods intervened between engorgement sessions.

Periods of rest lasting from between 0.4 and 1.7 s occurred between feeding. Probing, piercing and salivation took a very short time – 0.2 to 8.6 s. The engorgement period varied enormously, from 3.3 to 67.9 s.

TABLE I. FEEDING PATTERNS OF THREE SPECIES OF TSETSE FLIES ON A MOUSE

Species	Duration of events (seconds) ^a		
	PPS	F	R
<i>G. austeni</i>	8.6	0.0	1.4
	3.2	0.0	0.4
	4.0	33.5	1.3
	7.6	57.5	—
<i>G. morsitans</i>	4.8	67.9	0.5
	1.2	0.0	0.4
	2.1	0.0	0.6
	2.1	0.0	0.4
	5.5	9.3	0.5
	0.0	16.0	0.5
	2.2	0.0	1.5
	4.9	18.2	0.3
	3.4	59.9	—
	1.0	0.0	1.0
<i>G. pallidipes</i>	3.0	12.3	1.5
	0.2	3.3	0.7
	1.9	3.5	0.7
	2.8	5.6	1.7

^a PPS = Probing, piercing, salivation;

F = Feeding or engorgement;

R = Rest.

Rearing *G. morsitans* using batwing membrane for feeding

Young male and female *G. morsitans* were fed on a rabbit for three days and, beginning on the fourth day, they were fed artificially through *Rousettus aegyptiacus* wing membrane on defibrinated bovine blood. A record was kept of the number of flies surviving for a period of one month. The survival of membrane-fed flies was compared with that of stock laboratory colony (maintained on rabbits). Table II shows the mortality rate observed both in the membrane-fed

TABLE II. COMPARISON OF THE PERFORMANCE OF *G. morsitans* MAINTAINED ARTIFICIALLY ON BOVINE BLOOD (USING BATWING MEMBRANE) WITH THOSE MAINTAINED ON RABBIT (STOCK COLONY) DURING THE SAME PERIOD

Days after emergence	No. exposed to bovine blood	No. fed	Daily mortality (%)	
			Membrane-fed	Stock colony
4	36	14	—	0.9
5	27	13	25.0	0.9
6	24	22	11.1	0.5
7	19	18	20.8	0.4
9	19	14	—	1.0
10	15	13	21.1	1.1
11	12	10	20.0	1.9
12	12	12	—	0.6
13	12	12	—	0.9
14	11	11	8.3	0.7
16	10	10	9.1	0.6
17	10	10	—	0.4
18	10	10	—	1.7
19	10	10	—	1.4
20	9	9	7	1.0
21	9	9	—	0.7
23	9	9	—	1.9
24	9	6	—	1.3
25	9	8	—	1.4
26	9	6	—	1.0
27	9	8	—	0.4
28	9	9	—	0.9
30	9	7	—	1.6
31	9	9	—	1.1
32	9	6	—	0.4
33	9	8	—	0.7

and the laboratory colony. The number of *G. morsitans* in stock during the same period varied from 3069 to 4590. The table shows that the mortality of membrane-fed flies was fairly high during the first ten days, but thereafter they did remarkably well. It would appear therefore that once the flies became accustomed to membrane feeding, their survival was as good as those maintained on rabbits.

In a parallel experiment, a record was kept of the pupal weights of the pupae deposited during the experimental period. A comparison was made between pupal weights of the progeny of females maintained entirely by membrane feeding with those of the laboratory stock.

The mean weights of 32 pupae collected from 28 females over a 27-day period was 23.07 mg, whereas the mean weight of pupae collected from the laboratory colony was 27.09 mg.

DISCUSSION

Langley [9] working on the role of physical and chemical stimuli in the development of in vitro feeding techniques, noted that the tarsal contact with the feeding surfaces was not particularly important, and concluded that a humidity gradient close to the membrane surface stimulated the probing response in tsetse flies. On the other hand, thermosensitive cells have been found [10] in the hairs of the prothoracic tarsomeres of the tsetse fly that were involved in probing behaviour. The attractiveness of the batwing membrane to tsetse flies made it possible to examine the feeding behaviour of these flies. It was particularly interesting to note that probing response was elicited at an ambient (25°C) temperature without any apparent external stimulus. It was also important to note that as soon as the fly made tarsal contact with the membrane, the probing response was stimulated. It would appear therefore that other factors, apart from heat and texture of the substrate, are involved in eliciting the probing response in the flies.

Gordon and co-workers [11] showed that tsetse flies do not feed directly even when a small laceration of a blood vessel has been broached. The flies preferred to make a series of probing attempts so that a series of lacerated capillaries produce an accumulation of haemorrhages to ensure 'pool feeding'. Margalit and co-workers [8] similarly found that tsetse flies had to make an average of 2.7 probes for a successful gorging. Our observation that the flies, even after a successful probing, piercing and salivation, needed a short period of rest before the procedure is repeated, corroborates these earlier observations. In very few cases did flies manage to feed fully after only one probing, piercing and salivation attempt.

Rice and co-workers [5], having observed the attractiveness of *Eidolon helvum* wing membrane for tsetse flies, recommended the use of this membrane for tsetse fly synthetic feeding. Balogun [12] has subsequently maintained a small colony of *G. morsitans* on defibrinated bovine blood. He found that at least 70% of the flies maintained in this way survived for 62 days. He therefore suggested that bat's wing membrane appeared a suitable medium for artificially feeding tsetse. In our experience it appeared that the flies needed a brief period of adjustment to membrane feeding before they could be maintained successfully through the use of batwing membrane. The reduced pupal weights observed among the progeny of females maintained in this manner was another disadvantage with this system. The low pupal weights indicated that in vitro feeding with defibrinated bovine blood failed to allow a balanced nutritional and general regime to be maintained. Mews and co-workers [13] observed the inadequacy of bovine blood as a diet for tsetse compared with pigs blood, and thought that there were some nutritional differences between the two blood meals. However, the large differences between the pupal weights observed in the flies maintained artificially and those maintained on rabbits suggest that perhaps the flies maintained in vitro were not entirely healthy. It has been demonstrated in the case of some bacterial species that they cause the death of flies which ingest them [14]. It is therefore very possible that the high mortality seen initially and the constantly low pupal weights observed in our system were caused by some nutritional deficiency.

ACKNOWLEDGEMENTS

We appreciate useful advice from Professor R. Galun of the Israel Institute for Biological Research, who is also Director of the ICIPE Tick Research Programme. One of us (A.Y.) is grateful to the authorities of the University of Ibadan, Nigeria, for permission to travel to Nairobi, Kenya, for research. This work was financed by the Rockefeller Foundation, New York, and the International Atomic Energy Agency, Vienna, to whom we are greatly indebted.

REFERENCES

- [1] YORKE, W., BLACKLOCK, B., Food of *Glossina palpalis* in the Cape Lighthouse Peninsula, Sierra Leone, Ann. Trop. Med. Parasitol. 9 (1915) 363.
- [2] AZEVEDO, J.F.DE, SANTOS PINHÃO, R.C., GUERREIRO, J.P., Problems and prospects offered by the tsetse artificial breeding, Anales Esc. Nac. Saúde Publ. e Med. Osp. 3 (1969) 125.
- [3] LANGLEY, P.A., Further experiments on rearing tsetse flies in the absence of the living host, Trans. R. Soc. Trop. Med. Hyg. 66 (1972) 310.

- [4] MEWS, A.R., RUHM, F., "Maintenance of *Glossina morsitans* Westwood fed through an artificial membrane on defibrinated blood", Sterility Principle for Insect Control or Eradication (Proc. Symp. Athens, 1970), IAEA, Vienna (1971) 283.
- [5] RICE, M.J., KAMUGISHA, C.K., SEBUGWAWO, B.S., Bat's wing membrane for tsetse fly synthetic feeding, Trans. R. Soc. Trop. Med. Hyg. **66** (1972) 328.
- [6] YOUDEWEL, A., A simple technique for observing and collecting the saliva of tsetse flies (Diptera, Glossinidae), Bull. Entomol. Res. **65** (1975) 65.
- [7] MEWS, A.R., OFFORI, E., BAUMGARTNER, J., LUGER, D., Techniques for rearing the tsetse fly *Glossina morsitans* Westwood, Working paper for OAU/FAO/WHO RSCTI Meeting, Lagos, 1971.
- [8] MARGALIT, J., GALUN, R., RICE, M.J., Mouthpart sensilla of the tsetse fly and their function: 1: Feeding patterns, Ann. Trop. Med. Parasitol. **66** (1972) 525.
- [9] LANGLEY, P.A., The role of physical and chemical stimuli in the development of *in vitro* feeding techniques for tsetse flies *Glossina* spp., Bull. Entomol. Res. **62** (1972) 215.
- [10] REINOUTS VAN HAGA, H.A., MITCHELL, B.K., Temperature receptors on tarsi of the tsetse fly *Glossina morsitans* West., Nature (London) **255** (1975) 225.
- [11] GORDON, R.M., CREWE, W., WILLET, K.C., Studies on the deposition, migration and development of the blood forms of trypanosomes belonging to the *Trypanosoma brucei* group: 1. An account of the process of feeding adopted by the tsetse-fly when obtaining a blood-meal from the mammalian host, with special reference to the ejection of saliva and the relationship of the feeding process to the deposition of the metacyclic trypanosomes, Ann. Trop. Med. Parasitol. **50** (1956) 426.
- [12] BALOGUN, R.A., Bat's wing membrane for artificial feeding of tsetse flies, Nigeria J. Entomol. **1** (1975) 181.
- [13] MEWS, A.R., LANGLEY, P.A., PIMLEY, R.W., FLOOD, M.E.T., Large-scale rearing of tsetse flies (*Glossina* spp.) in the absence of a living host, Bull. Entomol. Res. **67** (1977) 119.
- [14] BAUER, B., WETZEL, H.W., "Effect of bacteria on tsetse flies fed through membranes", Sterility Principle for Insect Control 1974 (Proc. Symp. Innsbruck, 1974), IAEA, Vienna (1975) 495.

DISCUSSION

P.A. LANGLEY: The primary stimulus for probing activity by tsetse flies is temperature. We introduce an additional humidity gradient in order to augment this because we are interested in maintaining the flies in an optimum nutritional state. It is known that, when starving, a fly will probe a variety of surfaces in the absence of defined stimuli. I therefore suggest that flies which probe your batwing membrane in the absence of a temperature or humidity stimulus are in fact in a low nutritional state and this seems to be borne out by the high mortality and low pupal weights you have recorded using this membrane for feeding.

R. GALUN: I can see the point of using batwing membrane for saliva collection, but now that the silicone membrane is available for tsetse rearing, why use batwing membrane for this purpose?

L.H. OTIENO (*Chairman*): We were curious to see what would happen if we used the membrane for maintaining flies. Otherwise we are fully aware of the silicone membrane method and its advantages.

W.J. KLOFT: I was very interested to hear about your experiments with tsetse flies in which you have three phases during the feeding process. In our laboratory we use the electronic monitoring device for sheep keds (*Melophagus ovinus*). As I pointed out earlier¹, electronic monitoring devices are not very reliable for indicating the real beginning of food uptake in phloem-feeding aphids. The circuit contacts are closed as soon as an aphid reaches the phloem during probing and puncturing. I wonder whether similar effects can also be found with blood-feeders or not.

R. GALUN: With tsetse flies, 'bitometers' are reliable.

W.J. KLOFT: There might be a big difference between the very tiny aphids and the giant tsetse flies which are in any case pool-feeders.

¹ Discussion on KLOFT, W.J., CROMROY, H.L., KLOFT, E.S., "Use of isotopes as model substances to elucidate the mode of transmission of pathogens to animals and plants by arthropod vectors", IAEA-SM-240/10, these Proceedings.

FACTORS AFFECTING THE EFFICIENCY OF THE STERILE INSECT RELEASE METHOD FOR TSETSE

C.F. CURTIS

Ross Institute,
London School of Hygiene and Tropical Medicine,
London,
United Kingdom

Abstract

FACTORS AFFECTING THE EFFICIENCY OF THE STERILE INSECT RELEASE METHOD FOR TSETSE.

Data are reviewed on the levels of sterility, survival and competitiveness of *Glossina* males after irradiation with various gamma-ray doses delivered in air or nitrogen. A simple population model helps in the choice of the optimum dose. Field studies of mating competitiveness require a measure of the ratio of sterile to fertile males and of sterile to fertile matings, and both ratio estimates will be subject to sampling error. Data on multiple mating of female *Glossina* are reviewed. There is some degree of precedence for sperm from the first mating but sterile sperm are fully competitive for fertilization and, following the early death of an embryo, the timing of the next ovulation is only advanced to a slight extent. Thus in an isolated population the occurrence or non-occurrence of female polygamy would be of almost no consequence. Where mated females were immigrating into the release area female polygamy would be advantageous. The low recovery potential of tsetse populations is the main reason for thinking them suitable for control by the introduction of sterility. In trying to measure this recovery potential it is important to distinguish it from natural seasonal increase. If natural population fluctuations are studied to detect density dependent effects it is important not to confuse cause with effect. Non-isolation of the 'target' population is almost certainly the most serious obstacle to practical application of the sterile insect release method, and a steady 'rolling forward' of the release area may be a possible solution.

INTRODUCTION

Tsetse flies have long been considered suitable targets for control by the sterile insect release method. The first laboratory experiments based on the concept of using hybrid sterility for tsetse control were carried out in 1936 [1]. Vanderplank [2-4 and unpublished data] and Jackson [5] followed up this idea with further laboratory work, a field test of mating competitiveness and an apparently very successful population suppression experiment in a 25-sq-km area. Apart from some further laboratory work [6] there has been no further study of this 'low technology' form of sterility. However, following the early

successes with the screw worm fly (*Cochliomya hominivorax*), Simpson [7] and Knipling [8] made appraisals of the possibility of using radiation or chemically sterilized males for tsetse control. They emphasized that quite low density tsetse populations could cause a very serious cattle trypanosomiasis hazard. The almost unique larviparous reproductive system of tsetse probably causes the recovery potential of tsetse populations in response to control measures to be uniquely low so that, for example, the application of 50% zygote sterility to a tsetse population would most probably lead to population decline, whereas a similar sterility level applied to an oviparous insect might well be completely compensated by density dependent regulation at the larval stage. For these two reasons relatively small releases of sterile tsetses per unit area should be sufficient to produce control.

The production of even a few tens of thousands of tsetses per week is a difficult task which took years of preparatory work for the projects in Upper Volta and Tanzania. The autosterilization approach [9] would avoid the cost of rearing but, so long as this would involve leaving considerable quantities of aziridine chemosterilants unattended in the bush, it would, in the author's opinion, constitute an unacceptable health hazard.

Both the projects in Upper Volta and Tanzania have had remarkable success in demonstrating population suppression by releases in limited areas demarcated by specially constructed barrier zones. However, this is far from being a proof of the appropriateness of the sterile insect release method for practical tsetse control or eradication. Tsetse flies have not yet shown evidence of insecticide resistance, and large areas of Northern Nigeria have been completely freed of riverine tsetse by selective ground spraying [10]. Aerial spraying techniques are available for at least some of the circumstances where ground spraying is not applicable [11]. In areas where repeated aerial spraying for tsetse eradication could do serious damage to beneficial species such as pollenating insects, there is a strong argument for a biological technique for 'mopping up' of residual tsetse flies so that the number of preliminary sprayings can be minimized. However, as far as the author is aware, data have yet to be presented showing that the employment of scarce resources of skilled manpower on sterile male programmes is an appropriate way of achieving trypanosomiasis control. However, on the assumption that such a decision may eventually be made, this study reviews some published data on means by which the efficiency of the sterile insect release method could be maximized.

EFFECTS OF RADIATION ON SURVIVAL AND GENERAL BEHAVIOUR

Tsetse flies are more resistant to radiation sterilization than any other Diptera. Figure 1 summarizes dose/response data from several sources and

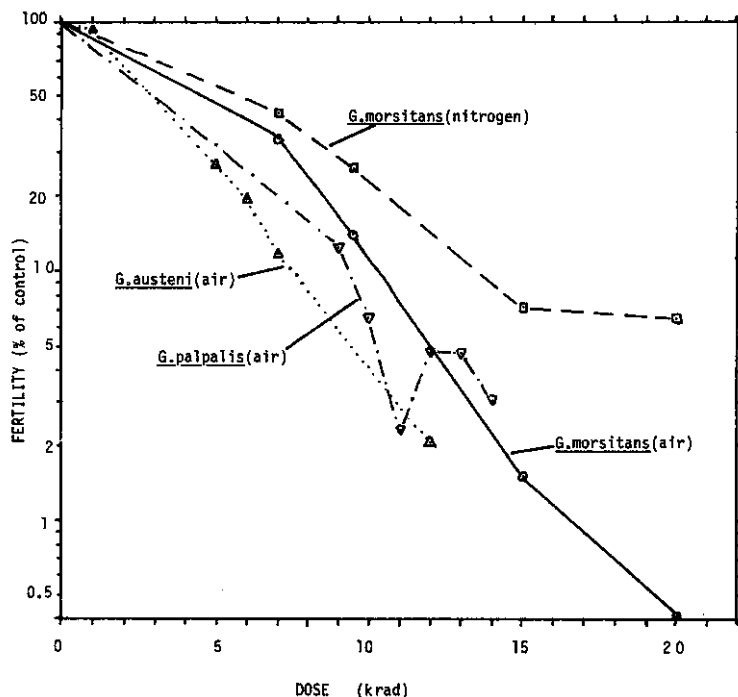


FIG.1. Relationship of residual fertility of males to gamma radiation dose. Data on *G. morsitans* from Ref. [15], on *G. palpalis* from Ref. [16] and on *G. austeni* from Ref. [32].

shows that tsetse species differ in radiosensitivity, and that irradiation in an atmosphere of nitrogen reduces the sterilization achieved at a given dose. In Fig.1 graphs of log. fertility against dose have been used instead of the more commonly used linear plot of sterility against dose. The former technique is preferred for presenting the data for the following reasons [12]. One cannot readily distinguish the effect of one or more than one dominant lethal mutation in a sperm — in either case the zygote dies. The overall mean number of dominant lethals per sperm rises with radiation dose so that at higher doses there are relatively few sperms which do not already contain at least one dominant lethal. Thus there is a spurious 'saturation' effect if sterility is plotted against dose. This is avoided if log. fertility is plotted instead, and such a graph has the additional advantage of stretching the scale in the area of interest, i.e. between 90% and 99.9% sterility.

After doses of over 10 krad required for greater than 90% male sterility in *Glossina*, life span is reduced [13–16]. Under optimal rearing conditions

TABLE I. STERILITY AND MEAN SURVIVAL IN THE LABORATORY OF *G. morsitans* MALES IRRADIATED WITH VARIOUS GAMMA-RAY DOSES IN AIR OR NITROGEN (data from Ref. [15])

Data given as percentages of unirradiated control values

Dose (krad)	Air		Nitrogen	
	Fertility	Survival	Fertility	Survival
9.5	13.8	83.3	25.4	101.3
15	1.5	69.4	7.1	93.0
20	0.4	43.0	6.4	95.8
30	—	—	0	76.4

Glossina morsitans males show little mortality for about the first 50 days of life and there is then relatively rapid death over the next 60 days. The effect of irradiation on survival of *G. morsitans* is not immediately apparent, but is expressed as premature senescence after about day 25 of adult life [15]. In *G. palpalis*, Taze and co-workers [16] reported a considerable reduction in maximum lifespan with increasing radiation dose, but little effect on mean lifespan up to a dose of 14 krad.

The effect of irradiation on lifespan in *G. morsitans* seemed sufficiently great to merit further study, and it was found (as in several other species of fly) that the use of an atmosphere of nitrogen during irradiation protected very effectively against premature senescence, as shown in Table I. Nitrogen protects against both the genetic (sterilization) and somatic (reduction in lifespan) effects of radiation and it is desirable to calculate the optimum 'trade-off' of the two effects. If these two effects were the only relevant factors one could use the simple equation shown in Fig. 2 to calculate the proportion of fertile matings to be expected with different treatments and numbers released. Figure 2 shows some numerical examples which indicate that a modest advantage is to be expected by the use of nitrogen.

In fact the equation used in Fig. 2 is seriously over-simplified. The fertilities shown in Fig. 1 and Table I are based on the rate of pupal production by the mates of males which received different dosages, but studies on these progeny have shown several abnormalities. Curtis [17] and Taze and co-workers [16] have reported opposite results with regard to the weights of such pupae in *G. austeni* and *G. palpalis*. In the former species the weights are lower than average and in the latter species they are higher than average. These results are a consequence of the viviparous mode of reproduction which allows the outcome

$$f = \frac{psR + W}{sR + W}$$

If R:W = 1:1 :-

$\frac{15 \text{ k rad in afr}}{f = 0.596}$	$\frac{20 \text{ k rad in } N_2}{f = 0.542}$

FIG.2. Equation linking survival (s) and fertility (p) of released males to the mean fertility of the population (f) and an example of its use with data from Table I. R and W = number of released and wild males.

of one fertilization to influence that of future ones via the physiology of the mother. In the case of *G. austeni* it appears that repeated pregnancy failures lead to over-accumulation of fat which is harmful to future maternal performance, and indeed usually leads to total sterility in later life. In *G. palpalis* it seems that the extra accumulation of fat in a partially sterile female is beneficial to the 'quality' of the pupa when a successful fertilization occurs.

Apart from such purely physiological effects, there are also a large number of genetic abnormalities in those progeny which, after high radiation dosages, 'escape' being affected by dominant lethals. One such effect is distortion of the sex ratio in favour of males which can occur in *G. morsitans* to the extent of over a 2:1 male:female ratio in the progeny of males which received 6–15 krad [18]. This is interpreted as being due to the greater vulnerability to dominant lethal induction of female-determining sperms because they carry the very large X-chromosome characteristic of tsetse. A similar effect has apparently been encountered in the *palpalis* group (though the deviation from an equal sex ratio was not statistically significant [16]). It appears that there are always sufficient males in a tsetse population to ensure insemination of all the mature females. Thus the number of females, and not the number of males, determines the future reproductive potential of the population, and distortion of the sex ratio in favour of males is equivalent to a delayed form of sterility.

In addition to sex ratio distortion, a more obvious form of delayed sterility is also important in the progeny of irradiated males of *G. morsitans*. This is due to the induction of semi-sterilizing chromosome translocations [18]. A fairly simple computer model, which takes account of these genetic abnormalities and predicts the overall population suppressing effect, has been developed [19]. As with any other computer model the credibility of the results depends on the accuracy of the data input.

The factors described above can all be measured fairly easily in the laboratory, but there are several important parameters that are much more difficult to measure. In the first place survival as measured in the laboratory may have little relation to survival in the field, and to measure this requires suitable mark-release-recapture experiments. By recording both the time and place of recaptures such experiments give information on survival times and dispersion distances. In *G. morsitans* it was concluded that the mean time and distance between release and recapture did not differ significantly for flies derived from wild collected pupae or from pupae from a seven-year-old laboratory colony fed on goats or through artificial membranes [20]. Similarly there was no significant difference in mean survival in the field or dispersion distance between chemosterilized and fertile males derived from wild collected pupae [21].

G. tachinoides adults transported to Africa from Europe, whether irradiated or not, showed reduced survival in the field compared with marked flies of wild origin [22]. Comparison of heavily irradiated (15 krad) with unirradiated flies, both of laboratory origin, showed an effect of irradiation in shortening the maximum longevity calculated by averaging the times of the last recapture over the twelve release experiments. Similarly the irradiation dose had a detectable effect in slowing the rate of dispersion, but eventually the irradiated males moved as far as the wild ones.

In *G. palpalis* a reduction in mean lifespan in the field of irradiated laboratory-reared males compared with wild males was found [23]. The order of magnitude of the effect was similar to that found in the same species in the laboratory [16]. During the first two days after release the sterile males dispersed less far than the wild ones, but by day 4 they had reached the same distances.

Simple observation of the phototactic response of irradiated *G. morsitans*, by counting the flies moving into daylight from a darkened cage, indicated that 15 krad leads to a marked degree of radiation-induced lethargy though there was less effect if the dose was given in nitrogen [15]. More detailed laboratory ethological studies by Brady [24] indicated several behaviour components in which flies irradiated with 11 krad in air differed significantly from unirradiated controls. There was evidence that higher doses given in an atmosphere of nitrogen had a less damaging effect. Field studies of feeding behaviour of unsterilized laboratory-reared *G. morsitans* showed no differences from flies of wild origin [20].

In *G. tachinoides* irradiated with 15 krad the immediate post-release period of low activity was associated with an abnormal number of feeds on humans, presumably in fact on the catching teams, but in later life their feeding behaviour became more normal [25]. The early inactive period may be considered as a time of adaptation to field conditions or perhaps, more correctly, as a period of selective elimination of the poorly adapted. It is noteworthy that a very large proportion of the total recaptures of males were made in the few days after release, suggesting heavy mortality at this time.

MATING COMPETITIVENESS

Competitiveness for mates is the essential requirement of released sterile males. Tests in the laboratory and in small field cages with *G. morsitans* showed full competitiveness of chemosterilized or irradiated males [26]. However, there seems a danger that such tests might give misleadingly high estimates of the competitiveness of sterile males because of the long copulation time of *Glossina*. Suppose a competition is set up between 20 sterile males and 20 fertile males for 20 virgin females. If in fact the sterile males have only one third the sexual aggressiveness of fertile males one might expect that after 16 couples have formed 12 would involve fertile males and four sterile males. The competition for the last four females would then be between eight fertile and 16 sterile males, i.e. a marked departure from the supposed 1:1 ratio. This effect would be more important if a larger ratio of females, say 30 in the above example, were used and in some circumstances the effect might mask the true magnitude of a difference in competitiveness. However, when tests were conducted to avoid this effect by using many successive replicate competitions between one sterile and one fertile male for one female, equal competitiveness was found [15].

Unreplicated tests in a large field cage with both irradiated and chemo-sterilized male *G. morsitans* suggested reductions to about 40% in competitiveness [26]. It is quite conceivable that, even if all the cage evidence is rather reassuring with regard to the 'quality' of reared and sterilized males, their mating competitiveness might be poor in the demanding conditions of the field when the wild males have been under intense natural selection for this character throughout their evolutionary history. Further tests in large field cages would be valuable, but better still are tests in the field in which the ratio of sterile to fertile males at the time and place of mating can be related to the proportion of females that received sterile inseminations. If such data are available, an equation similar in form to that shown in Fig. 2 can be re-arranged as shown in Fig. 3 to solve for the competitiveness coefficient. This is the method of Haisch [27], and is equivalent to that of Fried [28]. Sampling error must be expected in both the estimate of the proportion of sterile males and the proportion of sterile matings, and an estimate of the variance of the competitiveness coefficient and a method for testing the significance of an apparent difference between the estimated coefficients in two different circumstances was derived by Dietz [29].

It is a most important condition of this method of determining competitiveness that the estimated proportion of sterile males refers to the population which actually had access to the captured females; there would be a source of error if there were females present which mated before the releases began or immigrant females which mated outside the release area, assuming that such females will refuse subsequent matings in the release area. To avoid these errors in mosquito

$$e = \frac{W(q-f)}{R(f-p)}$$

When R:W = 2.3:1 and q = 1 :-

1) f = 0.736 and p = 0 ∴ e = 0.156

2) e = 1.0 and p = 0.1 ∴ f = 0.372

FIG. 3. Equation for mating competitiveness coefficient (e) and an example of its use with data from Ref. [23] and a hypothetical example. q = proportion of fertile females in control population; other symbols as in Fig. 2.

experiments, marked virgin females have been released into the male release area and then recaptured after mating [29]. This has not so far been done with *Glossina* and the only data known to the author from which an estimate of field competitiveness can be made are those of the team in Upper Volta working on *G. palpalis* [23]. Figure 3 shows an example of a calculation based on data from the month of November 1975 which indicates that competitiveness was markedly sub-normal, strongly suggesting that a reduction in radiation dose would improve the overall level of sterility introduced into the population, despite the slight increase in the fertility of the treated males which this would entail (see hypothetical example in Fig. 3).

MULTIPLE MATING OF FEMALES

There has been much concern about whether *Glossina* females mate more than once and, if so, whether this would have a serious adverse effect on the working of the sterile insect release method (see, e.g. Laird [30]; Pinhão and Grácio [31]).

Mating of *G. austeni* females to a sterile and to a fertile male followed by maintenance of the females individually, showed that individual females had intermediate fertility, indicating that they could use sperm from more than one mate [32]. The same point has been demonstrated in the same way for *G. palpalis* [16] and it has been demonstrated in *G. morsitans* using a visible marker gene [33]. Mating females first to sterile and then to fertile males has shown somewhat more average fertility than in females mated only to sterile males in *G. morsitans* [34] and *G. palpalis* [16]. The exact interpretation of this in terms of sperm precedence effects and/or competitiveness of dominant lethal-carrying sperm is not clear unless double mating in the reverse order (fertile then sterile male) is also carried out (only a single copulation being

TABLE II. EFFECTS OF MULTIPLE MATING OF FEMALES IN *G. austeni* (data from Ref. [32])

1st mating	2nd mating	Fertility	
		(% of control)	Averages
Unirrad.	Unirrad.	100.0	51.0 ^a
12 k rad	12 k rad	2.0	
Unirrad.	12 k rad.	84.0	62.8
12 k rad	Unirrad.	41.6	
			56.9 ^b

^a If R:W = 1:1 and all females mate once, $f = 51.0\%$.

^b If R:W = 1:1 and all females mate twice, $f = 56.9\%$.

allowed with each male). This has been done in *G. austeni* [32], and the results are summarized in Table II. It is clear that there is a strong tendency for precedence in usage of sperm from the first mating. Averaging the fertility of the two types of doubly mated females gives a value similar to the average of the fertility of females mated only to fertile or only to sterile males. This indicated that the sperm irradiated with 12 krad only carried dominant lethal mutations and was still competitive for fertilization and, under these conditions, the sterile insect release method will be effective whether or not multiple mating of females occurs [35]. This point is demonstrated by a numerical example in Table II; the essential point in the argument is that whatever the frequency of females mated first to a sterile then to a fertile male, females doubly mated in the reverse order will be equally frequent.

G. palpalis females were mated to males which had been irradiated with one of a series of different doses, and the females were then mated to fertile males [16]. The fertility was somewhat higher when the first males received 13 or 14 krad rather than 12 krad, indicating that at doses only slightly higher than that referred to in Table II sperm damage, in addition to dominant lethality, begins to enter the picture. In Table II the mean fertility of the doubly mated females is slightly higher than that of the singly mated ones. This does not indicate any failure of sperm competitiveness, as the effect can be satisfactorily explained as a consequence of the larviparous mode of reproduction [34]. When an embryo or young larva dies in the uterus the next ovulation occurs as soon as the egg is mature, i.e. seven days, and not the usual nine days, after the previous ovulation [36]. Thus partially sterile females have more ovulations per unit time than do fully fertile females. It is fortunate for the prospects of the sterile insect release method against *Glossina* that the

next ovulation cannot occur as soon as the uterus is emptied by an embryonic death. As an aside it might be pointed out that suggestions that have been made (e.g. Knipling [9]) for the use of genetic control against mammalian or bird pests raise questions about whether, in the species concerned, failure of one pregnancy or clutch will lead rapidly to another ovulation and whether this might lead to reproductive success if, in the meanwhile, the female has taken another mate.

It is concluded that in tsetse the occurrence of multiple mating of females need have no adverse effects on control of isolated populations by the sterile insect release method. By contrast, in an incompletely isolated population, multiple mating of females would be beneficial as it would allow the possibility that a female, which had received a fertile mating before immigrating into the release area, could be partially sterilized by re-mating after arrival. Perhaps the best way of finding out whether such multiple mating occurs under field conditions would be to capture females after a sterile male release and to determine whether an appreciable proportion of individuals produce one or more pupae and yet can be shown, by examining the ovarian configuration by the method of van der Vloedt [37], to have had one or more unsuccessful pregnancies.

EFFECT OF DENSITY-DEPENDENT REGULATION AND IMMIGRATION

The depression in mean population fertility by sterile male release is not necessarily simply related to the suppression of population density which will be achieved. Populations of highly fertile oviparous species, where there is ample opportunity for larval competition, show a recovery potential stimulated by reductions in the percentage of egg hatch and acting via density-dependent regulatory factors (see e.g. Rajagopalan and co-workers [38]). Such effects must be distinguished from natural seasonal increase which, even if it temporarily nullifies the effects of introduced sterility, can be relied upon to reverse itself at another season. In the absence of opportunity for inter-larval competition, density-dependent regulation of *Glossina* must act via pupal parasite populations or via the effects of high tsetse densities tending to drive away their host animals. Quantification of such effects would clearly be extremely difficult.

There is one method by which density-dependent regulation is sometimes studied and which, in the author's opinion, might be seriously misleading. This is to identify times or places at which the population density 'happens' to be high or low, and then to measure the rate of population increase and determine whether a significant negative regression of population increase on density can be established. This principle seems to run the risk of confusing cause and effect, i.e. the physical or biological factors that caused population density to be high at one time or place may still be one of the factors directly affecting

population increase or decrease at the time that this is measured. Thus it seems preferable to measure population recoveries where density has been artificially reduced by a method which does not disrupt the local ecology. What this amounts to is a recommendation that every effort should be made to correlate levels of sterility introduced into populations by sterile male release with the consequent change (or lack of change) in population densities [39]

It can be confidently assumed that the recovery potential of tsetse populations could not be as high as tenfold per generation since, even in the best laboratory conditions, mean yields of 20 pupae per female cannot be approached. Thus a radiation treatment which left 10% residual fertility would be capable of driving an isolated population to complete extinction, though this would not necessarily be the most efficient way of achieving this end. Ideally the most efficient dosage would be calculated in the light of information on the overall sterility and competitiveness in the field at various dosages, and also using information on the population recovery potential. It will certainly be some time before such data are available but in the meantime it would seem worthwhile to explore the possibility that lower radiation dosages than those used hitherto in an atmosphere of nitrogen might give more efficient control.

The immigration of already mated females into the release area is almost certainly the most serious obstacle to the successful use of the sterile insect release method for practical tsetse eradication. The use of barrier zones created with insecticides and/or bush clearance is justifiable for experiments on improving the sterile insect release technique, but for large practical projects, the necessary barrier zones would be very large and if they can be maintained tsetse free by methods not involving the use of sterile males, it is hard to see why such methods should not be used over the whole area. Thus, in the author's opinion, sterile males will only have a practical future if they can maintain their own 'biological barrier zones', the aim being to sterilize females before they enter the central eradication area. Such immigrants would still constitute a vector population which would be replaced from outside every generation if the release pattern remained unchanged. Therefore in the long term the aim should be to 'roll' the release area forward generation by generation so that what was the biological barrier zone becomes part of the eradication zone and a new barrier zone is established. Such a strategy should certainly be planned in relation to the natural boundaries of fly belts to avoid an ever-expanding requirement for sterile flies, but the areas to be covered by the releases would inevitably be large. The main justification for trying to improve the efficiency of existing sterilization and release methods is that it would allow larger areas of land to be treated from existing rearing facilities.

REFERENCES

- [1] POTTS, W.H., Tsetse hybrids, *Nature* (London) **154** (1944) 606.
- [2] VANDERPLANK, F.L., Hybridisation between *Glossina* species and a suggested new method for control of certain species of tsetse, *Nature* (London) **154** (1944) 607.
- [3] VANDERPLANK, F.L., Experiments in the hybridization of tsetse flies (*Glossina*, Diptera) and the possibility of a new method of control, *Trans. R. Entomol. Soc. London* **98** (1947) 1.
- [4] VANDERPLANK, F.L., Experiments in cross-breeding tsetse flies (*Glossina* species), *Ann. Trop. Med. Parasitol.* **42** (1948) 131.
- [5] JACKSON, C.H.N., Pairing of *Glossina morsitans* with *G. swynnerloni*, *Proc. R. Entomol. Soc. London* **A20** (1945) 106.
- [6] CURTIS, C.F., Sterility from crosses between sub-species of the tsetse fly *Glossina morsitans*, *Acta Tropica* **29** (1972) 250.
- [7] SIMPSON, H.R., The effect of sterilised males on a natural tsetse fly population, *Biometrics* **14** (1958) 159.
- [8] KNIPLING, E.F., Potential Role of the Sterility Principle for Tsetse Fly Eradication, WHO/Vector Control/27 (1973).
- [9] KNIPLING, E.F., Sterile male method of population control, *Science* **130** (1959) 902.
- [10] NA'ISA, B.K., "The possibility of applying the sterile male technique to the control of tsetse flies in Nigeria and the problems to be faced", The Sterile-Insect Technique and its Field Applications (Proc. Panel Vienna, 1972), IAEA, Vienna (1974) 75.
- [11] HAMON, J., BALDRY, D.A.T., PARKER, J.D., CHALLIER, A., STILES, A.R., "Status and future of control", Tsetse: the Future for Biological Methods in Integrated Control, (LAIRD, M., Ed.), IDRC, Ottawa (1977) 35.
- [12] CURTIS, C.F., Induced sterility in insects, *Adv. Reprod. Physiol.* **5** (1971) 120.
- [13] ITARD, J., Stérilisation des mâles de *Glossina tachinoides* par irradiation aux rayons gamma, *Rev. Elev. Méd. Vét. Pays Trop.* **21** (1968) 479.
- [14] DEAN, G.J.W., WORTHAM, S.M., Effect of gamma radiation on the tsetse fly *Glossina morsitans*, *Bull. Entomol. Res.* **58** (1969) 505.
- [15] CURTIS, C.F., LANGLEY, P.A., Use of nitrogen and chilling in the production of radiation induced sterility in the tsetse fly *Glossina morsitans*, *Entomol. Exp. Appl.* **15** (1972) 360.
- [16] TAZE, Y., CUISANCE, D., POLITZAR, H., CLAIR, M., SELLON, E., Essais de détermination de la dose optimale d'irradiation des mâles de *Glossina palpalis gambiensi* en vue de la lutte biologique par lâchers de mâles stériles dans le region de Bobo-Dioulasso (Haute-Volta), *Rev. Elev. Méd. Vét. Pays Trop.* **30** (1977) 269.
- [17] CURTIS, C.F., The expression of induced sterility in *Glossina austeni*, *Trans R. Soc. Trop. Med. Hyg.* **64** (1970) 186.
- [18] CURTIS, C.F., LANGLEY, P.A., MEWS, A.R., OFFORI, E.D., SOUTHERN, D.I., PELL, P.E., Sex ratio distortion and semi-sterility in the progeny of irradiated *Glossina morsitans*, *Genet. Res.* **21** (1973) 153.
- [19] CURTIS, C.F., "The use of simulation in the choice of an optimum radiation dose for control of *Glossina morsitans*", Computer Models and Application of the Sterile-Male Technique (Proc. Panel Vienna, 1971), IAEA, Vienna (1973) 177.
- [20] VALE, G.A., HARGROVE, J.W., JORDAN, A.M., LANGLEY, P.A., MEWS, A.R., Survival and behaviour of tsetse flies (Diptera, Glossinidae) released in the field: a comparison between wild flies and animal-fed and in vitro-fed laboratory-reared flies, *Bull. Entomol. Res.* **66** (1976) 731.

- [21] DAME, D.A., BIRKENMEYER, D.R., NASH, T.A.M., JORDAN, A.M., The dispersal and survival of laboratory bred and native *Glossina morsitans morsitans* in the field, Bull. Entomol. Res. 65 (1975) 453.
- [22] CUISANCE, D., ITARD, J., Comportement de mâles stériles de *Glossina tachinoides* West. lâchés dans les conditions naturelles-environs de Fort-Lamy (Tchad): II. Longévité et dispersion, Rev. Elev. Méd. Vét. Pays Trop. 26 (1973) 169.
- [23] CLAIR, M., POLITZAR, H., CUISANCE, D., LAFAYE, A., Observations sur un essai préliminaire de lâchers de mâles stériles de *Glossina palpalis gambiensis* (Haute-Volta), Rev. Elev. Méd. Vét. Pays Trop. 29 (1976) 341.
- [24] LANGLEY, P.A., CURTIS, C.F., BRADY, J., The viability, fertility and behaviour of tsetse flies (*Glossina morsitans*) sterilized by irradiation under various conditions, Entomol. Exp. Appl. 17 (1974) 97.
- [25] CUISANCE, D., ITARD, J., Comportement de mâles stériles de *Glossina tachinoides* West. lâchés dans des conditions naturelles environs de Fort-Lamy (Tchad): III. Lieux et hauteurs de repos, comportement alimentaire, Rev. Elev. Méd. Vét. Pays Trop. 26 (1973) 328.
- [26] DEAN, G.J.W., DAME, D.A., BIRKENMEYER, D.R., Field cage evaluation of the competitiveness of male *Glossina morsitans orientalis* sterilized with tepa or gamma irradiation, Bull. Entomol. Res. 59 (1968) 339.
- [27] HAISCH, A., "Some observations on decreased vitality of irradiated Mediterranean fruit fly", Sterile Male Technique for Control of Fruit Flies (Proc. Panel Vienna, 1969), IAEA, Vienna (1970) 71.
- [28] FRIED, M., Determination of sterile insect competitiveness, J. Econ. Entomol. 64 (1971) 869.
- [29] GROVER, K.K., CURTIS, C.F., SHARMA, V.P., SINGH, K.R.P., DIETZ, K., AGARWAL, H.V., RAZDAN, R.U., VAIDYANATHAN, V., Competitiveness of chemo-sterilized males and cytoplasmically incompatible translocated males of *Culex pipiens fatigans* in the field, Bull. Entomol. Res. 66 (1976) 469.
- [30] LAIRD, M., "Biocontrol prospects in future integrated tsetse control programs", Tsetse: the Future for Biological Methods in Integrated Control (LAIRD, M., Ed.), IDRC, Ottawa (1977) 177.
- [31] PINHÃO, R.C., GRÁCIO, A.J.S., The degree of spermathecal impregnation and the number of matings in *Glossina austeni*, An. Inst. Hig. Med. Trop. 1 (1973) 103.
- [32] CURTIS, C.F., Radiation sterilization and the effect of multiple mating of females in *Glossina austeni*, J. Insect Physiol. 14 (1968) 1365.
- [33] KAWOYIA, J., Demonstration of multiple insemination in females of *Glossina morsitans* Westwood, using a phenotype genetic marker OcrA, Z. Angew. Entomol. 84 (1977) 312.
- [34] DAME, D.A., FORD, H.R., Multiple mating of *Glossina morsitans* and its potential effect on the sterile male technique, Bull. Entomol. Res. 58 (1968) 213.
- [35] VON BORSTEL, R.C., Population control by release of irradiated males, Science 131 (1960) 878.
- [36] SAUNDERS, D.S., The ovulation cycle in *Glossina morsitans* and a possible method of age determination for female tsetse flies by the examination of their ovaries, Trans. R. Entomol. Soc. Lond. 112 (1960) 221.
- [37] VAN DER VLOEDT, A.M.V., TAHER, M., CZOCK, K.H., MALEKGHASSEMI, B., HASELBERGER, N., Laboratory studies on the sexual sterilisation of the tsetse fly *Glossina palpalis palpalis* by ionizing radiation: 2. Ovarian configuration and uterine content of females mated by irradiated males, IAEA, Vienna (1976), IAEA Document.

- [38] RAJAGOPALAN, P.K., CURTIS, C.F., BROOKS, G.D., MENON, P.K.B., The density dependence of larval mortality of *Culex pipiens fatigans* in an urban situation and prediction of its effect on genetic control operations, Indian J. Med. Res. 65 (Suppl.) (1977) 77.
- [39] WEIDHAAS, D.E., LABRECQUE, G.C., LOFGREN, C.S., SCHMIDT, C.H., Insect sterility in population dynamics research, Bull. World Health Organ. 47 (1972) 309.

DISCUSSION

D.J. ROGERS: I am afraid I do not quite understand your comments on the possibility of the spurious identification of density dependence. If population changes due to any factor whatsoever are not causally related to density, I doubt whether you will succeed in showing a density dependence. There are, however, more critical (i.e. statistically acceptable) tests for the presence of density dependence and its strength which eliminate the objection about the interdependence of the axes of the usual graphs drawn to show density dependence (i.e. percentage mortality versus density or the logarithmic equivalent.¹ The strength can be estimated by a least-squares method that overcomes the usual problem with the commonly used regression calculations (which of course assume errors only in the y-axis variable and if anything underestimate rather than overestimate the strength of the structural dependence).²

C.F. CURTIS: The point that I was trying to make could perhaps be put more clearly as follows. If we use a regression line of rates of population increase on naturally occurring densities to predict a population's 'resilience' to control procedures, we assume that a naturally occurring low-density population will behave in the same way as a population initially at high density which has been artificially suppressed. This may be so, but it seems to me possible that the harsh conditions which produced the natural low density may still be operative and tending to inhibit population recovery. This is unlikely in the temperate-zone species considered by Varley and Gradwell, where breeding and activity are seasonal, but is perhaps more likely in a continuously breeding tropical species. If my suggestion is correct, it would certainly not exclude the possibility of significant population increase or density regressions providing evidence for some degree of density dependence, but it would rather indicate that the true recovery potential of artificially controlled populations was stronger than one would deduce from such regressions.

¹ For example, VARLEY, G.C., GRADWELL, G.R., HASELL, M.P., Insect Population Ecology, Blackwell Scientific Publications, Oxford (1973).

² For example, KENDALL, M.G., STUART, A., The Advanced Theory of Statistics, Vol. II, Griffin, London (1973).

As I understand it, Varley and Gradwell's critical test for density dependence is designed to eliminate spurious regressions caused by sampling error which, for example, might give too high an estimate for a density and, accordingly, too low an estimate for the population increase in the interval beginning with that estimate. My objection does not concern sampling error and therefore I do not think that it can be met by more sophisticated statistical methods.

D.A. DAME: In the second paragraph of the introductory remarks in your paper you are right about the risks which unrestricted use of aziridinyl chemo-sterilants holds for the environment. It is interesting to note, however, that a new group of chemical sterilants has passed the Ames test for mutagenicity and will be studied for their effect on tsetse flies. Such compounds, if effective with tsetse, may prove to be a suitable means for sterilizing natural populations directly.

A.D. IRVIN: In the experiments in which flies were irradiated in a nitrogen atmosphere, how were these conditions achieved, and is it the presence of nitrogen or the absence of oxygen which is significant?

C.F. CURTIS: We blew nitrogen through the tube of pupae for five minutes before the irradiation and then throughout the period of irradiation [15]; in the field application of the technique in Tanga, nitrogen was blown over the pupae for five minutes and then the pupae were sealed in a nitrogen atmosphere by closing a tap before their exposure to the ^{137}Cs source. On the basis of the large amount of work on irradiation of *Drosophila* in different gases, I would guess that the effect on tsetse is due to the absence of oxygen. This could be tested by repeating the work with an inert gas such as argon.

D. CUISANCE: What conclusion have you yourself reached about the deviation in the sex ratio found in the progeny of females mated with sterile males?

C.F. CURTIS: In *G. morsitans* radiation doses of 6–10 krad to males gave an average sex ratio in their progeny of about two males to one female [18]. The explanation presumably lies in the greater susceptibility to dominant lethality of X-chromosome-carrying sperms, because the total length of functional chromosome is greater in such sperms than in Y-chromosome sperms. This suggestion was first made by Catcherside and Leo (1945) to explain sex ratio distortion in the progeny of irradiated *Drosophila*.

H. POLITZAR: In descendants of untreated females and sterile males we found a sex ratio deviation of less than 2:1 in favour of males. In the field we found after six months' release a ratio of males to females of 3:1. Do you think that this discrepancy is caused by other factors, such as lower longevity of sterile females?

C.F. CURTIS: It would certainly seem that the strong distortion of the sex ratio among unmarked flies in your field experiment cannot be explained by the relatively weak distortion found at emergence of the progeny of irradiated males in the laboratory. I suppose that reduced viability of the female progeny of irradiated males due to damage to their paternally derived X-chromosome

might possibly be an explanation. I wonder whether another possibility could be the loss of detectable marking on a small portion of your released males. When only males are being released and the released-to-wild ratio is high, only a small percentage of released flies would need to lose their markings to give a strong sex ratio distortion among the unmarked population.

P.A. LANGLEY: Is the precedence of sperm from the first mating due to a suppressing effect of the first arrivals upon the later arriving sperm, or is it simply that the first mating fills the major portion of the spermathecae, leaving little room for the sperm of the second mating, which are therefore lost?

C.F. CURTIS: I do not know, but your second suggestion is the simplest hypothesis.

T.G.T. JAENSON: In our investigations on *G. pallidipes* we dissected about 360 females 15–20 hours after copulation. Sixty per cent of the females had both spermathecae filled with sperm. Only six per cent had empty spermathecae. Therefore, there would normally seem to be little space left in the spermathecae for sperm from a second insemination. I believe this may explain why most fertilizations originate from the sperm of the first insemination.

A.M. VAN DER VLOEDT: I would also like to comment on the phenomenon of multiple mating. Our laboratory observations for different species have shown that up to 80% of previously mated females accept a second mate during the first few days after the first mating. Even so, it was usually less than 5% of the females that actually received and used sperm from two different mates. This proves that even under confinement in the laboratory multiple insemination is quite infrequent and of no practical value, especially when one considers that sperm from irradiated males (for example 12 krad in air to *G.p. palpalis* and *G.m. morsitans*) is competitive with the untreated sperm.

In the field, dissections of many nulliparous females (up to day 9) showed completely filled spermathecae in some cases (first mating) and a freshly deposited spermatophore in utero. Microscopic examination (after 1–2 hours) showed that no outflow of sperm into the spermathecae had occurred.

LA MULTIPLICITE DES ACCOUPLEMENTS CHEZ LES GLOSSINES ET LA METHODE DES LACHERS DE MALES STERILES

R.C. PINHÃO
Instituto de Higiene e
Medicina Tropical,
Lisbonne,
Portugal

Abstract-Résumé

MULTIPLE MATINGS AMONG GLOSSINA AND THE STERILE MALE TECHNIQUE.

The fact that multiple matings are a common phenomenon among glossina turns the sterile male technique into a competition not between adult insects but between two types of sperm, and the proportion of females inseminated with the one or the other is given by the binomial $(p + q)^n$, where p is the percentage of normal males, q the percentage of sterile males and n the average number of matings per female. However, multiple matings cannot damage the effectiveness of the technique unless two conditions are present either separately or simultaneously: precocious death of the spermatozoa and reduced inseminating potential among the sterile males. Study of the factors which can alter the inseminating potential is thus important for those who wish to use the sterile male technique. These factors are of three kinds: factors connected with quality, with quantity and with availability. The first are associated with the nature and intensity of the alterations brought about in the spermatozoa by the sterilizing agent, the second with possible variations in the amount of sperm reaching the spermatheca, the third with the behaviour of the sterile males in the nature — that is, the question whether sterilization has a favourable or unfavourable influence on their chances of mating with wild females. The author describes his observations of the quantity of sperm produced by *Glossina morsitans submorsitans* males from the colony reared at the Institute for Tropical Hygiene and Medicine in Lisbon, compares them with the observations of other authors and discusses their practical significance. He also makes some suggestions for research in this area. Finally, he stresses the practical advantages that would accrue from assessing the behaviour of colonies not just in terms of female productivity but also in terms of male inseminating potential, and from developing laboratory techniques to enhance that potential.

LA MULTIPLICITE DES ACCOUPLEMENTS CHEZ LES GLOSSINES ET LA METHODE DES LACHERS DE MALES STERILES.

Le fait que la multiplicité des accouplements soit un phénomène courant chez les glossines transforme la méthode des lâchers de mâles stériles en une compétition non entre insectes adultes, mais entre deux types de sperme, et la proportion des femelles inséminées avec l'un ou l'autre ou les deux types de sperme est donnée par le binôme $(p + q)^n$, où p représente le pourcentage de mâles normaux, q le pourcentage de mâles stériles et n le nombre moyen d'accouplements pour chaque femelle. Toutefois, la multiplicité des accouplements ne pourrait nuire à l'application de la méthode que si deux conditions se vérifiaient isolément ou simultanément: la mort précoce des spermatozoïdes et un potentiel inséminateur moindre des mâles stériles. L'étude des facteurs susceptibles de faire varier ce potentiel inséminateur est d'une

importance considérable pour ceux qui s'intéressent à l'application de la méthode. Ces facteurs sont de trois ordres: facteurs de qualité, de quantité et de disponibilité. Les premiers sont liés à la nature et à l'intensité des altérations produites dans les spermatozoïdes par l'agent stérilisant, les seconds aux variations possibles de la quantité de sperme parvenant aux spermathèques et les troisièmes dépendent du comportement des mâles stériles dans la nature, en ce que la stérilisation influe favorablement ou défavorablement sur les possibilités d'accouplement avec les femelles sauvages. L'auteur présente ensuite les résultats de ses observations sur la quantité de sperme produite par les mâles de *Glossina morsitans submorsitans* de la colonie élevée à l'Institut d'hygiène et de médecine tropicale de Lisbonne, les compare avec les observations faites par d'autres auteurs et discute leur signification pratique. Il fait également quelques suggestions concernant la recherche dans ce domaine. Enfin, il souligne l'intérêt pratique qu'il y aurait à évaluer le comportement des colonies non seulement en termes de productivité des femelles, mais aussi en termes de potentiel inséminateur des mâles, et même de mettre au point en laboratoire des techniques permettant d'accroître ce potentiel.

Lindquist [1] a écrit en 1969 que «pour pouvoir appliquer efficacement la méthode des lâchers de mâles stériles, une connaissance approfondie de la biologie de l'insecte considéré est sans doute la première condition requise».

C'est une vérité que l'expérience acquise au cours des dix dernières années n'a fait que confirmer et qui vaut pour tous les moyens de lutte contre les insectes, car même le plus «mécanique» exige une connaissance minimum de la biologie de l'espèce à combattre.

Inversement, chaque nouvelle méthode de lutte contre les insectes contribue à l'approfondissement des connaissances sur certains aspects de la biologie des insectes.

Les progrès accomplis dans la méthode des lâchers de mâles stériles confirment pleinement cette règle. Elle a été ces dernières années le point de départ d'une série de travaux qui ont fait beaucoup progresser nos connaissances sur la biologie des glossines, du développement des techniques d'élevage aux études écologiques.

La multiplicité des accouplements est certainement l'un des aspects de la biologie des glossines qui doit à la méthode des lâchers de mâles stériles le regain d'intérêt qu'il a suscité ces derniers temps. Mais il est aussi certainement celui qui a été l'objet des interprétations les plus diverses, dans la littérature, quant à son importance pour le succès de la méthode; ainsi, un auteur considère que tenir compte de cet aspect rendrait la méthode inapplicable, un autre auteur pense qu'il serait superflu d'en tenir compte.

J'aborderai donc ce sujet pour deux raisons. D'abord parce qu'il me semble utile d'éclaircir son importance véritable et toutes ses implications pour la méthode des lâchers de mâles stériles, et ensuite parce que j'aimerais présenter quelques résultats de mon expérience personnelle qui peuvent contribuer à cette compréhension. Nous examinerons pour commencer les connaissances que nous possédons actuellement sur ce phénomène chez les glossines.

Il semble que les glossines sont capables de dispenser plusieurs accouplements.

D'une part, elles ont un processus reproducteur très lent et une très basse productivité. Une trentaine de spermatozoïdes suffiront normalement à féconder tous les œufs d'une femelle de grande longévité. De plus, la présence dans l'utérus des glossines, pendant la plus grande partie de leur vie, d'une larve volumineuse réduit vraisemblablement les périodes où un nouvel accouplement est possible aux quelques rares périodes de courte durée qui suivent immédiatement une ponte.

D'autre part, les femelles disposent toujours d'un extraordinaire surplus de spermatozoïdes. En effet:

- elles possèdent deux spermathèques de grande capacité: chez les *G. submorsitans* de notre colonie de Lisbonne, cette capacité a été estimée à $4,5 \times 10^{-5} \mu/l$, ce qui leur permet d'emmagasiner non pas des dizaines mais plusieurs milliers de spermatozoïdes;
- Les spermathèques offrent aux spermatozoïdes un milieu optimal qui leur permet une survie aux moins égale à celle des femelles porteuses. En 1936 déjà, Nellanby avait identifié dans les spermathèques d'une glossine des spermatozoïdes encore vivants 200 jours après l'insémination; nos recherches ont abouti à des valeurs du même ordre, parfois même plus élevées [2];
- les glossines possèdent probablement des mécanismes qui règlent le passage des spermatozoïdes dans l'utérus de façon que, à chaque ovulation, seule une petite quantité de sperme pénètre dans l'utérus. Ces mécanismes ne sont pas encore parfaitement connus, mais Pinhão [3] et Roberts ont décrit des observations et émis des suggestions qui permettent de mieux comprendre ces mécanismes.

Il n'est pas surprenant que, pendant longtemps, l'hypothèse de la multiplicité des accouplements chez les glossines n'ait pas éveillé un grand intérêt chez les chercheurs; pourtant, le premier travail de recherche sur le sujet [4] fait déjà mention de la méthode des mâles stériles, alors à ses débuts, et à son application éventuelle aux glossines.

Aujourd'hui, on observe fréquemment ce phénomène en laboratoire. En 1952, Squire a observé que les femelles jeunes étaient «*repeatedly and vigourously mated*» et que les femelles vieilles s'accouplaient occasionnellement. En 1958, Jordan [4] a réussi à accoupler de jeunes femelles de *G. palpalis* jusqu'à cinq fois et en a conclu que les femelles âgées de 10 jours au maximum acceptaient plusieurs accouplements, que les accouplements multiples étaient plus fréquents chez les femelles plus jeunes et que de nouveaux accouplements chez les femelles plus âgées étaient très rares. Les travaux de Dame et Ford [5] et Curtis [6, 7] ont prouvé que les femelles au contact de mâles normaux et de mâles stériles s'accouplaient normalement avec les deux.

Certaines observations indiquent que dans la nature les accouplements multiples sont certainement la règle. Pinhão et Grácio [8] ont observé que le degré de remplissage des spermathèques des femelles accouplées à deux reprises étaient

nettement plus élevé que chez les mouches qui avaient été accouplées une fois seulement et sont parvenus à la conclusion que seuls des accouplements répétés pouvaient expliquer le taux élevé de spermathèques pleines observé dans la nature chez les femelles âgées [9]. Nos observations ont été confirmées par Taze et al. [10, 11] qui ont remarqué que le nombre de spermathèques totalement pleines augmentait avec le nombre d'accouplements. Plus récemment, au cours de travaux dont nous parlerons plus loin, nous avons observé qu'après une insémination unique le taux de spermathèques pleines ne dépassait jamais 40%, cette valeur étant d'ailleurs exceptionnelle.

Enfin, les récents travaux des chercheurs français qui, en Afrique, ont fait des observations particulièrement intéressantes sur la méthode des lâchers de mâles stériles, ont apporté la preuve que la multiplicité des accouplements est un phénomène courant dans la nature, en ce sens que la baisse constatée de la productivité des femelles sauvages ne peut s'expliquer que par des accouplements successifs avec des mâles stériles et des mâles normaux.

Tout ceci montre que la multiplicité des accouplements est une possibilité en laboratoire et la règle dans la nature. Voyons l'importance pratique qui en découle pour la méthode des lâchers de mâles stériles.

Les bases théoriques de la méthode des lâchers de mâles stériles sont bien connues [12]; on peut les résumer en disant que le lâcher d'un certain nombre de mâles stériles dans une population naturelle provoquera une réduction du nombre d'individus de la génération suivante correspondant à la réduction du nombre des mâles normaux dans l'ensemble de la population des mâles.

Il s'agit alors d'une compétition entre deux types de mâles, ou mieux, si l'on tient compte du mécanisme reproducteur chez les insectes, entre deux types de sperme [13].

Quelle incidence peut avoir la multiplicité des accouplements chez les femelles sur la méthode des lâchers de mâles stériles?

Il faut admettre tout d'abord que, en *elle-même*, la multiplicité des accouplements n'altère en rien la dynamique et les résultats du processus. Pourvu que les accouplements se fassent au hasard, ou mieux, que les deux types de sperme forment un mélange homogène dans les spermathèques, la réduction finale de la progéniture sera la même, indépendamment du nombre d'accouplements pour chaque femelle.

En effet, l'existence de deux types de mâles dans une même population fera qu'un certain nombre de femelles sera fécondé seulement par des mâles fertiles, un autre groupe sera fécondé uniquement par des mâles stériles et toutes les autres femelles par les deux types de mâles. Les effectifs et proportions de tous les groupes possibles obéiront aux lois des probabilités et se répartiront selon le développement du binôme $(p + q)^n$, où p est la proportion de mâles normaux, q la proportion de mâles stériles et n le nombre moyen d'accouplements par femelle.

L'analyse mathématique du développement du binôme

$$(p + q)^n = p^n + \binom{n}{1} p^{n-1} q + \dots + \binom{n}{n} p q^{n-1} + q^n$$

montre que n détermine le nombre et la valeur de chaque terme, mais non le résultat final en termes de réduction de la descendance, celle-ci étant déterminée uniquement par les valeurs relatives de p et q .

Il semble pourtant que la conclusion de von Borstel, selon laquelle le nombre d'accouplements ne jouerait aucun rôle, est justifiée. Elle ne le serait en réalité que *si le comportement des deux types de mâles était totalement identique*, soit par leur aptitude à s'accoupler avec les femelles, soit par leur pouvoir inséminant, soit par les caractéristiques mêmes de leur sperme. A toute diminution du pouvoir inséminateur des mâles stériles correspondrait ainsi une diminution réelle de leur nombre et, en conséquence, une efficacité réduite de la méthode. Le problème est donc de connaître tous les facteurs qui peuvent influencer négativement sur l'application de la méthode.

Puisqu'en effet, vu le mode de reproduction des glossines, le succès de la méthode dépend en réalité du rapport sperme stérile/sperme normal, comme l'ont fait remarquer récemment Taze et al. [10, 11], nous grouperons les facteurs qui influent sur ce rapport en trois grands groupes: facteurs liés à la *qualité* du sperme, facteurs liés à la *quantité* de sperme disponible pour féconder les oeufs et facteurs qui règlent la *disponibilité* du sperme, à savoir son aptitude à atteindre les spermathèques. Nous les passerons brièvement en revue; nous nous attarderons toutefois sur les facteurs de quantité afin de présenter quelques éléments nouveaux tirés d'une étude actuellement en cours dans notre laboratoire.

Facteurs de qualité

Les facteurs de qualité sont liés aux altérations provoquées dans le sperme par l'agent stérilisateur.

Ici la multiplicité des accouplements prend une importance toute particulière. En effet, si la stérilisation a provoqué la mort des spermatozoïdes, il est évident qu'une éventuelle insémination par un mâle fertile annulerait totalement l'effet stérilisant d'une insémination antérieure par un mâle stérile. Le succès de la méthode des mâles stériles exige donc que les spermatozoïdes des mâles stériles puissent féconder les oeufs, entraînant la mort de ces derniers, c'est-à-dire que se vérifie une véritable compétition entre deux types de sperme capables d'inséminer une femelle.

Chez les glossines, deux types d'agent stérilisateur ont été utilisés: les chimiostérilisants et les rayonnements gamma.

Les premiers ont été expérimentés en Rhodésie, par Dame et al. [14]; les seconds, employés pour la première fois par Potts [15] semblent être aujourd'hui

le moyen le plus répandu, grâce surtout aux travaux d'Itard [16–19], Taze et al. [10, 11] et d'autres auteurs.

L'expérience actuelle avec les deux processus montre que leur action se traduit par l'induction de mutations létales dominantes qui provoquent la mort de l'oeuf peu de temps après la fécondation [6, 7, 20].

Si un autre processus de stérilisation était envisagé à l'avenir cet aspect devrait être soigneusement considéré.

Facteurs de quantité

Ces facteurs sont liés au potentiel inséminateur, qui peut se mesurer de deux manières: quantité totale de sperme qu'un mâle peut produire et quantité de sperme injectée à chaque insémination.

Il est évidemment nécessaire que, lorsqu'une femelle a été fécondée par les deux types de mâles, les deux types de sperme forment un mélange homogène. Taze et al. [10, 11] ont vérifié chez des femelles accouplées avec des mâles stériles et des mâles normaux que «la stérilité induite chez les femelles décroît quand le nombre d'accouplements avec des mâles normaux augmente». Ceci est en faveur de l'hypothèse du mélange des spermatozoïdes dans les spermathèques avec fécondation des oeufs par un spermatozoïde provenant au hasard du sperme stérile ou du sperme normal.

Le potentiel inséminateur des mâles est encore très mal connu. On suppose généralement que les mâles ont déjà, au moment de l'éclosion, tout le stock de spermatozoïdes déjà complet, et qu'il ne se produit pas de spermatogenèse pendant la vie adulte [6, 7]. Mais on ne connaît pas encore la quantité totale du sperme produit, ni les quantités délivrées au cours des divers accouplements.

La seule étude en laboratoire ayant eu pour objet le potentiel inséminateur des mâles a été faite par Jordan en 1972 [21]. Cet auteur a mesuré l'efficacité inséminatrice des mâles d'après la production moyenne de pupes par femelle, selon le numéro d'ordre de l'accouplement effectué par le mâle. L'auteur a obtenu, chez *G. austeni* et *G. morsitans*, des valeurs élevées de pupes/femelle/jour jusqu'au 6^e accouplement du mâle, les valeurs allant en diminuant pour les accouplements ultérieurs. Chez *G. austeni* on est allé jusqu'au 9^e accouplement; chez *G. morsitans*, grâce à la longévité plus élevée de cette espèce on est parvenu à 12 accouplements, mais la baisse de productivité au cours des trois derniers accouplements a été très accentuée. Les femelles ont été disséquées après leur mort, et l'auteur indique que chez les femelles accouplées à des mâles plus «utilisés», non seulement le degré de remplissage des spermathèques était moindre, mais encore beaucoup d'entre elles présentaient des spermathèques vides. On a aussi observé que la productivité augmentait quand les femelles restaient 24 heures avec les mâles, au lieu du temps d'un seul accouplement.

Ces résultats indiquent donc que les mâles vont apparemment en épuisant leur réserve de sperme, mais ne nous informent ni sur la quantité initiale de ce sperme, ni sur le nombre d'accouplements efficaces réalisés par chaque mâle; en réalité, à un nombre d'accouplements de 12 peut très bien correspondre un nombre beaucoup plus réduit d'inséminations. On a constaté également que de nombreux mâles survivants, disséqués après le dernier accouplement, avaient encore des spermatozoïdes dans les testicules.

Curtis [6, 7] a lui aussi observé que les mâles de *G. austeni* pouvaient effectuer de 9 à 15 accouplements, mais ne donne aucune indication qui nous permettrait de nous faire une idée sur l'efficacité de tous ces accouplements.

Etant donné qu'il n'y a pas de spermatogenèse après l'éclosion, il est bien possible que la quantité totale de sperme puisse être déterminée par les conditions d'élevage ou d'autres facteurs, tels que, par exemple, l'âge auquel la stérilisation a eu lieu (à l'âge adulte ou au stade pupal) ou même l'agent stérilisateur utilisé.

Aux fins de comparaison, il nous a paru nécessaire de connaître le plus exactement possible la quantité de sperme produite par un mâle et la quantité moyenne délivrée à chaque insémination.

Dans une première expérience, nous avons utilisé un lot de mâles qui étaient accouplés tous les cinq jours, mais cet intervalle s'est révélé trop long et la plupart des mâles étaient morts avant que des conclusions définitives n'aient pu être tirées. Nous avons constaté néanmoins que chaque mâle pouvait remplir totalement les spermathèques d'au moins quatre femelles.

L'expérience actuellement en cours porte sur un lot de 14 mâles. A partir du 4^e jour de leur vie, les mâles sont accouplés tous les 2 ou 3 jours à une nouvelle femelle vierge. Après un accouplement d'au moins deux heures et la séparation spontanée, les couples sont séparés et les femelles sacrifiées 48 heures après pour permettre de déterminer le degré de remplissage des spermathèques. Celui-ci est mesuré par le rapport entre le diamètre de la spermathèque et celui de la masse de sperme. On obtient ainsi le degré de remplissage pour chaque spermathèque; la valeur finale pour chaque insémination est obtenue par la moyenne arithmétique de la valeur des deux spermathèques de chaque femelle. Ainsi, l'unité signifie que les deux spermathèques étaient pleines et zéro signifie qu'elles étaient vides. En additionnant les valeurs obtenues à chaque nouvelle insémination pour le même mâle, nous obtenons la quantité totale de sperme qu'il a délivrée.

Après les mesures, les spermathèques sont écrasées et seulement celles qui contiennent des spermatozoïdes sont considérées comme inséminées.

Jusqu'à présent, seulement un mâle est mort et tous les autres mâles en sont déjà à leur 9^e accouplement.

Il est encore trop tôt pour tirer des conclusions définitives mais on peut déjà faire un certain nombre d'observations.

L'expérience a déjà révélé un fait important: il existe des cas de stérilité congénitale chez les mâles. Dans un travail antérieur [2] nous avons déjà émis cette hypothèse, car nous avons observé que des femelles de *G. morsitans* accouplées au même mâle restaient stériles. Mais aujourd'hui nous en avons la preuve, car dans notre lot un mâle s'est révélé incapable, après avoir été accouplé à 9 femelles, d'en inséminer une seule, ce malgré que ses accouplements se soient déroulés normalement et que leur durée ait été normale (de deux heures à plus de huit heures).

L'existence d'un pourcentage élevé de mâles incapables d'inséminer les femelles dans un lot de mâles irradiés serait évidemment un sérieux inconvénient et je crois que cette question mérite d'être examinée.

Le taux moyen d'insémination après 8 accouplements est de 0,60. Les valeurs de chaque accouplement sont les suivantes:

1 ^{er}	0,41	6 ^e	0,69
2 ^e	0,60	7 ^e	0,68
3 ^e	0,49	8 ^e	0,62
4 ^e	0,75	9 ^e	0,41
5 ^e	0,63		

Dans toutes les séries d'accouplements on a observé des spermathèques vides, dans des pourcentages qui oscillent entre 7 et 23%.

Dans toutes les séries, sauf dans la 5^e, on a observé également des spermathèques pleines, dans des pourcentages oscillant entre 0 et 40%.

On n'observe pas de différence accentuée entre les accouplements successifs en ce qui concerne les taux d'insémination et les pourcentages de spermathèques vides et pleines.

Le degré de remplissage des spermathèques n'a aucun rapport avec la durée de l'accouplement.

Ces résultats, bien qu'incomplets, font ressortir deux éléments importants. Le premier est l'existence de la stérilité congénitale. Si ce phénomène était lié aux conditions d'élevage, son importance pratique pour la méthode des lâchers de mâles stériles serait considérable, surtout si des pourcentages de l'ordre des 7% se confirmaient. Il constituerait un inconvénient à ajouter à d'autres, comme la baisse du pouvoir inséminateur provoquée par l'irradiation. En effet, selon Taze et al. le «le degré de remplissage des spermathèques des femelles (*G. palpalis gambiensis*) mises en présence de mâles irradiés diminue progressivement au fur et à mesure

que la dose d'irradiation augmente». Ces résultats sont en opposition avec ceux d'Itard [18] qui, avec *G. tachinoides*, a observé que les mâles irradiés avaient généralement une capacité d'insémination supérieure aux mâles témoins et que la capacité d'insémination était indépendante de la dose d'irradiation. L'autre aspect important est l'occurrence régulière, dans des pourcentages de l'ordre de 20%, d'accouplements «ratés», c'est-à-dire non suivis d'insémination. Si ce phénomène était lui aussi provoqué par les conditions d'élevage, les mâles irradiés seraient alors très défavorisés par rapport aux mâles sauvages.

Facteurs de disponibilité

Ce sont les facteurs qui peuvent influencer, dans la nature, sur l'accouplement des femelles sauvages avec des mâles irradiés. Je ne m'attarderai pas sur ce sujet et je préfère renvoyer le lecteur intéressé aux travaux de Cuisance et Itard [22–25], Clair et al. [26], Taze et al. [10, 11] et Cuisance et al. [27], dans lesquels des essais de lâchers de mâles stériles sont décrits.

De tous ces travaux, comme de ceux de Dame et al. en Rhodésie, il ressort que la longévité et la dispersion sont diminuées par l'action des rayonnements et qu'un compromis est nécessaire entre un taux de stérilisation de 100% et une survie garantissant une compétitivité satisfaisante [10, 11, 26].

Conclusions

Pour tous ceux qui ont élevé des glossines en laboratoire, le principal centre d'intérêt a été la productivité des femelles. Celles-ci ont été le point de départ de presque toutes les observations — longévité, physiologie de la reproduction, nombre et rythme des pontes, qualité des pupes produites, etc.

L'existence d'élevages destinés à fournir des mâles pour des opérations de lâchers de mâles stériles devrait, à mon avis, encourager des chercheurs à orienter leurs travaux dans d'autres directions. La production de mâles plus robustes, d'une survie plus grande, doués d'un pouvoir inséminateur plus élevé, devrait être l'objectif principal de tous les éleveurs.

Les cas de stérilité congénitale constatés dans les élevages devraient faire l'objet d'une attention particulière et, si ce phénomène devait s'avérer non négligeable, il serait souhaitable d'étudier ses causes et de chercher à y remédier. Parallèlement ce phénomène devrait aussi être étudié dans la nature.

La capacité d'insémination en termes de production de sperme devrait aussi être étudiée chez les mâles d'élevage et chez les mâles sauvages. L'influence éventuelle des techniques de stérilisation, à savoir non seulement l'agent et la dose mais aussi la phase de la vie à laquelle elle doit avoir lieu, mérite également une attention particulière.

La compétition dans la nature devrait aussi faire l'objet d'études plus approfondies. Je pense que l'étude de la stérilité induite chez les femelles sauvages mérite une attention particulière parce qu'elle peut nous donner des renseignements théoriques importants. Quand Taze et al. disent avoir observé que la stérilité induite chez les femelles sauvages est de deux types, totale et partielle, ils ne font que confirmer l'hypothèse selon laquelle les divers types d'accouplement se font selon une distribution binaire.

Il est plus facile de mesurer le pourcentage des femelles totalement fertiles et celui des femelles totalement stériles en termes quantitatifs. Ces deux catégories sont représentées respectivement par p^n et q^n . Dans toute expérience de lâcher de mâles irradiés, il importe de connaître précisément l'importance respective de ces deux catégories. Cette indication, lorsqu'on connaît également le rapport mâles stériles/mâles fertiles, peut nous renseigner sur le nombre moyens d'accouplements dans la nature et constituer l'une des meilleures sources de renseignements sur les progrès du processus d'éradication.

REFERENCES

- [1] LINDQUIST, A.W., "Biological information needed in the sterile-male method of insect control", Sterile-male Technique for Eradication or Control of Harmful Insects (C. R. Groupe d'étude, Vienne, mai 1968), AIEA, Vienne (1969) 33.
- [2] PINHÃO, R.C., Contribuição para o estudo da reprodução e ciclo evolutivo da *Glossina morsitans* West., Ann. Inst. Med. Trop. 23 3/4 (1966) 311.
- [3] PINHÃO, R.C., "Anatomical bases of the mechanism of egg fertilisation in tsetse flies", 1st International Symposium - Criação da mosca tsé-tsé em laboratório e sua aplicação prática, Lisbonne, 1970.
- [4] JORDAN, A.M., The mating of *Glossina palpalis* in captivity, Bull. Entomol. Res. 49 (1958) 35.
- [5] DAME, D.A., FORD, H.R., Multiple mating of *Glossina morsitans* and its potential effect on the sterile male techniques, Bull. Entomol. Res. 58 (1968) 213.
- [6] CURTIS, C.F., Radiation sterilisation and the effect of multiple mating of females in *Glossina austeni*, J. Insect Physiol. 14 (1968) 1365.
- [7] CURTIS, C.F., Some observations on reproduction and the effects of radiation on *Glossina austeni*, Trans. R. Soc. Trop. Med. Hyg. 62 (1968) 124.
- [8] PINHÃO, R.C., GRACIO, A.J.S., The degree of spermathecal impregnation and the number of matings in *Glossina austeni*, Ann. Inst. Hig. Med. Trop. 1 (1973) 103.
- [9] CHALLIER, A., Etude de la présence des cicatrices copulatrices, du degré d'insémination et leurs variations saisonnières chez les femelles de *Glossina palpalis gambiensis* Vanderplank en zone de savane soudanienne (Haute-Volta), Cah. ORSTOM, Sér. Entomol. Méd. VI 1 (1972) 55.
- [10] TAZE, Y., CUISANCE, D., POLITZAR, H., CLAIR, M., SELLIN, E., Essais de détermination de la dose optimale d'irradiation des mâles de *Glossina palpalis gambiensis* (Vanderplank, 1949) en vue de la lutte biologique par lâchers de mâles stériles dans la région de Bobo-Dioulasso (Haute-Volta), Rev. Elev. Méd. Vét. Pays Trop. 30 (1977) 1269.

- [11] TAZE, Y., CUISANCE, D., POLITZAR, H., CLAIR, M., SELLIN, E., Essai de détermination de la dose optimale d'irradiation des mâles de *Glossina palpalis gambiensis* (Vanderplank 1949), Rev. Elev. Méd. Vét. Pays Trop. 30 (1977) 269.
- [12] KNIPLING, F., Sterile-male method of population control, Science 130 (1959) 3366.
- [13] VON BORSTEL, R.C., Population control by release of irradiated males, Science 131 (1960) 878.
- [14] DAME, D.A., DEAN, G.J.W., FORD, J., "Investigations of the sterile male technique with *Glossina morsitans*", Proc. 10th Meeting International Scientific Committee for Trypanosomiasis Research (Kampala, Uganda) (1964) 93.
- [15] POTTS, W.H., Sterilisation of tsetse flies (*Glossina*) by gamma radiation, Ann. Trop. Med. Parasitol. 52 (1958) 484.
- [16] ITARD, J., «Stérilisation des mâles de *Glossina morsitans morsitans* et de *Glossina tachinoides* West., par irradiation aux rayons gamma», Premier symposium sur l'élevage en laboratoire de la mouche tsé-tsé et ses applications pratiques, 22–23 avril 1963, Lisbonne, Portugal.
- [17] ITARD, J., «Premiers résultats d'un essai d'irradiation gamma sur des pupes et des mâles adultes de *Glossina morsitans morsitans*», Control of Livestock Insect Pests by the Sterile-male Technique (C.R. Groupe d'étude, Vienne, janvier 1967), AIEA, Vienne (1968) 59.
- [18] ITARD, J., Stérilisation des mâles de *Glossina tachinoides* West. par irradiation aux rayons gamma, Rev. Elev. Méd. Vét. Pays Trop. 21 4 (1968) 479.
- [19] ITARD, J., "Sterilisation by gamma irradiation of adult male *glossinae*. Low dosage irradiation (4000 to 6000 rads) of adult males *G. tachinoides*", I.S.C.T.R., 13^e réunion, Lagos, Nigeria (7–11 Sept. 1971) Doc. 4, Publ. n° 105 (1971) 321.
- [20] DAME, D.A., FORD, H.R., The effect of the chemosterilant tepa on *Glossina morsitans*, Bull. Entomol. Res. 56 (1966) 649.
- [21] JORDAN, A.M., The inseminating potential of male *Glossina austeni* Newst. and *G. morsitans morsitans* West., Bull. Entomol. Res. 62 (1972) 319.
- [22] CUISANCE, D., ITARD, J., Lâchers de mâles stériles de *Glossina tachinoides* West. dans un gîte naturel de faible densité (Bas-Logone, Cameroun), Rev. Elev. Méd. Vét. Pays Trop. 26 4 (1973) 405.
- [23] CUISANCE, D., ITARD, J., Comportement de mâles stériles de *G. tachinoides* West. lâchés dans les conditions naturelles — environs de Fort-Lamy (Tchad), I. Transport, lâchers, rythme d'activité, action sur la population sauvage, Rev. Elev. Méd. Vét. Pays Trop. 26 1 (1973) 55.
- [24] CUISANCE, D., ITARD, J., Comportement de mâles stériles de *G. tachinoides* West. lâchés dans les conditions naturelles — environs de Fort-Lamy (Tchad), II. Longévité et dispersion, Rev. Elev. Méd. Vét. Pays Trop. 26 2 (1973) 169.
- [25] CUISANCE, D., ITARD, J., Comportement de mâles stériles de *G. tachinoides* West. lâchés dans les conditions naturelles — environs de Fort-Lamy (Tchad), III. Lieux et hauteurs de repos; comportement alimentaire, Rev. Elev. Méd. Vét. Pays Trop. 26 3 (1973) 339.
- [26] CLAIR, M., POLITZAR, H., CUISANCE, D., LAFAYE, A., Observation sur un essai préliminaire de lâchers de mâles stériles de *Glossina palpalis gambiensis* (Haute-Volta), Rev. Elev. Méd. Vét. Pays Trop. 29 4 (1976) 341.
- [27] CUISANCE, D., POLITZAR, H., CLAIR, M., SELLIN, E., TAZE, Y., Impact des lâchers de mâles stériles sur les niveaux de deux populations sauvages de *Glossina palpalis gambiensis* en Haute-Volta (sources de la Volta Noire), Rev. Elev. Méd. Vét. Pays Trop. 31 3 (1978) 315.

DISCUSSION

M.E. LEEGWATER-VAN DER LINDEN: I have an experiment currently in progress for checking the insemination capacity of the *Glossina pallidipes* males I am rearing in Amsterdam. The males are able to fill up both spermathecae completely four times in succession in one morning. A fifth or sixth copulation occurs sometimes without sperm transfer but, after approximately one hour, the males are again able to fill up the spermathecae.

Copulation time for the first copulations is approximately 20 minutes and later lasts approximately 50 minutes. After a period of rest the copulation time is sometimes 20 minutes again, as with the first copulation. This demonstrates the ability of these males to replenish their sperm stores within a very short time. Could you give me more details on how you measure the diameters of spermathecae and sperm mass.

R.C. PINHÃO: First of all, let me congratulate you on your results and, in particular, your *G. pallidipes*! I measure both the spermathecal diameter and the sperm mass with a graduated eye-piece fitted into the microscope with low amplification (10 X objective). The dimensions are, of course, only relative. I also use a cover slip.

M.E. LEEGWATER-VAN DER LINDEN: What was the interval between the opportunities for mating that you gave the males?

R.C. PINHÃO: The males are offered the first opportunity to copulate on the fourth day of life and thereafter on alternate days or after three days if there is a Sunday in between.

R. GALUN: The question whether multiple matings affect sterile males keeps coming up. We have produced experimental evidence which supports Von Borstel's theory on competition at the sperm level. Where ticks mated many times, the sterility of the eggs showed the ratio of sterile to fertile males. For example, females that mated with ten sterile and one fertile male produced about 10% fertile eggs and 90% sterile, whereas with females mating with sterile males in a one-to-one ratio, half the eggs are sterile.

C.F. CURTIS: I doubt whether the ability of males to perform six or seven inseminations is relevant to sterile male release programmes. If the released-to-wild ratio is for example 5 : 1 and only males are released, the overall male-to-female ratio will be 6 : 1. Under these conditions, unless each female mates many times, few males would have the opportunity of more than one or two matings.

R.C. PINHÃO: Multiple mating is important only if the irradiated males are weaker or less competitive than wild males. When this is so the importance of multiple mating depends on the kind of weakness and not normally on the number of matings.

I also have the impression that in practice a young female may well not copulate more than twice. If you look at the figures presented by Clair and co-workers you will see that for a sterile-to-normal male ratio of 1 : 1 they obtained approximately 25% totally sterile wild females. Nevertheless, the high proportion of old wild females that some authors have found with full spermathecae suggests that at least one further insemination can occur late in life.

**MASS REARING OF TSETSE FLIES;
COMPUTER MODELLING
(Session 8)**

RECENT ADVANCES IN THE REARING OF *Glossina pallidipes* Austen

M.E. LEEGWATER-VAN DER LINDEN
Laboratory of Experimental Entomology,
University of Amsterdam,
Amsterdam,
The Netherlands

Abstract

RECENT ADVANCES IN THE REARING OF *Glossina pallidipes* Austen.

The rearing technique of *Glossina pallidipes* Austen in Amsterdam is described. The flies are kept at 25°C, 80% relative humidity in very dim light which operates 12 hours a day. Flies are fed on ears of lop-eared rabbits inside the colony room. Nine-day-old females are mass-mated with an equal number of 12-day-old males for approximately three days. Mated females are kept without males. Under these circumstances the interlarval period is nine days. The first larva is deposited on the 20th day after emergence and the mean longevity hereafter is 60 days average or 101 days with individual attention. Productivity has been observed from 1977 onward. The insemination rate gradually increased from 66 to 97%, and fecundity from about 0.5 to 0.8. Premating deaths remained at a level of 4%. The eclosion rate was 92% and pupal weight 39 mg. In the Amsterdam practice these figures imply that nearly one third of the weekly pupal production is needed to maintain the colony at a set number. The remainder can be used for other purposes. Judging from observations and experience it seems that prevention of chemical contamination and any disturbance, mass-mating at the appropriate time and a very high humidity are relevant points in the various techniques used in rearing *G. pallidipes*. Attention is now being given to the viability of the colony in Amsterdam for eradicating *G. pallidipes* in East Africa. Females emerging from pupae sent from a test area near Tanga, Tanzania, were readily inseminated by males from the Amsterdam colony, indicating the possibility of using the sterile male technique for reduction of *G. pallidipes* in East Africa.

In 1975 an attempt was made to start a colony of *Glossina pallidipes* Austen in the Laboratory of Experimental Entomology at the University of Amsterdam. Mrs E.S. Opyo from EATRO, Tororo, Uganda, kindly sent us three batches of pupae collected near Lugula, in the Lake Victoria area. From the 36 females which produced initially, the colony had grown to over 1500 producing females early in 1978 and is presently kept at a number of 500 producing females. Though results of experiments concerning optimal breeding conditions are not yet fully evaluated, a description of our present way of rearing seems justified.

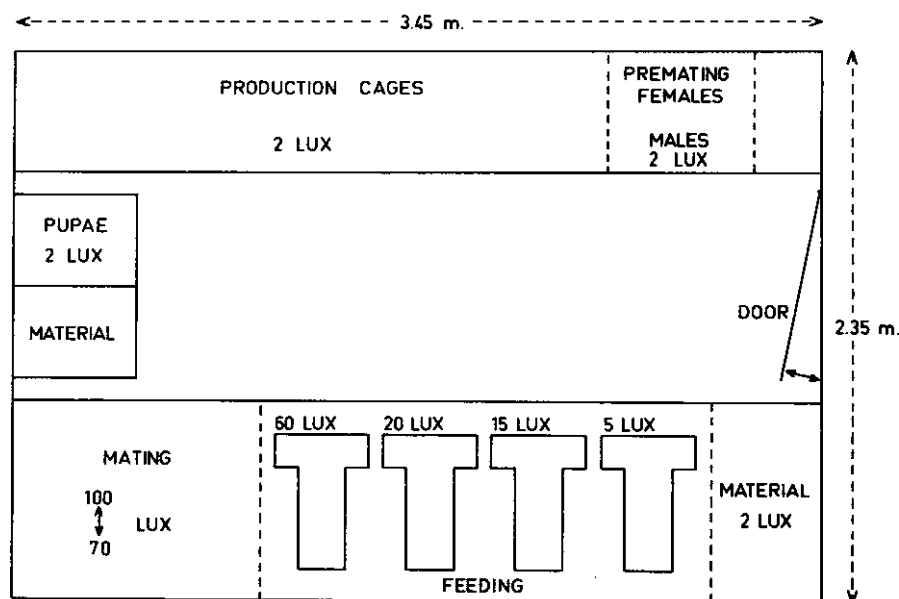


FIG.1. Housing of *Glossina pallidipes*. The light intensity in different parts of the room is indicated.

HOUSING THE COLONY

Flies are kept in a room measuring 3.45 X 2.35 X 2.00 m, in which temperature and humidity are controlled at 25°C and 80% relative humidity. In one corner of the room two fluorescent lamps (each 20 W) are lit for 12 hours daily. The lamps are covered by black paper in such a way that the bright light (70–100 lx) shines down on a bench only. At this bright spot males are added to females for mating. Mating cages are left there as long as males and females are held together. Away from the bright spot on the same bench, flies are fed on ears of lop-eared rabbits. At the other side of the room, which is lit very dimly (2 lx), young flies and cages with producing females are kept on three shelves one above the other. Pupae are also kept in a shady place (2 lx) in some seven containers above each other. Figure 1 shows the arrangement of the colony room.

STANDARD REARING TECHNIQUE

Flies that have emerged on the same day are held in Geigy-type cages [1] measuring 540 ml; sexes are kept separately, with up to six females, in a later stage

of the study, up to 12 in a cage; there are up to 20 males per cage. Mating takes place in these cages between females aged nine days and males aged 13 days or older (day of emergence is day 0) by putting an equal number of males and females in the cage; originally one to six pairs, later 12. They are left together for approximately three days. Separation is done manually without immobilization. Females are then transferred to production cages (540 ml) with up to 12 females in each cage. Males are used for a second mating or discarded. (As no work is done on Saturdays and Sundays, mating cannot always be done on the female's ninth day, but is often done earlier to ensure that there are males available for mating when the females are nine days old.) After birth of a larva it drops through the netting of the cage and pupates in the container below. Pupae are transferred to small glass jars covered with gauze, with filter paper at the bottom. After emergence flies are caught manually and transferred to Geigy cages. The flies are fed by strapping the cages to the ears of the rabbits [2] for approximately 10 minutes. Flies which are reluctant to feed are persuaded by activating them. Feeding is done on Mondays, Tuesdays and Wednesdays in the morning and on Fridays in the morning and afternoon. In this manner weekend feeding can be omitted without great losses.

Male and female flies which are to be mated are not fed until they have been together for approximately one hour. On the following days they are offered food according to the above scheme. The number of flies applied to each rabbit has varied from 50 to 350 a day. At present it is some 200 a day and a rabbit is used once or twice a week. The flies are preferably handled in the mornings.

In some instances mated females have been kept singly in a 42-ml Geigy-type cage to measure larviposition.

The following records have been kept daily except on Saturdays and Sundays:

- Number of premating deaths
- Number of deaths during mating procedure
- Number of deaths in production cages
- Age of females at their natural death
- Number of pupae per cage
- Pupal weight immediately after collection
- Emergence ratio
- Ratio of males and females at emergence

RESULTS AND DISCUSSION

Observations showed that the first possible larva was produced on the 20th day after emergence.

Longevity was calculated from deaths occurring in 43 production cages each with 12 females initially, from September 1977 onward. Longevity was 60 days

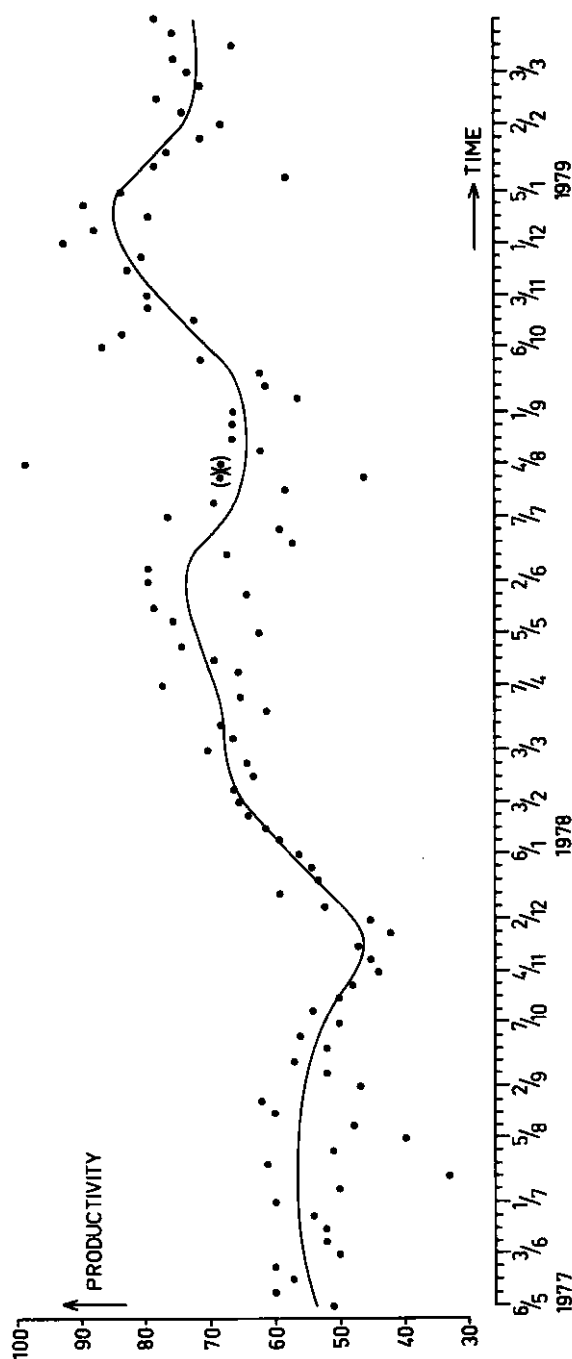


FIG. 2. Productivity: puparia produced/potential number of puparia; $(\cdot X \cdot)$ = average of the two irregular intervals.

after the 20th day (80 days after emergence), standard deviation 12.0 (data were obtained until 120 days after emergence).

In the same time a total of 71 females received individual care in individual cages. Their mean longevity was 101 days after the first potential larva (121 days after emergence), standard deviation 44.3.

A longevity record observed in flies kept singly is 239 days during which 21 larvae were produced.

Interlarval period

Starting in August 1977, 37 females were kept singly and checked daily for larviposition; 30 females produced larvae regularly; of their 185 interlarval periods the mean was 8.9 days, standard deviation 1.4. (If the 210 interlarval periods of the total number of 37 producing females were taken together the mean was 9.6 days, standard deviation 3.8.)

Starting in November 1977, 11 females were kept singly. Only four produced larvae regularly. The mean interlarval period over 49 periods was 9.3 days, standard deviation 1.4. (Taking together all 11 females, the mean interlarval period over 56 periods was 10.8 days, standard deviation 4.4.)

Subsequently nine days were considered the interlarval period for our circumstances.

Fecundity

Every Friday the number of producing females was counted and the number of pupae produced in the preceding week. From these figures fecundity has been calculated as follows:

$$\frac{\text{pupae produced}}{\text{producing females} \times 7}$$

The results are presented in Fig.2. In 1977 fecundity varied at about 0.5; in October 1978 there was a peak of 0.8.

Because of lack of personnel in October 1978 the mating scheme had to be altered slightly: some 12 females were held per cage to be mated with 12 males and were left together until the developing larvae were clearly visible in the females. When in December with the arrival of a new assistant the standard technique was restored, the fecundity declined slightly to the earlier level. Early 1977, when the colony was small, mating cages usually contained three to four females and an equal number of males, and mating opportunities were relatively low. Cages were separated after a maximum of three days. The insemination rate then was 66%. Later when six females per mating cage was normal, the insemination rate increased to

TABLE II. PERFORMANCE OF *G. pallidipes* IN TORORO AND AMSTERDAM

	Tororo	Amsterdam
	Means of different generations	Means in early 1978
Pupal weight	36.4–38.0 mg	38.6 mg
Total percentage of usable pupae which become producing females in the succeeding generation	18.9–43.1%	41%
Pupae per female	4.0–5.6	6

Present mating technique

The experiences mentioned above were reason to adjust the standard mating technique in such a way that there are plenty of mating opportunities for females and males, i.e. 12 females and 12 males per cage. Care has been taken that females and males are brought together early enough not to miss the first reproductive cycle, i.e. for females on the ninth day after emergence or earlier, for males on the 13th day or correspondingly earlier.

Performance of colony

Some figures calculated from data obtained early in 1978 are given in Table I. At present the colony is kept at a rate of 500 producing females by adding approximately 40 adult females a week to the producing colony. Females are kept some 130 days after emergence or until their natural death.

The weekly pupal production varies from 291–354. Of these roughly 130 become producing females which means that nearly one third of the weekly pupal production of the colony is needed to maintain the colony taking into consideration that one has to select 40 females from unsexed pupae.

The results can be compared with results of a colony in Tororo reported by Rogers and Kenyanjui [4]. Some data for both colonies are given in Table II. The better results in Amsterdam can possibly be attributed to rearing conditions. The differences in rearing technique for the two colonies are summarized in Table III.

TABLE III. DIFFERENCES IN REARING TECHNIQUE OF *G. pallidipes* COLONIES IN TORORO AND AMSTERDAM

	Tororo	Amsterdam
Feeding	Oxes in a crush daily; cages are covered with a black cloth to prevent distraction of flies by light	Rabbits in the colony room Mo, Tu, We, — Fr, Fr, — —
Light intensity in colony room	Window light in the morning	No window; 12 hours dim light, 12 hours dark
Temperature	25°C at daytime 20°C at night	25°C day and night
Relative humidity	75%	80%
Mating technique	1 single female mated to 2 males for 24 hours; 1 male:10 females in production cages	1–12 females mated together to as many males for 3 days; no males in production cages
Premating females	Held singly	Females of the same emergence date held together
Adult females	Cages contain flies selected by weight	No selection made
Pupae	Pupae weighing less than 30 mg are discarded	No pupae are discarded
Visual stimuli	Reduced by cabinets and drop-down flaps	Very dim light and the general rule to behave quietly
Quality control	Weak flies at emergence were discarded	Initially all flies alive on the day of mating were used; later any selection was avoided

Importance of differences in the rearing conditions

Recently some *G. pallidipes* pupae have been transported to the IAEA laboratories in Seibersdorf, to start a daughter colony fed on artificial membranes. The initial reduced survival and productivity, attributed to the artificial membranes filled with cattle blood, disappeared when humidity in the colony room could be raised to 80% (W. Takken, pers. comm.). This probably indicates the importance of a high humidity rather than the source of food. A mating technique which offers

plenty of mating opportunities appeared essential. In Amsterdam one was reluctant to do so initially because of the many deaths which occurred in mating cages. A closer look at the mating cages revealed that dead flies were situated in cul-de-sacs of ill-fitting cages from which they could not reach the rabbit's ear during feeding. Sewing the netting in such a way that flies could no longer become trapped reduced mortality at mating to incidental cases. Similarly too many flies were lost when cages with their bowed ends were situated towards the light source instead of towards the dark side of the room. This illustrates the necessity of attentive care. Death and production must be watched carefully, unusual events in the colony and conditions must be registered and not taken for granted.

Though mating technique, humidity, light intensity and feeding technique are important, contamination with chemicals or their vapours nullifies all efforts, as in other *Glossina* colonies [5].

Validity

Considering that the Amsterdam colony originates from flies from Tororo, Uganda, and that trials for reduction of *G. pallidipes* by the sterile male technique are likely to be carried out in Tanga, Tanzania, the question arose whether the Amsterdam flies were of any operational value. To test this we obtained some pupae deposited by *G. pallidipes* trapped at Mkwaja Ranch near Tanga. After emergence Tanga females were mated to Amsterdam males and vice versa. The results were as follows:

F Tanga × M Amsterdam: 5 dissected after death at mating contained spermatheca full of sperms;
5 out of the remaining 7 females produced larvae though there were many abortions.

F Amsterdam × M Tanga: 4 out of 5 produced larvae which appeared healthy.

In the laboratory the two strains apparently inseminate each other easily, though this still does not provide information about the competitiveness of males (or attractiveness of females) in the field. (The vitality of hybrids is doubtful.)

The weekly surplus of *G. pallidipes* males is about one quarter of the colony size, a figure corresponding to figures given by Nash [5] for *G. austeni* (21–30%) and *G. morsitans* (18–25%), which justifies envisaging the sterile male technique for control of *G. pallidipes* in the field.

ACKNOWLEDGEMENTS

I wish to acknowledge the help of H.R. Bolland who succeeded in establishing the colony, and T.G.C. Overzier who maintained the colony.

This work is partly subsidized by the International Atomic Energy Agency.

REFERENCES

- [1] GEIGY, R., Elevage de *Glossina palpalis*, Acta Tropica 5 (1948) 201.
- [2] NASH, T.A.M., JORDAN, A.M., BOYLE, J.A., A promising method for rearing *Glossina austeni* (Newst.) on a small scale, based on the use of rabbits' ears for feeding, Trans. R. Soc. Trop. Med. Hyg. 60 2 (1966) 183.
- [3] SAUNDERS, D.S., The ovulation cycle in *Glossina morsitans* Westwood (Diptera : muscidae) and a possible method of age determination for female tsetse flies by examination of their ovaries, Trans. R. Entomol. Soc. Lond. 112 9 (1960) 221.
- [4] ROGERS, A., KENYANJUI, E.N.F., The maintenance of a colony of *Glossina pallidipes* Austen, Ann. Trop. Med. Parasitol. 66 2 (1972) 267.
- [5] NASH, T.A.M., JORDAN, A.M., TREWERN, M.A., "Mass rearing of tsetse flies (*Glossina* spp.): Recent advances", Sterility Principle for Insect Control or Eradication (Proc. Symp. Athens, 1970), IAEA, Vienna (1971) 99.

DISCUSSION

A.M. VAN DER VLOEDT: Your pilot mating tests have shown that *G. pallidipes*, and not only the 'super-males', are capable of inseminating several females within a very short time. Why are you still using the 1 : 1 ratio of females to males for your group mating in the stock colony? It must be possible to increase the ratio to at least two females to one male, thereby economizing on blood where the membrane system is used or reducing the challenge to the animal stock.

M.E. LEEGWATER-VAN DER LINDEN: I agree that it would be possible to reduce the number of males in the laboratory mating procedure and that this should be done for the sake of economy. I did not do this, however, because I preferred to maintain certain standard conditions over a long period of time.

W. TAKKEN: *Glossina pallidipes* is a very large fly and it is my experience that on membranes it takes up huge meals frequently. Thus the challenge to rabbits might be greater than with other tsetse species. What is the challenge you expose your rabbits to, and how often do you use them? Have you ever estimated the maximum number of flies you can feed on one rabbit?

M.E. LEEGWATER-VAN DER LINDEN: I have never measured the quantity of blood taken up per meal nor have I run experiments specifically with a view to testing rabbits, so I can only answer you on the basis of the experience I have had. The challenge at present is 150 flies per rabbit, the rabbits being used once or twice a week. The maximum challenge to the rabbits occurred in 1978 with 350 flies per rabbit, the rabbits being used three times a week. This challenge continued for approximately five months and I did not notice any negative effect on the rabbits' health.

A.M. VAN DER VLOEDT: Does pupal weight affect receptivity and the time of egg maturation and ovulation in the adult females ensuing from the pupae?

M.E. LEEGWATER-VAN DER LINDEN: I have no idea whether pupal weight affects maturation time but this could be investigated experimentally.

M.J. MUTINGA: Have any experiments been done which would indicate whether the willingness of females to mate is diminished or increased by the first mating? Could this be of relevance if one were trying to economize on the numbers of males needed in mass rearing because, if mating occurs on a random basis, large numbers of males would be needed?

M.E. LEEGWATER-VAN DER LINDEN: I have not done experiments to elucidate the increase or decrease in the willingness of females to mate after a first mating. The males are able to inseminate females at least four times in succession and I expect that one would need fewer males than females in mass-rearing plants. Some work has been done on this subject by T.G.T. Jaenson at the International Centre of Insect Physiology and Ecology (ICIPE) in Kenya and by A. Rogers at the East African Trypanosomiasis Research Organization at Tororo in Uganda.

T.G.T. JAENSON: In our experiments on *G. pallidipes* we found that more than 90% of virgins 9–10 days old were sexually receptive. Only 8% of females paired each day after emergence were sexually receptive on days 9–10. Thus, receptivity is higher in virgins than in non-virgins, which suggests that some aspect or aspects of copulation do affect subsequent sexual receptivity.

I would also like to comment on the choice of flies to be used for mating. Vanderplank (1949) and Maudlin (1970) cross-mated *G. pallidipes* from various areas in Africa. There were no mating barriers between flies of the different populations under laboratory conditions, and the hybrids were apparently fully fertile. However, data have indicated recently that the behaviour of *G. pallidipes* differs genetically between allopatric populations. I believe it is therefore advisable not to use *G. pallidipes* from Uganda, but to use flies of this species originating from an SIT programme at the Mkwaja Ranch.

Moreover, human sleeping sickness is transmitted by *G. pallidipes* at Lugala but the disease does not occur in the Mkwaja Ranch area. Until it has been established that this is not due to a greater vectorial capacity of the *G. pallidipes* from Lugala I believe one should be careful not to release such flies at the Mkwaja Ranch.

M.E. LEEGWATER-VAN DER LINDEN: I agree with your comments.

P.A. LANGLEY: What is the significance of the different light intensities above each feeding station as illustrated in Fig.1?

M.E. LEEGWATER-VAN DER LINDEN: The light intensity illustrates the actual light conditions in the laboratory. On ears nearest to the bright spot flies are sometimes distracted from feeding by the light. Flies feed best on ears away from the bright spot.

CROSS-BREEDING EXPERIMENTS WITH *Glossina morsitans morsitans* (Westwood) WILD GENOTYPE AND PHENOTYPE GENETIC MARKER *ocra*

A.M.V. VAN DER VLOEDT

Joint FAO/IAEA Division of Atomic Energy
in Food and Agriculture,
International Atomic Energy Agency,
Vienna

Abstract

CROSS-BREEDING EXPERIMENTS WITH *Glossina morsitans morsitans* (Westwood) WILD GENOTYPE AND PHENOTYPE GENETIC MARKER *ocra*.

Some cross-breeding experiments with *Glossina morsitans morsitans* wild type and phenotype genetic marker *ocra* are reported. The laboratory fitness, as expressed by mating frequency, fecundity, F_1 survival, phenotypic and sex ratio, is evaluated. The possible usefulness of the marker strains in a sterile male release programme is also considered.

INTRODUCTION

Theoretical considerations on vector control [1], the results of related laboratory experiments [2] and in particular the outcome of continuing field trials in Upper Volta [3] and Tanzania [4] indicate that the concept of utilizing the sterile male release method against tsetse flies is technically sound and shows promise for large-scale implementation.

The successful use of the SIRM depends upon a number of well-known requirements including economical rearing, optimal sterilization, adequate handling and subsequent releases leading to effective overflooding of the natural insect population. One of the important conditions for an efficient enterprise is the availability of reliable information regarding the ecology of the target species. Thus, population estimates and monitoring movement and survival abilities are essential before any population suppression measures are contemplated. Various marking techniques are applicable for collecting specific ecological information on dispersal, flight range and resting sites. The most conventional method used for mark-release-recapture studies consists in dye-marking adult flies with fluorescent powders or paints which do not interfere with behavioural patterns of the insects. During recent years, the application of radioisotope labelling

TABLE I. RESULTS OF INTRA-STRAIN CROSSES^a OF WILD TYPE *Glossina morsitans morsitans* (Tanga strain) AND PHENOTYPE MARKER STRAINS *ocra*

Type of cross	♀	♂	No. of mated females	Females reaching reproductive age (= day 18) (%)	Females surviving at day 50 (%)	Mean no. of puparia per initial female	Mean pupal weight ^b per reproductive cycle (N) (mg ± SD)	Emergence rate (%)	Offspring phenotype and sex ratio		
									Wild type	Mutant <i>ocra</i>	
									Female (%)	Male (%)	
A ^c +/+ × +/+			240	95.4	75.0	2.86	I 100 II 100 III 100 IV 100	95.2	51.6	48.4	
							25.2 ± 2.79 25.7 ± 2.80 26.8 ± 3.27 27.5 ± 3.03				
							I 100 II 100 III 100 IV 100	94.1			51.0 49.0
							25.6 ± 1.85 25.9 ± 1.98 25.2 ± 2.19 26.2 ± 2.06				
B $\sigma T/\sigma T \times \sigma T/\sigma T$			200	94.0	74.0	2.12	I 120 II 125 III 100 IV 70	93.7			
							28.4 ± 1.33 28.6 ± 1.32 28.5 ± 1.19 28.2 ± 1.68				
C $\sigma R/\sigma R \times \sigma R/\sigma R$			290	96.5	70.7	2.63					

^a Group-mating 10:10 and females regrouped per 15–20 in standard holding cages: experimental period 50 days.

^b Based upon the individual weight of random samples of pupae produced during the first four reproductive cycles.

^c A = Pooled data for production units of the Tanga wild-type stock colony fed on membranes (> 20 generations).

B = Pooled data for F₁ and F₂ experimental units of the Tanga *ocra* strain fed on guinea pigs.

C = Pooled data for F₁ through F₃ experimental units of the Rhodesia *ocra* strain fed on guinea pigs.

methods was considered by several workers [5–9] who demonstrated that some tagging substances when injected or administered in the diet of the laboratory-reared adult fly might be useful tools for studying various aspects of the ecology and behaviour of tsetse flies.

With increased interest in formal genetics of insects of veterinary and medical importance more research has been devoted to genetic mechanisms governing morphological differences and certain physiological characteristics [10, 11]. Genetic markers became available in many orders of insects and their applicability has been considered [12] but not practically explored, mainly because of reduced fitness for survival of the organisms carrying the mutant gene and their expected poor penetration into the natural population. As more and more insects are colonized and subjected to specific treatments the possibilities for finding good mutations that could be used as either biological markers or direct control measures will increase.

In recent years, visibly detectable phenotypes, i.e. the 'ocra' [4, 13] and the 'salmon' [14] mutations, have been isolated from *Glossina morsitans morsitans* Westwood reference strains. The genetic basis for the phenotypic character has been established and successful attempts at laboratory rearing of the *ocra* marker have been made [4, 15, 16].

The purpose of this study is to report on some cross-breeding experiments with *G.m. morsitans* wild type and phenotype genetic marker *ocra*. The laboratory fitness, as expressed by mating frequency, fecundity, F_1 survival, phenotypic- and sex-ratio of different crosses is studied. Furthermore, some considerations are given regarding the possible usefulness of the marker strains in a sterile male release programme.

MATERIALS AND METHODS

The wild type *Glossina morsitans morsitans* used in this study are from a colony that has been reared continuously at Seibersdorf since it was initiated in 1974 from pupae sent from the Tanga Tsetse Fly Project, Tanzania. In addition to this wild type stock colony reared on the membrane feeding system [17], experimental colonies of pure bred lines of two *ocra* strains have been maintained since 1977. One strain has been isolated by the workers of the Laboratory of Experimental Entomology, Amsterdam, The Netherlands, from a *Glossina morsitans morsitans* reference strain of Rhodesian origin. This marker strain is hereinafter referred to as (*oR*). The second one is a similar colour variation detected in the field at the Mkwaja Ranch, Tanga, Tanzania, and hereinafter referred to as (*oT*). Both *ocra* strains have been derived from a single male fly that when crossed with wild type females led to uniform wild type F_1 offspring.

TABLE II. RESULTS OF SINGLE-PAIR INTRA- AND INTERSTRAIN CROSSES OF WILD TYPE *Glossina morsitans morsitans* (Tanga strain) AND VISIBLE MARKERS

Type of cross	♀	♂	No. of replicate pair matings ^a	Females ^b found inseminated	Females surviving at day 50 (%)	Mean number of pupae ^c	First larva (days) ^d	Inter-larval period (days) ^e	Mean pupal weight (mg ± SD)	Emergence rate (%)	Offspring phenotype and sex ratio	
											Wild type (%)	Mutant <i>ocra</i> (%)
	+/+	X +/+	100	98	83.0	2.56	19.7	9.3	28.1 ± 3.26	94.0	51.8	48.2
	oT/oT	X oT/oT	100	97	79.0	2.19	20.0	9.3	27.9 ± 3.02	95.4	—	—
	oR/oR	X oR/oR	30	30	83.3	2.46	19.2	9.2	29.1 ± 2.32	92.1	—	—
	+/+	X oT/oT	30	30	85.4	2.85	19.2	9.7	29.2 ± 2.58	94.4	48.8	51.2
	+/+	X oR/oR	60	59	85.0	2.25	19.0	9.3	29.5 ± 2.94	93.4	46.1	52.9
	oT/oT	X +/+	30	29	80.9	2.61	19.8	9.6	27.9 ± 3.14	93.3	39.3	—
	oR/oR	X +/+	30	29	88.6	2.63	18.9	9.0	28.7 ± 3.08	97.0	42.5	—
	oT/oT	X oR/oR	30	30	83.3	2.56	18.6	9.2	27.1 ± 3.42	94.8	—	—
	oR/oR	X oT/oT	30	29	86.6	2.90	18.4	9.2	28.7 ± 3.01	96.5	—	—
											50.2	49.8
											56.6	43.4
											—	—
											—	—
											—	60.7
											—	57.5
											45.2	54.8
											49.4	50.6

^a Mating period 48 h.

^b Females dissected at day 50 and spermathecae examined for presence of sperm.

^c Mean number of pupae per initial female during 50-day period.

^d Minimum period between emergence and first larviposition was 16 days in all combinations.

^e Average length of period.

Results of intercrosses within the F_1 and F_2 generations indicated that the colour variant is a sex-linked recessive trait residing at the differential part of the X-chromosome. The mutation, which is very conspicuous, has been described in detail by Bolland and co-workers [13].

Adult flies emerging from pupae received from Amsterdam and Tanga formed the parental generation of the experimental colonies. All material was kept at $25^\circ-25.5^\circ\text{C}$, 60–70% relative humidity and a daily 12 h photophase. Morphological examination of the emerging (*oT*) and (*oR*) adults did not reveal any difference in general appearance.

The flies were fed six times weekly on guinea pigs' flanks. All possible pairing combinations of the available strains were made. Insects used were isolated as pupae to ensure virginity, and single pair matings were used to obtain all progeny data and all classifications were made by sex and phenotype. Using the above-mentioned genetic strains and in spite of some limitations associated with the sex-linked recessive character of the trait, the occurrence of multiple mating and possible multiple insemination was examined.

RESULTS

Performance of *G. m. morsitans* wild type and mutant phenotype in intra- and interstrain crosses

It is clear from Table I that there was very little difference in overall survival, insemination and emergence rate when wild type or mutant phenotype females were group-mated to their own type of males. However, during the 50-day test period the (*oT*) cross showed a markedly lower productivity than the (*oR*) cross in which the weight of the produced pupae was also consistently higher. On the other hand, no significant (at $p = 0.05$) departure from an expected 1:1 sex ratio was found in either of the cross groups.

Additional intrastrain pair matings were conducted together with reciprocal crosses of all phenotypic classes. Data on survival, insemination, average time elapsed between emergence and first larviposition, mean duration of the inter-larval period, emergence rate and frequency of progeny phenotypes and sexes are summarized in Table II. They indicate that all matings within and between the strains occurred readily and resulted in a nearly identical high degree of insemination. Consequently, all types of interstrain crosses exhibited normal fertility. However, taking the $+/+ \times +/+$ cross as control, it becomes obvious that most of the combinations in which material of Rhodesian origin was contributing showed the best performance in terms of pupal production and pupal weight.

TABLE III. OFFSPRING PHENOTYPES RESULTING FROM WILD TYPE, (*oT*) AND (*oR*) FEMALES KEPT TOGETHER WITH THREE TYPES OF MALES DURING 24 h (Ratio 1:1:1:1)

Type of female	Test number	Types of males ^a accepted at indicated sequence	No. of puparia and mean weight (mg)	Phenotype and sex of emerging flies		
				Wild type	Mutant <i>ocra</i>	
		+/+	<i>oT/oT</i>	<i>oR/oR</i>	Female	Male
+/+	1	(3)	(2)	(1)	2	3
	2	—	—	(1)	3	0
	3	—	(1)	(2)	4	0
	4	(2)	—	(1)	2	4
	5	(1)	(2)	—	2	0
					Two spermatophores in utero	
<i>oT/oT</i>	6	(1)	(2)	—	0	0
	7	—	(1)	(2)	0	2
	8	(2)	(1)	—	1	0
	9	(1)	(2)	—	1	0
	10	—	(1)	(2)	1	4 (<i>xx</i>)
					Female found inseminated and with second spermatophore	
<i>oR/oR</i>	11	(1)	—	—	2	0
	12	(2)	(1)	—	0	1
	13	—	(1)	—	0	3
	14	(2)	—	(1)	1	0
	15	(1)	—	(2)	2	3 (<i>xx</i>)
					0	0

^a Multiple mating observed under competitive conditions; females seen in copula within the first 7 h following confinement of sexes.
 (*xx*) Female using sperm from 2 matings.

The observed differences in fecundity of certain crosses involving Tanga material can neither be attributed to differential mortality nor to changes in first larviposition time or duration of the interlarval period. The resulting progeny from any particular reciprocal mating and results of some backcrosses confirmed that the *ocra* trait is sex-linked recessive. Furthermore, it is noteworthy that in the *oT/oT* or *oR/oR* $\times$ $+/+$ cross, progeny males considerably exceeded females.

Observations on selective and multiple mating of wild type and mutant flies

The fact that all females in the above-mentioned crosses showed a normal larviposition pattern and produced viable hybrids is indicative that no crossing barriers exist between the strains. Therefore it was investigated further whether some tendency for assortative mating under competitive circumstances occurred. Individual virgin wild type, (*oT*) or (*oR*) females were introduced into a standard oblong PVC cage (16 $\times$ 8 $\times$ 4.5 cm) containing sexual mature males each of a different type. As (*oT*) and (*oR*) flies are morphologically indistinguishable, males of both strains were differentiated by topical dye marking. Sexes were left together for 24 h and the mating pairs recorded according to the phenotype of the male. After separation, the females were allowed to produce six pupae and the phenotype of the emerging adults was scored.

The results in Table III show no evidence of discrimination by the females in favour of their own type of male. Moreover, it appears that any type of female was willing to remate with males of the other strain(s), and some females could be found to accept more than one spermatophore within a short interval. The question whether such multiple matings resulted in production of mixed progeny required further study.

Without using electrophoretic techniques, it is not possible to examine whether a wild type female is using sperm of different males. On the contrary, in the case of a mixed population containing both wild type and *ocra* individuals, segregation of *ocra* females and morphological examination of the offspring can give direct evidence for the use of different kinds of sperm by the same female. However, since *ocra* is a sex-linked trait it is only the phenotypes of the female offspring that provide such information.

The results for (*oT*) and (*oR*) females given in Table III show that in spite of their willingness to accept different males, most of them produced the theoretically expected phenotypes only. Apparently, two of the double-mated females used sperm from both matings.

In order to obtain more quantitative evidence regarding the impact of multiple mating, additional tests were conducted. From each strain, groups of five virgin females were mated to five males of a particular phenotype and left together for 24 h. Two days later, i.e. on day four following emergence, the same females were confined with five males of another strain. Their production was followed

TABLE IV. EFFECT OF SUCCESSIVE GROUP MATINGS OF WILD TYPE (+/+) *Glossina morsitans morsitans* AND MUTANT (*oT/oT* or *oR/oR*) FEMALE FLIES WITH BOTH TYPES OF MALES AT 48 h INTERVAL

Sequence of mating		No. of females in test	No. of females accepting first male	No. of females accepting second male	No. of puparia and mean no. per initial female by day 60	Mean pupal weight (mg $\pm$ SD)	Emerging flies	Offspring phenotype	
$\varnothing$	(1) δ	(2) δ						Wild type	Mutant <i>ocra</i>
+/+	X	+/+	X	<i>oT/oT</i>	5	5	5	10	8
+/+	X	<i>oT/oT</i>	X	+/+	5	5	5	6	10
+/+	X	<i>oR/oR</i>	X	+/+	5	5	5	8	10
<i>oT/oT</i>	X	<i>oT/oT</i>	X	+/+	5	5	5	(3) ^a	0
<i>oT/oT</i>	X	+/+	X	<i>oT/oT</i>	5	5	5	4	0
<i>oR/oR</i>	X	<i>oR/oR</i>	X	+/+	45	45	11	(1)	0
<i>oR/oR</i>	X	+/+	X	<i>oR/oR</i>	37	37	37	54	0
								0	(9)
								0	64

^a Figures in brackets refer to offspring fathered by the second mate.

TABLE V. RESULTS OF SINGLE-PAIR MATING OF *ocra* RHODESIA FEMALES TO BOTH WILD TYPE MALES AND *ocra* RHODESIA MALES
Interval between 2 matings: 48 hours

Sample No. ^a	Weight of pupae according to successive reproductive cycles ^b (mg)					Phenotype and sex of emerging flies			
						Wild type		Mutant <i>ocra</i>	
	I	II	III	IV	V	Female	Male	Female	Male
1	27.8	25.4	27.4	30.4	32.5	2		0	3
2	28.0	29.3	31.4	—	32.8	2		0	2
3	24.0	23.9	26.4	(27.4)	28.2	2		0	2
4	27.1	29.5	33.7	30.2	30.2	3		0	2
5	24.8	(23.4)	22.4	29.7	32.3	2		0	2
6	28.8	27.9	34.1	30.5	30.7	3		0	2
7	28.7	27.3	36.9	30.9	30.3	3		0	2
8	29.1	31.3	27.8	30.3	29.8	2		0	3
9	(26.3)	26.5	25.6	26.1	28.7	3		0	1
10	29.4	25.4	(26.7)	27.7	29.3	0		2	2
11	28.7	(25.3)	29.8	28.3	30.6	2		0	2
12	27.9	29.8	29.7	29.4	32.3	2		0	3
13	31.4	31.4	26.1	29.1	30.7	3		0	2
14	27.4	29.7	26.9	29.7	26.6	0		2	2
15	25.4	29.4	27.1	27.8	(27.7)	0		2	2
16	34.1	28.5	30.1	30.4	32.1	3		0	2
17	27.5	30.1	29.7	30.2	32.3	2		0	3
18	30.4	28.3	30.4	29.7	32.1	3		0	2
19	26.5	27.9	30.0	32.4	30.6	3		0	2
20	26.4	29.4	29.7	29.3	30.0	3		0	2
21	26.9	31.7	31.3	30.8	31.8	2		0	3
22	25.8	26.1	(25.8)	25.6	—	2		0	1
23	28.0	(25.4)				1		0	0
24	27.1	27.4				0		1	1
25	23.6	25.7				0		0	2
26	(23.9)	25.7				0		1	0
27	27.9	27.7				1		0	1
28	26.9	27.9				0		1	1
29	c								
30	c								
Mean weight	27.4 ± 2.27	27.7 ± 2.25	29.0 ± 3.28	29.3 ± 1.65	30.5 ± 1.73	49	—	6	52

^a All females observed in copula with each type of male.

^b Bracketed figures = no emergence.

^c Premature death.

TABLE VI. INSEMINATION CAPACITY OF IRRADIATED (12 krad) *ocra* AND WILD TYPE MALES

Single males caged with two untreated females for 72 hours

Type of treated male ^a	Total number of females of particular phenotype found inseminated			Number of males surviving 30 days post treatment
	+/+	oT/oT	oR/oR	
+/+ (10)	19	17	18	26
oT/oT (10)	18	16	17	21
oR/oR (10)	19	17	16	23

^a Three batches of 10 males of each phenotype.

up for 60 days and their offspring classified. In another test, 30 (*oR*) females, all emerged on the same day, were mated alternately to wild type Tanga males and to males of their own type. The interval between both matings was 24 hours. Results are summarized in Tables IV and V.

The results of double-mating tests with *ocra* females (Table IV) show that 63% of previously mated females were observed in copula with a second type of male. The pooled offspring contained 332 individuals (i.e. mean of 3.6 per female), but only 4 wild type females (versus 83 mutant females) and 10 mutant females (versus 59 wild type females) were due to the second mating. This confirmed that for most of the multiple-mated females the first mate was predominant. The data in Table V prove that the impact of multiple mating is limited, and the use of sperm from two matings is not a common phenomenon. None of the 30 females forced to accept a second mate produced mixed progeny. Some of them (20%) produced progeny fathered by the second male because the first mating apparently must have been ineffective through lack of sperm transfer.

Comparative observations on insemination capacity and post-treatment survival of irradiated *ocra* males and wild type males

Thirty males of each phenotype were irradiated when five days old with 12 krad of a cobalt-60 source and one day later distributed singly over standard cages containing untreated virgin females of a particular phenotype. After 72 h, the sexes were separated, the females dissected and their spermathecae examined for the presence of motile sperm. The males were regrouped with 30 in larger cages, fed six times weekly on guinea pigs and their survival monitored until day 30 post treatment.

The results in Table VI show that, irrespective the type of female with which they were confined, all irradiated males showed a nearly identical mating response, and the resulting insemination rate was not significantly different. Post-treatment survival was also very satisfactory but some of the *ocra* males showed reduced flight activity because of breakage at the wing base.

DISCUSSION

For practically every insect species whose genetics have been studied in any detail, organ-specific and pigmentary mutants have been found. It has been recognized that genetic markers of neutral selection value might be used to monitor the effect of released males upon the wild population. The competitiveness of the released material can be measured by the rate of recovery of the marker gene in field-collected flies. Many examples can be found in the literature of mutants whose practical use is hampered by lethal or sublethal conditions which prevent or reduce their penetration and expressivity in a native population.

The data presented on the visible marker mutation *ocra* found in *G.m. morsitans* reference strains reveal some interesting features. This body colour mutation is quite different from the other known eye colour mutant *salmon* [14] which under some conditions appears to be a maternally influenced post-pupal lethal mutant. The *ocra* flies of both Tanzanian and Rhodesian origin were found to be fully compatible with the wild type *G.m. morsitans* from Tanga, but some differences in fecundity were observed for certain reciprocal crosses. It has been demonstrated by Jordan and co-workers [15] that there are significant differences in laboratory performance between wild type strains of *G.m. morsitans* from Rhodesia and Tanga, and it has been postulated that the differences are related to genetic diversity. Possibly a similar degree of diversity exists between the Tanzanian and Rhodesian *ocra* strains, and influences the intrinsic fecundity.

Another interesting finding is that under competitive laboratory circumstances, mating was non-assortative and neither male proved to be a superior competitor. Further information gained on the occurrence of multiple mating is consistent with that given by Kawooya [16] and Gooding (personal communication). Multiple mating occurs frequently, but uptake and use of two kinds of sperm by the same female is fairly infrequent and most likely is made possible when the female accepts a spermatophore from a second male immediately after termination of the previous mating.

The *ocra* progeny from interstrain crosses and reciprocal backcrosses was found to have a very good initial fitness and fertility. However, it was noticed that in the pure bred lines of both the Tanga and Rhodesia strain up to 10% of the emerging *ocra* flies showed weakness of the wings. With aging of the flies, i.e. after day 15–20 following emergence, a considerable number of them showed

breakage at the wing basis. This phenomenon may have a genetic basis but requires further study.

Whether the sex-linked visible marker mutation will be a useful tool in a sterile male release programme remains to be determined. Its viability and reproductive performance, and in particular its compatibility with wild type strains of different origin, indicate that this easily recognizable marker could prove useful in field studies, e.g. for barrier testing.

ACKNOWLEDGEMENTS

The author thanks L. Williamson of the Tanga Tsetse Project and W. Helle of the Laboratory of Experimental Entomology, Amsterdam, who furnished *ocra* pupae. Thanks are due to R. Acs for his assistance in handling the experimental material.

REFERENCES

- [1] KNIPLING, E.F., Vector Control (WHO) 27 (1963) 17.
- [2] INTERNATIONAL ATOMIC ENERGY AGENCY, "Application of sterility principle for tsetse fly suppression: Review of a panel", At. Energy Rev. 10 1 (1972) 101.
- [3] I.E.M.V.T., Rapport d'activités, 1976, 1977, Institut d'Elevage et de Médecine Vétérinaire des Pays Tropicaux, Centre de Recherches sur les Trypanosomoses Animales, Bobo-Dioulasso, Haute-Volta.
- [4] WILLIAMSON, D.L., Monthly and Annual Reports of Tsetse Research Project, Tanga, 1975-78 (unpublished).
- [5] CURTIS, C.F., CURTIS, J., HAMANN, H.J., Int. J. Appl. Radiat. Isot. 24 (1973) 535.
- [6] CUISANCE, D., ITARD, J., «Techniques de marquage des glossines au moyen de radio-isotopes», Sterility Principle for Insect Control or Eradication (Proc. Symp. Athens, 1970), IAEA, Vienna (1971) 111.
- [7] HAMANN, H.J., LANGLEY, P.A., Int. J. Appl. Radiat. Isot. 29 (1978) 315.
- [8] TAYLOR, S., Bull. Entomol. Res. 68 (1978) 1.
- [9] HAMANN, H.J., IWANNEK, K.H., Z. Angew. Entomol. 86 (1978) 434.
- [10] WORLD HEALTH ORGANIZATION, Technical Reports Series, No. 398 (1968).
- [11] PAL, R., LACHANCE, L.E., Annu. Rev. Entomol. 19 (1974) 269.
- [12] BARTLETT, A.C., J. Hered. 58 (1967) 159.
- [13] BOLLAND, H.R., VAN BUREN, A., VAN DER GEEST, L.P.S., HELLE, W., Entomol. Exp. Appl. 17 (1974) 522.
- [14] GOODING, R.H., ROLSETH, B.M., IAEA Research Co-ordination Meeting, Antwerp, 1978 (unpublished).
- [15] JORDAN, A.M., TREWERN, M.A., SOUTHERN, D.L., PELL, P.E., DAVIES, E.D.G., Bull. Entomol. Res. 67 (1977) 33.
- [16] KAWOoya, J., Z. Angew. Entomol. 84 (1977) 321.
- [17] BAUER, B., WETZEL, H., A new membrane for feeding *Glossina morsitans* Westw. (Diptera, Glossinidae), Bull. Entomol. Res. 65 (1976) 563.

DISCUSSION

C.F. CURTIS: First, I would just like to point out that the *ocra* mutant is sex-linked and the genotype of males should be represented, for example, by (*oT*) and not by (*oT/oT*) because they have only one X-chromosome.

Secondly, the term 'polymorphism', which you used in your oral presentation, according to its precise definition, refers to the existence of distinct variants within one population. It therefore should not be used for variation between the (*oT*) and (*oR*) stocks.

Thirdly, I think it likely that any morphological mutant which is rare in wild populations would show poor fitness if released and its use would therefore not provide a satisfactory way of allowing released flies to be recognized. Since we clearly have good methods of marking with fluorescent dust I think that it would be unwise to take the risk of using a mutant, even if it seems to perform well in the laboratory.

P.A. LANGLEY: I should like to amplify on Mr. Curtis' third remark. We find our colony of the *ocra* strain of *G. morsitans* has greater fertility and produces larger puparia than the wild type under the same laboratory conditions. Hence, the non-persistence of this mutant in the field must indicate a lack of fitness in the natural environment which is not seen under laboratory conditions. One feature of this lack of fitness may be the tendency for approximately 30% of the insects to lose their wings during early adult life in the laboratory. The phenomenon has been investigated by N. Hillen at Manchester University using electron microscopy. He has found that the leading edge of the wing in the *ocra* strain is slightly deformed during expansion and hardening. A fracture line develops just distal to the point of insertion of the wing, and, even in wings which are not immediately lost, the presence of melanized haemolymph marks the existence of a fracture. This is probably due to inadequate tanning of the cuticle which gives the *ocra* mutant its characteristic colour.

A.M. VAN DER VLOEDT: It is well-known that, in some other insects, for example *Aedes aegypti*, wing mutants constitute the largest class of aberrations. Most such aberrations make the use of the marker for field studies inappropriate. I agree with your and Mr. Curtis' opinion that the non-persistence of the *ocra* strain in the field is most likely the result of intense selection pressure against the marker showing reduced fitness.

We have also observed some weakness at the wing base under the normal optical microscope. However, the frequency with which the flies showed this phenomenon differed markedly in the various inter- and intra-strain crosses. We believe that the mutants provide interesting material for work on reproduction biology (i.e. multiple mating and multiple insemination) and for some compatibility studies involving different but related species. e.g. *Glossina morsitans centralis*.

D.A. DAME: At Tanga the *ocra* strain has been produced on a regular basis since its origin about 1974. For 13 months about 10% of the sterile insects released during the release trials at Mkwaja were from the *ocra* strain. The recovery of these flies in routine fly surveillance activities was less than 20% of the recovery rate of the regular colony flies; this reveals that the fitness of this strain under field conditions is in fact significantly lower. Although the strain is easy to maintain in the laboratory and offers a useful visible marker for genetic studies, unfortunately it does not appear to be as useful for field population studies because of its lack of fitness in the field.

A.M. VAN DER VLOEDT: Thank you for the information on the performance of the *ocra* strain under insectary conditions at the Tanga Tsetse Project. I was not aware that the marker had been tested in field releases and had been found to be of no particular value because of its clear lack of fitness.

C.F. CURTIS: Mr. Langley and I are using the *ocra* mutant as a laboratory marker for the X-chromosome. It has been shown in *Anopheles gambiae* that the sterility of hybrid males produced by crossing sibling species is due to a genetic interaction involving the X-chromosome. Sterile hybrid males are produced when *G. morsitans morsitans* and *G.m. centralis* are crossed, and we are carrying out crosses and back-crosses involving *ocra* to see whether the X-chromosome is involved in causing this sterility.

M.E. LEEGWATER-VAN DER LINDEN: In view of your ideas about the occurrence of multiple mating, do you know of any evidence showing when and where most copulations take place in nature?

A.M. VAN DER VLOEDT: Dissections of field-caught females in the O_a-O_b stage (nulliparous flies younger than 5–7 days old) yielded more than 95% insemination. This indicates that, as far as *G. palpalis* and *G. tachinoides* are concerned, mating arrangements are excellent and females are mated very soon after emergence. I don't know what the situation is in field populations of *G. pallidipes*.

R.C. PINHÃO: I would like to comment on your difficulties in measuring the degree of insemination. During your oral presentation you showed us a slide of an apparently full pair of spermathecae. It was probably taken a short time after insemination. My experience has shown me that if you look at the spermathecae during the first 24 hours after insemination, they usually look full but have an irregular aspect like the one in the slide you showed us. If you then crush the spermathecae you will see that the mass of sperm becomes concentrated and looks normal, as in the one that you showed us in the next slide. But if you only look at the spermathecae after 48 hours, the mass of sperm already looks normal. That means that during the first 24 hours there is inside the spermathecae something in addition to the spermatozooids, probably material from the accessory glands or the spermathecae themselves. The use of a cover slip in my opinion does not influence the aspect of the spermathecae.

REARING OF *Glossina* *palpalis palpalis* Robineau-Desvoidy ON FREEZE-DRIED BLOOD*

H. WETZEL

Joint FAO/IAEA Division of
Atomic Energy in Food and Agriculture,
International Atomic Energy Agency,
Vienna

Abstract

REARING OF *Glossina palpalis palpalis* Robineau-Desvoidy ON FREEZE-DRIED BLOOD.

Tsetse flies have been reared by the membrane feeding method since 1973 in the Laboratory of the Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture. The flies obtain blood by piercing an artificial membrane which is easy to prepare and suitable for different insects and long-term use. Approximately 100 ml of blood are needed to feed up to 1700 tsetse flies on a 45 X 45 cm surface. Feeding fresh blood through the membrane technique has become routine. The advantage of not needing to keep host animals is offset by the need to have a regular supply of fresh blood. However, it was demonstrated that even haemolysed blood, stored by deep freezing, could be used for tsetse fly feeding once it had been processed. This was one of the main steps towards the use of freeze-dried blood. In a series of experiments, it was demonstrated that *Glossina p. palpalis* can be successfully maintained by feeding on freeze-dried, reconstituted bovine blood. The powdered blood was stored for more than six months and reconstituted by adding distilled water equal in volume to the amount lost during the freeze-drying process. Defibrinated and heparinized blood are suitable. The addition of the feeding stimulus ATP is necessary. Evaluations of performance of flies fed on freeze-dried blood show no difference compared with those fed on guinea pigs with respect to longevity and female fertility but the mean weight of puparia are sometimes not as high. At present, about 14 000 flies are fed daily by the membrane technique and freeze-dried blood is used exclusively.

DISCUSSION

H. POLITZAR: Can you give me an indication of the price of a litre of freeze-dried blood?

H. WETZEL: Running costs, which include electricity and water consumption, are negligible, since some 20 litres of blood can be freeze dried within 24 hours.

* The abstract only is printed here since the paper is to be published in *Tropenmedizin und Parasitologie*.

H. POLITZAR: Does the use of freeze-dried blood lower rearing costs?

H. WETZEL: Yes, certainly, because it means fresh blood can be collected on an irregular basis (the powder has a long storage time).

D.A. DAME: Can you comment on the acceptability of freeze-dried blood for other *Glossina* species?

H. WETZEL: My work is based on my experience with *G. palpalis* only. However, studies have shown that a similar procedure can be used for rearing *G. pallidipes*, although for *G. morsitans* some modifications might be necessary.

P.A. LANGLEY: We have reared *G. morsitans* for many generations using a diet of freeze-dried pig blood. Little success has been achieved with cow blood. We do not find it necessary to add adenosine triphosphate (ATP) to the freeze-dried product before feeding, but, of course, cow blood may be deficient in this respect.

H. WETZEL: We were not aware of your experiments, but I should point out that our data are based on almost two years of rearing on freeze-dried blood.

D.A. LINDQUIST: If a membrane-fed tsetse colony is accidentally contaminated with bacteria, can this be cured?

H. WETZEL: Contamination which leads to high mortality in females cannot be cured by treating the flies because of the side-effects on reproduction.

D.A. LINDQUIST: What methods are available for quality control of freeze-dried blood?

H. WETZEL: The best method being used at the moment for colony flies is the feeding test.

D.A. LINDQUIST: Can quality control tests be performed before the tsetse flies are fed?

H. WETZEL: The feeding test is performed after freeze drying but before storage.

J.L. GRINGORTEN: I assume that the freeze-drying process results in a complete disruption of cell-membrane integrity, and you end up with haemolysed blood upon reconstitution. Is that right?

H. WETZEL: Yes, it is.

H.J. HAMANN: What are your intentions as far as the applications of your method in Africa is concerned? Do you plan to send the freeze-dried blood to Nigeria or will you obtain it from African donor animals?

H. WETZEL: At present we are obtaining the blood in Europe and shipping the powder.

**SERUM SUBSTITUTES FOR FEEDING *Glossina palpalis palpalis* Robineau-Desvoidy
(Diptera: Glossinidae):**

**II. The influence of serum albumin
on the fecundity and weight
of the progeny***

W. TAKKEN

Joint FAO/IAEA Division of
Atomic Energy in Food and Agriculture,
International Atomic Energy Agency,
Vienna

Abstract

SERUM-SUBSTITUTES FOR FEEDING *Glossina palpalis palpalis* Robineau-Desvoidy
(Diptera: Glossinidae): II. THE INFLUENCE OF SERUM ALBUMIN ON THE FECUNDITY
AND WEIGHT OF THE PROGENY.

Females of a membrane-fed colony of *Glossina palpalis palpalis* (food source: defibrinated bovine blood) were fed on derivatives of bovine blood in which the red cell fraction remained unchanged whereas the serum fraction had been replaced by an artificial solution 'X'. The composition of this solution 'X' was exactly known. It consisted of a simple saline, isotonic to serum and enriched by bovine serum albumin with increasing concentrations ($X_1, 2, \dots, n$). Preliminary to feeding, care was taken that physical parameters such as pH and osmolarity of all media X_i did not differ markedly from controls. It was found that the absence of serum albumin in the food medium results in sterility of the females and gives rise to a high mortality. The presence of increased levels of serum albumin in the medium increased the fecundity until it was similar to that of flies fed on the standard diet of fresh defibrinated bovine blood. The offspring size was positively correlated with the concentration of the serum albumin in the mother diet. However, a high concentration of albumin (3%) resulted in an increased mortality. From the results it is suggested that the presence of free serum albumin in bovine blood is necessary for the development and production of viable larvae of the tsetse fly *Glossina p. palpalis*.

DISCUSSION

R. GALUN: Haemoglobin, which is the major protein of red blood cells (RBC), is deficient in isoleucine, so the RBC alone are not sufficient for tsetse reproduction. Did you try to supplement the saline with isoleucine rather than with albumin?

* The abstract only is printed here since the paper is to be published in *Entomologia Experimentalis et Applicata*.

W. TAKKEN: No, I have never fed the flies on a mixture of RBC and saline supplemented with isoleucine. However, when the diet was supplemented with 50 mg amino-acid mixture (which contained isoleucine) per 100 ml of saline, I did not find any improvement over RBC and saline.

M.E. LEEGWATER-VAN DER LINDEN: Could you include emergence rates in addition to the results you present? I ask this because from experiments I did on feeding regimes I noticed that increased puparial production per female is sometimes outweighed by reduced emergence rate.

W. TAKKEN: I can give you figures for emergence rates, but I did not include them in the paper as they did not differ from those of control groups when the puparial weights were close to those of the control groups. As I have already indicated, the puparia from the group fed on RBC and saline did not emerge.

GROWTH OF LABORATORY POPULATIONS OF TSETSE FLIES

Some mathematical investigations

W. TIMISCHL

Arbeitsbereich für Mathematik in
den Biowissenschaften,
Technische Universität Wien,
Vienna,
Austria

Abstract

GROWTH OF LABORATORY POPULATIONS OF TSETSE FLIES: SOME MATHEMATICAL INVESTIGATIONS.

The growth of laboratory populations of *Glossina palpalis* is described by a discrete time model. The model parameters are calculated from a life and fertility table derived from records of 85 control groups of *Glossina palpalis*. Various measures of productivity are discussed together with the problem of how to make predictions of the productivity of a colony when only data for a few age groups are available. Another investigation concerns which life table parameters have the largest influence on the growth rate.

1. INTRODUCTION

Calculations of the productivity of tsetse fly colonies have been performed by several authors, e.g. by Buxton [1], Glasgow [2] and more recently by Jordan and Curtis [3, 4]. Using a formula derived from a well-known continuous growth model by Lotka, they were able to obtain an estimate of the so-called intrinsic rate of natural increase for *Glossina austeni* colonies as well as of the productivity in terms of the weekly output of disposal pupae. In this study a modified approach to calculations of productivity of *Glossina palpalis* is presented, based on a discrete growth model. The proposed procedure seems to be more satisfying both from the theoretical and from the practical point of view. In particular it shows that the previously mentioned procedure tends to overestimate the population's rate of increase. A further examination concerns the maximum percentage of pupae that can be taken from the colony so that a stationary population is maintained, and how both this percentage and the doubling time change if higher (less productive) age groups are eliminated. Various measures for productivity are discussed and the problem is examined on how to estimate the future productivity of a cohort, when only the life-table

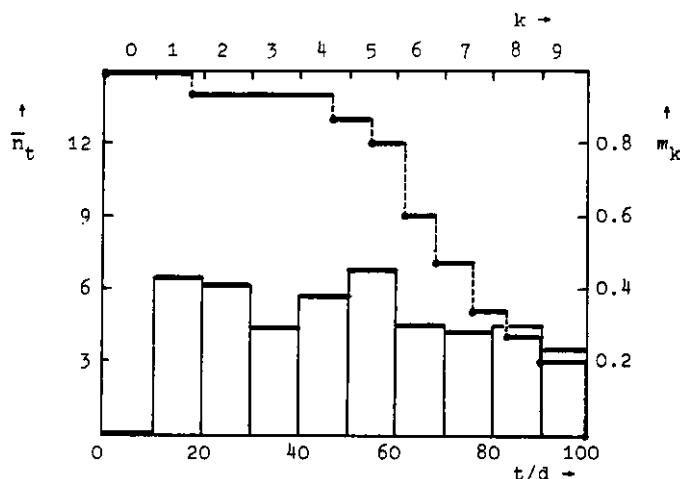


FIG.1. Survivorship curve and fertility diagram for experiment K1E602.

parameters of the early age groups are available. Finally, an analysis is performed to show the different influences of the life-table parameters on the population's increase.

2. LIFE AND FERTILITY TABLES

This mathematical investigation is based on data from the IAEA Laboratory at Seibersdorf, Austria. Records of 85 control groups of a tsetse fly colony (*Glossina palpalis*) were available. Each cage was started with a cohort of 15 females. The maintenance of the colony and the feeding technique (membrane feeding on defibrinated bovine blood) have been described by Wetzel and Luger [5]. According to the ovulation cycle, time t is measured in units of 10 days by using a (dimensionless) time parameter s . Instead of time t we shall simply speak of time s . The same time unit is used to measure the age of the females. A female is said to belong to age group k if its age a (in days) satisfies the inequality $10k \leq a < 10(k+1)$. An individual, being in age group k at time s , will then be in age group $k+1$ at time $s+1$. To cover the 'fly stage' 10 age groups ($k = 0, 1, \dots, 9$) are necessary and 3 more for the pupal stage ($k = -3, -2, -1$) so that each generation is subdivided into 13 age groups giving a generation time of 130 d.

TABLE I. LIFE AND FERTILITY TABLE OF *Glossina palpalis* BASED ON ALL DATA AVAILABLE

k	$\bar{\ell}_k$ ($\pm 99\%$ conf. limit)	$\bar{m}_k$ ($\pm 99\%$ conf. limit)
0	1	0
1	0.9817 (± 0.0097)	0.2363 (± 0.0306)
2	0.9615 (± 0.0139)	0.3925 (± 0.0356)
3	0.9257 (± 0.0189)	0.3688 (± 0.0362)
4	0.8760 (± 0.0238)	0.3625 (± 0.0371)
5	0.7984 (± 0.0289)	0.3230 (± 0.0378)
6	0.6782 (± 0.0337)	0.3094 (± 0.0405)
7	0.5777 (± 0.0356)	0.2694 (± 0.0421)
8	0.4648 (± 0.0360)	0.2222 (± 0.0440)
9	0.3471 (± 0.0343)	0.2070 (± 0.0496)
10	0.2368 (± 0.0306)	0

Figure 1 shows the 'survivorship curve' for a special experiment (K1E602) referring only to the fly stage. This graph expresses the number $\bar{n}_t$ of female flies alive at age t . The proportion of the total entering the fly stage which survive to age k ($k = 1, 2, \dots, 9$) is given by $\bar{\ell}_k = \bar{n}_k / \bar{n}_0$ ($\bar{\ell}_0 = 1$). Obviously the chance of survival to age k can be described for any female by a random variable S_k being 1 (if it survives) or 0 (if it dies). Assuming that the mortality of one female does not affect the mortality of another (which is most likely justified for the rearing methods applied), the number of females alive at time k has a binomial distribution with parameter $p_k = E(S_k)$ and $\bar{\ell}_k$ can be regarded as a (maximum likelihood) estimate of p_k . A very good estimation of p_k is reached if all $\bar{n}_k$ -values are added, giving n_k which is then divided by n_0 . The estimates $\bar{\ell}_k = n_k / n_0$, together with the corresponding 99% confidence limits, are listed in Table I.

The fertility in the colony can be characterized in several ways. The cumulative PPF-value f_k , for example, is defined as the average number of living offspring born per female up to age group k . The age-specific fertility $\bar{m}_k$ records the number of living female offspring born per female (alive at the beginning of age group k) in age group k . The $\bar{m}_k$ -diagram for the experiment K1E602 is shown in Fig.1. Because the age groups correspond to successive reproductive cycles and assuming a sex ratio of 1 : 1, the inequality $0 \leq \bar{m}_k \leq 1/2$ can be expected to hold. If fertility differences between females of the same

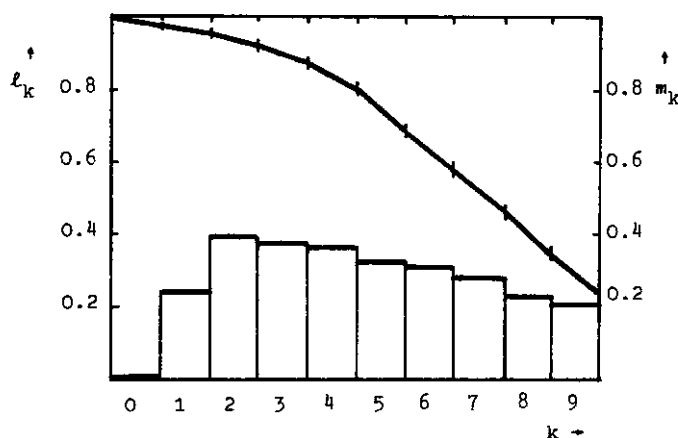


FIG.2. Survivorship curve and fertility diagram for *Glossina palpalis* (based on all data available).

age groups are neglected, the ratio m_k of the female offspring (born by the females of all cages in age group k) and the number of all females alive at age k can be taken as a good estimate for the probability that a female alive at age k will produce a female pupa in age group k . These m_k -values as well as the corresponding 99% confidence limits are also shown in Table I. The l_k - and m_k -columns of Table I represent a so-called life- and fertility table for the population of *Glossina palpalis* under consideration. An illustration of the mortality and fertility behaviour is given in Fig.2.

The l_k - and m_k -values have been interpreted as estimates for the probability that a female will survive to age k , and the probability that a female alive at age k will produce a female pupa in age group k , respectively. Another point of view is possible. Consider one cage as the 'population unit' and consequently the population as a population of cages. Then l_k expresses the probability that a cage will 'survive' to age k , and m_k is the probability that a cage alive at age k will produce a female 'offspring cage' in age group k . Obviously the life and fertility table for the 'cage population' is the same as for the fly population (only the confidence limits have to be changed), and since the concept of the cage population corresponds exactly to the rearing conditions, it will be adopted.

3. GROWTH RATES

A simple parameter characterizing the growth of a population is the so-called net reproductive rate $R_k = l_0 m_0 + l_1 m_1 + \dots + l_k m_k$, which is equal

to $f_k/2$. In particular R_0 expresses the total number of female individuals produced per female in one generation. From Table I it can be found that $R_0 = 2.0668$. If the ratio of the number of individuals living at the end and the beginning of one generation is approximated by R_0 it is possible to estimate the so-called finite rate of natural increase λ which is defined as the ratio of the number of individuals at time $s+1$ and at time s . Assuming λ to be independent of s (which is justified when the population has reached the stable age distribution), the relation $\lambda \cong \hat{\lambda} = R_0^{1/13} = 1.0574$ holds. Taking $\hat{r} = \ln \hat{\lambda} = 0.0558$ gives an estimate of the so-called intrinsic rate of natural increase r (defined by $r = \ln \lambda$), and taking $\hat{T} = \ln 2 / \hat{r} \cdot 10d = 124 \cdot 2d$ gives an estimate of the doubling time T (defined by $T = \ln 2 / r \cdot 10d$). These results are only rough approximations. Better results can be expected by applying the following model.

Consider a cohort of N (female) offspring cages produced at time $s = -3$. If the probability to survive the pupal stage is expressed by E then NE cages will enter the fly stage at time $s = 0$. Let the births be uniformly distributed within each age group. Then without making a large error one can assume that all births occur in the middle of each age group, that is, NEm_0 female cages are produced at time $s = 3.5$, NEl_1m_1 at time $s = 4.5$, ..., NEl_9m_9 at time $s = 12.5$. Provided the age distribution is stable, the age-group-0 cages at time $s = 0$ will increase to $N\lambda^{13}$ age-group-0 cages after one generation has passed. Consequently, the NEl_km_k age-group-0 cages at time $s = k + 3.5$ will increase to $NEl_km_k\lambda^{9.5-k}$ age-group-0 cages at time $s = 13$. Adding up these contributions for $k = 0, 1, \dots, 9$ must, of course, yield $N\lambda^{13}$. Thus the equation

$$E(l_0m_0\lambda^{-3.5} + l_1m_1\lambda^{-4.5} + \dots + l_9m_9\lambda^{-12.5}) = 1 \quad (1)$$

is obtained. In order to obtain an estimate of λ , the real positive root $\tilde{\lambda}$ of Eq.(1) must be determined. By using the l_k - and m_k -values of Table I and by setting $E = 0.96$ the following result was found: $\tilde{\lambda} = 1.0978$, $\tilde{r} = \ln \tilde{\lambda} = 0.0933$ and $\tilde{T} = \ln 2 / \tilde{r} \cdot 10d = 74.3 d$. An equation like Eq.(1) has also been used in Ref. [3] to obtain an estimate of r for *Glossina austeni*. A theoretically more satisfying growth formula is based on the so-called Leslie model, which will be briefly outlined in the following section (see also Ref. [6]).

4. THE GROWTH MODEL

Let $x_{k,s}$ be the number of female cages being in age group k at time s , $\mathcal{P}_k$ the probability that a cage being in age group k at time s will be in age group $k+1$ at time $s+1$ and $\mathcal{F}_k$ the average number of female offspring cages

produced per female cage (being in age group k at time s) between time s and $s+1$. Then the relations

$$\begin{aligned}x_{k,s+1} &= \mathcal{P}_{k-1} x_{k-1,s} \quad (k = 1, 2, \dots, m) \\x_{0,s+1} &= \mathcal{F}_0 x_{0,s-3} + \mathcal{F}_1 x_{1,s-3} + \dots + \mathcal{F}_m x_{m,s-3}\end{aligned}\quad (2)$$

hold where m is the index of the highest age group with $\mathcal{F}_m > 0$. These equations refer only to the fly stage. The pupal stage, lasting 30 days, is taken into account by the time lag -3 in the last part of Eq.(2). From Eq.(2) a difference equation of order $m+1$ can be derived for $y_s = x_{0,s}$. The dominant solution of this equation is formed by $y_s = c_0 \bar{\lambda}^s$, where c_0 is a constant and $\bar{\lambda}$ the root with largest modulus of the characteristic equation

$$\lambda^{m+4} - \mathcal{A}_0 \lambda^m - \mathcal{A}_1 \lambda^{m-1} - \dots - \mathcal{A}_{m-1} \lambda - \mathcal{A}_m = 0 \quad (3)$$

with the coefficients $\mathcal{A}_k$ defined by $\mathcal{A}_0 = \mathcal{F}_0$ and $\mathcal{A}_i = \mathcal{F}_i \mathcal{P}_0 \mathcal{P}_1 \dots \mathcal{P}_{i-1}$ ($i = 1, 2, \dots, m$). It can be shown that exactly one real root with largest modulus exists. An immediate consequence is that

$$\begin{aligned}\lim_{s \rightarrow \infty} \frac{y_s}{\bar{\lambda}^s} &= \lim_{s \rightarrow \infty} \frac{x_{0,s}}{\bar{\lambda}^s} = \lim_{s \rightarrow \infty} \frac{x_{1,s}}{\bar{\lambda}^{s-1} \mathcal{P}_0} = \dots \\&= \lim_{s \rightarrow \infty} \frac{x_{m,s}}{\bar{\lambda}^{s-m} \mathcal{P}_0 \mathcal{P}_1 \dots \mathcal{P}_{m-1}} = c_0\end{aligned}$$

By calling the $(m+1)$ -tuple $(x_{0,s}/x_{m,s}, x_{1,s}/x_{m,s}, \dots, 1)$ the age distribution at time s , it follows that for any initial age distribution finally the so-called stable age distribution

$$\left(\frac{\bar{\lambda}^m}{\mathcal{P}_0 \mathcal{P}_1 \dots \mathcal{P}_{m-1}}, \frac{\bar{\lambda}^{m-1}}{\mathcal{P}_1 \mathcal{P}_2 \dots \mathcal{P}_{m-1}}, \dots, 1 \right)$$

is reached. If otherwise the initial age distribution is already the stable one, the initial numbers $x_{k,0}$ will increase according to $x_{k,s} = x_{k,0} \bar{\lambda}^s$.

The problem remains to relate the model parameters $\mathcal{P}_k$ and $\mathcal{F}_k$ to the life-table parameters l_k and m_k . In Fig.3 the Lexis diagram is shown for a cohort of cages. Assuming that as many cages die in the time interval $(s-1, s)$

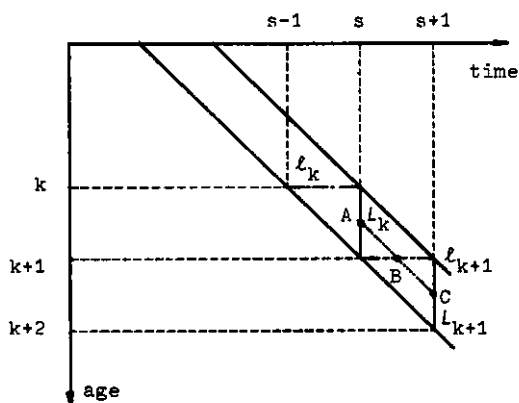


FIG.3. Lexis diagram to show the relationship between the Leslie and the life-table parameters.

TABLE II. LESLIE PARAMETERS $\mathcal{P}_k$ AND $\mathcal{F}_k$
Partial derivatives expressing the influence of perturbations in l_k and m_k on $\bar{\lambda}$

k	$\mathcal{P}_k$	$\mathcal{F}_k$	$\partial \bar{\lambda} / \partial l_k \cdot 10^2$	$\partial \bar{\lambda} / \partial m_k \cdot 10^2$
0	0.9806	0.1102	-----	-----
1	0.9712	0.2937	1.18	9.67
2	0.9547	0.3541	2.97	8.61
3	0.9293	0.3356	3.33	7.54
4	0.8819	0.3079	2.93	6.42
5	0.8505	0.2788	2.76	5.19
6	0.8301	0.2535	2.13	4.05
7	0.7788	0.2104	1.79	3.08
8	0.7192	0.1765	1.40	2.20
9	-----	0.0985	1.12	1.45

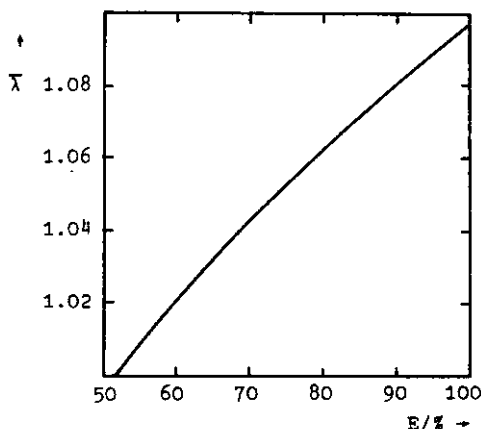


FIG.4. Relationship between growth rate $\bar{\lambda}$ and probability E to survive the pupal stage.

as in $(s, s+1)$, the number of cages being in age group k at time s can be expressed by $\mathcal{L}_k = (\ell_k + \ell_{k+1})/2$. Thus $\mathcal{P}_k$ can be estimated by the ratio $\mathcal{L}_{k+1}/\mathcal{L}_k$.

In order to estimate $\mathcal{F}_k$ let us assume that the $\mathcal{L}_k$ members of the cohort at time s are all of age $k + 0.5$. Then the cohort thus idealized follows the line from A to C in Fig.3 while one time unit is passing. Let us furthermore assume now that all deaths in the time interval $(s, s+1)$ occur while passing point B so that the number of surviving members of the cohort jumps from $\mathcal{L}_k$ to $\mathcal{L}_{k+1} = \mathcal{P}_k \mathcal{L}_k$ in B. The number of female offspring cages produced between A and B is then given by $\mathcal{L}_k m_k/2$ and between B and C by $\mathcal{L}_{k+1} m_{k+1}/2$ so that between time s and time $s+1$ on the whole $(m_k + \mathcal{P}_k m_{k+1}) \mathcal{L}_k/2$ female offspring cages are produced. These cages survive the pupal stage with probability E and spend then on an average 5 days before being counted in age group 0 at time $s+4$. If the probability of surviving these 5 days is approximated by $\mathcal{L}_0/\ell_0$, the formula $\mathcal{F}_k = E \mathcal{L}_0 (m_k + \mathcal{P}_k m_{k+1})/2\ell_0$ is obtained. The $\mathcal{P}_k$ - and $\mathcal{F}_k$ -values are listed in Table II (assuming $E = 0.96$). To obtain $\bar{\lambda}$, Eq.(3) with $m = 9$ has to be solved, which leads to $\bar{\lambda} = 1.0908$, $\bar{r} = \ln \bar{\lambda} = 0.0869$ and $\bar{T} = \ln 2/\bar{r} \cdot 10d = 79.7d$.

5. CONCLUSIONS FROM THE MODEL

First the problem is considered how $\bar{\lambda}$ depends on E introduced as the probability to survive the pupal stage. By setting $E = 0.96$ the fact was taken

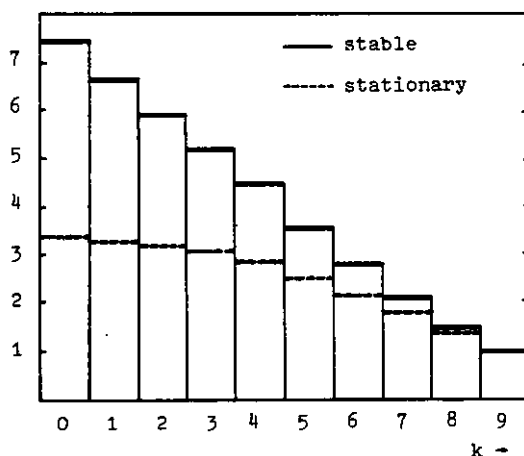


FIG. 5. Stable and stationary age distribution for *Glossina palpalis*.

into account that the emergency rate lies somewhat below 1. The relationship between $\bar{\lambda}$ and E , which is pictured in Fig. 4, is of some importance in connection with the question of how many pupae can be taken from the colony without causing the population to collapse. It is seen that the size of the colony is expected to become stationary if E approaches the value 0.51. This means that 51% of the pupae must enter the fly stage in order to hold the colony size fixed. Since the emergency rate is 0.96, only the proportion $Y = 1 - 0.51/0.96 \cong 47\%$ of the deposited pupae can be removed to be used for some purpose such as sterilization and release. Y can be calculated directly by using the formula $Y = 1 - 1/(\mathcal{A}_0 + \mathcal{A}_1 + \dots + \mathcal{A}_9)$. If the colony considered has become stationary on a certain population level by removing 47% of the offspring per time unit, the stable age distribution has changed into the so-called stationary one. Both distributions are shown in Fig. 5. The graph of the stable age distribution declines almost linearly from more than 7 in age group 0 to the value 1 in age group 9.

Productivity is often measured by the PPF-value f_k and, as a practical rule, a good productivity is expected if $f_2 > 1$. The connection between f_k and R_k has already been emphasized. Of course, there are other possible measures of productivity, e.g. the age-specific rate of natural increase λ_k . This quantity is defined as λ with the exception that now only the age groups up to age group k are taken into consideration. The behaviour of λ_k with respect to k is illustrated in Fig. 6, where besides $\bar{\lambda}_k$ (calculated from Eq. (3)) also $\hat{\lambda}_k = R^{1/(k+4)}$ (closely related to the PPF-value f_k), $\tilde{\lambda}_k$ (calculated from Eq. (1)) and f_k are shown. It can be seen that the different λ -estimates agree quite well for λ -values about 1,

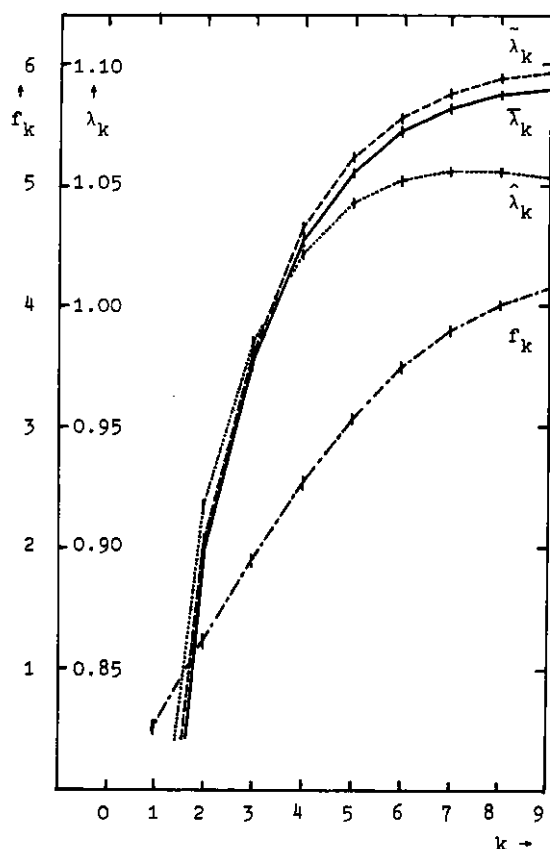


FIG. 6. Comparison of the different measures of productivity.

whereas larger λ -values will be overestimated by $\tilde{\lambda}_k$ and underestimated by $\hat{\lambda}_k$. Figure 7 contains the doubling times $\bar{T}_k$ (corresponding to $\bar{\lambda}_k$) and $\tilde{T}_k$ (corresponding to $\tilde{\lambda}_k$) and Y_k , another possible measure of productivity which is defined by $Y_k = 1 - 1/(\mathcal{A}_0 + \mathcal{A}_1 + \dots + \mathcal{A}_k)$ and which expresses the proportion of offspring that can be removed per time unit from each cage (cut short after age group k) in order to hold the colony size fixed. Which of these different measures is best suited to allow predictions about the future productivity of a cage is a matter still under investigation.

Obviously $\bar{\lambda}$ is a function of the parameters ℓ_k and m_k . It has been said that $\bar{\lambda}$ becomes 1.0908 if the values of Table I are substituted for ℓ_k and m_k . Let us assume now that these values are slightly changed by some increments $\Delta\ell_k$ and Δm_k . What change $\Delta\bar{\lambda}$ of $\bar{\lambda}$ will be caused by such 'perturbations'?

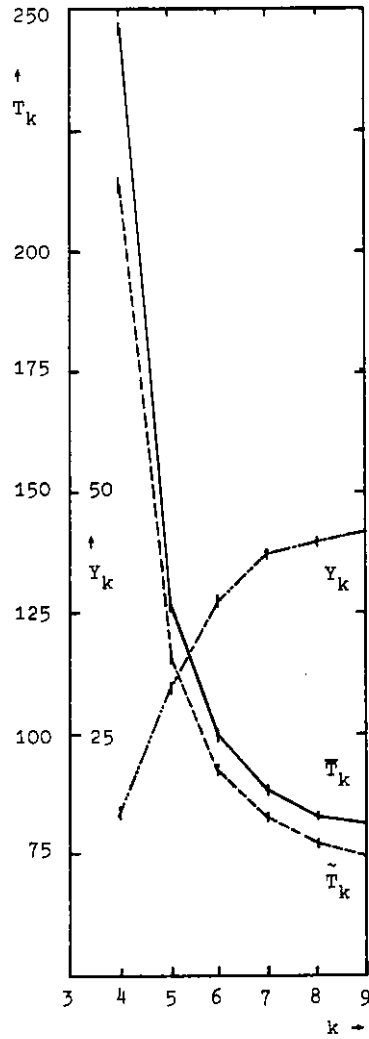


FIG.7. Doubling times and output of disposal pupae if experiments are terminated after age group k .

To give an answer to this question, $\Delta\bar{\lambda}$ is approximated by the total differential $\partial\bar{\lambda}$, i.e. we write

$$\Delta\bar{\lambda} \cong \sum_{k=1}^9 \left(\frac{\partial\bar{\lambda}}{\partial\ell_k} \Delta\ell_k + \frac{\partial\bar{\lambda}}{\partial m_k} \Delta m_k \right) \quad (4)$$

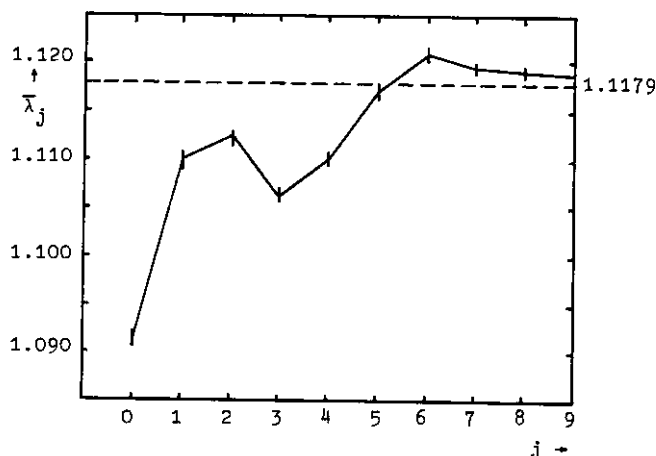


FIG.8. Successive approximation of the growth rate of experiment K1E602.

The partial derivatives, being calculated for the ℓ_k - and m_k -values of Table I, are listed in Table II. They express the magnitude of the influence of changes in ℓ_k and m_k upon $\bar{\lambda}$. It can be seen from Table II that high m_1 - or m_2 -values will have a high positive influence on $\bar{\lambda}$ and that high ℓ_k -values are in general not as effective as high m_k -values.

According to Eq.(4), $\Delta\bar{\lambda}$ can be split up into a sum of contributions of the different age groups. Let $\Delta\bar{\lambda}_j$ be the contribution of age group j to $\Delta\bar{\lambda}$. Then $\bar{\lambda}_j = \bar{\lambda} + \Delta\bar{\lambda}_j$ can be used to estimate the rate of increase of an experiment whose life-table parameters are only known up to age group j . An example is given in Fig.8 for experiment K1E602 with a finite rate of increase of 1.1179.

ACKNOWLEDGEMENTS

I wish to thank H. Wetzel and his staff of the Seibersdorf Laboratory for their co-operation, and F. Reschenhofer for his assistance in performing some calculations.

REFERENCES

- [1] BUXTON, P.A., Mem. London Sch. Hyg. Trop. Med. 10 (1955) 816.
- [2] GLASGOW, J.P., The Distribution and Abundance of Tsetse, Pergamon Press, Oxford (1963).
- [3] JORDAN, A.M., CURTIS, C.F., Bull. Entomol. Res. 58 (1968) 399.
- [4] CURTIS, C.F., JORDAN, A.M., Bull. Entomol. Res. 59 (1968) 651.
- [5] WETZEL, H., LUGER, D., Tropenmed. Parasitol. 29 (1978) 239.
- [6] NOBAUER, W., TIMISCHL, W., Mathematische Modelle in der Biologie, Vieweg, Wiesbaden (1979).

DISCUSSION

D.J. ROGERS: In your paper we are given three approximations to the real situation where the laboratory colony is concerned. Although one particular approximation may be more attractive than the others, it does not necessarily provide the most accurate description. Can you say which one does?

Such a description is only useful in practice if it can help us to manage the laboratory colony better. What would be your advice to laboratory personnel whose aim is to maximize colony output per unit time within the constraint of a fixed total number of cages or of a fixed total number of female flies? In other words, at what age should you discard your old cages or flies?

Finally, a caution: life-table treatments of the sort you describe are suitable only for age-dependent death rates, which are likely to be of relevance only to the laboratory and not to the field.

W. TIMISCHL: I would say that colony output (under the constraint of a fixed total number of cages) is not likely to be increased by stopping production at a given age group with index $k < 9$. However, I cannot give a definite answer to your questions without a more detailed study of the specific points you raised.

H. WETZEL: I believe that the data you used relate to a phase in which we were trying to expand our colony.

M. FRIED: Can your programme answer operational questions, such as whether it is more advantageous in terms of yield to stop production at a given age group? In a mass-production colony all cages will be in use either with old or young flies and it takes longer before the first pupae are produced; in addition, there is an increase in handling each time an older group is replaced by newly emerging flies.

W. TIMISCHL: Two cases must be distinguished: that of the expanding colony and that of the steady-state colony. For a colony in the expanding phase the answer is given in Fig.6. The case of a steady colony will be considered in a separate investigation.

C.F. CURTIS: I envisage that a computer system might be used for record-keeping in the large-scale rearing of tsetse with input of data on deaths and pupal production daily, or even as the data are collected. It would be useful to obtain frequently up-dated predictions of the output from the colony one month or more in the future so as to give early warning if something is going seriously wrong. Are suitable hardware and software for such a system available?

W. TIMISCHL: There are certainly no hardware problems. To my knowledge, there is as yet no software available. It is planned, however, to work out an appropriate software package.

CHAIRMEN OF SESSIONS

Session 1	F.M.J. EVENS	Belgium
Session 2	I.R. TIZARD	Canada
Session 3	F.E.G. COX	United Kingdom
Session 4	R. GALUN	Israel
Session 5	D.A. LINDQUIST	United States of America
Session 6	C.F. CURTIS	United Kingdom
Session 7	L.H. OTIENO	ICIPE
Session 8	G. NOGGE	Federal Republic of Germany

SECRETARIAT OF THE SYMPOSIUM

Scientific Secretaries:	G.C. LABRECQUE	Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, International Atomic Energy Agency, Vienna
	J.D. DARGIE A. HASSAN	
	P. FINELLE	Food and Agriculture Organization of the United Nations, Rome
Administrative Secretary:	G. SEILER	Division of External Relations, International Atomic Energy Agency, Vienna
Editor:	S.M. FREEMAN	Division of Publications, International Atomic Energy Agency, Vienna
Records Officer:	H. ORMEROD	Division of Languages, International Atomic Energy Agency, Vienna

FRANCE

- Cuisance, D. Institut d'élevage et de médecine vétérinaire des
pays tropicaux,
10, rue Pierre Curie,
F-94700 Maisons-Alfort
- Itard, J. Institut d'élevage et de médecine vétérinaire des
pays tropicaux,
10, rue Pierre Curie,
F-94700 Maisons-Alfort
- Luffau, G.B. Station de virologie et immunologie (I.N.R.A.),
F-78850 Thiverval-Grignon

GERMAN DEMOCRATIC REPUBLIC

- Rösseler, M. Zentralinstitut für Isotopen- und Strahlenforschung
der Akademie der Wissenschaften,
Permoserstrasse 15,
DDR-705 Leipzig

GERMANY, FEDERAL REPUBLIC OF

- Hamann, H.-J. Institut für Strahlenbotanik,
Herrenhäuser Strasse 2,
D-3000 Hannover 21
- Kloft, E.S. Institut für Angewandte Zoologie,
Universität Bonn,
An der Immenburg 1,
D-5300 Bonn 1
- Kloft, W.J. Institut für Angewandte Zoologie,
Universität Bonn,
An der Immenburg 1,
D-5300 Bonn 1
- Nogge, G. Institut für Angewandte Zoologie,
Universität Bonn,
An der Immenburg 1,
D-5300 Bonn 1
- Politzar, H. Deutsche Gesellschaft für Technische
Zusammenarbeit,
Dag-Hammarskjöld-Weg 1,
D-6236 Eschborn

INDIA

Aiyar, A.S.

Biochemistry and Food Technology Division,
Bhabha Atomic Research Centre,
Trombay, Bombay – 4000 85**ISRAEL**

Galun, R.

Hebrew University,
Department of Zoology,
Jerusalem**MADAGASCAR**

Razafindrakoto, C.

Division Parasitologie,
Département de recherches zootechniques
et vétérinaires,
BP 4, Tananarive**NETHERLANDS**

Leegwater-van der Linden, M.E.

University of Amsterdam,
Laboratory of Experimental Entomology,
Kruislaan 302,
N-1098 SM Amsterdam**PORTUGAL**

Pinhão, R.C.

Disciplina de Entomologia,
Instituto de Higiene e Medicina Tropical,
Junqueira 96,
P-1300 Lisbon**SAUDI ARABIA**

Trabulsi, I.Y.

College of Agriculture,
Riyad University,
Riyad**SUDAN**

Hussein, H.S.

Faculty of Veterinary Science,
University of Khartoum,
BP 32, Khartoum North

UNITED KINGDOM

- Bourn, D.M. Zoology Department,
Oxford University,
Oxford
- Cox, F.E.G. Department of Zoology,
King's College,
University of London,
Strand,
London WC2R 2LS
- Curtis, C.F. Ross Institute of Tropical Hygiene,
London School of Hygiene and Tropical Medicine,
Keppel Street,
London WC1E 7HT
- Langley, P.A. Tsetse Research Laboratory,
School of Veterinary Science,
University of Bristol,
Langford, Bristol BS18 7DU
- Molyneux, D.H. Department of Biology,
University of Salford,
Salford, M5 4W5
- Phillips, R.S. Department of Zoology,
University of Glasgow,
Glasgow G12 8QQ
- Purnell, R.E. ARC Institute for Research on Animal Diseases,
Compton,
Newbury, Berks
- Rogers, D.J. Hope Department of Entomology,
University Museum,
Parks Road,
Oxford OX1 3PW
- Terry, R.J. School of Biological Sciences,
Brunel University,
Uxbridge, Middlesex

UNITED STATES OF AMERICA

- Bram, R.A. United States Department of Agriculture,
Federal Center Building,
Hyattsville, MD 20782

- Dame, D.A. Federal Research Science and Education
Administration,
P.O. Box 14565,
Gainesville, FL 32604
- Groocock, C.M. Plum Island Animal Disease Center,
P.O. Box 848,
Greenport, NY 11935
- Lindquist, D.A. United States Department of Agriculture,
Science and Education Administration,
Agricultural Research,
Bldg 005, Room 207, BARC-West,
Beltsville, MD 20705
- Patterson, R.S. United States Department of Agriculture,
Insects Affecting Man and Animals
Research Laboratory,
1600 S.W. 23rd Drive,
P.O. Box 14565,
Gainesville, FL 32604
- Ristic, M. College of Veterinary Medicine,
University of Illinois,
Urbana, IL 61801

UPPER VOLTA

- Boubakar, H. Laboratoire de diagnostics et recherches
vétérinaires,
Direction de l'élevage,
Ouagadougou

ZAIRE

- Dimundu, P. Centre régional d'études nucléaires de Kinshasa,
Commissariat général à l'énergie atomique,
BP 868, Kinshasa XI

ORGANIZATIONS**FOOD AND AGRICULTURE ORGANIZATION OF THE
UNITED NATIONS (FAO)**

- Baumgartner, H. Tse Tse Research Project,
Joint FAO/IAEA Division,
P.O. Box 1026,
Tanga, Tanzania

**FOOD AND AGRICULTURE ORGANIZATION OF THE
UNITED NATIONS (FAO) (cont'd)**

Fried, M.	Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, P.O. Box 100, A-1400 Vienna
LeRoux, J.G.	Animal Health and Agricultural Health Division, FAO, Via delle Terme di Caracalla, I-00100 Rome

INTERNATIONAL ATOMIC ENERGY AGENCY (IAEA)

Calkins, C.	Division of Research and Laboratories, P.O. Box 100, A-1400 Vienna
Greiling, J.	Division of Research and Laboratories, P.O. Box 100, A-1400 Vienna
Gringorten, J.L.	Division of Research and Laboratories, P.O. Box 100, A-1400 Vienna
Jaenson, T.G.T.	Division of Research and Laboratories, P.O. Box 100, A-1400 Vienna
Lamm, C.G.	Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, P.O. Box 100, A-1400 Vienna
Oladunmade, M.	Federal Livestock Department, IAEA Project, Kaduna, Nigeria
Serghiou, C.	Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, P.O. Box 100, A-1400 Vienna
Takken, W.	Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, P.O. Box 100, A-1400 Vienna

**INTERNATIONAL CENTRE OF INSECT PHYSIOLOGY AND
ECOLOGY (ICIPE)**

**INTERNATIONAL LABORATORY FOR RESEARCH ON
ANIMAL DISEASES (ILRAD)**

UNITED NATIONS ENVIRONMENTAL PROGRAMME (UNEP)

Alozie, O.U. P.O. Box 30552,
Nairobi, Kenya

AUTHOR INDEX

*
Italic numerals refer to the first page of a paper by the author concerned.
Upright numerals denote the page numbers of comments and questions in discussions.
Literature references are not indexed.

Abendroth, H.-C.: 219	Gates, D.B.: 267
Adamsky, G.: 319	Giannetti, M.: 313
Aiyar, A.S.: 233, 244, 245	Gopalaswamy, U.V.: 233
Akol, G.W.O.: 15, 129, 130, 131	Gringorten, J.L.: 278, 440
Alozie, O.U.: 14, 33	Grocock, C.M.: 161, 177
Bauer, B.: 319	Hamann, H.J.: 319, 325, 326, 440
Baumgartner, H.: 267	Hasibeder, G.: 367
Ben-Eliahu, M.N.: 207	Hassan, A.: 218, 231, 245
Ben-Tamar, D.: 207	Hudson, K.M.: 133
Bourdoiseau, B.: 249	Hussein, H.S.: 31, 55, 57, 65, 66, 75, 104, 117, 146, 197
Brocklesby, D.W.: 77	Irvin, A.D.: 94, 104, 105, 107, 117, 395
Bursell, E.: 327	Iwannek, K.H.: 319
Clair, M.: 249	Jaenson, T.G.T.: 325, 369, 396, 423
Cobb, P.E.: 267	Kloft, E.S.: 299
Cox, F.E.G.: 65, 95, 104, 105, 130, 131, 146	Kloft, W.J.: 189, 217, 299, 326
Cromroy, H.L.: 299	Koch, H.: 219
Cuisance, D.: 249, 264, 265, 266, 395	LaBrecque, G.C.: 283
Curtis, C.F.: 218, 279, 294, 335, 381, 394, 395, 396, 408, 437, 438, 455	Langley, P.A.: 197, 265, 279, 318, 327, 334, 335, 336, 379, 396, 423, 437, 440
Dame, D.A.: 218, 267, 278, 279, 280, 326, 395, 438, 440	Leegwater-Van der Linden, M.E.: 130, 245, 408, 413, 422, 423, 438, 442
Dargie, J.D.: 66, 121, 146, 177, 190	LeRoux, J.G.: 281
Darji, N.: 191	Lewis, D.: 77
Emery, D.L.: 15	Lindquist, D.A.: 33, 280, 440
Evens, F.M.J.: 264, 266	Luther, D.: 219
Faghihi Shirazi, M.: 133	Masake, R.A.: 15
Février, J.: 249	May, D.: 133
Finelle, P.: 3, 14, 217, 264, 326	Mella Lizama, C.: 117, 245, 318
Fried, M.: 279, 455	Mellors, A.: 149
Galun, R.: 31, 33, 190, 197, 207, 217, 218, 245, 266, 294, 318, 336, 379, 408, 441	Moloo, S.K.: 15

AUTHOR INDEX

- Molyneux, D.H.: 130, 158, 179,
189, 190, 197, 198, 204, 295,
339, 353
- Morrison, W.I.: 15
- Mtuya, A.G.: 267
- Murray, Max: 15, 31, 32, 33, 105,
130, 158, 189, 279
- Mutinga, M.J.: 105, 199, 204, 217,
281, 423
- Nielsen, K.: 149
- Nogge, G.: 14, 313, 317, 318, 326
- Olobo, J.: 191
- Onyango, P.: 191
- Otieno, L.H.: 32, 158, 191, 197,
198, 294, 317, 353, 368, 371, 379
- Patterson, R.S.: 283, 294, 295
- Phillips, R.S.: 32, 55, 67, 75, 76,
116, 146, 177
- Phondke, M.P.: 233
- Pinhão, R.C.: 281, 397, 408, 438
- Politzar, H.: 249, 265, 295, 395,
439, 440
- Purnell, R.E.: 54, 77, 94, 104, 107,
129, 280
- Razafindrakoto, C.: 245
- Ristic, M.: 37, 54, 55
- Rogers, D.J.: 264, 334, 335, 359,
367, 368, 394, 455
- Rösseler, M.: 219, 232, 244, 245
- Ryan, L.: 339
- Sellin, E.: 249
- Simkin, J.: 207
- Soulsby, E.J.L.: 161
- Takken, W.: 369, 422, 441, 442
- Taze, Y.: 249
- Terry, R.J.: 31, 76, 104, 133, 146,
147, 159
- Timischl, W.: 443, 455
- Tizard, I.R.: 105, 149, 158, 159, 177
- Van der Vloedt, A.M.: 198, 264, 265,
335, 370, 396, 422, 425, 437, 438
- Vercoc, J.: 130, 318
- Warner, P.V.: 267
- Weiss, M.: 265, 369, 370
- Wetzel, H.: 265, 278, 317, 325, 335,
370, 439, 440, 455
- Williams, D.F.: 283
- Williamson, D.L.: 267
- Youdeowei, Y.: 371
- Young, E.R.: 107

HOW TO ORDER IAEA PUBLICATIONS

An exclusive sales agent for IAEA publications, to whom all orders and inquiries should be addressed, has been appointed in the following country:

UNITED STATES OF AMERICA UNIPUB, 345 Park Avenue South, New York, NY 10010

In the following countries IAEA publications may be purchased from the sales agents or booksellers listed or through your major local booksellers. Payment can be made in local currency or with UNESCO coupons.

ARGENTINA	Comisión Nacional de Energía Atómica, Avenida del Libertador 8250, RA-1429 Buenos Aires
AUSTRALIA	Hunter Publications, 58 A Gipps Street, Collingwood, Victoria 3066
BELGIUM	Service du Courrier de l'UNESCO, 202, Avenue du Roi, B-1060 Brussels
CZECHOSLOVAKIA	S.N.T.L., Spálená 51, CS-113 02 Prague 1
FRANCE	Alfa, Publishers, Hurbanovo námestie 6, CS-893 31 Bratislava Office International de Documentation et Librairie, 48, rue Gay-Lussac, F-75240 Paris Cedex 05
HUNGARY	Kultura, Hungarian Trading Company for Books and Newspapers, P.O. Box 149, H-1389 Budapest 62
INDIA	Oxford Book and Stationery Co., 17, Park Street, Calcutta-700 016 Oxford Book and Stationery Co., Scindia House, New Delhi-110 001
ISRAEL	Heiliger and Co., Ltd., Scientific and Medical Books, 3, Nathan Strauss Street, Jerusalem
ITALY	Libreria Scientifica, Dott. Lucio de Biasio "aeiou", Via Meravigli 16, I-20123 Milan
JAPAN	Maruzen Company, Ltd., P.O. Box 5050, 100-31 Tokyo International
NETHERLANDS	Martinus Nijhoff B.V., Booksellers, Lange Voorhout 9-11, P.O. Box 269, NL-2501 The Hague
PAKISTAN	Mirza Book Agency, 65, Shahrah Quaid-e-Azam, P.O. Box 729, Lahore 3
POLAND	Ars Polona-Ruch, Centrala Handlu Zagranicznego, Krakowskie Przedmiescie 7, PL-00-068 Warsaw
ROMANIA	Ilexim, P.O. Box 136-137, Bucarest
SOUTH AFRICA	Van Schaik's Bookstore (Pty) Ltd., Libri Building, Church Street, P.O. Box 724, Pretoria 0001
SPAIN	Diaz de Santos, Lagasca 95, Madrid-6 Diaz de Santos, Balmes 417, Barcelona-6
SWEDEN	AB C.E. Fritzes Kungl. Hovbokhandel, Fredsgatan 2, P.O. Box 16356, S-103 27 Stockholm
UNITED KINGDOM	Her Majesty's Stationery Office, P.O. Box 569, London SE 1 9NH
U.S.S.R.	Mezhdunarodnaya Kniga, Smolenskaya-Sennaya 32-34, Moscow G-200
YUGOSLAVIA	Jugoslovenska Knjiga, Terazije 27, P.O. Box 36, YU-11001 Belgrade

Orders from countries where sales agents have not yet been appointed and requests for information should be addressed directly to:



Division of Publications
International Atomic Energy Agency
Wagramerstrasse 5, P.O. Box 100, A-1400 Vienna, Austria

INTERNATIONAL
ATOMIC ENERGY AGENCY
VIENNA, 1980

SUBJECT GROUP: I
Life Sciences/Entomology
PRICE: Austrian Schillings 680,—