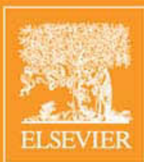

ADVANCES IN PARASITOLOGY

Volume 5

Ben Dawes

Advances in

PARASITOLOGY

VOLUME 5

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Advances in PARASITOLOGY

Edited by

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PREFACE

In this volume of *Advances in Parasitology* more than usual emphasis has been placed on the Protozoa, but many readers will agree that this is equitable. Of the twenty-four contributions already published in four volumes, five dealt with protozoan parasites, five with trematodes, two with cestodes, six with nematodes, four with helminths in general, one with snail control in trematode disease and one with feeding in ticks. In this the fifth volume, four contributors deal with various Protozoa, one with post-embryonic developmental stages of cestodes and one with the biology of the Acanthocephala.

In his review on *Toxoplasma* and toxoplasmosis, Leon Jacobs has been concerned mainly with work published during the past three to four years, earlier work providing historical perspective. He begins with electron microscopy and goes on to discuss in some detail mechanisms of transmission, serology, the association of *Toxoplasma* with certain diseases, epidemiology, pathology, immunity, physiology and therapy. The study of fine structure of trophozoites of *Toxoplasma* from *in vivo* and *in vitro* sources has cleared up some points about reproduction. The rosette-like bodies which appear repeatedly are incompletely separated daughter parasites developing by a special form of schizogony ("endodyogeny"), which occurs also in encysted forms. Matters concerning the cytostome, "toxonemes" and other organelles are also clarified by discussion. The cyst develops within a vacuole in a host cell, and it may attain an enormous size without rupturing the cell as does a rapidly growing mass of trophozoites. The transmission of *Toxoplasma gondii* to man and animals is widespread but sources of infection are difficult to recognize, except in the case of carnivores which eat the raw meat of infected sheep or swine. The investigation of possible invertebrate carriers of cysts or vectors of trophozoites has placed nematodes under suspicion. Widespread toxoplasmosis in sheep both in this country and far abroad may be explicable in terms of transmission by nematodes in their eggs. However, the hygienic habits of felines militate against the view that nematode eggs in the faeces of cats are sources of such considerable human infection as is known. Jacobs believes that "we will have much work to do to identify the characters in this play, and to ascribe to them their proper importance", although there is a promise of exciting research results to come. Progress in serology indicates the need to find new methods of obtaining preparations for antigen-production, and for freeing antigens of contaminating substances. In the diagnosis of toxoplasmosis indirect fluorescence has proved successful, but some cases are difficult to diagnose by serological methods and suitable techniques are not yet available in many laboratories. Medical parasitologists will be very interested in the association of toxoplasmosis with various disease conditions, some of which may approach malignancy. The parasite may be present in the tissues of the heart in some cases of myocarditis and pericarditis, and there is a notable association of toxoplasmosis with habitual abortion in pregnant women. Epidemiological data refer to man and animals such as sheep, cattle

and horses, cats and chickens, and what features of transmission remain hidden in contacts between man and domesticated animals awaits future elucidation. Therapy in the treatment of toxoplasmosis depends on the use of a few drugs, which are named, and we note only that the side effect of teratogenesis in pregnancy is a drawback to the use of pyrimethamine and sulfa drugs but outside pregnancy side effects of these drugs can be avoided by the use of folinic and yeast extract.

Cecil A. Hoare brings to the exposition of his subject, evolutionary trends in mammalian trypanosomes, an expertise which is unrivalled. Out of a vast literature bearing on the practical problems of control of trypanosomiases he has recognized the efforts of some protozoologists to study problems of general biological interest, and he explains and expounds the evidences which have thus arisen concerning the evolution of trypanosomes. What has become known forms the basis of a natural classification of trypanosomes on phylogenetic lines which expresses affinities clearly and groups together species with similar physiological characteristics, antigenic constitution and host–parasite relations, thus improving prospects in studies on chemotherapy, immunology and pathology by the appropriate systematic apposition of related forms. The review begins with the delineation of trypanosomatid genera and characterization of the genus *Trypanosoma*. Questions of the origin of mammalian trypanosomes has involved the discussion of a number of hypotheses, all of which depend on the close resemblance between some of their stages of development within the body of the insect that serves as vector and monogenetic trypanosomatid flagellates which occur only in insects that do not suck blood. These flagellates formerly constituted three main genera, *Leptomonas*, *Herpetomonas* and *Crithidia*, but it is species of *Blastocrithidia* which show the closest affinity with the genus *Trypanosoma*, the epimastigote stage of the former differing only slightly from the trypomastigote stage of *Trypanosoma*, in which the kinetoplast and the origin of the flagellum are posterior to the nucleus. Hoare discusses in significant detail the origin of the haematophagous insects and the ancestry of the trypanosomes, the trypomastigote stage of which has no counterpart among the insect flagellates, first appearing in the genus *Trypanosoma* as characteristic blood forms in the mammalian host, being represented in the insect vector by so-called metacyclic trypanosomes, or "metatrypanosomes", which arise from the epimastigote forms. In the later parts of this review, the affinities and phylogeny of vertebrate trypanosomes are discussed in such detail as can scarcely be summarized here, followed by discussion of microevolutionary divergence in respect of biological races, variation in a number of subgenera (*Trypanozoon*, *Duttonella*, *Schizotrypanum* and *Herpetosoma*), infrasubspecific variation and speciation by mutation, and a section devoted to the taxonomic treatment of biological variants.

In writing on problems in the cultivation of some parasitic Protozoa, Ann Bishop points out that in spite of such effort as has so far been forthcoming, it is still not possible to maintain trypanosomes from the mammalian bloodstream in culture, and at most the erythrocytic stages of malarial parasites can be cultivated only for short periods. Although trypanosomiasis is a disease of

economic importance and malaria is still a great scourge in the less highly developed countries of the world, little is known of the mode of action of trypanocidal and antimalarial drugs. In both trypanosomiasis and malaria drug resistance is an important problem, and both haemoflagellates and plasmodia exist in different forms in vertebrate hosts and invertebrate vectors, thus providing special problems of morphogenesis. For the solution of problems of therapeutic action, drug resistance and morphological transformations it is necessary to learn more about the biological, physiological and biochemical characteristics of these protozoan parasites, and in order to achieve this end it is essential to develop methods of cultivation of all stages of the life cycles involved. At present, *in vitro* experimentation with trypanosomes from the blood-stream of mammals must be carried out by means of short-lived suspensions obtained from experimental animals. Even worse than this limitation to short-term results is the position in respect of malaria on account of difficulties of transmission. It is claimed, therefore, that successful methods for the cultivation of protozoan parasites should make it possible to open up new fields of investigation in respect of both trypanosomes and malarial parasites, not to mention other Protozoa. In this contribution Miss Bishop has given herself the task of reviewing some achievements in this field of protozoan culture, and some of the difficulties encountered, taking the trypanosomes (including Leishmanias) and malarial parasites in turn. In the case of haemoflagellates some of the problems of cultivation will be solved only by methods using chemically defined media, and strains within species have shown such different growth requirements as to suggest for parasitic protozoans having exacting nutritional requirements that "the more defined the medium, the more restricted may be the organisms which will grow in it". The cultivation of malarial parasites presents different problems: exoerythrocytic forms depend on the development of tissue culture, erythrocytic forms require media in which they can grow and multiply in erythrocytes, or else corresponding extracellular conditions, and the insect stage requires cultures of mosquito stomach tissue or else a medium suitable for extracellular development of oocysts. These stages are dealt with separately and there is some discussion of limited progress which has been made in a subject with outstanding technical problems.

In the introduction to his contribution on malaria in mammals excluding man, P. C. C. Garnham mentions that it was necessary to consider human malaria briefly because man can be infected with two species of simian malaria in nature and eight species in the laboratory, and because recent intensive studies on ultrastructure concern human and non-human species alike. His review is limited to the genus *Plasmodium* s.s. and it deals with modern advances in respect of such topics as new species and taxonomic problems, life cycles, vertebrate host susceptibility and the zoonoses problems, ultrastructure of the various developmental stages, pathogenesis, and matters related to immunity and serology. The approach is strictly biological, no special effort having been made to deal with chemotherapeutic problems or biochemical information of special kinds. New species dealt with include forms from gibbons, the "Cynomolgi" complex, parasites of oriental monkeys,

rodent parasites and other species of the subgenus *Vinckeia*. A complete account is given of the life cycle of the important species *P. berghei* along with other data on rodent parasites, simian parasites and discussion of the topic of relapses. The question of zoonoses is the subject of an exciting passage recounting human infection with species formerly believed to be exclusively simian parasites, and a number of species are named. The climax was reached when the blood of a surveyor diagnosed as a case of *P. falciparum* malaria was inoculated into rhesus monkeys which subsequently died of typical *P. knowlesi* malaria. Although examples are known from Malaya, Brazil and other places, it is concluded that monkey malaria is not likely to interfere seriously with the strategy of malaria eradication. The study of the ultrastructure of malarial parasites has proceeded beyond the point reached when Clay Huff reviewed avian malaria in the first volume of this series. A progress report is now given dealing with erythrocytic and sporogonic stages of mammalian species of *Plasmodium*, and human or avian forms also when comparisons are necessary. It seems natural that there should be some doubt about the interpretation and function of some of the organelles studied and that this should await development of cytochemical techniques allied to electron microscopy. The pathology of malaria is not now regarded as a common effect irrespective of species; the diversity of the morbid processes has been discovered with increased interest in the host's reactions to its parasites, and modern beliefs and theories are here discussed in some detail. The subject of immunity is of interest because if it could be established by vaccination or by natural means, current methods of eradication which rely on insecticides might be thrown aside in favour of now traditional methods used successfully against smallpox, poliomyelitis and other diseases. Much still remains to be learned about immunity to mammalian malaria, so that research continues on a large scale and is considered by Garnham in terms of serological measurement of immunity, cross-immunity reactions, passive immunity and malaria antigens.

In reviewing the biology of the Acanthocephala, W. L. Nicholas has covered studies in embryology and post-embryonic development, histology and cytology, histochemistry, biochemistry and host–parasite relationships. He also considers the practical importance of these parasites in human and veterinary medicine and in so far as they affect fishes and wild birds. Morphological questions are considered so as to provide a background for other topics. These thorny-headed worms—so called because of the spinous proboscis—are parasites of vertebrate animals, living in the alimentary canal and sometimes deeply embedded in its wall. The sexes are separate and reproduction is entirely sexual, internal fertilization following copulation. The females release shelled larvae into the lumen of the host's intestines and these acanthors reach the external world, infect arthropodan animals and develop through another larval stage (acanthella) to the final resting stage (cystacanth) that marks the end of development within the intermediate host. Vertebrates are infected by ingestion of cystacanths, but in many paratenic hosts these forms may penetrate the issues without undergoing further development. Nicholas elaborates this theme of interpolated hosts and

illustrates it neatly before considering details of histology and cytology and then histochemistry and ultrastructure. Much up-to-date information here concerns food reserves, enzymes, structural materials, the nature of the tegument, hooks and spines, and in a section devoted to biochemistry matters such as composition, assimilation, intermediary metabolism and respiration. The account of host–parasite interaction refers to both arthropodan and vertebrate hosts in respect of specificity, pathogenicity, resistance to infection and other topics. In a concluding passage it is affirmed that acanthocephalans resemble cestodes in their sensitivity to the level of carbohydrate in the intestines of their hosts and in the kinetics of amino-acid assimilation, but in their respiratory metabolism they show interesting similarities with other worm-like parasites and significant differences from the higher animals.

Marietta Voge has written an informative review on the post-embryonic developmental stages of cestodes which lays emphasis on overall resemblances and differences between the main groups of cestodes and indicates where future research is essential or may be profitable. She begins by considering broadly life histories and development of separate orders of cestodes, then deals with the morphology of the various post-larval forms (procercoids, plerocercoids, cysticercoids and cysticerci), certain aspects of host–parasite relations, temperature and development, post-embryonic stages and excystment *in vitro*, and finally gives her conclusions. The review of life histories and development in intermediate hosts show how little we know about some orders of cestodes and how slightly knowledge gained about this subject has been allowed to modify an orthodox systematics, in spite of much progress. Some pseudophyllids, including proteocephalids, require only one intermediate host, some trypanorhynchs utilize a crustacean first intermediate host, and cladocerans and psocids have been added to the list of arthropodan groups which harbour developmental stages of cestodes. Much more has become known about hymenolepidid life histories and cysticercoid development, and new approaches of experimental kinds have given data on the variability of developmental processes. Certain patterns of development have emerged and are summarized with the aid of diagrams. The literature dealing with post-embryonic stages of cestodes commonly concerns gross morphology, studies of histology being few and analyses of cellular or nuclear structure and arrangement virtually non-existent. What is known about procercoids, plerocercoids, cysticercoids and cysticerci is admirably stated and supplemented by studies of histochemistry. It is concluded that these stages have certain features in common and comparable patterns of organization, the cuticular hairs of plerocercoids, tetrathyridia and cysticerci, the fibrous layers around the withdrawn scolex of the cysticercoid, the sequence of layers in cuticle and subcuticle and the distribution therein of certain enzymes and substances. In conclusion, there is consideration of new questions and problems which have arisen from the progress made by research (which has prompted the G.B.S. quotation). Little is known about factors controlling the process of differentiation in cestode development, and less about agencies that "trigger" the transition from one stage to the next. Some of the factors which form the basis of physiological host specificity are

tabulated and discussed briefly. The existence of interspecific competition within the host has been established but the mechanisms underlying competition are undetermined. Areas of study which seem to have been most neglected are indicated and it is emphasized that further progress is dependent upon new techniques and approaches which emanate from various biological disciplines.

Once more, I give thanks to these writers who have given substantial amounts of their time and energy and thereby generous help in my aim to provide a fund of reliable information and ideas in the field of parasitology. I am happy to acknowledge with gratitude also the great care which has been devoted to the production of this book by members of staff of the Academic Press.

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August 1967

CONTENTS

Contributors to Volume 5 v
Preface vii

Toxoplasma and Toxoplasmosis

LEON JACOBS

I. Introduction 1
II. Electron Microscopy 2
A. The Trophozoite (Proliferative Form) 2
B. The Cyst 6
III. Transmission 15
IV. Serology 20
V. Toxoplasmosis Associated with Various Disease Entities 25
A. Myocarditis 25
B. Malignant Disease 26
C. Habitual Abortion 27
D. Other Conditions 29
E. Antibody Response to Infection 29
VI. Epidemiological Data 32
A. Human Populations 32
B. Animals 33
VII. Pathogenesis and Immunity 34
VIII. Physiology 35
IX. Therapy 35
X. Conclusion 36
References 37

Evolutionary Trends in Mammalian Trypanosomes

CECIL A. HOARE

I. Introduction 47
II. Relationship of Trypanosomes to other Trypanosomatids 48
A. Characteristics of Trypanosomatidae 48
B. Origin of Trypanosomes 50
III. Course of Evolution in Trypanosomes 57
A. Affinities of Vertebrate Trypanosomes 57
B. Phylogeny of Mammalian Trypanosomes 59
IV. Microevolutionary Divergence 66
A. Biological Races 66
B. Variation in *Trypanozoon* 67
C. Variation in *Duttonella* 74
D. Variation in *Schizotrypanum* 75
E. Variation in *Herpetosoma* 75

F. Infrasubspecific Variation 76
G. Speciation by Mutation 79
V. Taxonomic Treatment of Biological Variants 82
VI. Summary 85
References 85

Problems in the Cultivation of some Parasitic Protozoa

ANN BISHOP

I. Introduction 93
II. The Cultivation of Mammalian Trypanosomes 94
A. The Classification of Mammalian Trypanosomes 94
B. The Cultivation of Trypanosomes belonging to the Salivaria 95
C. The Cultivation of Trypanosomes belonging to the Stercoraria 110
D. The Cultivation of the Genus *Leishmania* and the Relationship of the Stage of Development of the Organism to the Type of Culture Medium and the Environmental Temperature 116
E. Discussion 119
III. The Cultivation of Malaria Parasites 121
A. Introduction 121
B. The Cultivation of the Erythrocytic Stage of the Life Cycle of the Malaria Parasites 121
C. The Cultivation of the Exoerythrocytic Stage of the Life Cycle of Malaria Parasites 125
D. The Cultivation of the Sporogonic Cycle of Malaria Parasites 127
E. Discussion 128
References 129

Malaria in Mammals Excluding Man

P. C. C. GARNHAM

I. Introduction 139
II. Taxonomic Problems and New Species 140
A. Taxonomic Problems 140
B. New Species 142
III. Life Cycles 150
A. Rodent Parasites 150
B. Simian Parasites 153
C. Relapses 155
IV. Host Susceptibility and the Zoonosis Problem 156
A. Zoonoses 156
B. Other Host Susceptibilities 159
V. Ultrastructure 160
A. Erythrocytic Stages 160
B. Exflagellation 166
C. Ookinete 172
D. Oocyst 174
E. Sporozoites 176

VI. Pathogenesis 181
VII. Immunity and Modern Serological Reactions 184
A. Introduction 184
B. Serological Measurement of Immunity 185
C. Cross Immunity Reactions 189
D. Passive Immunity 190
E. Malaria Antigens 192
References 195

The Biology of the Acanthocephala

W. L. NICHOLAS

I. Introduction 205
II. General Morphology 206
A. External Features 206
B. Internal Anatomy 207
III. Development 209
A. Reproduction 209
B. Embryology 210
C. The Role of the Acanthor 214
D. Development within Intermediate Hosts 215
E. Development within the Definitive Host 220
IV. Histology and Cytology 222
A. General Histology 222
B. Histochemistry 222
C. Ultrastructure 224
V. Biochemistry 228
A. Composition 228
B. Assimilation 229
C. Intermediary Metabolism 229
D. Respiration 231
VI. Host–Parasite Interaction 232
A. The Arthropod Host 232
B. The Vertebrate Host 233
VII. Practical Importance for Man 235
A. Human Medicine 235
B. Veterinary Medicine 235
C. Parasites of Wild Birds and Fishes 236
VIII. Conclusion 236
References 237

The Post-embryonic Developmental Stages of Cestodes

MARIETTA VOGE

I. Introduction 247
II. Life Histories and Development 248
A. Trypanorhyncha and Tetraphyllidea 248
B. Pseudophyllidea 249

C. Cyclophyllidea ... 252
D. Asexual Multiplication ... 262
E. Summary ... 264
III. Structure ... 267
A. Procercoids and Plerocercoids ... 268
B. Cysticercoids ... 270
C. Cysticerci ... 272
D. Studies in Histochemistry ... 276
E. Summary ... 277
IV. Certain Aspects of Host–Parasite Relations ... 278
V. Temperature and Development ... 282
VI. Post-embryonic Stages *in vitro* ... 285
VII. Excystment *in vitro* ... 288
VIII. Conclusions ... 289
References ... 290

AUTHOR INDEX ... 299
SUBJECT INDEX ... 311

Toxoplasma and Toxoplasmosis

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I. Introduction 1
II. Electron Microscopy 2
  A. The Trophozoite (Proliferative Form) 2
  B. The Cyst 6
III. Transmission 15
IV. Serology 20
V. Toxoplasmosis Associated with Various Disease Entities 25
  A. Myocarditis 25
  B. Malignant Disease 26
  C. Habitual Abortion 27
  D. Other Conditions 29
  E. Antibody Response to Infection 29
VI. Epidemiological Data 32
  A. Human Populations 32
  B. Animals 33
VII. Pathogenesis and Immunity 34
VIII. Physiology 35
IX. Therapy 35
X. Conclusion 36
References 37

I. INTRODUCTION

A number of reviews of the entire body of knowledge of toxoplasmosis in the last few years (Jacobs, 1963; Beattie, 1964; Kirchhoff and Kraubig, 1966) are adequate guides to the field and to various unresolved problems in it, and there is no point in another of the same type. The discussion in this review will be focused mainly on literature appearing during 1963–66, older papers being cited only to bring points into historical perspective. It will be concerned primarily with advances in the electron microscopy of *Toxoplasma*, knowledge of mechanisms of transmission and serological work.

Additional attempts will be made to summarize and comment on new epidemiological data, observations on the association of *Toxoplasma* with various disease conditions, papers concerned with anti-*Toxoplasma* drugs, and a few papers on physiology, tissue culture and toxin production of *Toxoplasma*.

II. Electron Microscopy

A. The Trophozoite (Proliferative Form)

Continuing work on the trophozoite of *T. gondii*, grown in tissue culture as well as harvested from the peritoneal exudate of mice, has clarified some of the points about the reproduction of the parasite.* Despite exhaustive search, various investigators (e.g. Ogina and Yoneda, 1966) have failed to find any forms which are inconsistent with endodyogeny. The occurrence of rosette-like formations (Gavin *et al.*, 1962) may, therefore, be regarded as due to the repeated sequential production of daughter parasites without complete separation of the posterior ends. The connections of organisms to residual material, or to each other, appear now to be at the posterior pole of the parasites, opposite the conoid. This is in contrast to the idea of Gavin *et al.* that the connections are lateral, the individual organisms being arranged around a central residual mass like the carpels of an orange.

I am willing to accept these new interpretations, as well as to consider that the other more filamentous connections between parasites, described by Gavin *et al.* as another indication of rosette formation and possibly schizogony, are also derived because of repeated endodyogeny without complete separation at the posterior pole. I do, however, continue to regard endodyogeny as a special form of schizogony, in which only two merozoites are produced at the same time. The process may be described as given by Gavin *et al.*, with the formation of dense conical sheaths within the cytoplasm of the parental organism close to the anterior surface of the nucleus. Within these sheaths, the conoid and granular bodies which may be precursors of "toxonemes" appear. The maternal nucleus becomes horseshoe-shaped, its two portions becoming surrounded by the sheaths as the latter elongate enclosing other cytoplasmic components such as granules, vesicles, and endoplasmic reticulum. Eventually the nucleus separates and two almost complete filial organisms, each with a single membrane and its cytoplasm in communication with the maternal cytoplasm at the posterior pole, lie within the parent structure. If one were to imagine this sequence being altered to the extent of new sheath formation and cytoplasmic and nuclear partition being initiated before the previous divisions are completed, we would have an accumulation of various stages of filial developmental forms associated or connected together. Endodyogeny is consistent with schizogony because of similarity in the organization of cytoplasmic components prior to nuclear partition, as has been shown by Rudzinska and Trager (1961) and others for *Plasmodium*

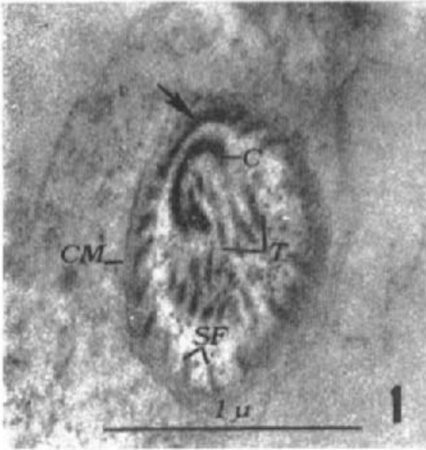
* I am relying here on some work being done by Dr Harley G. Sheffield, in collaboration with Miss Marjorie L. Melton, Laboratory of Parasitic Diseases, NIAID, NIH, which I have had the opportunity to see.

lophurae, by Scholtysek (1965) for *Eimeria perforans* and *E. stiedae*, and by Hepler *et al.* (1966) for *P. fallax*. The difference is in the number of progeny formed; and, as has just been conjectured, this may be a matter of the relation between and the timing of sequential developments. Overlapping of the production of daughter cells, by the initiation of the division process prior to completion of the previous one, would result in the type of rosette formation described by Gavin *et al.* and would explain the interrelation of the rosettes and endodyogeny.

The juxtaposition of organisms described by Gavin *et al.* as possibly indicative of binary fission or sexual union can be interpreted as merely fortuitous sections through two or three closely packed organisms. Such appearances as the incomplete membranes we described, with apparently associated vesicular structures, may be explained as due to tangential sectioning of the membranes. Olisa's (1963) interpretation of some electron micrographs as binary fission may also be explained as a late endodyogeny in which the daughter cells are still attached. Kikkawa and Gueft (1964) also suggest binary fission as a mode of division within the cyst, basing their interpretation on the relative position of double truncated cones "well anterior to the peripheral portion of convoluted tubules and not near the nucleus". However, they had limited material and saw only this one structure. I hold no great brief for the interpretation of these structures as Gavin *et al.* originally offered it. As we stated, they were the least frequently observed forms in our study, and we were merely conjecturing about their significance. Possibly the interpretation was somewhat subjective, because with the increasing evidence bearing on the relationships of *Toxoplasma*, the idea of demonstrating sexual union would indeed be attractive. Obviously, however, our judgments should be made as objectively as possible, and we certainly cannot propose our findings as a demonstration of such a phenomenon.

Goldman *et al.* (1958) in their description of the silver staining of *Toxoplasma* pointed out the presence of a posterior polar granule. This has not been observed in our studies, and Sheffield (1966) has not seen such granules despite exhaustive search, his interpretation being that the silver-stained "granule" is an artifact representing the terminal thickening of the inner membrane of the organisms to which may be attached the submembranous fibrils that extend between the anterior polar ring and the posterior pole.

The existence of a micropyle, or cytostome, as described by Garnham *et al.* (1962a), has been confirmed by Sheffield (1966) in both *Toxoplasma* and *Besnoitia* and by Wildführ (1964, 1966) in *Toxoplasma*. Akinshina and Bykovsky (1964) depict, in a schematic drawing, one structure on the surface of *Toxoplasma* which they designate a "pinocytotic vacuole" and which may indeed be the micropyle; its position is similar to that described by the other workers cited. Sheffield also considers, in analogy with what he has seen in *Besnoitia*, that the micropyle in *Toxoplasma* may be the site of exchange of material with the milieu outside the parasite, because of the arrangement of vacuoles near this structure. The cytostome and its function in malarial parasites was well discussed by Aikawa *et al.* (1966). The Russian authors also described various projections of the outer membrane and the breaking-off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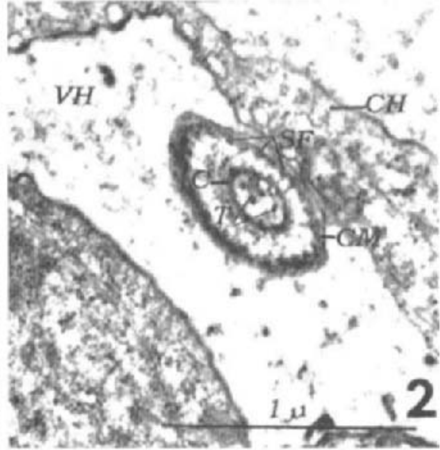
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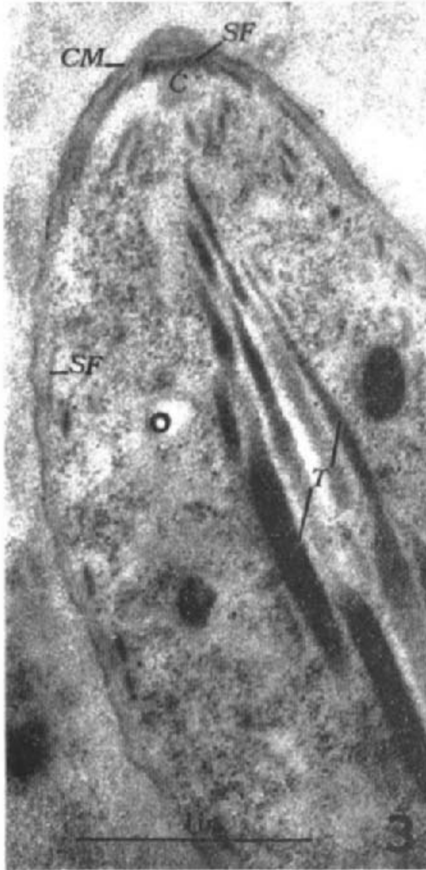
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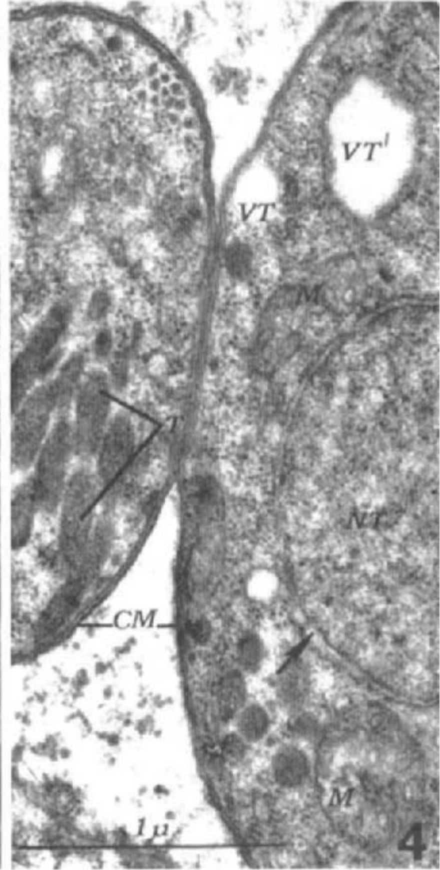
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of cytoplasm to the outside in small membrane-enclosed globules. The "waviness" of the membranes they observed has appeared from time to time in some of our material, and in that of Hogan *et al.* (1960). It may be associated with the ability of the parasite to perform gliding movements. When it has been very exaggerated, we have interpreted it as due to osmotic changes or to differences in fixation.

The nomenclature and relationship of "toxonemes" originally named by Gustafson *et al.* (1954) and paired organelles (Garnham *et al.*, 1962b) is a matter of some discussion. As structures resembling toxonemes have been found in a number of Sporozoa, we would prefer to adopt a more general term, but Garnham's proposal of "convoluted tube" may not be satisfactory because Wildführ (1964) has seen fibrillar elements within them. Ogina and Yoneda (1966) describe a mesh-like internal structure in toxonemes of *Toxoplasma* fixed in glutaraldehyde. The term "microneme" here proposed for these organelles in the variety of organisms within which they occur should not conflict with terms in use for other groups of organisms.

Gustafson *et al.* (1954) described the micronemes (their "toxonemes") as progressively increasing in uniform diameter posteriorly. However, in some of our preparations, some of those of Hogan *et al.* (1960) and in Wildführ's (1964) there appears to be an abrupt enlargement of the posterior portions of the micronemes. Furthermore, Wildführ's description of them as "schmale langgestreckte keulenformige Gebilde oder . . . sackartige Erweiterungen" (slender, extended, club-shaped structures or . . . sack-like dilatations) and his picture of four such organelles in a longitudinal section, suggests that there may be a functional relationship between micronemes and the "paired organelles" of Garnham *et al.* (1962b). Garnham and his co-workers (1960, 1961, 1962a, b) have described paired organelles in malaria parasites, *Toxoplasma*, and *Lankesterella*, but the picture is most confusing in *Toxoplasma* and *Besnoitia* because of the abundance of micronemes in these

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FIG. 1. An oblique section through the anterior pole of a *Toxoplasma* trophozoite. The conoid (*C*) appears as a dark semi-circle. The radially diverging submembranous fibrils (*SF*) extend between the conoid and the cell membrane (*CM*). A few oblique sections of micronemes (*T*) are seen.

FIG. 2. A region similar to Fig. 1 in cross-section. The conoid (*C*) appears as a ring encircling the anterior ends of a few micronemes (*T*). Cross-sections of submembranous fibrils (*SF*) abut the cell membrane (*CM*). The vacuolar space (*VH*) appears to contain some particulate material. *CH* is host cell cytoplasm.

FIG. 3. Longitudinal section through the anterior half of a trophozoite. The protuberating front end illustrates the intimate association of the cell membrane (*CM*) with the submembranous fibrils (*SF*) at their origin and with the tangentially sectioned conoid (*C*). The cytoplasm contains longitudinally sectioned micronemes (*T*) which show the characteristic progressive increase in diameter posteriorly.

FIG. 4. Portions of two trophozoites. On the left, the micronemes (*T*) exhibit a delicate lacunary structure. The parasite on the right contains a few vacuoles, one of which (*VT′*) shows the remains of some contents in its periphery while the contents of the other (*VT*) have apparently completely sublimed. The parasite nucleus (*NT*), perinuclear cistern (arrow), mitochondria (*M*) and cytoplasmic granules are all clearly seen.

(Figs. 1–4 from Gavin *et al.*, 1962.)

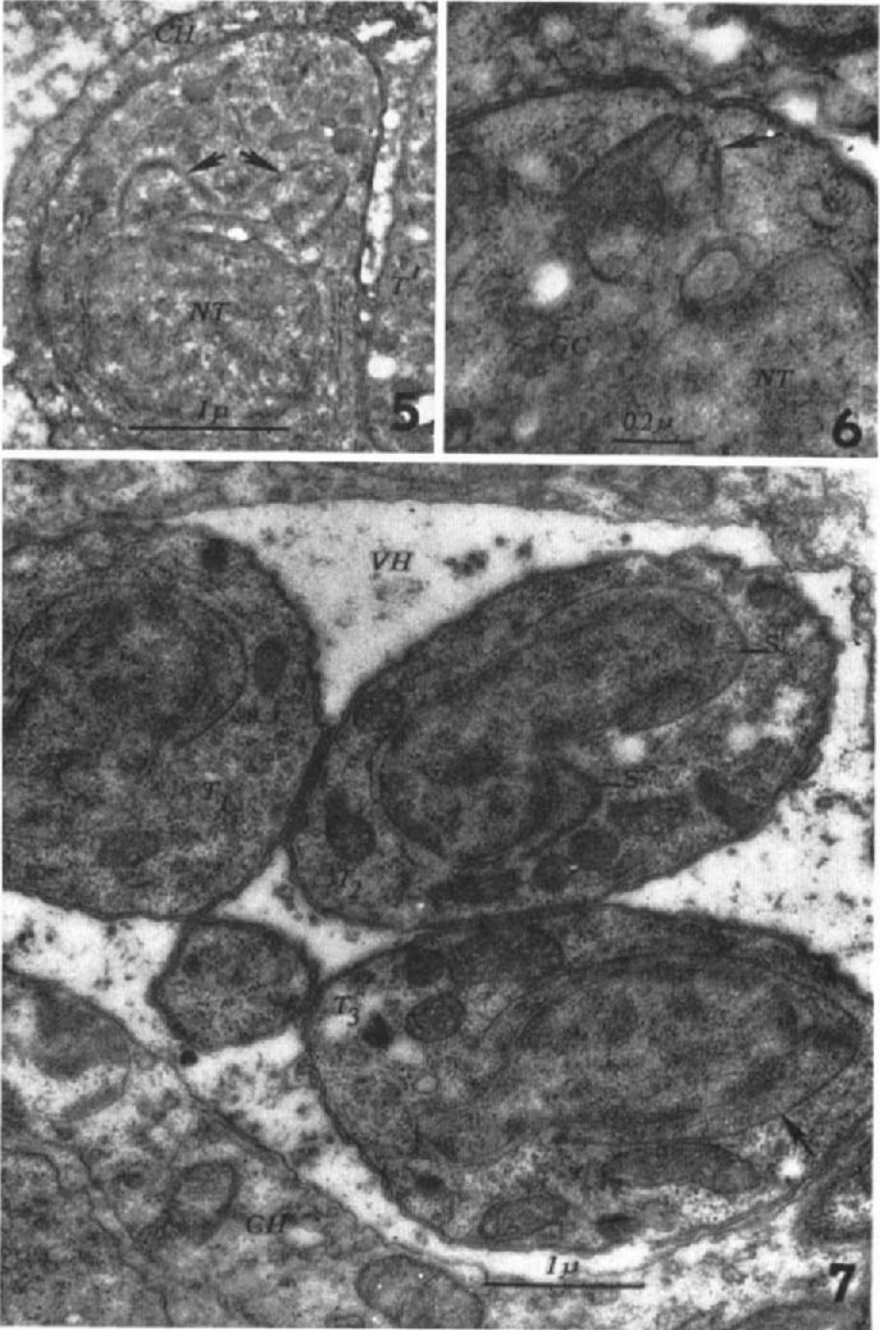
species. It is possible, however, since these structures exist in close proximity to the conoid, and portions of some of them lie within it, that they all have a part in the secretory function ascribed to the paired organelles by Garnham *et al.* (1962a, b). Sheffield (1966) demonstrated, in *Besnoitia*, a short-stalked vesicular structure just below the conoid with the stalk entering the conoid, and this supports the suggestion of a secretory function. The structure also appears somewhat similar to the paired organelles of malaria parasites and *Lankesterella*. Wildführ (1966) stated that despite different techniques of fixation and other methods of obtaining contrasts, he could not find any difference between "toxonemes" and paired organelles. The idea now suggested for further consideration is that we are dealing with similar structures when we are talking about micronemes or paired organelles, and are finding them in different functional states and in varying numbers. Ogina and Yoneda (1966) described "radial clubs", which have an internal resemblance to micronemes and may be rudimentary micronemes. This suggests that we are studying structures that are continually in a process of formation and functional change. In opposition to this idea, it is difficult to reconcile only one paired organelle with the large number of micronemes seen in *Eimeria* (see Sheffield and Hammond, 1966) and *Plasmodium* (see Aikawa *et al.*, 1966). It is also difficult to see how a structural function can be ascribed to any of these bodies, as has been done by Mossevitch and Cheissin (1961, cited by Garnham *et al.*, 1962a), for *Eimeria intestinalis*, because they lie apparently unattached at their posterior ends, are convoluted, and some enter (or originate in) the conoid. The pictures of protruded conoids (Wildführ, 1964, 1966; Olisa, 1963) suggest an aggressive function for the structure. The micronemes do not appear, structurally, to be able to effect such protrusion. However, Wildführ (1966) contends that this possibility is not ruled out. Lund *et al.* (1966) suggest that the micronemes or paired organelles may be the site of lysosomal enzymes. Hepler *et al.* (1966) state that these structures are the first to disappear in newly penetrated merozoites of *P. fallax*, which suggests they are involved in invasion of the cell.

B. THE CYST

Studies of encysted forms of *Toxoplasma* have revealed the occurrence of endodyogeny here also. No other form of reproduction has been observed, except for the single structure described by Kikkawa and Gueft (1964). Wanko *et al.* (1962) described the wall as partly of parasite origin. The cyst wall is demonstrably not the wall of the host cell, because the rough-surfaced endoplasmic reticulum, mitochondria, and nucleus of the host cell have been demonstrated around the cyst, even when large cysts were studied. The loose organization of granular material forming the cyst wall, its growing density in larger cysts, and its appearance between organisms as septa, are inconsistent with the hypothesis of Garnham *et al.* (1962a) that the wall is derived from an enormously extended parental cell membrane within which repeated divisions have occurred. It seems that the cyst develops intracellularly within a new wall of its own. However, the existence of parasites in the cytoplasm of a host cell, without any separating membrane, as described by Gavin

et al. (1962) was questionable. Matsubayashi and Akao (1963) and Sheffield have found that the developing cyst does have a vacuolar membrane around it. Matsubayashi and Akao (1963) believe that the material which comprises the wall originates from the parasites and is deposited on the vacuolar membrane. They have shown in additional electron microscope studies (1966) that ferritin-conjugated antibody is deposited on the limiting membrane of the *Toxoplasma*-containing vacuoles of the host cell. The particulate precipitates usually seen in the vacuole have an antigenic property as evinced by the ferritin labeling. This, they consider, confirms their interpretation that the cyst wall is produced by the accumulation of *Toxoplasma* substances on the vacuolar membrane. The blebs we described on the surface of encysted parasites (Wanko *et al.*, 1962), seen also by Matsubayashi and Akao (1963), would be consistent with this view. It is also possible that the cyst wall material diffuses out of the parasites and precipitates in the vacuolar milieu by reaction with substances produced by the host cell and deposited in the vacuole. The dense accumulation of host endoplasmic reticulum and mitochondria around the cyst (Wanko *et al.*, 1962; Matsubayashi and Akao, 1963) would support this idea. According to various authors (Matsubayashi and Akao, 1963; Van der Zypen and Piekarski, 1966) the original vacuolar membrane enclosing the cyst wall is difficult to discern in large cysts. As already stated, the wall appears to be intimately connected with host cell endoplasmic reticulum and mitochondria. (I cannot agree with Van der Zypen (1966) that cysts develop extracellularly, although they may appear so situated because of destruction of the cell when the cyst attains a large size.) The ground substance of the cyst wall is amorphous or granular and at the surface has a "serrated profile" (Garnham *et al.*, 1962a) of "vesicular and membranous profiles" (Wanko *et al.*, 1962) that has been described by Van der Zypen and Piekarski (1966) as a many folded membrane. Kikkawa and Gueft (1964) describe the cyst wall as made up of minute tubules which may represent another interpretation of the vesicular and membranous profiles of Wanko *et al.*, but may also be related to differences in cysts formed in muscle as compared with nervous tissue cells. It seems likely, now, that the ground substance is the precipitate applied to the vacuolar membrane, and the latter becomes so intimately associated with the host cell cytoplasmic structures that it cannot be clearly demarcated. The irregularity of the outer surface is probably due to these adherent materials and possibly also to some folding of the original membrane.

The material, continuous with the cyst wall substance, that extends among the encysted parasites has been described by most authors as a dense amorphous or granular substance. Wanko *et al.* (1962) described pore-like formations in these septa which we conjectured could indicate the presence of inter-organismal connections. Matsubayashi and Akao (1963) recognized structures of the same type in their pictures, and were circumspect about their importance. On further study, it is possible to explain gaps in the interparasitic septal substance as merely areas where the parasites are pressed closely together so that very little substance is present. Similarly, the pore-like connections may be artifacts resulting from failure to reveal the membranes of two such closely approximated organisms, which failure could be due to


CH
T
NT
1μ
5
GC
NT
0.2μ
6
VH
St
T1
S
T2
T3
CH
1μ
7

tangential sectioning. Unless such structures can be established more definitely as distinct entities, and some function ascribed to them, they appear to be of little significance. The canaliculi described by Van der Zypen and Piekarski (1966) as a network within the septal substance have not been seen by others. These structures must be distinctly differentiated from unit membranes of tangentially sectioned parasites or other possible artifacts before they can be accepted.

The factors influencing cyst formation are still a problem. I suggested in an earlier review (1956), on the basis of the report by Gustafson *et al.* (1954) of granular material on the inner surface of host cell vacuoles containing *Toxoplasma*, that the cyst wall was formed from these materials. However, we were still not exactly certain then of the distinction between the trophozoite, terminal colony (or pseudocyst) and cyst, and it was difficult to understand how the cyst wall could develop in one situation while under other conditions the parasite ruptured the cell and escaped to invade others.

It could be suggested by hypothesis that the immune mechanisms of the host resulted in retardation of cell rupture and mediated the development of cysts. However, Lainson (1958) pointed out that structures he identified as cysts in rabbit lung alveolar cells and in brain cells began to appear as early as 7 or 8 days after infection. This is very early for immune mechanisms to be adequately developed. Also, Stahl *et al.* (1965) demonstrated cyst production in mice in which the immune response was suppressed by 6-mercaptopurine. Furthermore, Hogan *et al.* (1960) demonstrated cysts developing in retinoblastoma cells *in vitro*, where immune mechanisms could not be operative (except for the possibility of interferon-like substances which have not yet been adequately demonstrated in *Toxoplasma*-infected cells). While some workers have expressed doubt concerning the true nature of the cysts developed *in vitro*, I believe they are cysts on the basis of another criterion employed by Jacobs and Melton (1965)—the ability of encysted parasites to withstand action of artificial gastric juice, which destroys trophozoites very rapidly. In our studies, we cultivated the Beverley strain of *Toxoplasma* in monkey kidney cells, and at various intervals scraped the cells off the glass into the pepsin-HCl solution. Digestion-resistant forms were found

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FIG. 5. Initial phase of development of two filial organisms within a *Toxoplasma* trophozoite. Two opaque parabolic profiles are apposed on their concave surfaces to the parent nucleus (*NT*). An adjoining organism (*T′*) and a small rim of host cell cytoplasm (*CH*) are labeled.

FIG. 6. A somewhat later stage. Within the primordial sheath (arrow) another dense contour resembling a conoid (*C*) is apparent. Parent cell nucleus (*NT*) and Golgi complex (*GC*) are labeled.

FIG. 7. Three parental trophozoites (T_1–T_3) containing filial organisms in various phases of development. The earliest stage is seen in T_2 where a primordial sheath (*S*) extends over one pole of the nucleus. The second sheath (*S′*) is more advanced, approximates the character of a membrane and encloses a larger area around the other nuclear pole. A similar phase exists in T_1. The most developed filial organism is in T_1, where the newly formed membrane (arrow) almost entirely encloses the nuclear segment and cytoplasm of the future organism. The vacuole (*VH*) and host cell cytoplasm (*CH*) are labeled.

(Figs. 5–7 from Gavin *et al.*, 1962.)

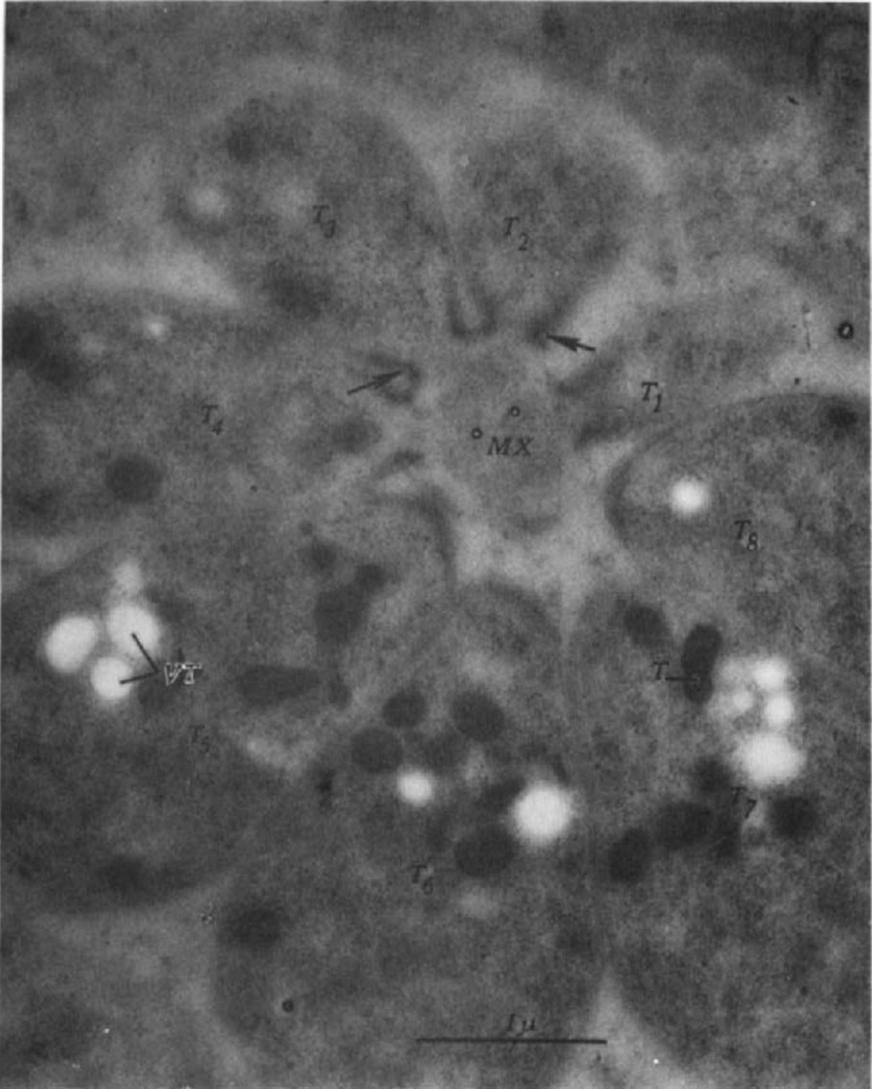

FIG. 8. Dividing trophozoites in rosette arrangement. Around a central matrix (*MX*) eight organisms (T_1–T_8) are radially oriented. The parasites T_1–T_4 are in continuity with one another through cytoplasmic communications with the central matrix and appear to have a common membrane. This boundary surrounds each organism except for a small sector. There the central projection of the membrane appears thickened, possibly representing the posterior termination of the submembranous fibrils; then the membrane reverses its course (arrows) and encircles the adjoining parasite. Cross-sectioned micronemes (*T*) led originally to the idea that the organisms were connected at their sides (see text), but the above interpretation of the membrane thickenings is not consistent with this. *VT* are vacuoles. (From Gavin *et al.*, 1962.)

occasionally as early as 8 days after infection of the tissue culture. Table I summarizes these results. Attempts to use immune serum in the tissue culture medium did not hasten cyst production in our tests.

Therefore, some other mechanism must be sought to explain cyst formation. Like the trophozoite, the cyst grows within a host cell vacuole. In the first case the mass of trophozoites ruptures the cell, but in the second the cyst

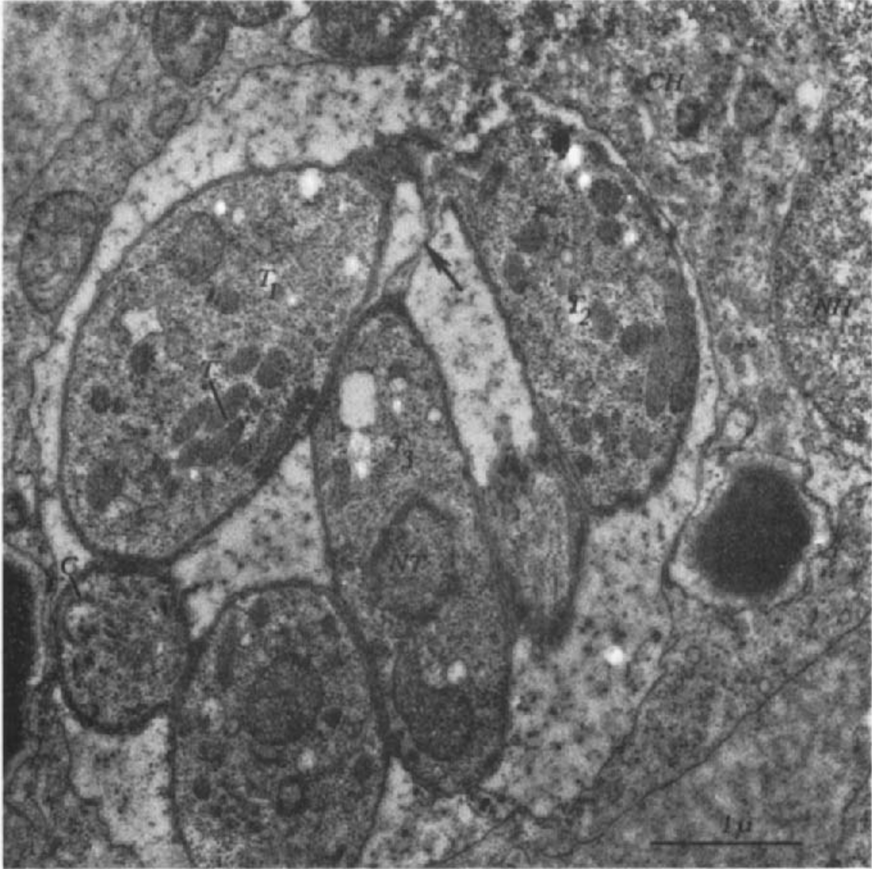

FIG. 9. Incompletely separated organisms. T_1 and T_2 are almost separated from one another, and only a small membranous structure (arrow) connects T_3 to the area of the central matrix. Other structures seen are micronemes (*T*), parasite nucleus (*NT*), mitochondria (*M*), a conoid (*C*), host cell nucleus (*NH*), and cytoplasm (*CH*). (From Gavin *et al.*, 1962.)

may attain a relatively enormous size while still within the host cell. Matsubayashi and Akao (1963) associate cyst production with slow growth of parasites and, conversely, rupture of cells and dispersal of the trophozoites with rapid growth. However, there are two observations which indicate that such a relatively simple explanation is not adequate. One observation is their

own, that in tissue culture cells infected with the relatively "slow" Beverley strain of *Toxoplasma*, the limiting membrane of the vacuole "often appears to be thick even in the one-cell stage of the parasite", in contrast to the

TABLE I

*Frequency of occurrence of digestion-resistant** Toxoplasma *in tissue cultures*** *infected for various periods*

Duration (days) of tissue culture infection	No. of trials	No. of trials showing digestion-resistant parasites
6–8	7	1*** (14·3%)
9–14	14	4 (28·5%)
15–26	23	16 (69·5%)

* Digestion for 30 min or 1 h in pepsin-HCl. Undigested controls positive on all trials.
** Monkey or rabbit kidney epithelium.
*** Tissue culture infected for 8 days.

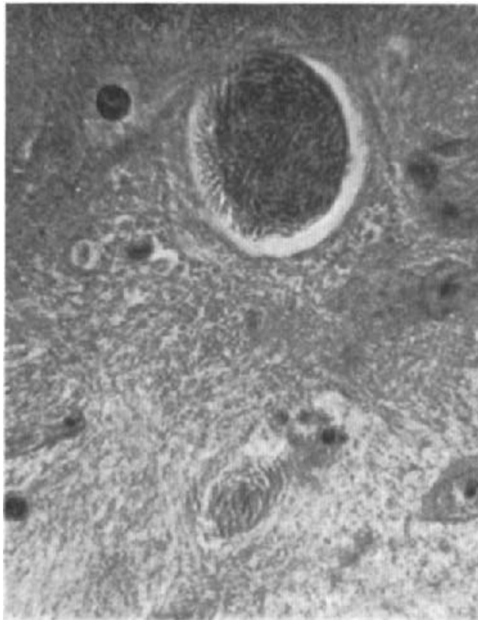

FIG. 10. Two *Toxoplasma* cysts in a section of mouse brain. The space around the larger cyst is probably an artifact due to shrinkage.

extremely thin membrane of the vacuole in RH-infected cells. This observation suggests an altered physiology of the parasite, with the production of

more cyst wall-forming material very early, rather than merely an accumulation because the parasites remain within the vacuole. The other observation is an old one recorded by Sabin and Olitsky (1937). These authors stated that, accompanying increasing pathogenicity of the parasite on passage in mice, there was a spontaneous change whereby invaded cells disintegrated when the total number of parasites in them was still quite small. Thus, it appears that trophozoite forms need not rely on rapid growth to distend the host cell in order to be released from it, and this observation must represent another physiological difference between the cyst form and the trophozoite. It would be interesting to extend the work of Lund *et al.* (1966) to see if differences in concentrations of lytic enzymes exist between the two forms.

It is possible that we are missing an important event in the life cycle of the parasite, which is necessary to explain the differences between trophozoites and cyst forms and to allow us to understand mechanisms of cyst formation.

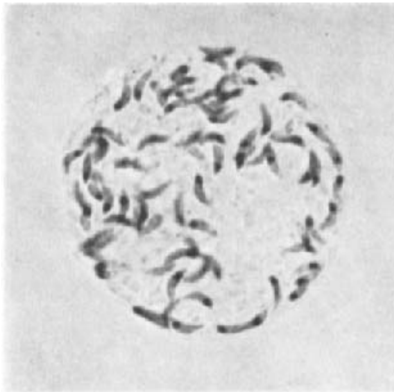

FIG. 11. A micro-isolated *Toxoplasma* cyst. This was allowed to degenerate partially, by standing in hypotonic solution; then it was stained to show some of the lanceolate enclosed parasites. The "ghosts" of other parasites occupy the remainder of the cyst.

One explanation would be the existence of two morphologically indistinguishable populations of the organism, a relatively slow-growing form with cyst-forming capabilities, the other rapid-growing and capable of bursting cells before the parasite population within them really distends the cells. If this hypothesis were true, one would expect that a rapidly growing strain of *Toxoplasma*, like RH, which has been passaged every 3–4 days in mice for over 20 years, would have lost the slow-growing cyst-forming population by a dilution process. However, Hogan *et al.* (1960) used the RH strain in their *in vitro* studies and for some unexplained reason were able to maintain their infected retinoblastoma cell cultures for extended periods of time, with the production of *Toxoplasma* cysts. Moreover, numerous workers have observed cysts in RH-infected animals treated with drugs at levels adequate to obtain survival but not radical cure. It therefore appears that the cyst-forming capacity of the RH strain has not been lost. This information speaks against

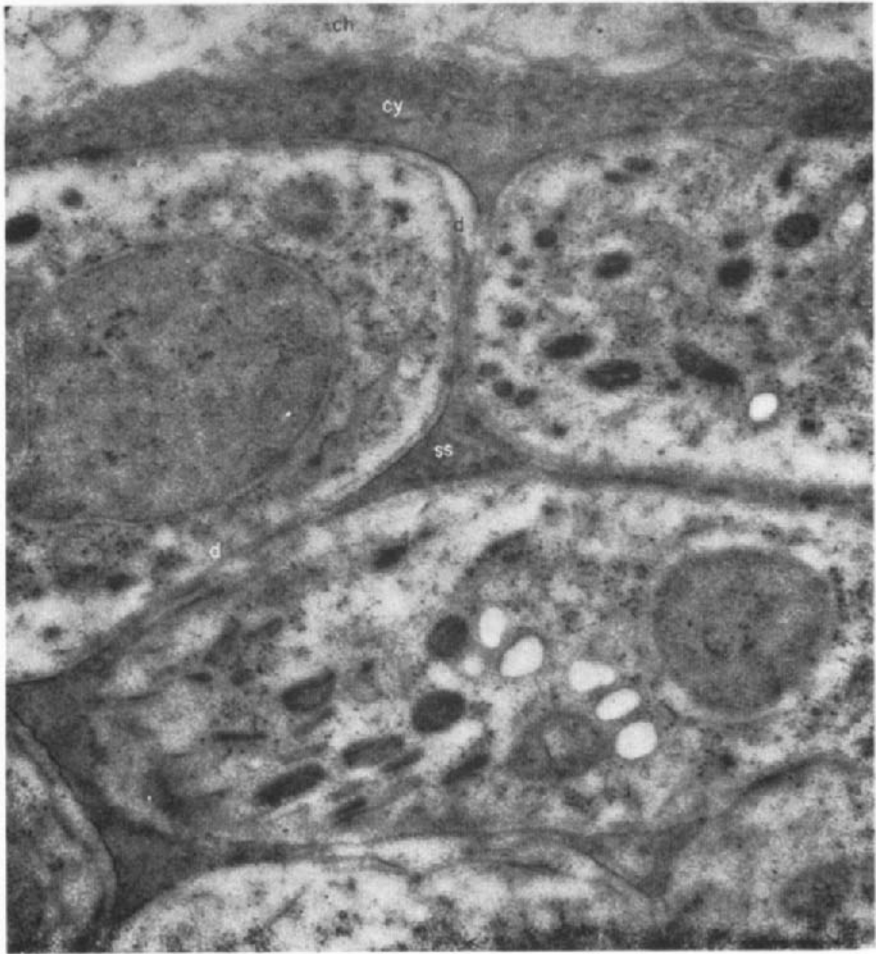

FIG. 12. Portion of a large *Toxoplasma* cyst. The cyst wall (*cy*) exhibits membranous and vesicular profiles embedded in opaque material. It closely resembles the septal substance (*ss*) which extends between the outer membranes of adjoining parasites. Discontinuities (*d*) in the septal substance can be seen. Host cell cytoplasm (*ch*) appears beyond the cyst wall. The difficulty of discerning an original host vacuolar membrane is obvious. Approx. ×43 000. (From Wanko *et al.*, 1962.)

the possibility that establishing clones of the parasite would reveal population differences as to cyst formation among them.

The idea of some event that "triggers" cyst production is intriguing, but more refined methods than we yet have are necessary in order to reveal it. If we could develop some genetic "markers" for *Toxoplasma* strains, it is conceivable we could obtain evidence of a sexual union of parasites. This so far we have failed to do in a convincing manner, as mentioned above, but

more electron microscopy of *Toxoplasma* under different conditions, *in vitro* and *in vivo*, may be very rewarding.

III. Transmission

In the course of many years of research effort, tests of transmission of *T. gondii* by bloodsucking arthropods and by various other means have been performed by many investigators. These and new studies have been adequately reported in earlier papers (Jacobs, 1956; Pestre *et al.*, 1962; Beattie, 1964; Soliman *et al.*, 1963; Piekarski, 1966) and need not be detailed here. Although *T. gondii* is widespread in animals, it is difficult to identify sources of infection among them except for the oral route in carnivores. The cyst form is resistant to digestion, and animals or human beings who consume raw flesh can become infected. As the skeletal muscles of sheep and swine frequently contain *Toxoplasma* cysts (Jacobs *et al.*, 1960a, 1963; Ishii *et al.*, 1962; Sergent, 1963), it is quite possible that the ingestion of raw meat is a source of human infection. Desmonts *et al.* (1965a) consider this practice the reason for the high prevalence of toxoplasmosis in France and for a very high incidence of sero-conversion in hospitalized children. There is little reason to doubt this means of transmission, but it is obviously not the only method of spread because of the high prevalence of toxoplasmosis in herbivorous animals. Since neither the cyst form nor the trophozoite seems capable of surviving under conditions which would allow efficient spread of these forms by a contaminative route, and since epidemiological information does not lend much support to a respiratory route of infection, we have not been able to explain many aspects of the transmission of this versatile parasite. This is an exceedingly important facet of the biology of *Toxoplasma gondii*, because it may be that with a full knowledge of its transmission we can complete our understanding of the life cycle of the organism.

Because of the lability of the cyst and trophozoite to the external environment, I have, in past (unpublished) work, investigated a wide variety of invertebrate hosts as possible carriers of cysts, or as vectors of trophozoites. Our early work with concomitant infections of hookworm, *Ancylostoma caninum*, and *Toxoplasma* in dogs (Jacobs *et al.*, 1965) was done with the idea of testing the possibility of spread of *T. gondii* by nematodes; it was part of a study, done in collaboration with Dr Paul P. Weinstein, in which we used also *Nippostrongylus muris* and *Trichinella spiralis*. Except for one instance, in which we found infection in a mouse force-fed a macerated earthworm (*Lumbricus terrestris*) after the worm had been fed *T. gondii* cysts, which was not repeated in numerous subsequent experiments, all of these attempts gave negative results. Feeding of feces of chronically infected animals, either fresh or after storage, to other animals was tried many times, also with negative results.

The report by Hutchinson (1965) of transmission of *T. gondii* by material recovered by zinc sulfate flotation of feces of a cat that had 2 weeks previously been fed *Toxoplasma* cysts, came, therefore, as a welcome clue to the transmission of this protozoan. The fecal float contained *Toxocara cati* eggs and *Isopora* sp. oocysts. It was washed and stored in tap water at room

temperature. Three months later and at intervals thereafter for over 12 months some of the sediment was fed to mice, which became infected with *Toxoplasma*. Hutchinson was justifiably circumspect about this single experiment, in regard to the question of whether the infection induced by the material was transmitted within the nematode eggs or by other means. Nevertheless, his work has been a stimulus for others and has resulted in corroborative findings.

Jacobs and Melton (1966a) have been able to repeat Hutchinson's findings with the feces of cats containing only *Toxocara cati* eggs; no *Isospora* cysts or other protozoan cysts or helminth eggs were present. Three cats with natural *Toxocara cati* infections were fed two or three mice which were chronically infected with *Toxoplasma gondii* strains derived originally from chickens or sheep. Flotations of their feces, collected on various days, were prepared in $ZnSO_4$ solution, and the floated material was washed and resuspended in tap water. Part of the float was fed immediately to mice, and the remainder was stored at room temperature. No float produced toxoplasmosis when fed to mice immediately after collection and washing. Stored floats of fecal material, obtained on days 8–14 after feeding the cats the *Toxoplasma*-infected mice, produced toxoplasmosis when fed to mice 48 days to 7 months later.

Numerous floats of feces from these cats obtained earlier than 1 week or later than 2 weeks after feeding with *Toxoplasma* failed to produce toxoplasmosis when fed to mice. Hutchinson (personal communication) also originally observed a rather short period of time, that he termed "narrow shed", during which the feces of his first cat were infective. Hutchinson (1967) fed *T. gondii*-infected mouse carcasses to cats on 5 successive days, and obtained infective floats from the cat feces from the 13th day after the first feeding through the 30th day. These would correspond to the 8th through 25th day after the last feeding of *Toxoplasma* cysts to the cats. Thus, Hutchinson's later results indicate a longer period of infectivity of the cat feces. Since he did not arrive at a negative end-point (collections of feces terminated 30 days after the first feeding of the cats), we do not know how long the feces may have continued to contain infective forms of *Toxoplasma*.

Differences in size of *Toxoplasma* inoculum and in worm load may account for the discrepancies between Hutchinson's findings and ours. Dubey (1966) reports that he was able to produce *Toxoplasma* infection of mice with infected *T. cati* eggs laid only over a maximum period of 10 days, from 5 to 14 days after feeding the cat. In these tests of the infectivity of the fecal floats, Dubey fed large numbers of eggs.

Neither Hutchinson nor Dubey reported on any attempts to feed *T. cati* eggs prior to embryonation of the larvae. They excluded this because they were apparently anxious to be assured that any forms of *Toxoplasma* possibly collected along with the nematode eggs would be destroyed by the long incubation period. In our experiments, we regularly fed mice unembryonated eggs immediately after flotation and washing in water. In no instance did these unembryonated eggs produce infection in the mice, even when the same batch of eggs did so after embryonation.

These results suggest that the form of the protozoan associated with the nematode egg when it is shed is either not yet infective or that the hatching of the egg is necessary to liberate it. Since in all three laboratories attempts to isolate *Toxoplasma* from the tissues of *Toxocara cati* after feeding of the feline host have failed, it appears that we may be dealing with a developing stage of *Toxoplasma* within the nematode egg.

It would appear that the parasite was within the egg, on the basis of experiments we have performed with infective eggs treated with 5% NaOCl for 30 min. This treatment did not reduce the ability of the eggs to infect mice with *Toxoplasma.* Dubey performed similar experiments with undiluted "Chloros" which removed the protein coat and shell of the *T. cati* eggs. Such eggs were also still infective, as were larvae obtained by shaking treated eggs with sand. Similar experiments should be done with unembryonated eggs.

TABLE II

Results of feeding floats to mice
Data on cat no. 11. No *Toxocara cati* eggs seen.
Fed *Toxoplasma gondii* strain M-7741 (2 mice).

Day feces collected*	Day float fed**	Results
3	50	–
4	49, 63	+
5	48, 62	+
6	47	–
7–8	45	+
10	43	–
11	42	+
21–21	32–40	–

* Day of infection with *T. gondii* is day 0.
** Day of flotation is day 0.
Fresh feces fed and inoculated into mice on days 3, 4, 5 and 10 were all negative.

Dubey claims that 1% formalin in the water in which *T. cati* eggs were embryonated had a deleterious effect on infectivity of *Toxoplasma*. In parallel experiments with eggs incubated in water or in 1% formalin only the former produced toxoplasmosis in mice. The significance of this observation is not yet apparent.

Hutchinson (1967), in designing appropriate controls for his experiments, fed *Toxoplasma*-infected mice to cats without *Toxocara* infections and obtained negative results on subsequent feeding of incubated floats of these cats' feces to mice. He also used a filter to remove embryonated *T. cati* eggs from float material which was *Toxoplasma*-infective. This filtrate did not produce toxoplasmosis when fed to mice. However, we have had some confusing results with two cats in which we could not demonstrate a patent *T. cati*

infection. One of these cats was fed two mice chronically infected with *T. gondii*, and its feces were collected daily from day 3 to 21. Fresh feces from some collections were suspended in saline and fed to mice, all of which remained negative for *Toxoplasma*. These fresh fecal preparations suspended in saline were also inoculated into mice intraperitoneally, again with negative results. Floats of feces collected on days 4, 5, 7–8 and 11 after the feeding of *Toxoplasma* to the cat, and incubated for 6–9 weeks, did produce toxoplasmosis in mice. The details of this test are given in Table II. It is noteworthy that no nematode eggs were seen in the floats from any of the fecal collections. These floats were examined immediately after they were prepared, and the water suspensions were examined at the time of feeding to the mice. The cat was killed 27 days after it had received the meal of *Toxoplasma*, and only one small, immature male *T. cati* was found in its intestine.

The other cat in which similar results were obtained was fed 300 embryonated eggs of *T. cati* 3 months prior to receiving a *Toxoplasma* infective meal. However, the nematode infection was apparently abortive because no *T. cati* eggs were found in its feces, and when it was killed 5 months later no worms were found in its intestine. However, here again floats of feces collected on days 8 and 9 after the infective meal, and incubated for 8 weeks, produced toxoplasmosis in mice. The float of the day 8 collection was filtered through a sintered glass filter of large pore size, and its infectivity was lost in the filtrate. Fresh feces of the day 9 collection did not produce toxoplasmosis in mice when suspended in saline and fed to or inoculated into mice.

In both of these cases it is possible that small numbers of *T. cati* eggs were present in the infective floats and were missed on microscopic examination of the preparations; otherwise we must resort to the hypothesis that a developmental form of *Toxoplasma gondii* is produced in the intestinal tract of cats fed the encysted protozoan, that this form is not immediately infective to mice when it is discharged in the cat feces but requires an incubation period in water, and that it is small enough to be easily missed on microscopic examination. Furthermore, this form must be resistant to $ZnSO_4$ and must float in a solution of this salt at a specific gravity of 1·180. It would also have to be resistant to a 30-min exposure to 5% NaOCl. These characteristics seem remarkably similar to those of the nematode egg. At the present time, there is no explanation available to account for these apparently discrepant findings. We can only wait for further work to determine whether or not the infective form of *T. gondii* in the feces of cats is always associated with *T. cati* eggs.

Certainly the cyst of *Toxoplasma* could not have been the infective form in these instances. The cyst wall is rapidly destroyed by digestive enzymes. Furthermore, if cysts were present in the cat feces, the fresh saline suspensions of feces would have been infective to mice. We have tested micro-isolated cysts of *T. gondii* in $ZnSO_4$ solution. The cysts do float in this solution and remain infective, even after a short time, up to 10 min, in water. However, if after floating they are left in water for 20 min or longer they are apparently destroyed. Other than the indication that the flotation procedure may provide us with a practical technique for harvesting large numbers of cysts free

of other material, these experiments do not offer us any clue to the transmission of *T. gondii*. (Dubey (1966) obtained *Toxoplasma* infection in mice by injection of saline suspensions of feces 22 and 25 days after feeding a cat a *Toxoplasma*-infected meal. These were the only two positive results in tests of 111 fecal samples from six experimentally fed cats. Since he suspended the feces in saline, he may have been dealing with cysts or trophozoites liberated from the intestinal mucosa of the cats.)

In the course of our studies on transmission of toxoplasmosis by the feces of cats, we carried out a survey of 158 stray animals. These were brought to the laboratory for use by another investigator, but we were able to obtain serum specimens for the *Toxoplasma* dye test, and feces or worms from them. In those cases in which feces were obtained (the first 112 animals) a total of twenty-eight specimens containing *T. cati* eggs and derived from dye test-positive cats were kept for feeding to mice. Adult *T. cati* were obtained at autopsy from twenty-six of the next forty-six cats, twenty-five of which were also positive in the dye test. The worms were dissected and the eggs derived from them were incubated in water without flotation.

Of the total 158 animals, ninety-three were positive for *Toxocara*, and fifty-three (57·3%) of these had *Toxoplasma* antibodies. Of the sixty-five *Toxocara*-negative cats, twenty-five (38·5%) had *Toxoplasma* antibodies. The association of the worm and the protozoan is easily understood since both can be acquired by the ingestion of infected mice.

Of the fifty-three samples of incubated *T. cati* eggs, from *Toxoplasma*-positive cats, two produced toxoplasmosis in mice. Both of these samples were derived from fecal floats, stored for 8 or 9 weeks after collection. Thus, it has been demonstrated that the transmission of *Toxoplasma* may occur in nature in the same way that it has been demonstrated in the laboratory. Unfortunately, because we were so impressed with our earlier positive experimental results, and because we had not yet obtained the discrepant findings with cats without patent *T. cati* infections described above, we did not retain and feed to mice fecal floats from *Toxocara*-negative, dye test-positive cats. However, even had we done so, only a positive result would have been significant since we had only twenty-five animals in this category, too small a number for statistical significance were they negative. Obviously more work along these lines is necessary.

In the light of Hutchinson's (1967) negative experimental results with at least five *Toxocara*-negative cats fed *Toxoplasma*-infected meals, I am inclined to accept the idea of nematode transmission of *T. gondii*. Our discrepant results were obtained with floats of feces from one cat that was found to have an immature male worm and from another which had been experimentally infected with the nematode, although no worms were found at autopsy. As stated earlier, we cannot rule out the presence of so few eggs in some floats that they were not detected, even though this seems unlikely because of the multiple microscopic examinations done.

In any case, Hutchinson's reports have initiated a new chapter in the study of the transmission of *Toxoplasma gondii*. Although we have thus far failed to repeat such findings in dogs infected with *Toxocara canis*, more work will

be necessary with many other nematodes and other hosts. The idea of spread of *T. gondii* by a contaminative route, with or without the intervention of a nematode egg, fits in with certain aspects of the epidemiology of toxoplasmosis, such as the high prevalence rates in populations having poor sanitation. The finding by Verma and Dienst (1965) that transmission of *T. gondii* occurred only in pigs kept penned with other animals infected by mouth, not by parenteral ingestion, may be explained by this newly described mechanism of transmission, and it will be important to investigate pig-to-pig transmission via *Ascaris lumbricoides* eggs. In this connection, the work of Spindler *et al.* (1946) on *Sarcocystis* in swine, in which they observed infection in pigs that ate their own feces after ingesting *Sarcocystis*-infected meat, is suggestive of a similar life cycle for this parasite as well. Early work on *Sarcocystis muris* (Négre, 1907) reported transmission from mouse to mouse via feces of animals previously fed infected flesh. We have tried a number of times to repeat this work with *S. muris* and have not been able to do so. This again leads to the conjecture that it may be necessary also to have the proper nematode egg involved in the cycle.

The epidemiology of toxoplasmosis in human and animal populations may, hopefully, be explicable on the basis of increasing knowledge of the transmission of *T. gondii*. Considering the hygienic habits of cats, it appears unlikely that cat feces are the source of human infection to the great extent that this occurs. The increasing prevalence of *Toxoplasma* antibodies with increasing age in human populations in most temperate climates is not consistent with transmission from such a source. It is much more consistent with more opportunities to acquire the infection, say, in raw meat the longer an individual lives. The high prevalence of toxoplasmosis in sheep in England, New Zealand, and other countries, and its occurrence in "abortion storms" in New Zealand, may be explained by nematode transmission, but we will have much work to do to identify the characters in this play, and to ascribe to them their proper importance. In addition, the demonstration of the fecal or nematode route of transmission of *Toxoplasma gondii* indicates that we can anticipate new knowledge of stages in the life cycle of this parasite. All in all, it seems that we are on the road to a series of exciting findings in regard to the parasite and its epidemiology.

IV. Serology

A number of papers have appeared, since 1963, comparing the results of hemagglutination and dye tests (Thiermann *et al.*, 1964; Chordi *et al.*, 1964a; Smit, 1964; Ben Rachid, 1965; Prakash, 1966). In general, the authors found good qualitative agreement between the two procedures. However, certain discrepancies are apparent between the tests. Patients with acute toxoplasmosis are more likely to have a positive dye test than hemagglutination test, indicating the more rapid appearance of dye test antibodies. These data support earlier findings relating the two tests temporally. Thiermann and her co-workers (1964) present some interesting data on this aspect, and also on the complement fixation test, which appears to lag behind the other two in titer and to be the least enduring. They conclude that the two tests

measure different antibodies, as do various other authors. Also, they believe that, once HA antibodies appear, they tend to last longer than dye test antibodies, or at least do not undergo the spontaneous fluctuations in titer which they have sometimes observed in the dye test. Qualitative disagreements in cases of chronic infections occurred only rarely, and they concluded that such differences are not attributable to lack of specificity, low sensitivity, or to technical errors. Chordi *et al.* (1964a) demonstrated that non-specific reactions in the hemagglutination test were due to anti-sheep red cell antibodies. When these were removed by absorption, the agreement between the dye test and the HA test was 93%. Among 1 020 sera they tested by both procedures only seven were positive by the dye test and negative by hemagglutination. One of these instances was a fatal case of congenital disease, with hydrocephalus. In a personal communication, Desmonts has supplemented Ben Rachid's (1965) reports, showing that congenital cases may have negative HA tests for months after birth. Some of his results have been confirmed in my laboratory. Smit (1964) also observed two congenital cases in which the dye test was positive at 1:4 096 and the HA test was negative. It is apparent that the HA test cannot be offered as a substitute for the dye test in suspected cases of acute toxoplasmosis, either congenital or acquired. It does, I believe, have excellent usefulness in the diagnosis of chronic *Toxoplasma* infection, especially presumed ocular toxoplasmosis, and as an adjunct to the dye test in acute infections.

Some of the discrepancies between the dye and HA tests in cases of chronic or latent infection may be technical, related to the multiplicity of materials present in peritoneal exudates from which the parasites are harvested for preparation of *Toxoplasma* hemagglutination or complement fixing antigens. Chordi *et al.* (1964b) have pointed out the presence of mouse serum and leucocyte antigens in HA antigen. By gel diffusion, and absorption techniques, they found four to seven bands of host origin and one to three bands of parasitic origin. By immunoelectrophoresis, they found up to fourteen host components and three to four parasite components. Lunde and Jacobs (1959), in a preliminary experiment, found that purified HA antigen produced one rather wide continuous band on electrophoresis, which we interpreted as a mixture of antigenic substances. In later work Lunde (personal communication) has found two components. Lunde and Jacobs (1967) have shown that the HA antigen appears different from dye test antigen and have also been able, incidentally, to show that only small amounts of mouse proteins are present in a preparation of *Toxoplasma* derived from peritoneal exudates and separated from mouse leucocytes by the filtration technique of Fulton and Spooner (1957), followed by careful washing. Chordi *et al.* (1964b) attempted to wash their "purified" *Toxoplasma* preparation very carefully, after first centrifuging at low speed for 5 min to remove leucocytes; they did not use a filter. They did not state if their procedure included the use of siliconized glassware for centrifuging the parasites. We have found that this is a requirement for maintaining viable organisms (see the dye test procedure detailed in Frenkel and Jacobs, 1958). The loss of an "exotoxin-like antigen which is very important in diagnostic tests" was reported by

Chordi *et al.* in their antigen produced from repeatedly washed parasites. This may have been due to decrease in the number of viable organisms prior to lysis, with a concomitant, quantitatively important loss of this particular substance, to a level undetectable by their techniques.

At any rate, the above discussion points out the need for exploration of new methods of producing *Toxoplasma* preparations for antigen-production, or at least for the careful handling of parasites derived from peritoneal exudates before antigens are prepared. In the Laboratory of Parasitic Diseases we have been using the filtration method of Fulton and Spooner (1957) for several years to obtain clean preparations of parasites prior to lysis. We have also tried tryptic digestion to remove mouse serum proteins adherent to *Toxoplasma* derived from peritoneal exudate. Since the organisms can survive at least 6 h in 1% trypsin, this would be an ideal way of removing foreign proteins from them before lysis. The problem of removing residual traces of the enzyme, even after use of only 0·25% trypsin, has kept us from adopting this method as a routine. The *Toxoplasma* HA antigen is easily destroyed on storage, probably because it contains some proteases or ribonucleases, and the possible presence of traces of trypsin may enhance loss of potency. Our results with trypsinized parasites were inconsistent in that some antigens were potent and others were weak or inactive, probably because of residual enzymatic activity. Other attempts along these lines should be made, however, in an attempt to produce a highly purified and potent product. It will only be possible to assess differences between dye and HA tests, as reported by different laboratories, when comparably purified antigens are in use in all laboratories.

This applies also to the complement fixing antigen. Fleck (1963) has distinguished "heavy" and a "light" complement fixing antigens on the basis of differential centrifugation. The denser antigen had a sensitivity close to that of the dye test, giving titers up to 1:1 024. It also inhibited dye test antibody when added in equal volume to the serum in the dye test. The light antigen was much less sensitive and non-inhibitory in the dye test. Fulton and Sutton (1962) and Fulton and Fulton (1965) have used preparations of pure, formalinized suspensions of *Toxoplasma* washed and filtered from the peritoneal exudate of cotton rats, as originally described by Fulton and Spooner (1957, 1960) for complement fixation, and claim good correlations with the dye test and the direct agglutination test of Fulton and Turk (1959). Fleck and Payne (1963) obtained similar correspondence between the dye test and complement fixation test with mouse peritoneal fluid antigen, and found the hemagglutination test somewhat less sensitive than the dye test. Obviously, we are again confronted with technical differences in preparation of antigens, which must be standardized and purified. Schmidt-Burbach and Schwarz (1965) are in agreement with the need for some international standard *Toxoplasma* antisera and antigens.

The use of antigens prepared from a source other than peritoneal exudates is one possible way of freeing the antigens of contaminating substances from host serum, although of course we will then have to deal with tissue culture cell and media components. Stewart and Feldman (1965) have described the

use of tissue culture-cultivated *Toxoplasma* in the dye test. They were able, by adjusting their inocula, to obtain high yields of viable parasites from Hep-2 cells after 7 or 8 days of incubation. This may be important and useful in relation also to the preparation of toxoplasmin skin test antigen; the elimination of the need for demonstrating absence of latent viruses in mice used for production of such antigens would reduce the need for the extensive safety-testing required for commercial products. Akinshina and Gracheva (1964) have reported the preparation of an antigen from tissue cultures. Bickford and Burnstein (1966) have also described maintenance of *T. gondii* in Hep-2 cultures.

In a later paper, describing a micromodification of the dye test, Feldman and Lamb (1966) returned to the use of mouse peritoneal exudates rather than tissue cultures, but Feldman (personal communication) has stated that tissue culture-derived parasites have also been used in this procedure. Tissue culture maintenance of *T. gondii* for the dye test requires continued passages at carefully determined intervals. It would certainly not add to the facility with which the dye test could be performed except in laboratories which can devote full time to the procedures. The micro-method of Feldman and Lamb offers an advantage in the use of microtiter loops instead of pipettes for dilution, direct reading of the results in the plates, and small quantities of materials. The saving of accessory factor may be very important in places where acceptable serums for this use are difficult to obtain.

The question of satisfactory accessory factor sera has continued to plague some workers who are performing the dye test. Some workers assume that undiluted sera rich in the activator, or accessory factor, have the capacity of lysing *Toxoplasma*. Aagard (1960) used 50% or 75% concentrations of accessory factor sera in his preliminary assays of the proper concentrations of AC serum and antigen to use in the test. Folkers (1964) examined five human sera by this technique, and found that one could be used undiluted and two when diluted 3:1; the other two could not be used at all. TePunga and Penrose (1965) found that they could inhibit non-specific anti-toxoplasma activity in human sera by either sodium citrate or calcium. They suggest that non-specific activity is magnesium-dependent and that calcium competes with magnesium, while citrate chelates magnesium. For the accessory factor used in the Laboratory of Parasitic Diseases, we ordinarily employ blood collected in ACD dilution as our source. However, a small sample of serum is first obtained from the donor and checked for its usefulness before the large quantity is drawn. We have had experience similar to that of TePunga and Penrose, in that an occasional serum negative for *Toxoplasma* antibodies, but having the ability to cause cytoplasmic modification of about 20–30% of fresh parasites, can be rendered free of this capacity if blood is collected in ACD solution rather than allowed to clot. Since we have no difficulty in obtaining sera which have no anti-toxoplasma activity, we have not exploited the use of citrate nor investigated it further. However, it may be worth while for those who do encounter difficulty in obtaining satisfactory accessory factor sera to investigate such techniques further. Considerable care must be taken in all such studies to be certain that non-specific anti-toxoplasma

activity of sera is indeed non-specific and not merely representative of very low levels of specific antibody (Gronroos, 1960).

Within the last few years, the use of indirect fluorescence for diagnosis of toxoplasmosis has been the subject of reports by a number of authors (Zardi, 1963; Camargo, 1964; Fulton and Voller, 1964; Kramar *et al.*, 1964; Fletcher, 1965; Van Nunen and Van der Veen, 1965; Walton *et al.*, 1966a). According to Fletcher, the indirect FA test is capable of revealing titers as high as those in the dye test, when initial care is taken to produce and standardize potent reagents. It has a sharp end point, and it can be performed without the tedious counting of stained and unstained organisms required in the dye test. (Interestingly enough, among his sera was one which reacted strongly in FA but was negative in the dye test at dilutions up to 1:128. A repeat dye test (done in Ludlam's laboratory) revealed a prozone between 1:128 and 1:256 and a titer of 1:4 096 in this serum. This is one of the best-documented instances of a prozone in the dye test of which I am aware; although others have mentioned their occurrence (Eichenwald, 1956) they have not given the details in their reports.) Fletcher's results are, therefore, much better than those reported by Kelen *et al.* (1962), or Angelillo and Mandras (1962) who found a good correlation between the IFA and the dye test with high-titered sera but a poor correlation with weakly reacting sera. Kramar *et al.* (1964) compared indirect fluorescence with the fluorescence inhibition test (Goldman, 1957) and found the former more sensitive. Fulton and Voller (1964) used the clean suspensions of *Toxoplasma* prepared by the method of Fulton and Turk (1959). They found the IFA test sensitive and specific, but they prefer the direct agglutination test as more rapid and convenient. Wéry (1965) does not recommend the IFA test for routine work, since in his hands it lacks precision.

Archer *et al.* (1966) have used Goldman's direct technique of immunofluorescence in the examination of tissue from lambs with experimental congenital toxoplasmosis. Archer *et al.* believe that the fluorescing units they observed, which correlated well with dye tests and isolations from the tissues, were reticulo-endothelial cells containing *Toxoplasma* antigen in the form of subcellular particles. "If this is so, the . . . method should prove to be more sensitive than either isolation . . . or histological methods in the diagnosis of abortion due to toxoplasmosis." Tsunematsu *et al.* (1964) used direct examination of tissue stained with fluorescein-labeled antibody in three cases of human lymphadenopathic toxoplasmosis, and demonstrated the parasite in the lymph nodes of two. They observed other fluorescing particles but did not identify them as cell-associated *Toxoplasma* antigen. Certainly, this technique should be exploited in the study of cases of animal and human abortion, but careful controls are necessary until it is adequately evaluated.

The use of the indirect fluorescence test does indeed look promising on the basis of the above reports. Further work is necessary with it, in relation to the detection of antibody at various stages of infection. Suzuki *et al.* (1965) claim good agreement with dye test results in experimentally infected pigs, as to time of appearance and decline of antibodies. The same work should

be done with the direct agglutination procedure of Fulton and Turk (1959) which has not yet received adequate attention from other investigators. Fulton (1965a, b) has given good evidence of its usefulness. Various workers have used sensitized bentonite particles (Garin and Despeignes, 1964) or sensitized latex particles (Bozdech and Jira, 1961) for agglutination tests. Lunde and Jacobs (1967a) have also used latex particles. While these have given some promise, they appear to be less sensitive or less specific than dye or hemagglutination tests. Since they require more manipulations than the direct agglutination test, it is surprising that no more attempts have been made to evaluate the latter.

Regardless of the test used, there are some cases of toxoplasmosis which are difficult to diagnose serologically because of the presence of stable titers. Differences between dye and HA or complement fixation titers may be helpful in such instances, since the latter two may be low or rising while dye test antibodies are already present at high titer. Remington and Miller (1966) have developed the technique of identifying 19S and 7S globulin antibody as an additional aid in the diagnosis of acute cases. They have found 19S globulins in both acute congenital and acute acquired human toxoplasmosis, whereas only 7S globulins were found in infants born of mothers with chronic toxoplasmosis and in adults with longstanding infection. The use of dimercaptoethanol to degrade 19S globulin antibody from sera has also been used by Remington's group. This reductive cleavage technique resulted in loss or reduction of dye test and HA antibody titers in patients with early toxoplasmosis. Remington *et al.* (personal communication) also experimented with the use of indirect fluorescence, with a fluorescein-tagged anti-19S human globulin serum. This procedure may prove to be especially useful in cases of congenital toxoplasmosis. Since only 7S globulins are transmitted across the placenta, any 19S antibodies found in an infant must represent the infant's own antibody response to the infection. There is some evidence, reported by Remington and Miller (1966) that 19S antibodies may reappear during a new unrelated febrile episode in individuals with a past history of toxoplasmosis. This decreases the value of the demonstration of the macroglobulin antibody in adult cases, although it may still have some usefulness.

It can be seen that considerable progress has been made in serological techniques for the diagnosis of toxoplasmosis. Unfortunately, these techniques are still in the hands of only a few laboratories, and serological diagnosis is difficult to obtain in many areas. Hopefully, some stable reagents will eventually become available for routine use in hospital laboratories equipped for other serological procedures.

V. Toxoplasmosis Associated with Various Disease Entities

A. Myocarditis

There is no question that in acute toxoplasmosis the myocardium may be invaded and a definite myocarditis ensue. Theoretically, it may be expected that latent *Toxoplasma* organisms in the heart muscle may occasionally cause a hypersensitivity reaction because of the release of antigen into

sensitized tissue or conceivably also multiply in some foci. Therefore, myocarditis may conceivably be a manifestation of chronic toxoplasmosis. Niedmann *et al.* (1963, 1964) have paid particular attention to this question in Chile. They have reported a small study (1963) comparing serological test results on cases of myocarditis with those on sera of blood donors of the same age, showing a higher percentage of positive reactions in the cardiac patients. They have also isolated the parasite from the heart on at least two occasions. Similar reports, based on serological tests and on treatment results, have been contributed by Shee (1964) and Ward *et al.* (1964) in England. Jones *et ul.* (1965) have reported a case of pericarditis associated with toxoplasmosis. Harvey *et al.* (1966) report three clinical cases with some changes in titer on HA and complement fixation tests.

B. MALIGNANT DISEASE

A report of fatal toxoplasmic myocarditis in an individual with acute lymphocytic leukemia has also been published recently by Wertlake and Winter (1965). This patient had undergone intensive chemotherapy with antileukemia agents. Numerous cysts surrounded by cellular infiltrates were seen in the heart. The authors state that clinical manifestations of toxoplasmic myocarditis are not distinctive from those due to other causes.

There was no way of determining, in this case, whether the *Toxoplasma* infection was recently acquired or was reactivated because of the immunosuppressive effects of the chemotherapeutic agents used for the leukemia. It is interesting that, in another report of toxoplasmosis complicating Hodgkin's disease (Cheever *et al.*, 1965) in which the patient also had received therapy with prednisone, alkylating agents, and X-rays, *Toxoplasma* cysts were present in the heart, but no trophozoites were seen. There was very little association between the cysts and lymphocytes in the interstitial spaces. In the brain, however, there was a necrotizing encephalitis with trophozoites in the tissue, similar to that described by Frenkel in hamsters with toxoplasmosis reactivated by treatment with cortisone. These authors also cannot decide on whether the toxoplasmosis in this case was an initial infection or recrudescent; all they can report is that a dye test carried out 2 years before death was negative.

These two cases are obviously dramatically different from the chronic myocarditis reported by Niedmann and the other authors cited above, and by others. The case of Cheever *et al.* (1965), showing some mild focal myocarditis, may represent what could occur in patients with a subacute or chronic toxoplasmosis without concomitant disease. The question arises as to whether or not, or how often this may occur in individuals without debilitating disease or a therapeutically produced immunologic deficiency. As stated in an earlier review (Jacobs, 1963) we need much more statistical data and more histopathological evidence concerning such cases than is presently at hand. The evaluation of the importance of *Toxoplasma* in myocarditis, therefore, still depends on the development of information along the same lines as was done for ocular toxoplasmosis (Frenkel and Jacobs,

1958). Certainly, there is at present no definitive way of diagnosing chronic myocardial toxoplasmosis.

Various case reports of toxoplasmosis associated with leukemia, Hodgkin's disease, or other malignant disease, in addition to those cited above, should be mentioned (Hooft *et al.*, 1962; Keel *et al.*, 1963; Connolly, 1963). Cases of encephalitis, as yet unreported, have been observed at the Clinical Center, NIH. Theologides *et al.* (1966) report one case of toxoplasmic encephalitis with multiple myeloma. *Toxoplasma* may find especially vulnerable sites for its proliferation in such conditions. There is no reason, however, to believe the association is anything other than coincidental; the only equivocation would be that there is a slight possibility that toxoplasmosis may be acquired from blood transfusions. The use of immunosuppressive agents in therapy is very likely to result in the compilation of more case reports such as those cited above.

C. HABITUAL ABORTION

The subject of congenital transmission during the chronic stage of toxoplasmosis in the mother has received increasing attention in recent reports (for review of literature prior to 1963, see Jacobs, 1963; Remington, 1963). To supplement Langer's (1964, 1966) recent reports of isolation of *Toxoplasma* from products of abortion, etc., those of Remington (1964), Remington *et al.* (1964) and Robertson (1966) can be added. Langer has recently (1966) admitted some doubt concerning cases in which a direct demonstration of *Toxoplasma* cysts was made in tissues of the fetus or in material from the mother in fifteen cases; the identification of pollen grains as cysts appears possible since he found, in the vicinity of his laboratory, pollen grains which are similar in appearance. Werner (1966a) presents pictures comparing pollen grains and cysts, and indicating difficulties in distinguishing them. However, Langer considers that the isolations in mice of *Toxoplasma* from twenty-three other cases are definite proof. Werner (1966b) has presented some photomicrographs showing structures in the endometrium of women that closely resemble *T. gondii*. The report of Remington *et al.* (1964) appears to be subject to no doubt. That of Robertson (1966) is of special interest since the mother had either very low (1:8) dye test titers or the dye test was completely negative; the tests were done in very competent laboratories. In addition, her skin test was negative. (Langer also reported that four of the twenty-three women, whose aborted fetuses or body discharges were positive for *Toxoplasma* on isolation in mice, had negative serological tests.)

The thesis of Langer and Robertson is that when antibody levels drop to moderate or low levels in women with chronic infection, cysts in the uterus may liberate *Toxoplasma*, either to invade the fetus or to produce an endometritis. These ideas are also expressed by Van der Waaij (1964), who also concluded, on the basis of statistical calculations, that only 25% of cases of congenital toxoplasmosis can result from maternal infection during pregnancy and that the rest must be due to chronic infection of the mother. Jones *et al.* (1966) report different findings. A significantly greater incidence of abortions occurred in patients who had *higher* titers than in those with

low titers or negative tests. Jirovec's (1964) reports of the intradermal test and its correlation with abortion and fetal malformations, stillbirths, etc., have been the basis for recommendations of therapy during pregnancy. The use of the skin test for such studies has not been done elsewhere other than in central and eastern Europe.

Entirely different findings have been reported by Desmonts *et al.* (1965b) and by Thalhammer (1966). Desmonts *et al.* have not found a single case of congenital transmission during chronic infection among 15 000 women studied. Of these women, 84% had dye test antibodies prior to pregnancy; it was only among children of the group without antibodies prior to pregnancy that cases of neonatal toxoplasmosis occurred. Thalhammer investigated eighty-nine women with abortions and found no positives in mice inoculated with the suspect materials. Thalhammer's statistical studies also gave no significant correlation between abortion and serological tests. The report of Sever (1966) on 23 000 pregnant women indicates that there were fifty-nine pregnancies in which HA titers of $\geqslant 1:4096$ or significant increases to $\geqslant 1:256$ occurred, and among these there were five definite cases of congenital transmission and ten suspected cases. Perinatal mortality, prematurity, low mental and motor scores of the children, were all associated to some degree with the highest titers in the mothers and/or with rises in antibody titers. His data as analyzed thus far do not indicate a causal relation between chronic toxoplasmosis and abortion. A greater incidence of *Toxoplasma*-positive reactions associated with abnormal pregnancy outcomes does not necessarily establish the connection in any case. Acute toxoplasmosis is a cause of stillbirth or malformation, and there is no reason to believe a woman with another cause for abortion may not also experience acute toxoplasmosis during a pregnancy. Thus, there could exist two causes for abortion in successive pregnancies in the same woman. Coupled with this is the age factor: those women with histories of abortion may be several years older than those chosen as controls, and this may operate to give an apparently statistically significant difference if not adequately corrected. Sever's data show a very steep slope, with age, in the prevalence of HA titers in child-bearing women.

Eventually these discrepant findings will be explained, either by differences in technique, local epidemiology or other factors. No interpretations made on the basis of present data would be anything but speculative. The principal question to be resolved at present is whether or not findings such as those of Langer (1966) justify the use of antitoxoplasma drugs during pregnancy in women with positive serological tests (Glowinsky *et al.*, 1964; Sikorski, 1964). In an addendum to Desmonts *et al.* (1965b), it is reported that up to 1966, forty-seven cases of acute toxoplasmosis in pregnant women resulted in four neonatal deaths, three living children with signs of congenital toxoplasmosis, thirteen children proven to be infected on the basis of serology but *without* signs or symptoms of disease, and twenty-seven uninfected infants. Thus, even in *acute* infection of the mother, over 50% of fetuses escaped infection. When one considers, therefore, the teratogenic effects of drugs, together with the difficulty of eradicating *Toxoplasma* during chronic

infection, Remington's (1964) words of caution against treatment appear justified. This was the consensus of a symposium on toxoplasmosis held at the Seventh International Congress on Tropical Medicine and Malaria, Rio de Janeiro, in 1963. Hickl *et al.* (1964) have presented experimental evidence of teratogenesis in rats treated with pyrimethamine during pregnancy.

D. OTHER CONDITIONS

Labzoffsky *et al.* (1965) found no correlation between mental retardation and toxoplasmosis in a study of 345 mentally retarded children compared with a normal control group of the same age. Remington (1965) concludes, from studies done at institutions for mentally retarded children in California and Massachusetts, that a small but significant percentage of such children are victims of congenital toxoplasmosis. Remington calculates, then, that the incidence of cases of mental retardation due to toxoplasmosis is greater than cases due to biochemical defects such as phenylketonuria, and deserves similar attention.

Occasional cases of blood dyscrasia or hemolytic anemia associated with chronic or acute toxoplasmosis have also been reported (Kalderon *et al.*, 1964; Olbing, 1963).

E. ANTIBODY RESPONSE TO INFECTION

I do not propose to discourse extensively on the large number of papers that have been contributed to the literature on lymphadenopathic and ocular toxoplasmosis during the last few years. However, there are a few papers of interest in relation to the phenomenon of the persistence of *Toxoplasma* infection in the absence of demonstrable specific antibodies. Engelbrecht and Franceschetti (1963) demonstrated the parasite in mice inoculated with subretinal fluid from a patient, 18 years of age, with chorioretinitis. The dye, complement fixation, and hemagglutination tests done on the serum and aqueous humor from this patient were consistently negative, in three European laboratories and in our laboratory at NIH. Another similar instance, in which the dye test was positive only with the undiluted serum, is the case reported by Zscheile (1964).

Walls *et al.* (1963) isolated *Toxoplasma* from the brain of a patient with a dye test titer of 1:4. Remington and Cavanaugh (1965) obtained the parasite from skeletal muscle and brain of five additional patients, two with titers of 1:4, one each with a titer of 1:16, 1:64 and 1:256 (some of these had received corticosteroid therapy). This is reminiscent of our earlier findings of *Toxoplasma* cysts in the uterus of women with low titers (Remington *et al.*, 1960) and in ocular infections (see Jacobs, 1963).

Instances of infections in birds or mammals with negative serological tests have ben summarized previously (Jacobs, 1956, 1963). To add to these, Walton and Walls (1964) reported the isolation of *T. gondii* from some raccoons and squirrels that were negative in the dye test.

The reasons for such findings have generally been considered to be decreased antibody production with time since acute infection, lack of antigenic

stimulus from encysted parasites, and seclusion of active trophozoites in small foci in the brain or eye where they do not produce a large antigenic stimulus. There is no question that these factors are influential. However, another mechanism is conceivable for explaining persistence of infection in the absence of humoral antibody. This is that the immune response may be altered by exposure to the parasite or to parasite antigens during fetal life. Since congenital transmission during chronic infection has been demonstrated in mice (see Werner, 1966c, for another review of this subject), Jacobs and Melton (1964) did serological tests on young born of mice with chronic Beverley strain infections, and then performed isolation studies after passively transferred antibody was no longer demonstrated.

We tested eighty-eight litters and found sixteen with some positive young. This is considerably less than the percentage of litters we found positive in earlier studies (Remington *et al.*, 1961a), possibly because in this investigation we had to wait several months prior to studying the young and the mothers may have destroyed some of the affected newborn. Of the sixteen positive litters, eleven had only one offspring surviving, four had two, and one had three. Table III shows the comparative results of serological and parasitological tests on these young. Of nineteen that were serologically positive, eighteen were found positive parasitologically. This points out the good correlation we usually find between the two tests.

TABLE III

Comparison of serological and parasitological tests on litters of chronically infected mice

No. of young positive on dye test	No. positive parasitologically	No. of young negative on dye test	No. positive parasitologically
19	18	357	4 (in 3 litters)

However, the data in the right-hand column of Table III show that the presence of the parasite was not always indicated by serological findings. In four mice, of three litters, humoral antibodies were not demonstrable on repeated dye tests, but parasites were found on subinoculation of their tissues.

It seemed possible, on the basis of these results, that some mice born of *Toxoplasma*-infected mothers may have a lowered responsiveness to antigens prepared from the parasite, either because of exposure *in utero* to the organism itself or to parasitic antigenic materials in the maternal circulation. In order to test this, our next step was to inoculate formalinized *Toxoplasma* suspensions into serologically negative young. The results are shown in Table IV.

Most of the dye test titers developed in the mice receiving dead antigens fell within the range of 1:64 to 1:1 024. We did, however, find sixteen instances in which serological unresponsiveness was demonstrated after two to three, or six injections of dead antigens. These mice were killed and tested

for *Toxoplasma* and found negative. Therefore, we cannot state that their failure to produce antibody was due to immune tolerance resulting from infection during fetal life.

In these experiments the chances were small of demonstrating immune tolerance in the mice. The percentage of congenitally infected young, lower than we had found in previous work, gave us only a small number of animals with which to work. It was because some of our most interesting results occurred early in the study that we were encouraged to persist with it as long as we did. The instances of serologically negative *Toxoplasma*-infected young stimulated the study of response to dead antigens. In this work we had to proceed "blind", i.e. without any way in which to test the animals for infection prior to vaccinating them, and we did not demonstrate any infected animals among those which failed to respond to the antigen.

Nevertheless, it appears possible that those young animals which were found negative serologically and positive parasitologically may represent occasional instances of some immune tolerance to a congenitally transmitted

TABLE IV

Results of injection of antigen into serologically negative young

No. of injections	No. and percent of mice developing titers of: < 16	16	64	256	1 024
2–3	11 (9·2%)	25 (21·0%)	42 (35·3%)	25 (21·0%)	16 (13·4%)
4		8 (12·9%)	18 (29·0%)	20 (32·2%)	16 (25·8%)
5		3 (5·3%)	29 (50·9%)	22 (38·6%)	3 (5·3%)
6	5 (7·4%)	2 (2·9%)	12 (17·6%)	16 (23·5%)	23 (33·8%)

infectious agent or to antigens of the agent. The explanation for the occurrence of congenitally infected young which did produce antibody may be the degree of exposure or the time of exposure during fetal life. Those young that were exposed early and experienced greater proliferation of the parasite or greater concentrations of parasite antigen may have had more chance of becoming unresponsive. Here again, the odds were against our demonstrating such cases, because among these would be most of the young which would succumb to the infection *in utero* or soon after birth. In this light, the finding of four unresponsive young out of a total of twenty-two congenitally infected mice, or 18%, is not unimpressive.

In some respects, therefore, it appears that congenital toxoplasmosis may have similarities to lymphocytic choriomeningitis in mice, in which immune tolerance has been demonstrated in young infected *in utero*. LCM-infected young tolerate infection that is lethal to mice infected as adults, continue to have latent infection throughout their lives, and seem more resistant to other viral infections. The mechanism by which they tolerate the LCM infection and resist other viruses has been sought in interferon, but this has not been proved.

Until we can reproduce, with facility, the phenomenon of absence of humoral antibodies in the presence of latent infection we will not be able to study the mechanism of resistance in such infected young, nor will we be able to ascertain such possible features as the continued presence of organisms in the blood which occurs with the LCM virus. Successive congenital transmission through several generations of mice has been reported. It is conceivable that this occurs in "tolerant" young in each generation. In this connection it is noteworthy that Beverley (1959) terminated one line of congenitally infected mice because "the one surviving female was killed when a low antibody titer was, erroneously, taken to signify absence of infection."

These data may be only of academic interest, but they may also provide some clue to the occasional case of human or animal toxoplasmosis which is characterized by serological unresponsiveness to the infection. It is not a necessary hypothesis to explain low levels of antibody in latent infection, but may be useful to explain such levels in active disease, such as Engelbrecht and Franceschetti's case. Certainly the phenomenon was not seen very frequently in mice, and it may occur much less often in human beings, if at all. Nevertheless, it seems that efforts to elucidate these phenomena would be rewarding.

VI. Epidemiological Data

A. Human Populations

A review of survey work on toxoplasmosis in various countries would require more extensive treatment of the subject than is possible here. However, a few points can be made on the basis of some selected surveys. Feldman (1965) has reported on dye test results on sera from United States Army recruits. These men ranged in age from 17 to 26 years and 64% were under 20 years of age. The percentage of those with antibodies at a titer of 1:16 or higher was 13. There were no significant differences in prevalence of antibodies between urban, town or rural areas. A comparison of those from rural areas in various regions of the United States revealed the highest prevalences in the east and south and the lowest rates in the mountain and Pacific zones. Ludlam (1965) and Ludlam and Somers (1966) did dye test surveys in Africa, in the Niger Delta and in Uganda. In the Niger Delta, about 60% of eighty-four adults were positive. The rate was 83% in a southern area where the men fish in salt-water mangrove swamps, and 53% in the north where the occupations are agriculture or trade. In Uganda, the rates were surprisingly low for a tropical area, 22·5% overall among a group of 120 patients with various cardiac diseases and 11·7% of blood donors aged 14–25 years. The incidence of antibodies was low below age 30. (In no age group did the results suggest a relation between antibodies and cardiopathy.) Mitdvedt (1965) reported a 5% positive rate in 100 children from northern Norway as compared to 15·5% in 200 children from the temperate zone of Norway. These reports emphasize the differences found from one geographic region to another, as has been pointed out in previous reports. Climatic factors or

altitude seem to have a bearing, as has been pointed out earlier (Jacobs, 1956, 1963); altitude has recently been the subject of a report by Walton *et al.* (1966b). McCulloch *et al.* (1963) found an association between contact with animals and skin sensitivity, in 775 veterinary and medical students in Iowa, and a higher rate among those from a farm background compared with lifetime city dwellers. In contrast to these results, Midtvedt discussed the fact that the Lapp children he studied in northern Norway have more intimate contact with animals than children in the south of the country. Occupational factors may be important (Kobayashi *et al.*, 1963; Orestenko, 1965). These observations all indicate that, despite the new clues as to transmission of *T. gondii*, the special circumstances of its spread will require much study in different environments.

B. ANIMALS

Here again, without proceeding on an extensive discussion of prevalence rates in animals, a few particularly notable findings will be mentioned. The report of Mayer (1962) is of particular interest because of failure to isolate *Toxoplasma* from cattle in two previous surveys, one in the United States and the other in New Zealand (Jacobs *et al.*, 1960a, 1963). Mayer studied 304 animals and states that he isolated *T. gondii* from the retina of seventy-four, or 24·3%, of these. Zardi *et al.* (1964) also reported isolation of *T. gondii* from the eyes of eight cattle, as well as swine, sheep, horses and cats. It is unfortunate that we have no information on other tissues of the same animals, because *T. gondii* does not seem to be very specific in its localization solely in the eye of other hosts. In an additional study, as yet unpublished, Melton and I have failed to find the parasite in the udders of about 100 cows, examined by digesting large amounts of the tissue in artificial gastric juice, washing in saline, and inoculating the sediments into mice.

In a recent paper (Jacobs and Melton, 1966b) we have recorded some new observations in regard to latent toxoplasmosis in chickens. Pools of tissues, each from the ovaries and oviducts of ten apparently healthy hens, obtained at a poultry processing plant, were examined for *Toxoplasma gondii* cysts by the digestion-inoculation technique. Twelve of a total of sixty-two pools were found positive. A second survey of 108 individual birds revealed four with latent infection of the reproductive tract. To supplement these observations, we examined 327 eggs laid by sixteen chickens with experimentally induced chronic infections, and found one positive egg. The epidemiological significance of this finding may be assessed in relation to the number of eggs consumed raw by human beings. Kimball *et al.* (1960) and Schnurrenberger *et al.* (1964) discuss their epidemiological data in this respect. I am inclined to believe that the rarity of this finding, together with the fact that most eggs are consumed cooked in various ways which would destroy *T. gondii* (Kunert and Werner, 1963; Jacobs *et al.*, 1960b), indicates that this method of acquisition of the infection by human beings would be operative only in limited situations. For an extensive review of toxoplasmosis in domestic animals, see Siim *et al.* (1963).

VII. Pathogenesis and Immunity

Recurrent low-grade parasitemia in the chronic infection in animals and man has been recorded in papers by Remington *et al.* (1961b, 1962). An additional instance of persistent parasitemia is the subject of still another report by Miller *et al.* (1966). This case concerned a woman who delivered a congenitally infected infant and whose dye test titer at delivery was 1:65 000. It ranged even higher than 1:65 000 over a 9-month follow-up period. Both 19S and 7S *Toxoplasma* antibodies were present in the serum. *T. gondii* was isolated from her blood at 1, 4 and 5 months after delivery. Possibly this is a case of persistent subacute rather than chronic infection.

A study of serum protein alterations in mice with chronic toxoplasmosis by Remington and Hackman (1966) has shown a state of "chronic" hyperglobulinemia lasting at least 8 months. The authors suggest that this is a response to continued activity of the parasite, and also that this activity may be due to a latent form of *Toxoplasma* which is not encysted but protected from antibody by an intracellular localization, so that we need not postulate frequent rupture of cysts.

This idea has always been attractive (Jacobs, 1963). Despite the fact of some evidence that cysts may break open, the density and resilience of the cyst wall suggest that rupture is unlikely. If organisms are liberated from cysts, it is possibly because of enzymatic activity of the enclosed parasites. The appearance of cysts of different sizes in clusters does not necessarily indicate that the smaller ones are derived from parasites liberated from larger cysts. Stahl *et al.* (1965) have observed cysts of different sizes developing 30 days after infection in mice with Beverley strain infections treated with 6-mercaptopurine. It seems likely that trophozoites, from which cyst-producing forms are most likely derived, may persist for varying lengths of time in cells without rupturing them, and that persistent activity in chronic infections may be due to residual trophozoites rather than to rupturing cysts. The discussion of cyst formation in Section II, concerning factors involved in "triggering" this event, has bearing here. Possibly by the use of histochemical techniques it will be feasible to detect some differences between cysts and agglomerations of intracellular trophozoites, or to reveal the latter more easily in chronic infections.

Two additional studies on toxic exudates in *T. gondii* infections have contributed new information on "toxotoxin". Velasco and Varela (1965) inserted collodion sacs filled with *Toxoplasma* peritoneal exudates of mice into the abdominal cavity of uninfected mice. No toxic effects were observed in these animals. Fulton (1965c) did an extensive study of toxic effects of the peritoneal exudates of cotton rats when injected intravenously into mice and characterized the material producing them. No toxic effects were observed following injection by other routes. He concluded that *T. gondii* does not produce a true toxin. Lunde and Jacobs (1964) described the effects of *Toxoplasma* lysates in rabbits. We were circumspect about the use of the word "toxin". I do not believe the material is important in pathogenesis.

Lycke *et al.* (1965a) have studied the effect of humoral immune factors on the infectivity of *T. gondii* in tissue culture. Penetration of the parasites into cells was inhibited if they had been exposed to immune serum alone. If activator was added, the inhibitory effect was enhanced. This is not surprising, because of the lysis of parasites which occurs in the presence of antibody and activator if incubated long enough. The interesting thing is the effect of antibody in the absence of activator, which the authors say they can discern because of the greater sensitivity of the cell culture system, compared with the dye test, for antibodies of low avidity.

VIII. Physiology

Capella and Kaufman (1964) and Lund *et al.* (1966) have examined *T. gondii* by histochemical techniques and the latter authors have also used manometric methods with the ampulla diver technique. Both of these reports indicate that mitochondrial enzymes are fully represented in *T. gondii.* The metabolic requirements which make this parasite unable to survive extracellularly are not, therefore, associated with energy production. Lund *et al.* also demonstrated lysosomal enzymes histochemically and suggest that the site of these enzymes is in the micronemes or paired organelle. Lycke *et al.* (1965b) have shown that lysozyme and hyaluronidase have an enhancing effect on the penetration of *T. gondii* into cultured cells. There was also evidence of a penetration-enhancing effect in preparations of lysed parasites; this could possibly be due to the release of lysozymes from them.

IX. Therapy

The usefulness of pyrimethamine and sulfa drugs in the treatment of toxoplasmosis has been generally accepted as well demonstrated. The area of doubt about the efficacy of this chemotherapy is in ophthalmic disease. Here, to a great extent, the evaluation of the drugs depends on diagnostic criteria, on treatment regimens, and on methods of assessing clinical improvement. For a review of the literature in this respect, the reader is referred to Kaufman (1966).

One disadvantage of pyrimethamine, already mentioned above, is teratogenesis. In addition, hematological side effects are frequently seen and must be monitored (Kaufman and Geisler, 1960). Weissbach (1965) has reported on anemia, granulocytopenia, and thrombocytopenia in three babies treated with pyrimethamine and sulfonamides. Weissbach's paper also affords a good resumé of the literature on these drugs. Ten Pas and Abraham (1965) report on two uveitis patients who developed severe thrombocytopenic purpura and anemia on treatment with pyrimethamine and sulfonamide. One patient also showed evidence of myocarditis and a peculiar bronzing of the skin. Bronzing of the fingers in monkeys was reported by Schmidt *et al.* (1953) in an early report of the pharmacology of pyrimethamine.

Ebringer *et al.* (1965) have obtained encouraging results with a new antibiotic, trypacidin, isolated from *Aspergillus fumigatus*. Treatment of infected HeLa cell cultures with trypacidin, at doses of 40 and 50 μg per ml of medium, caused complete clearance of viable parasites from the cultures. Lower doses

also had an inhibitory effect on *T. gondii.* Trypacidin administered intraperitoneally in a total dose of 75 mg/kg, 25 mg/kg on the first day and 12·5 mg/kg on the next 4 days, apparently effected radical cure of all the treated mice as revealed by negative results of subinoculation of their tissues. The administration of drug was started 4 h after infection with 2×10^5 parasites of a virulent strain.

One interesting point in this report is that the authors were required to use penicillin along with trypacidin in their *in vivo* experiments to avoid secondary bacterial infections. Even with penicillin dosage, the majority of control mice not treated with trypacidin died in 3 days. This is a surprisingly short survival period even with a virulent strain of *T. gondii* and an inoculum of 2×10^5 organisms. Furthermore, the subinoculated tissues of treated animals were prepared with penicillin and streptomycin to avoid bacterial infections. One wonders if the virulence of the *T. gondii* strain was enhanced by a concomitant bacterial infection. This would not necessarily detract from the demonstration of efficacy of trypacidin, but nevertheless should be noted. Trypacidin appears to deserve more extensive trials.

Garin *et al.* (1965) report that dimethylchlortetracycline given orally at doses of 50–200 mg/kg daily for 3 weeks permitted the survival of 50–70% of mice given a dose of 500–1 000 RH parasites intranasally or intraperitoneally. They suggest the drug may have some value in treatment of human toxoplasmosis.

Spiramycin has been tested by various workers in animal experiments, since the original report of its efficacy in mice by Garin and Eyles (1958). Chrusciel *et al.* (1965) found it effective alone and in combination with sulfa drugs. MasBakal and In't Veld (1965) found spiramycin effective even when given 24–48 h after injection of a small inoculum of 100 organisms. They note excitation of the mice when the drug was injected. Cassady *et al.* (1964) report the drug to be toxic to mice and ineffective. Giles *et al.* (1964a) obtained some improvement in experimental anterior uveitis of rabbits produced by injection of *T. gondii* into the anterior chamber of the eye and treated subcutaneously with spiramycin. However, the results were not very satisfying, and the drug was markedly irritating. I would not recommend it for any more clinical trials in man than have already been given it.

Except possibly for trypacidin and dimethylchlortetracycline, there does not appear to be a great deal new in the armamentarium of drugs against *T. gondii* to be used as substitutes for pyrimethamine and sulfas. Since there appears to be a way to avoid the side effects of pyrimethamine by the use of folinic acid and yeast extract (Frenkel *et al.*, 1960), the use of this drug in human beings need not be approached fearfully except during pregnancy. Giles *et al.* (1964b) have confirmed Frenkel and Hitchings's (1957) original report of the efficacy of folinic acid in this respect, and Giles (1964) has used folinic acid in uveitis cases treated with pyrimethamine and sulfas.

X. Conclusion

As stated in the introduction, this review does not pretend to take into account all recent papers in the field of toxoplasmosis. Nevertheless, it is

hoped that an adequate summary of various important aspects has been presented. Certainly, the preparation of such a contribution as this is exciting because it reveals so dramatically the gaps in our knowledge and the need for more research. If, in this respect, readers are stimulated as the author has been, the effort of writing will have been worth while.

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Evolutionary Trends in Mammalian Trypanosomes

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Similarity implies affinity, and this in turn is evidence of descent from common ancestors.
Sir Gavin de Beer, *Observer*, 23 March 1956

I. Introduction ... 47
II. Relationship of Trypanosomes to other Trypanosomatids ... 48
A. Characteristics of Trypanosomatidae ... 48
B. Origin of Trypanosomes ... 50
III. Course of Evolution in Trypanosomes ... 57
A. Affinities of Vertebrate Trypanosomes ... 57
B. Phylogeny of Mammalian Trypanosomes ... 59
IV. Microevolutionary Divergence ... 66
A. Biological Races ... 66
B. Variation in *Trypanozoon* ... 67
C. Variation in *Duttonella* ... 74
D. Variation in *Schizotrypanum* ... 75
E. Variation in *Herpetosoma* ... 75
F. Infrasubspecific Variation ... 76
G. Speciation by Mutation ... 79
V. Taxonomic Treatment of Biological Variants ... 82
VI. Summary ... 85
References ... 85

I. Introduction

Owing to the medical and veterinary importance of trypanosomiases in tropical and subtropical countries of the Old and New Worlds, the mammalian trypanosomes have been studied extensively in the field and in the laboratory, with the result that a vast body of information regarding these parasites is available. Although most of the investigations have been, of necessity, concerned with practical problems of control of the diseases caused by trypanosomes, protozoologists have not lost the opportunity, which the abundant material provided, to pursue studies on problems of more general biological interest. Although such studies are sometimes frowned upon by medical and veterinary workers as being too academic, they have nevertheless furnished them with useful information of a practical nature. Thus the available evidence concerning the evolution of trypanosomes provided a basis for

their natural classification on phylogenetic lines, which not only gives expression to their affinities but also brings together related species that show similarities in their physiology, antigenic constitution and host–parasite relations. These features in their turn have a direct bearing on the response of trypanosomes to chemotherapy, on the immunological relations of the host and on the clinical manifestations of the diseases caused by them.

One of the difficulties in tracing the affinities of trypanosomes is that—in the absence of palaeontological evidence—the historical process of their evolution must be deduced from data presented by existing forms. On this account the phylogenetic considerations are bound to be largely hypothetical and justifiable only in so far as the available facts support them. But, as James Frazer has put it in "The Golden Bough", "What we call truth is only the hypothesis which is found to work best." Fortunately the family Trypanosomatidae (to which the genus *Trypanosoma* belongs) is one of the most natural groups among the flagellates, since the structure and life cycles of the genera belonging to it point to a close affinity between them, and supply a clue to the main phases of evolution through which they have probably passed.

II. Relationship of Trypanosomes to other Trypanosomatids

A. Characteristics of Trypanosomatidae

The Trypanosomatidae comprise a number of genera, some of which are monogenetic intestinal parasites of invertebrate animals, while others are digenetic parasites, the life cycle of which alternates between two hosts: one, an invertebrate, representing the intermediate host or vector, in which the flagellates develop primarily in the gut; the other, a vertebrate or plant, in which they inhabit the blood and/or tissues (genera *Leishmania*, *Endotrypanum*, *Trypanosoma*) and the sap (genus *Phytomonas*) respectively.

The monogenetic parasites were formerly divided into three main genera, *Leptomonas*, *Herpetomonas* and *Crithidia*, but recent electron microscopic and taxonomic studies (Wallace, 1943, 1963, 1966; Clark, 1959; Laird, 1959) have necessitated a radical revision both of these genera and of the terms used to denote their developmental stages (Hoare and Wallace, 1966), which are now re-defined and classified as follows (Fig. 1).

1. Genus *Leptomonas*: comprises (a) promastigote (= leptomonad) stage, represented by elongated flagellates with an antenuclear kinetoplast situated in the anterior end of the body, where the flagellum emerges from a short reservoir; undulating membrane absent; and (b) amastigote (= leishmanial) stage (free and cysts), without free flagellum.

2. Genus *Herpetomonas*: comprises (a) typical promastigote (= leptomonad) stage (as in 1) and (b) opisthomastigote stage, represented by similar forms, but with postnuclear kinetoplast situated at variable distances behind the nucleus; flagellum emerging at anterior end of the body from the bottom of a long reservoir which extends as far back as the kinetoplast; undulating membrane absent. Since those opisthomastigote forms in which the kinetoplast lies near the posterior extremity bear a superficial resemblance to the true

trypanosomal (= trypomastigote) stage (see 5 below), they were erroneously regarded as such; (c) amastigote (= leishmanial) stage (as in 1).

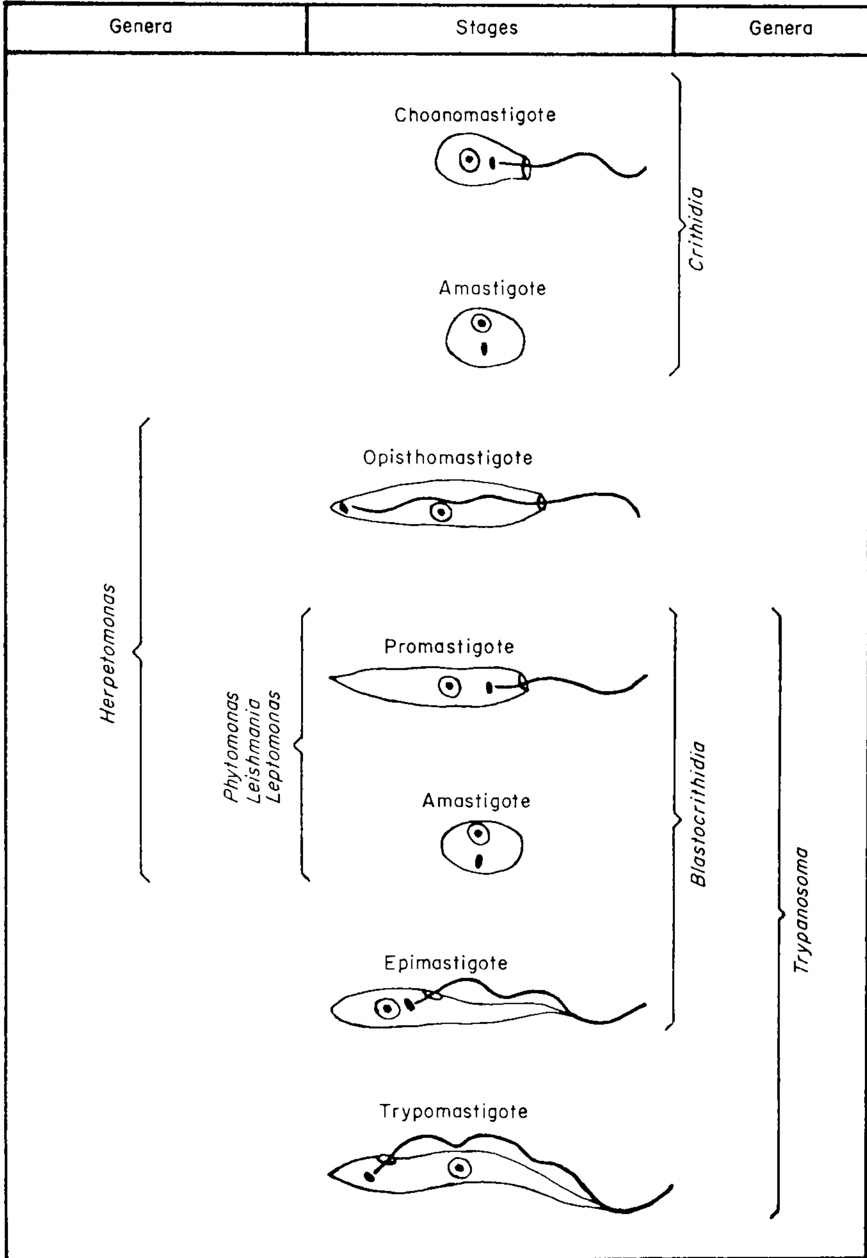


FIG. 1. Diagram of developmental stages and genera of trypanosomatid flagellates.

3. Genus *Blastocrithidia* (syn. *Crithidia* pro parte): comprises (a) promastigote (= leptomonad) stage (as in 1, 2); (b) epimastigote (= crithidial) stage represented by elongated flagellates with juxtanuclear kinetoplast; flagellum emerging at the side of the body out of a short reservoir and running along a short undulating membrane. Similar developmental stages of *Trypanosoma* in the vector are conventionally called "crithidial" forms; (c) amastigote (= leishmanial) stage (as in 1, 2).

4. Genus *Crithidia* (syn. *Strigomonas*): comprises (a) choanomastigote stage, represented by short broad (barleycorn-like) flagellates with truncated anterior end; kinetoplast antenuclear; flagellum emerging from short funnel-shaped reservoir; undulating membrane absent; (b) amastigote (= leishmanial) stage (as in 1, 2).

As is seen, these four genera differ from each other in the structure and type of morphological stages through which they pass in the alimentary tract of the invertebrate host. While the promastigote (= leptomonad) and amastigote (= leishmanial) stages are common to the first three genera (*Leptomonas*, *Herpetomonas*, *Blastocrithidia*), the barleycorn-like choanomastigote forms are peculiar to the fourth genus (*Crithidia*) only. In all these flagellates the rounded amastigote (= leishmanial) forms represent the infective stage; they are voided with the droppings and are transmitted from donor-insect to recipient-insect, when the latter ingests food contaminated with the infected faeces. This can be described as the direct contaminative method of transmission (Hoare, 1957a).

As this review is concerned primarily with the species of *Trypanosoma* parasitic in mammals, the genera *Leishmania*, *Endotrypanum* and *Phytomonas* are not considered.

5. The genus *Trypanosoma* is characterized by the appearance of a new stage, represented by the trypomastigote (= trypanosomal) forms. They resemble the epimastigote stage of *Blastocrithidia* commonly known as the "crithidial" form, but differ from it in the postnuclear position of the kinetoplast and of the starting point of the flagellum, which likewise emerges from a short lateral reservoir and runs along an undulating membrane. The trypomastigote stage occurs both in the blood and/or tissues of the mammalian hosts and in their intermediate insect hosts.

B. ORIGIN OF TRYPANOSOMES

1. *Hypotheses*

All the hypotheses regarding the phylogeny of mammalian trypanosomes are based on the close resemblance of some of their stages of development in the insect-vector to the monogenetic trypanosomatid flagellates specific to insects, especially *Blastocrithidia*. The first to speculate about the phylogenetic relations of trypanosomes was Léger (1904), who suggested that the ancestors of trypanosomes were originally monogenetic parasites of non-bloodsucking insects. When some of these acquired bloodsucking habits, their parasites adapted themselves to life in vertebrate hosts. Having established themselves in their blood the insect-flagellates underwent certain morphological changes, such as the development of an undulating membrane, which Léger regarded

as an adaptation to life in the blood-stream. He concluded that "Les trypanosomes du sang ne représentent donc qu'une adaptation . . . secondaire d'un parasite, primitivement intestinal . . . d'invertébré". This hypothesis in its essentials was adopted and amplified by a number of authors (Novy *et al.*, 1907; Brumpt, 1908; Roubaud, 1909; Laveran and Mesnil, 1912; and others: see Hoare, 1925, 1948; Baker, 1963, 1965; Nicoli and Quilici, 1964).

In the meantime Minchin (1908) advanced a diametrically opposite hypothesis. While agreeing that the developmental forms of trypanosomes pointed to their affinity with the corresponding stages in the intestinal flagellates of invertebrates, he believed "that the ancestors of trypanosomes were primitively parasites of the gut of vertebrates . . . and that from the gut they passed into the blood of the vertebrate and finally into the gut of the bloodsucking invertebrate". One of Minchin's main arguments in favour of this view was an assumption that the ancestral forms of trypanosomes were originally intestinal flagellates of vertebrates and were disseminated by "cysts" voided with their faeces. When these hypothetical flagellates eventually penetrated from the gut into the blood, they were taken up by bloodsucking invertebrates, and adapted themselves to life in their gut, where they continued to produce cysts which were transmitted to new vertebrate hosts by contamination. In support of this hypothesis Minchin refers to his discovery of "cysts" in the crocodile parasite, *Trypanosoma grayi.* In order to account for the presence of trypanosomatid flagellates in non-bloodsucking insects, such as the house-fly, Minchin was inclined to accept the view of Prowazek (1904) that *Musca domestica* had descended from bloodsucking ancestors which acquired *Herpetomonas muscarum* from vertebrate blood. However, later the force of evidence in favour of Léger's hypothesis led Minchin to change his views. At first (Minchin, 1912) he merely admitted its plausibility, but finally (Minchin, 1914) he accepted it unreservedly, stating that "the crithidial [= epimastigote] phase in the development of a trypanosome is to be interpreted as a reversion to, or recapitulation of, the type of development that occurred in the ancestral form which was originally a parasite of the invertebrate alone".

Nevertheless, some authors (e.g. Lavier, 1943) still adhered to Minchin's original concept, which was recently revived by Wallace (1966), whose main arguments in its favour are that (a) "the probability of accidental transfer [of trypanosomes] from the blood of a large animal to a bloodsucking vector is more likely than the reverse", (b) "experimental infection . . . of a vertebrate by an insect flagellate . . . has never been accomplished", and (c) these flagellates occur mainly in groups of insects which include bloodsucking forms, whereas they are virtually absent in other groups.

The present writer has previously committed himself to the view that the mammalian trypanosomes are descended from monogenetic trypanosomatid flagellates of non-bloodsucking insects, (Hoare, 1925, 1948, 1957a, b, 1966) but, in view of the continuing controversy regarding their origin, it is necessary to consider more fully the existing evidence regarding the course of their evolution. One of the strongest arguments against the descent of the insect-flagellates from parasites of vertebrates is the complete absence of evidence

of any trypanosomatid flagellates inhabiting the alimentary tract of these animals, with the possible exception of some reptilian *Leishmania*, with which we are not concerned here (cf. Hoare, 1948). As regards the first two points raised by Wallace, it should be borne in mind that the process of adaptation of an insect-flagellate to life in the blood of a vertebrate must have been very gradual and measured in terms of geological time. Nevertheless, even at present there is some indication that certain trypanosomes have apparently not yet fully adapted themselves to the blood medium but are still essentially insect-parasites. Thus, it is possible that some of the more primitive species have not entirely lost their ability to encyst in the vector. For instance, according to Silva (1965), *Trypanosoma cruzi* has a dual developmental cycle in its triatomine vector: one, with the production of cysts for transmission from bug to bug; the other, with the production of metatrypanosomes for transmission from bug to mammalian host.

In other species, e.g. in *T. melophagium* and *T. grayi*, practically 100% of the vectors (*Melophagus ovinus* and *Glossina palpalis*) are found to be infected, whereas in the sheep and in the crocodile the infection is so scanty that the presence of the trypanosomes can be detected only in thick blood films or indirectly—by haemoculture and/or xenodiagnosis (Hoare, 1923, 1931). In this connexion it may be recalled that the so-called "cysts" described from the insect-hosts of these two trypanosomes by Minchin (1908) were shown by the present writer (*loc. cit.*) to be artifacts. A still more remarkable case is presented by *T. theodori*, whose occurrence in goats was revealed by xenodiagnosis, which produced infection in the vector (*Lipoptena capreoli*), whereas it has never been detected in the mammalian host (Theodor, 1928). Since the trypanosomes in question are more intimately associated with their insect-vectors but are still ill-adapted to their vertebrate hosts, they provide examples of the initial steps in the establishment of insect-flagellates as blood-parasites of vertebrate animals.

Another argument advanced by Wallace in support of the derivation of the haemoflagellates from vertebrate hosts is the "virtual absence" of insect-flagellates in non-haematophagous insects. However, if we restrict ourselves to parasites of the genus *Blastocrithidia*, which shows the closest affinity with the genus *Trypanosoma*, it will be seen—from the check-list given by the American author, as well as from recent papers by Tuzet and Laporte (1965) and Lipa (1966)—that the hosts of these flagellates actually comprise only sixteen genera of bloodsucking insects, as compared with thirty-six genera of non-haematophagous insects. Apart from a single record from a predatory fly (*Dioctria*), the latter are represented entirely by bugs of the suborder Heteroptera, the great majority of which are phytophagous, feeding on plant juices (e.g. *Microvelia*, *Arilus*, *Oncopeltus*, *Orthaea*, *Oxycarenus*, *Cenaeus*, *Euryophthalmus*, *Pyrrhocoris*, *Cletus*, *Leptocoris*, *Erythesina*, *Eurydema*, *Pentatoma*, *Euschistus*, *Holopterna*, *Mesocerus*), while the rest are predatory, feeding mainly on insects (e.g. *Nepa*, *Gerris*, *Apiomerus*, *Leogorrus*, *Microtomus*, *Rhinocoris*, *Spiniger*). It is also significant that six of these genera (*Arilus*, *Apiomerus*, *Leogorrus*, *Microtomus*, *Rhinocoris*, *Spiniger*) belong to the family Reduviidae which includes the bloodsucking triatomine vectors

of a number of New World mammalian trypanosomes (e.g. *Trypanosoma cruzi* and *T. rangeli*).

2. *Origin of Haematophagous Insects*

According to Léger's hypothesis, the transition from the monogenetic trypanosomatids to the digenetic trypanosomes became possible when the hosts of the former acquired bloodsucking habits. Indeed, this view finds support in the evolution of the haematophagous insects, which proceeded on somewhat similar lines in the ancestors of the intermediate hosts of these haemoflagellates. In the case of the Stercoraria, i.e. the trypanosomes whose development in the insect-host terminates in the posterior station (Hoare, 1966), the vectors are represented mainly by Hemiptera (Reduviidae Triatominae and Cimicidae), Diptera (Tabanidae, Hippoboscidae) and Siphonaptera, while the Salivaria, i.e. trypanosomes whose development in the insect-hosts is completed in the anterior station (Hoare, 1966), are transmitted by muscid Diptera (genus *Glossina*) exclusively.

We now turn to a consideration of the phylogenetic relations in these insects. All bugs have similar food habits, reflected in the structure of their mouth parts, which are adapted exclusively for piercing and sucking. As already mentioned, the hosts of *Blastocrithidia* comprise phytophagous forms feeding on plant juices and predaceous forms feeding chiefly on insects. Though not bloodsucking by nature, these bugs may occasionally "bite" mammals, including man (cf. Imms, 1957). With time some of these bugs adapted themselves to predation on mammals and became obligatory bloodsuckers, giving rise to the Triatominae and Cimicidae, in which haematophagy is a relatively recent acquisition (Beklemishev, 1951, 1955). As is known, the triatomine bugs transmit the trypanosomes to their mammalian hosts by contamination, when the infected faeces are deposited on a mucous membrane or on the wound inflicted by the proboscis, whence the metatrypanosomes gain access to the blood-stream. Since defaecation at the site of a bite is a common feature among haematophagous insects, there can be no doubt that, when reduviid bugs infected with *Blastocrithidia* first acquired the bloodsucking habit, they followed this practice. It is also conceivable that the flagellates voided in their droppings occasionally found their way into the body of the mammal, where they eventually established themselves as haemoparasites, giving rise to the trypomastigote (= trypanosomal) stage, while the bugs became the intermediate hosts of the trypanosomes.

The development of a bloodsucking habit in the Diptera can be visualized more clearly in the Muscidae, in which there is a gradual transition from an omnivorous to a sanguivorous mode of nutrition, which is correlated with changes in the structure of their mouth parts (cf. Cragg, 1913; Thomson and Lamborn, 1934; Beklemishev, 1951, 1954, 1955, 1957; Theodor, 1957). Thus in the genus *Musca* many flies are omnivorous, subsisting on the excrements of mammals, while others attack these animals and feed on the exuded fluids from the surface of their body, such as sweat, mucus, pus, as well as blood oozing from sores and wounds. In the absence of biting mouth parts, these insects are incapable of piercing the integuments of the animals on which they

feed, but merely lick up the liquid discharge. However, in the course of evolution there have probably arisen forms with a greater predilection for blood and a stronger proboscis, which enabled them first to scrape an abraded surface more effectively and finally to pierce the skin. Once such a habit had developed, it would be a matter of time for this method of feeding to be firmly established and for the structure of the proboscis in succeeding generations to become modified in adaptation to active ingestion of blood from the host's body. It is thought that those muscid flies in which haematophagy is obligatory (e.g. *Glossina*) had evolved in this manner from *Musca*-like ancestors. This evolution of nutrition is in agreement with the accepted classification of muscid flies based on anatomical and biological features. On these grounds, Cragg (1913) concluded that "The theory that the flagellates of insects are descended from haemoflagellates finds little support from entomological facts."

As in the case of the Hemiptera, it is conceivable that both the purely haematophagous Diptera and their blood-licking ancestors contaminated the open sores and wounds of their mammalian food-hosts, thereby providing opportunities for their flagellates to gain access to the blood-stream. Having secured a footing in the mammal, these flagellates would presumably be in an impasse unless taken up by their insect-hosts again. In the case of non-bloodsucking flies the chances of this happening would be slender, but not impossible. Indirect evidence of this was provided by Thomson and Lamborn (1934), who showed that *Musca spectanda* was capable of ingesting trypanosomes (*Trypanosoma brucei*) with the blood exuding from the wounds of infected animals, and then transmitting these parasites to mammals by faecal contamination of their wounds. However, when true bloodsucking flies appeared on the scene, the chances of their successful infection from mammals harbouring the haemoflagellates increased, until eventually these insects became the intermediate hosts of the trypanosomes, whose cycle of development then proceeded with an alternation of hosts. At this stage of evolution it also became possible for any other bloodsucking insects to become secondarily parasitized by ingesting trypanosomes from infected animals. As will be shown below, this was probably how tsetse flies became the intermediate hosts of salivarian trypanosomes.

We can now consider the evolution of the tabanid vectors of mammalian trypanosomes. According to Oldroyd (1964), flies of the family Tabanidae were originally flower-feeders which later took to haematophagy. As is known, the male horse-flies feed on the nectar of flowers, while the females have retained this mode of nutrition in addition to bloodsucking. Furthermore, in the closely related Pangoninae some species feed only on nectar, others on blood exclusively, and others again on both (Downes, 1958). Thus, though *Pangonia* is primarily phytophagous, it occasionally also takes a blood meal (Oldroyd, 1964). Among the Brachycera the nearest relatives—and presumably ancestral forms—of the tabanid flies are the snipe-flies, Rhagionidae (Imms, 1957; Oldroyd, 1964). Although these flies are entomophagous predators, members of some genera (*Austroleptis*, *Dasyomma*, *Rhagio*, *Spaniopsis*, *Symphoromyia*) also suck blood. In the words of Oldroyd, "In

this family there is no clear division between the genera or species that suck blood, and those that cannot". Similarly the robber-flies, Asilidae—to which one of the hosts of *Blastocrithidia*, viz. *Dioctria rufipes*, belongs—regularly feed on the body fluids of insects, but some of these flies may occasionally bite human beings (Oldroyd, 1964).

The phylogenetic relations of the other insect-vectors of mammalian trypanosomes are not so clear. Oldroyd thinks that the hippoboscid vectors of ungulate trypanosomes (*Melophagus*, *Lipoptena*) and the tsetse flies (*Glossina*), to which the former are related, have arisen from common muscoid ancestors. As regards the siphonapteran vectors of rodent trypanosomes, their origin is even more obscure, but it is believed that they too have descended from Diptera.

3. *Ancestry of Trypanosomes*

From the foregoing account it is seen that the weight of evidence is in favour of the origin of the haematophagous insects from phytophagous and/or predaceous ancestors, the trypanosomatid parasites of which eventually gave rise to the trypanosomes. It was previously thought that the ancestral forms of the latter were derived from either *Herpetomonas* or *Crithidia* (*sensu lato*), or from both (cf. Hoare, 1948). This view was based on two erroneous assumptions: (a) that the trypomastigote (= trypanosomal) stage in *Trypanosoma* corresponded to the opisthomastigote stages in the former genus, and (b) that the "crithidial" (= epimastigote) stage of *Trypanosoma* was represented in the latter genus. However, in the light of the revised morphology of *Herpetomonas* and taxonomy of *Crithidia* discussed above, it is now evident that the ancestors of the trypanosomes should be sought among flagellates of the genus *Blastocrithidia*, whose stages are all represented in the genus *Trypanomonas* (Fig. 1). Indeed, trypanosomes may assume the promastigote (= leptomonad), epimastigote (= crithidial), as well as the amastigote (= leishmanial) stages. These stages appear in different proportions and at various periods of the invertebrate cycle, according to the species of *Trypanosoma*. Thus, in the mongoose parasites, *T. ichneumonis* and *T. helogaleae*, promastigote forms are preponderant (Grewal, 1960, 1961), while in *T. cruzi* epimastigote forms predominate. However, the most characteristic and constant stage, through which all species of *Trypanosoma* pass, is the epimastigote form.

As regards the trypomastigote (= trypanosomal) stage, it has no counterpart among the insect flagellates, but makes its first appearance in the genus *Trypanosoma*, where it is represented in the mammalian host by the characteristic blood forms, and in the vector by the special infective forms, known as the metacyclic trypanosomes or metatrypanosomes which arise from the epimastigote forms. The term "metatrypanosome", which was first proposed by Chatton (1913) and recently revived by Grassé (1952), will be used throughout this paper to denote the final stage of development of trypanosomes in the intermediate host. In addition to the metatrypanosomes, in some stercorarian species (e.g. *T. lewisi* and *T. rangeli*) the developmental stages in the vector include a special kind of trypomastigote form, while in the Salivaria

(e.g. *T. congolense*, *T. brucei* and allied species) trypomastigote forms represent the only intestinal stage (Fig. 5).

Already Léger (1904) suggested that the trypomastigote form had evolved as an adaptation to life in the blood-stream. However, no explanation of this morphogenetic transformation was available until recently, when electron microscopic studies revealed the presence in trypanosomatid flagellates of mitochondrial tubes connected with the kinetoplast. In the blood trypanosomes there is a single mitochondrial tube extending from the postnuclear kineto-

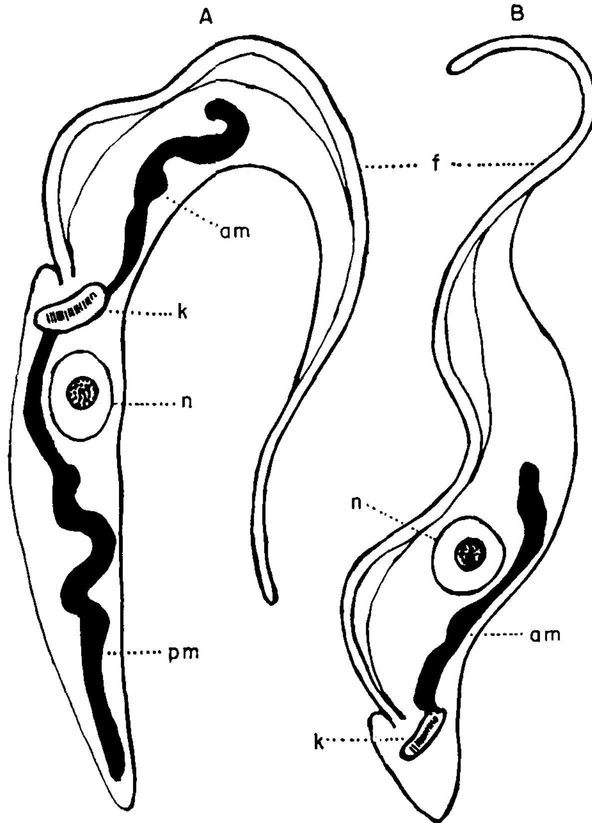


Fig. 2. Schematic ultrastructure of (A) epimastigote (= "crithidial") and (B) trypomastigote (= trypanosomal) stages in trypanosomes. *am*, Anterior mitochondrial tube; *f*, flagellum; *k*, kinetoplast; *n*, nucleus; *pm*, posterior mitochondrial tube. (Based on Vickerman, 1962.)

plast forwards into the anterior part of the body, whereas the epimastigote forms possess a second mitochondrial tube projecting into the posterior end of the body. Vickerman (1962) has shown that the transformation of the trypomastigote blood stage into the epimastigote stage (in culture and in the vector) is effected by growth of a posterior mitochondrion, which pushes the kinetoplast forwards until it assumes the typical juxtanuclear position (Fig. 2). According to this author, these changes in the ultrastructure are correlated

with the respiratory metabolism of the trypanosomes. Thus the strongly developed mitochondrial apparatus in the epimastigote forms is associated with the anaerobic conditions in the insect gut and a correspondingly greater demand for oxidative enzymes, while the reduced mitochondrion in the trypomastigote forms satisfies the respiratory needs of the blood forms in a medium rich in oxygen.

In the light of these observations it is conceivable that the trypomastigote (= trypanosomal) forms had evolved from the ancestral insect flagellates when these first established themselves in mammalian hosts, in which they underwent adaptive morphological and physiological changes in response to the new environmental conditions in the blood. Conversely, when the blood forms are taken up by the vector, they revert to the epimastigote stage by proliferation of the posterior mitochondrion.

The stages of development of trypanosomes in the vector can be regarded as a recapitulation of the forms of their monogenetic trypanosomatid ancestors, thus providing an example of Haeckel's biogenetic law among the Protozoa.

The close affinity between trypanosomes and the trypanosomatid parasites of insects can be illustrated by a number of historical examples, when flagellates discovered in the gut of bloodsucking insects were at first regarded as specific parasites of these insects, until it was proved that they were actually trypanosomes of some vertebrate, while the insects themselves were their vectors. Examples of such cases are provided by *T. grayi* of the crocodile, first described from tsetse flies; *T. melophagium* of sheep from the sheep-ked; *T. cruzi*, the causative agent of Chagas' disease and *T. rangeli* of man, both of which were first known only from stages in triatomine bugs.

Here it may be added that the evolution of trypanosomes is traced further back in a hypothesis proposed by Grassé (1952), who suggested that the Trypanosomatidae were originally parasites of aquatic invertebrates. These included leeches which transmitted the flagellates to fishes, amphibians and reptiles. At some stage, bloodsucking insects feeding on amphibious vertebrates might have acquired their parasites and eventually passed them on to terrestrial animals (birds and mammals), the trypanosomes of which were taken up and subsequently transmitted by haematophagous arthropods.

III. Course of Evolution in Trypanosomes

A. Affinities of Vertebrate Trypanosomes

The restriction of this review to mammalian trypanosomes is dictated mainly by the fact that, because of the medical and veterinary importance of these parasites, their evolution has been studied more fully than that of trypanosomes of the lower vertebrates. However, there is no hard and fast line dividing the parasites of vertebrates, for some of the stercorarian trypanosomes of mammals have their counterparts among the trypanosomes of lower vertebrates. The best examples are provided by the crocodile parasite *Trypanosoma grayi* (see Hoare, 1931) and by the bird parasite *T. avium* (see Baker, 1956), both of which are morphologically like mammalian trypano-

somes of *Megatrypanum*, and have similar life cycles, on account of which they might be classified in this subgenus (Fig. 3, a).

The morphological changes undergone by the blood forms of trypanosomes in the course of evolution have been traced by Lavier (1943), according to

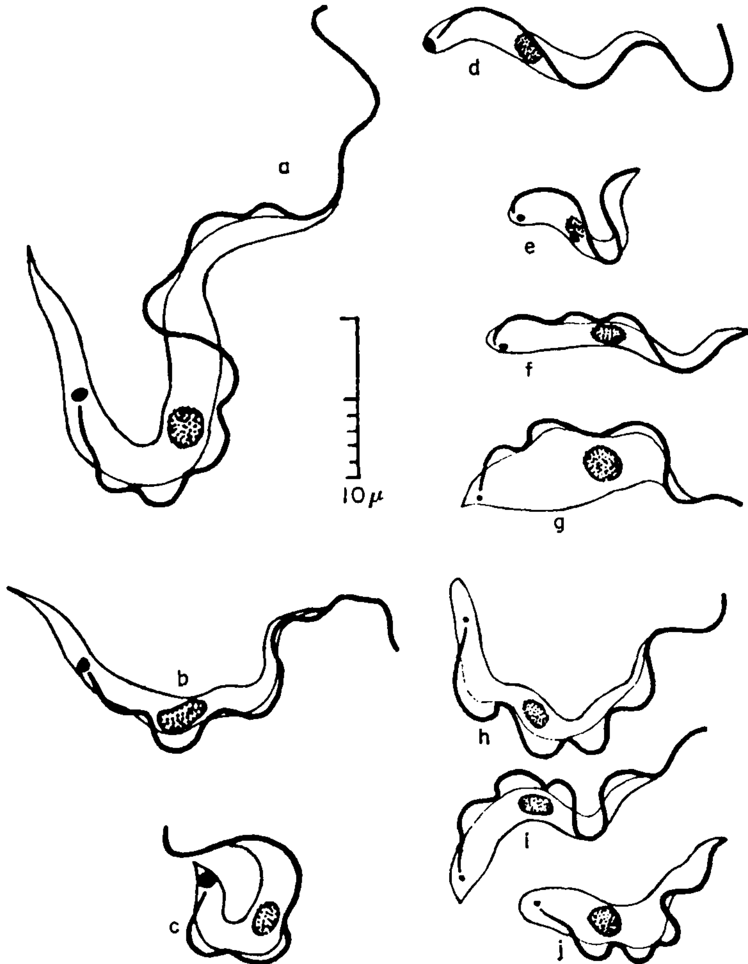


FIG. 3. Type-species of subgenera of mammalian trypanosomes. a–c, Stercoraria: a *Trypanosoma* (*Megatrypanum*) *theileri*; b, *T.* (*Herpetosoma*) *lewisi*; c, *T.* (*Schizotrypanum*) *cruzi*. d–j, Salivaria: d, *T.* (*Duttonella*) *vivax*; e and f, *T.* (*Nannomonas*) *congolense*; g, *T.* (*Pycnomonas*) *suis*; h–j, *T.* (*Trypanozoon*) *brucei*. (After Hoare, 1963.)

whom the earliest trypanosomes were of a uniform type, represented by the fish parasites (e.g. *T. rajae*). Later they became more diversified in some amphibians and reptiles (e.g. *T. rotatorium*, *T. boueti*), while in birds and mammals they underwent regression and again assumed a more uniform appearance. Finally, in some of the mammalian parasites there have evolved

special forms which do not correspond to any of the trypanosomes found among the lower vertebrate hosts. These forms comprise all the Salivaria, while among the Stercoraria they are represented by the subgenera *Schizotrypanum* (e.g. *T. cruzi*) and *Herpetosoma* (e.g. *Lewisi*-like trypanosomes) (Fig. 3).

B. PHYLOGENY OF MAMMALIAN TRYPANOSOMES

After the trypanosomes adapted themselves to life in the blood of the vertebrate host and became their specific parasites, in the course of time they underwent further evolution, clues to which are provided by the cycle of development in the vector and by the method of their transmission, as well as by the emergence of diverse groups or subgenera differing from each other in morphological and/or biological features. As is known, all species of *Trypanosoma* have a similar pattern of development in the vector, namely the trypanosomes ingested with the blood are earlier or later transformed into epimastigote (= crithidial) forms, and these eventually again give rise to trypomastigote forms, viz. metatrypanosomes. The latter represent the final stage of development in the intermediate host and are the infective forms, which are transmitted by the vector to new individuals of the mammalian host (Fig. 5 *me*,).

In many species, e.g. *T. melophagium* of sheep, *T. theileri* of cattle, *T. lewisi* of rats, *T. cruzi* of man and others (Figs. 3, a, b, c; 4, B), the trypanosomes undergo in the gut of the insect-vector their entire cycle of development, which terminates in the production of the infective metatrypanosomes in the hindgut or "posterior station". In these parasites transmission of the infection to the mammalian host takes place when the infected faeces of the vector are deposited on the mucous membranes or abraded skin of the vertebrate, as well as when the vector itself is devoured by the latter. In both cases, infection is produced by the contaminative method and the parasites actively penetrate into the body of the mammalian host. Parasites with this life cycle are referred to Section Stercoraria (= former *Lewisi* group *sensu lato*) (Hoare, 1964, 1966). In the tsetse-borne pathogenic African trypanosomes (e.g. *T. congolense* and *T. brucei* of domestic animals; *T. gambiense* and *T. rhodesiense* of man: Fig. 3, e–j) only the initial stages of development take place in the gut of the vector, after which the flagellates migrate forwards to the mouth parts and complete the cycle by the production of metatrypanosomes in the proboscis or salivary glands of the insect, i.e. in the "anterior station" (Fig. 4, E, F), but in the case of *T. vivax* the entire development is restricted to the proboscis (Fig. 4, D). This cycle of development in the vector determines the method of transmission of these trypanosomes to the mammalian host, which is effected by the inoculative method, i.e. through the bite of the vector. Parasites with this life cycle are referred to Section Salivaria (Hoare, 1964, 1966).

Of the two cycles of development the stercorarian pattern, in which the parasites are transmitted by the contaminative method, is undoubtedly the more primitive and ancient type, since it corresponds to that in the specific trypanosomatid parasites of insects (Fig. 4, A). Indeed, in both cases the infective stages develop in the posterior station of the insect, but while in the

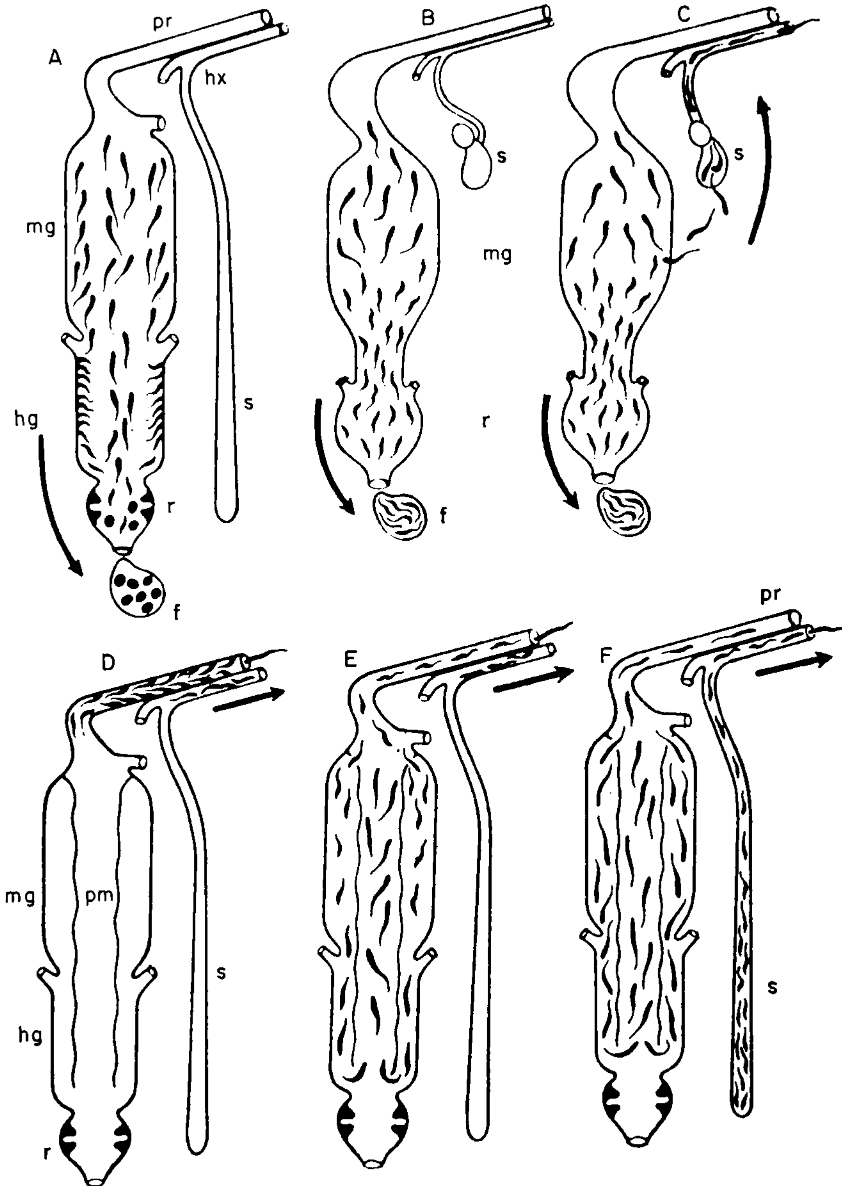


FIG. 4. Patterns of life cycles of trypanosomatid flagellates in insect-hosts, illustrating their evolution. Arrows indicate sites of development and release of final infective stages: posterior station in A–C, anterior station in C–F (cysts in A, metatrypanosomes in B–F). A. *Blastocrithidia*, specific insect parasite. Stercoraria: B, *Trypanosoma cruzi*; C, *T. rangeli*. Salivaria: D, *T. vivax*; E, *T. congolense*, F, *T. brucei*. *f*, Drop of faeces containing infective stages (cysts/metatrypanosomes); *hg*, hindgut; *hx*, hypopharynx; *mg*, midgut; *pm*, peritrophic membrane; *pr*, proboscis; *r*, rectum; *s*, salivary glands. (Adapted from Geigy and Herbig, 1955.)

purely insect flagellates they are represented by resistant forms or cysts which survive in the external environment and are transmitted directly, in trypanosomes they are represented by unprotected flagellates (metatrypanosomes) which are transmitted indirectly through the intervention of a vector (Fig. 4).

The groups in which the cycle of development terminates in the anterior station of the vector, namely in a salivary medium (= Salivaria), are represented by the pathogenic African trypanosomes which are transmitted by the inoculative method through the bite of tsetse flies (*Glossina*). The origin of these trypanosomes and their connexion with insect-trypanosomatids are not quite clear, since the latter provide no example of development in the anterior station. However, it is conceivable that this course of development was a secondary acquisition among some trypanosomes that originally completed their life cycle in the posterior station of their vector. It is interesting that this idea has recently been confirmed in the case of *T. rangeli*, the non-pathogenic parasite of man, dogs and some wild animals in South America, which is transmitted mainly by *Rhodnius prolixus*, as well as by some other reduviid bugs. The initial development of *T. rangeli* in its vector takes place in the midgut, after which it may proceed in two directions (Fig. 4, C): (a) the flagellates pass into the hindgut and produce metatrypanosomes in the posterior station, or (b) they also penetrate through the intestinal wall into the body cavity of the bug and invade its salivary glands, with the production of metatrypanosomes in the anterior station. Although it has a dual course of development, this trypanosome is now apparently transmitted chiefly by the inoculative method. The transition of *T. rangeli* from the primitive stercorarian pattern of development to the more specialized salivarian pattern is thus taking place under our eyes. However, as pointed out by Hoare (1967), the invertebrate cycle of this trypanosome normally proceeds in the intestine of the triatomine vector and terminates in the posterior station with the production of metatrypanosomes, whereas the extra-intestinal cycle is an inconstant and variable phenomenon, depending upon the species and/or geographical race of vector as well as upon the strain of parasite. The involvement of the haemolymph and salivary glands in the life cycle of *T. rangeli* is thus not yet fully tolerated by the vector. This is evident from the fact that the flagellates in the body cavity may be inhibited or eliminated by phagocytosis, and, even when they succeed in establishing an infection in this site, the insect is frequently unable to keep the parasites in check, with the result that they exert a harmful or even lethal effect upon the bug. The pathogenicity of this otherwise innocuous trypanosome to its insect-vector is a reflection of the unbalanced host–parasite relations between them under unusual conditions. Since *T. rangeli* possesses all the essential characteristics of the Stercoraria, it can be regarded as an aberrant species of the subgenus *Herpetosoma*, comprising strains differing in their host–parasite relations and developmental cycle in triatomine bugs. In some of them this development is confined to the gut (posterior station), while others exhibit various degrees of adaptation to a dual cycle in the posterior and anterior stations, in the course of which the contaminative method of transmission is being gradually superseded by the inoculative method. In this connexion it is significant that some closely

related *Rangeli*-like species from wild mammals (*T. diasi*, *T. saimiri*, *T. myrmecophagae*) are typical Stercoraria whose development in triatomine bugs is restricted to the alimentary canal.

The evolution of the true Salivaria, represented by the pathogenic African trypanosomes (Figs. 3, d–j; 4, D–F) has apparently followed a different course, because it has been proved by innumerable investigations that in tsetse flies the flagellates migrate directly from the gut into the mouth parts, without ever invading the body cavity of these insects. It is conceivable that these pathogenic trypanosomes were originally parasites of wild ruminants, whose development was completed in the posterior station of some other insect vector, which transmitted them by the contaminative method. It may be further assumed that, in addition to cyclical transmission by their specific intermediate host, they were at the same time transmitted mechanically by tsetse flies. At first they underwent no development in these insects, but in the course of time the trypanosomes might have adapted themselves to *Glossina* so well that they began to develop cyclically in these insects, with the result that the tsetse flies were transformed from mechanical inoculators into true intermediate hosts of the pathogenic trypanosomes, which began to be transmitted cyclically by the inoculative method. Although this hypothesis cannot be verified, it is not entirely speculative for there are a number of facts that provide circumstantial evidence of its correctness. Indeed some existing species of trypanosomes appear to be relict forms, the evolution of which remained fixed at certain phases of their adaptation to tsetse flies. Thus, *T. vivax*, which undergoes its entire development in the proboscis and does not survive in the gut of the fly, illustrates the initial phase when the trypanosomes, instead of merely surviving in the proboscis of the mechanical vector, began to develop there (Figs. 4, D; 5). The next phase is represented by *T. congolense*, the development of which has already extended to the gut (Figs. 4, E; 5); and finally the last phase of evolution is exemplified by *T. brucei* and *T. gambiense*, in which the development involves also the salivary glands (Figs. 4, F; 5). From these considerations it would appear that trypanosomes transmitted by inoculation (Salivaria) are of more recent origin than those with a contaminative method of transmission (Stercoraria). However, it is not implied here that there is a direct phylogenetic succession between trypanosomes of the subgenera *Duttonella* (*T. vivax*), *Nannomonas* (*T. congolense*) and *Trypanozoon* (*T. brucei* etc.): they merely represent landmarks on branches of the genealogical tree, indicating the stages of evolution reached by the subgenera in question.

As already mentioned (p. 52), presumptive evidence of the antiquity of the stercorarian trypanosomes is provided by the fact that some of the species (e.g. *T. melophagium*, *T. theodori*) are more intimately associated with their ancestral insect-hosts than with their mammalian hosts. In addition to this, the plausibility of the hypothesis outlined above is supported by certain other facts. Thus, it may be stated as a general rule that the more perfect the adaptation between parasite and vector, the older the association between them. Indeed, while in species of Stercoraria (*T. lewisi*, *T. cruzi*, *T. melophagium*) practically 100% of the vectors may become infected, the infectivity of the

pathogenic salivarian trypanosomes for *Glossina* is considerably lower, namely about 20% in *T. vivax*, about 10% in *T. congolense* and less than 1% in the *T. brucei*-complex. As is seen, the infection rates diminish in the order

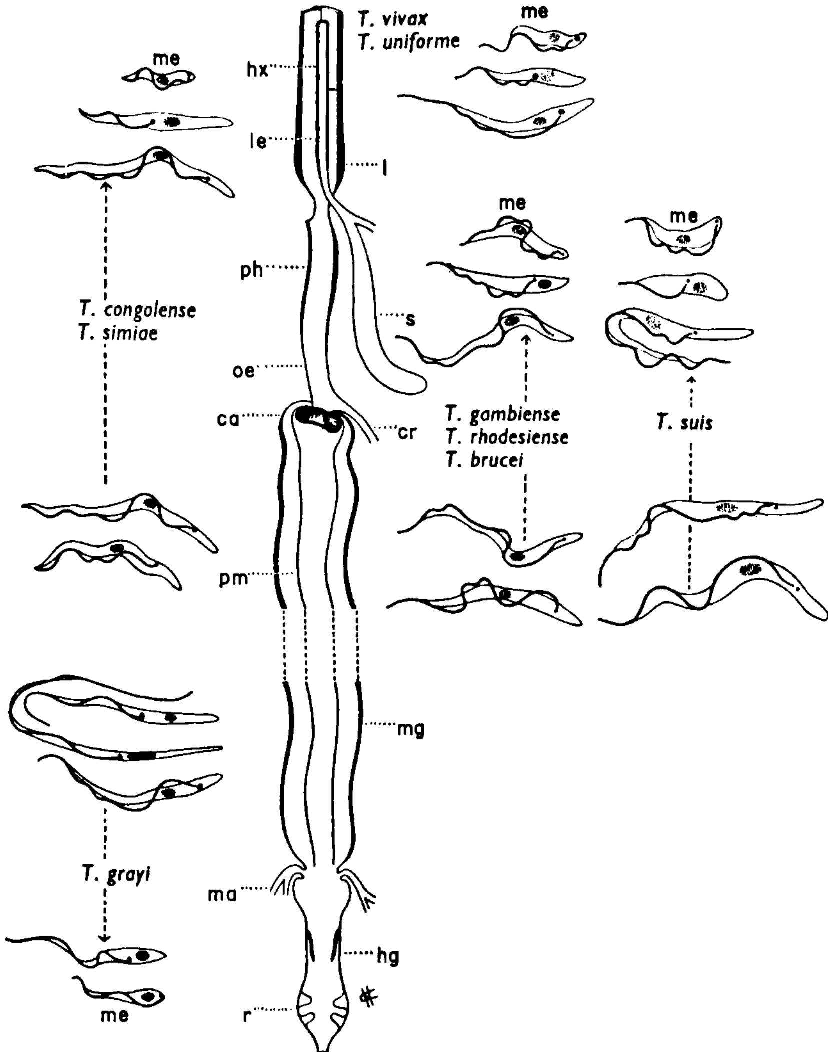


FIG. 5. Developmental patterns of trypanosomes in tsetse fly. *ca*, Cardia (= proventriculus); *cr*, crop duct; *hg*, hindgut; *hx*, hypopharynx; *l*, labium; *le*, labrum-epipharynx ($hx + l + le$ = proboscis); *ma*, Malpighian tubes; *me*, metatrypanosomes; *mg*, midgut; *oe*, oesophagus; *ph*, pharynx; *pm*, peritrophic membrane; *r*, rectum; *s*, salivary glands (one cut off). *T. grayi* illustrates stercorarian pattern; others are salivarian. Magnification of flagellates: approx. × 1 500. (Adapted from Hoare, 1949.)

of the postulated evolution of these species. The imperfect adaptation of the salivarian trypanosomes to their vectors is also reflected in the ease with

which they lose their power to develop in tsetse flies. This occurs when their cyclical development is interrupted by loss of contact between the parasite and vector, as in the case of prolonged direct passages of the trypanosomes from vertebrate to vertebrate (e.g. in laboratory strains) and in chronic infections. In such cases the transmission of the trypanosomes depends entirely on mechanical inoculators. An example of this phenomenon is provided by a bovine strain of *T. vivax* which had been introduced from Africa into South America, where it is propagated—in the absence of tsetse flies—by horse-flies (Tabanidae). It has also been demonstrated by laboratory experiments that the American strain of this trypanosome (sometimes described as *T. viennei*) is incapable of developing in its original intermediate host, *Glossina* (Hoare, 1947, 1957c).

Another argument in favour of this course of evolution of mammalian trypanosomes is supplied by their virulence. From the biological point of view, pathogenicity indicates a low degree of adaptation between host and parasite and points to their association in recent times. The ideal relations are found where there is complete harmony between both components of this system, as in the case of healthy carriers. Such relations are actually found in the majority of trypanosomes with contaminative transmission (Stercoraria), which have a restricted range of hosts to which they are not pathogenic. On the other hand, the trypanosomes that are transmitted by inoculation (Salivaria) are infective to a wide host-range. The evolution of these trypanosomes is also reflected in the course of their development in the mammalian host. Thus, in species of the Stercoraria multiplication is discontinuous, being either limited to the initial (reproductive) phase of the infection (e.g. *T. lewisi*), or taking place periodically (e.g. *T. cruzi*), but in both cases terminating in the production of so-called "adult" forms which do not divide any more. On the other hand, in the Salivaria multiplication is continuous throughout the mammalian cycle of development. Lavier (1943) interprets this phenomenon as a process similar to neoteny, in which the cycle of development had been reduced at the expense of the "adult" forms, leaving only the "young" forms which multiply continuously. Lavier connects this peculiarity with the pathogenicity of trypanosomes and points out that in artificially produced virulent strains of *T. lewisi* multiplication also becomes continuous. This phenomenon also conforms to the concept of paedomorphosis, according to which in the course of evolution of some organisms the adult forms have retained the characters of the young forms of their ancestors (de Beer, 1959). The evolutionary antiquity of the Stercoraria is also indicated by the retention in *Megatrypanum* (e.g. *T. theileri*) and *Herpetosoma* (e.g. *T. lewisi*) during their reproduction in the mammalian host of a transient epimastigote (= crithidial) stage. This stage is absent in the more recently evolved Salivaria, the blood forms of which multiply in the trypomastigote (= trypanosomal) stage.

Finally, the phylogenetic relations between mammalian trypanosomes are also supported by recent data regarding their physiology, which have established a certain correlation between the biochemical properties of these trypanosomes and their systematic position, and have shown that each group

possesses a distinct pattern of metabolic activities (von Brand, 1956; Hoare, 1948, 1958). In this respect the difference is greatest between *T. cruzi* and *T. brucei*, while *T. vivax* and *T. congolense* occupy an intermediate position. The physiological differences between these groups of trypanosomes are of great practical importance, since they determine the selective action of various chemotherapeutic agents upon them. Thus, the stercorarian *T. cruzi* (Chagas' disease) is not affected by any of the drugs used in the treatment of infections with the pathogenic salivarian species of the Old World. The latter can be divided roughly into two categories: one comprising diseases caused in livestock by trypanosomes of the subgenera *Duttonella* (*T. vivax*) and *Nannomonas* (*T. congolense*), which respond to treatment with phenanthridine compounds but are not affected by suramin; the other comprising species of the subgenus *Trypanozoon* (*T. gambiense*, *T. brucei*), which are cured by suramin (Bayer 205), tryparsamide and diamidines.

The foregoing facts seem to provide plausible arguments in favour of the course of evolution outlined above. However, in a recent discussion of this question an alternative hypothesis regarding the origin of the tsetse-borne mammalian trypanosomes was advanced by Baker (1963), who suggests that the evolution of trypanosomes proceeded in two directions. The first line, represented by the stercorarian trypanosomes of terrestrial vertebrates, evolved in the manner described above from trypanosomatids of insects developing in the posterior station. The second line, comprising trypanosomes developing in the anterior station of the vector, is represented, on the one hand, by parasites of aquatic vertebrates transmitted by leeches, and, on the other, by tsetse-borne salivarian trypanosomes of the subgenera *Duttonella*, *Nannomonas* and *Trypanozoon*. According to Baker, both these subdivisions are descended from parasites of the common ancestors of the annelids and arthropods, in which the development of the flagellates terminated in the anterior station. It is postulated that, after these two groups of animals became differentiated, they gave rise, on the one hand, to leeches and, on the other, to insects. In the course of evolution the former became the vectors for trypanosomes of aquatic vertebrates, while the latter (tsetse flies) became the vectors of the pathogenic mammalian trypanosomes (Salivaria). Although there can be no conclusive evidence one way or another, it would seem that of the two hypotheses outlined above the one advanced in the present review, being based on a certain amount of circumstantial evidence, is less speculative than Baker's.

If the different methods of transmission of the Trypanosomatidae are compared, it will be seen that their effectiveness—assessed by the degree to which they ensure infection of new hosts—improves in the course of evolution from the insect-flagellates to mammalian trypanosomes (Hoare, 1957a). Thus in the monogenetic parasites of insects the amastigote cysts, escaping from the hindgut with the droppings, are widely scattered in the external environment so that only some of them are accidentally eaten by and infect new insect-hosts. In the case of indirect contamination by this method there is a considerable element of chance, since the majority of the parasites perish without finding a new host. However, in the stercorarian trypanosomes the chances

of successful transmissions are considerably increased, for infection of the mammalian host depends upon direct contamination of its mucous membranes, which enables the metatrypanosomes to invade its body. Transmission in this case is effected by contamination with the infected faeces deposited by the vector (e.g. in *T. cruzi*) or by the ingestion of the vector itself by the host (e.g. in *T. melophagium*), whereas the infective forms deposited on less vulnerable parts of the body (such as fur or skin) perish. A further improvement is found in the Salivaria, in the transmission of which nothing is left to chance, since the metatrypanosomes are injected by the tsetse fly directly into the mammalian body through the bite of the insect.

IV. Microevolutionary Divergence

A. Biological Races

In the foregoing account we have attempted to trace the origin and evolution of the main subdivisions (subgenera) of the mammalian trypanosomes which are separable on morphological grounds. In addition to these, there are groups of species bearing distinct names, as well as strains within a single species, which are morphologically indistinguishable but differ from each other only in biological or physiological features, especially in various aspects of their host–parasite relations. Such groups, which are known collectively as biological races and strains, are examples of microevolutionary variation. They differ in the degree of isolation from each other and in stability, as well as in their bionomics, which determine the level of divergence reached by them and provide criteria for their classification.

Since trypanosomes are asexual organisms, their populations represent clones, therefore any diversifications undergone by them are determined by gene mutations or chromosome alterations, which are at once fixed and isolate such clones from others. The heterogeneous biological races have thus presumably evolved from originally homogeneous parental species by segregation of clones possessing new characters, the perpetuation of which was determined by favourable natural selection of those variants that were best adapted to a new hostal environment (cf. Dobzhansky, 1941; Mayr, 1942; Hoare, 1943, 1955; Huxley, 1963; Lambrecht, 1965). There can be no doubt that further evolution of biological races may lead to full speciation. In some cases there first appear cryptic structural changes (e.g. in the chromosomal constitution or in ultrastructure) which are gradually succeeded by visible characters, until eventually such races become recognizable as good morphological species. In other cases complete speciation of biological races may be accomplished without any morphological differentiation, thereby giving rise to purely biological species. The mammalian trypanosomes provide examples of variation at different levels of the evolutionary process.

These evolutionary trends have been more fully studied among the salivarian species of the subgenus *Trypanozoon* and the stercorarian species of the subgenera *Schizotrypanum* and *Herpetosoma*.

B. VARIATION IN *Trypanozoon*

1. Brucei-*like Trypanosomes*

In the subgenus *Trypanozoon* there is a group of tsetse-borne African trypanosomes, which are attributed to three species: *Trypanosoma gambiense* and *T. rhodesiense*, causing sleeping sickness in man, and *T. brucei*, causing nagana in domestic mammals. Since these species are morphologically indistinguishable, there can be no doubt about their close phylogenetic affinity. The only difference between them is biological, and is manifested in the relationships of these parasites to their hosts. All three are infective to diverse mammals, but only *T. gambiense* and *T. rhodesiense* are capable of infecting man. In their turn, the two human parasites differ from each other nosologically, i.e. in the type of disease produced by them: while the Gambian sleeping sickness due to *T. gambiense* is a chronic disease, the Rhodesian type caused by *T. rhodesiense* is an acute disease. The course of the disease caused by these two species is a reflection of differences in their virulence to man; it is paralleled in their effect upon experimentally infected rodents, which are highly susceptible to *T. rhodesiense* but relatively refractory to infection with *T. gambiense*. In addition to these types of sleeping sickness, it has recently been demonstrated (Ormerod, 1961; Apted *et al.*, 1963) that in the Zambezi basin and Botswana (= Bechuanaland) there occurs a third type, which occupies an intermediate position between the two classical forms of the disease. It resembles the Gambian form in its chronic and sometimes symptomless course, as well as in the pathological changes produced in man, but—like *T. rhodesiense*—the Zambezi-Botswana strains are very virulent to laboratory mammals.

There is historical evidence showing that the typical epidemic form of the Rhodesian disease found in the north (e.g. in former Tanganyika which is now incorporated in Tanzania) originated in the south from the Zambezian disease. Prolonged contact with this type of the disease experienced by man in the Zambezi basin and in Botswana brought about mutual adaptation between the human host and the parasite, with the result that in these endemic areas the infection in the immune population runs a benign and often symptomless course, but when people there are exposed to infection for the first time the disease may again become acute. Similarly, as the infection spread northwards through Zambia, Malawi and Tanzania it assumed the epidemic and lethal form of typical Rhodesian sleeping sickness. However, with time the outbreaks of the diseases in the north subsided, leaving only residual foci of infection (as in Tanzania), but these are liable to flare up under favourable conditions. On the other hand, in territories that had been more recently invaded by the disease, such as the area north of Lake Victoria (Kenya and Uganda) and Ruanda-Urundi (Evens, 1956), the disease remains acute.

Apart from the features noted above, the Gambian disease differs significantly from the Rhodesian-Zambezian type in the part played in their epidemiology by the vectors and other mammalian hosts of the trypanosome. In the various forms of sleeping sickness the role of lower animals as reservoirs of human infection is different, because each of these types of disease has its

specific vectors, which have their own ecological requirements, including distinct biotopes and food preferences. Thus, *Glossina morsitans* and related species, which are the chief vectors of *T. rhodesiense*, inhabit savannah-like country where they are restricted to biotopes represented by groves with a definite type of woody vegetation. This terrain also abounds in game animals, especially antelopes, on which the flies feed. That these tsetse flies and wild ruminants are linked in a food-chain was repeatedly demonstrated by precipitin and agglutinin-inhibition tests for the analysis of blood ingested by these insects (Weitz, 1960). Moreover, it has been demonstrated that antelopes in such country may be naturally infected with trypanosomes of the *Brucei* group, to which *T. rhodesiense* belongs. In the past there was considerable circumstantial evidence that man (e.g. in Tanzania) may acquire the infection from wild mammals (cf. Hoare, 1962). There is also convincing epidemiological evidence of the existence in Tanzania and in the Zambezi basin of enzootic foci of Rhodesian sleeping sickness (Apted *et al.*, 1963). The correctness of this hypothesis was confirmed in East Africa by experimental inoculation of volunteers: first, with trypanosomes isolated from bushbuck (*Tragelaphus scriptus*) in Kenya (Heisch *et al.*, 1958), and, second, with a strain isolated from wild tsetse (Southon and Robertson, 1961). From these facts it is evident that Rhodesian sleeping sickness is a typical anthropozoonosis (cf. Hoare, 1962). Under natural conditions in the bush, the infection circulates independently of man between donor-antelopes, their vectors and recipient-antelopes, while in endemic zones the circulation of the parasite involves man as well, with the result that he becomes both donor and recipient of the infection.

Vectors of the Gambian type of sleeping sickness are represented by *Glossina palpalis* and allied species. They are riverine insects that live among the trees forming the gallery forest along the edges of the waterside, to which their activities are restricted. The zones occupied by *G. palpalis* are characterized by a scanty mammalian fauna, but are usually situated near well-populated places, the inhabitants of which frequently come in contact with the vectors when visiting the river banks. Although under these conditions tsetse flies of the *palpalis* group will attack man, their main food-hosts are reptiles, especially crocodiles. Evidence of intimate association between *G. palpalis* and crocodiles was provided by a number of authors (Hoare, 1931; Weitz, 1960; Cott, 1961). On account of these peculiarities of the biotopes of *G. palpalis*, it is thought that the disease caused by *T. gambiense* is essentially an anthroponosis, which is transmitted mainly from man to man, though there is both experimental and epidemiological evidence that some domestic animals, such as pigs and goats, living in close association with man in endemic areas, might serve as reservoir hosts of human infection (Duke, 1928; van Hoof, 1947; Fairbairn, 1954).

We can now consider the relationship between the two main types of sleeping sickness, which are usually separated on the basis of differences in the course of the infection, geographical distribution, vectors, virulence and other features mentioned above. However, there is no hard and fast line separating the diseases caused by *T. gambiense* and *T. rhodesiense* (cf. Hoare, 1949). Thus, in West Africa and in the Congo, where only the Gambian type

is supposed to occur, the disease typically runs a prolonged chronic or even symptomless course, but in a minority of cases it is acute and has all the characteristics of the Rhodesian disease (Lester, 1933; Duggan, 1962). Conversely, in Tanzania, where the acute Rhodesian form is prevalent, the disease sometimes assumes a chronic course indistinguishable from the Gambian type (Buchanan, 1929). Moreover, as already noted, the two extreme types can now be linked by the Zambezian form, which occupies an intermediate position between them. Some of the other differential characters of the classical types of sleeping sickness are also of relative value. Thus, it was demonstrated that, by passages through experimentally infected mammals, the virulence of *T. gambiense* can be enhanced (Sandground, 1947) and that of *T. rhodesiense* reduced (Fairbairn and Burtt, 1946). As regards the vector, it is known that under experimental conditions both human trypanosomes are capable of developing in any species of *Glossina*, and it has been demonstrated that under field conditions either of these trypanosomes is transmissible by flies of both the *palpalpis* and *morsitans* groups (Willett, 1965).

There can thus be no doubt that the two human parasites are very closely related, but there is no agreement about the exact nature of their affinities. Thus Willett (1965) maintains that *T. rhodesiense* was derived from a virulent strain of *T. gambiense*, whereas others (Hoare, 1949, 1965a, *et alibi*; Lambrecht, 1964; Ormerod, 1967) believe that *T. gambiense* originated from *T. rhodesiense*. A clue to the evolution of sleeping sickness is provided by *T. brucei*, the causative agent of nagana in domestic animals and also harboured in natural foci by antelopes which are the reservoir hosts of this disease. Since *T. brucei* is morphologically indistinguishable from *T. rhodesiense* and *T. gambiense*, there can be no doubt about the close phylogenetic affinity between these three species. The only essential feature distinguishing *T. brucei* from the other two species is its inability to infect man, a fact established by numerous unsuccessful attempts to infect volunteers. However, on one occasion van Hoof (1947) produced experimental infection in man with a Congo strain of *T. brucei*, but the infection was very slight and transient, lasting only 3 weeks.

At one time it was thought that by passages through lower mammals, the human trypanosomes could be converted into *T. brucei*, i.e. revert to the ancestral type. However, experimental investigations carried out at Tinde (Tanzania) have demonstrated that after cyclical passages of *T. rhodesiense* through ruminants over a period of 23 years this trypanosome had not lost its ability to infect man, though on many occasions during this experiment man failed to become infected (Fairbairn and Burtt, 1946; Willett and Fairbairn, 1955; Ashcroft, 1959). In assessing the value of these experiments it should be borne in mind that the susceptibility of the human organism to infection even with his own specific trypanosomes may vary according to the physiological state of the host, parasite or vector, and may also be influenced by other factors. It is therefore conceivable that in the past—or even at present—exceptional virulence of a strain produced by mutation or a lowering of resistance on the part of the human organism might have created conditions enabling *T. brucei* to establish itself in man. An example of such a case is provided by van Hoof's experiment; moreover there is some epidemiological

evidence that sporadic cases of human infection with *T. rhodesiense* in the Zambezi basin must have been derived from *T. brucei* (see Ormerod, 1961, 1967). The difficulty involved in the differentiation of the two parasites was clearly summarized by Corson (1946) in the following passage: "if a strain of *T. rhodesiense* had been isolated from a naturally infected animal . . . and had been tested on very resistant persons, it would probably have been called *T. brucei*".

We can now turn to the genesis of the trypanosomes causing sleeping sickness in Africa. In tracing their evolution Lambrecht (1964) goes back to prehistoric times in the late Miocene or Pliocene, when certain branches of the primates left the forest environment and settled in the savannahs. Having abandoned their arboreal mode of life and become ground-dwellers, they eventually gave rise to the species of hominids, including man. In this habitat early man was continually exposed to attacks by the savannah-inhabiting tsetse flies of the *morsitans* group, which carried *T. brucei* acquired by them from naturally infected mammals such as antelopes. As already pointed out, contemporary human beings exposed to similar conditions do not normally acquire an infection, but in those exceptional cases when this took place *T. brucei* probably caused in the non-immune human hosts an acute disease, and became known as *T. rhodesiense*, which still retains its connexion with the game tsetse as vectors and with antelopes as reservoirs of the infection in enzootic foci. The close phylogenetic relationship between these two species is supported also by their possession of common antigens (Cunningham and Vickerman, 1962; Weitz, 1963). However, in waterside zones the trypanosome became better adapted to man and produced in him a chronic disease attributed to *T. gambiense*. In such endemic areas the role of vectors was taken over by tsetse flies of the *palpalis* group, which are associated with reptiles as food hosts and do not depend upon wild mammals. The chronic course of the infection caused by *T. gambiense* indicates that in the Gambian form of sleeping sickness the parasite and host are better adapted to each other than in the Rhodesian form. Moreover, the different virulence of *T. gambiense* and *T. rhodesiense* is to some extent correlated with the possession of distinct vectors—*Glossina* of the *palpalis* and *morsitans* groups respectively. This assumption finds some support in the early history of sleeping sickness in Uganda. When it first appeared on the shores of Lake Victoria at the beginning of this century it caused an epidemic outbreak of a disease corresponding to the Rhodesian type, which decimated the population, and only later—when the people became tolerant to the infection—did it acquire the characteristics of the Gambian type (Buxton, 1955; Hoare, 1965a).

In the light of this hypothesis, *T. brucei* may be regarded as the ancestral form, which gave rise to *T. rhodesiense*, while the latter evolved to *T. gambiense*. Furthermore the evolution of the respective diseases apparently proceeded from a purely enzootic animal infection (nagana) to an anthropozoonosis (Zambezian and Rhodesian sleeping sickness) and finally to a pure anthroponosis (Gambian sleeping sickness). This group of trypanosomes thus presents an interesting case of a dynamic evolutionary process that is still in progress.

2. Evansi-*like Trypanosomes*

A more complicated problem is posed by another representative of *Trypanozoon*, *T. evansi*, which has many features in common with *T. brucei* but, unlike the latter, occurs only outside the area of distribution of *Glossina*. Nevertheless, there are reasons to suppose that *T. evansi* has originated from this tsetse-borne species, but in the absence of direct proof of the phylogenetic relations between these two species this hypothesis can only be supported by circumstantial evidence (Hoare, 1947, 1957c). As is known, *T. evansi* is the causative agent of surra in domestic animals, especially camels, horses and cattle. In the Old World this disease has a wide geographical distribution in countries with hot or warm climates, mostly in arid deserts and semi-arid steppes, including North Africa, south-eastern U.S.S.R., Asia and Malaysia. Surra (under different local names, viz. desrengadera and murrina) is also prevalent in the New World, in Central and South America. Its distributional range thus includes the Palaearctic, Ethiopian, Madagascar, Oriental and Neotropical zoogeographical regions. The affinities between *T. evansi* and *T. brucei* are evident from their close morphological similarity. Although *T. evansi* is typically monomorphic, some strains of this species occasionally exhibit the same type and degree of polymorphism as *T. brucei*, and are then indistinguishable from the latter species (Hoare, 1956). On the other hand, when *T. brucei* is maintained by direct passages through laboratory animals it becomes monomorphic, like typical *T. evansi*, and also loses its power to develop in *Glossina*. In fact these two species are morphologically identical, differing only in the constancy of their polymorphism. The parallelism between *T. evansi* and *T. brucei* leaves no doubt that they are phylogenetically related, and although nothing is known about the course of their evolution, the facts mentioned above provide a strong argument in favour of the origin of *T. evansi* from *T. brucei*.

The specific differentiation of *T. evansi* must have taken place in mammalian hosts after they had been in contact with tsetse flies infected with *T. brucei*. In fact, the clue both to such contact and to the spread of surra outside tropical Africa is provided by camels, which are the chief hosts of *T. evansi* throughout the vast region of Africa lying to the north of a line which runs roughly from 15°N. lat. in the west and nearly to the Equator in the east, and also marks the northern boundary of the area of distribution of *Glossina* (see Fig. 6). Although the southern boundary of the area of cameline surra and the northern limit of nagana almost coincide, the line of demarcation is subject to fluctuation, while at a number of points the ranges of the two diseases even overlap. Owing to the danger of trypanosomiasis and unsuitable climatic conditions, camels are usually not taken across the boundary, but it is known that occasionally caravans penetrate into the "tsetse-belt" or come into contact with these flies in isolated foci lying north of the boundary, thereby exposing the camels to infection with *T. brucei*, which may eventually acquire the characteristics of *T. evansi* (Hoare, 1940a; Lewis, 1949; Mornet, 1945; Godfrey and Killick-Kendrick, 1962). There is thus no theoretical objection to the view that the species now known as *T.*

evansi had evolved from *T. brucei.* Nagana could have been contracted by camels temporarily brought into a "tsetse-belt", whence—on their return to areas free of *Glossina*—the infection was spread among other camels by horse-flies. Having in the course of time adapted itself to mechanical transmission, *T. brucei* lost its polymorphism and assumed the characteristics of *T. evansi.* From this point of view the sporadic occurrence of polymorphism in *T. evansi* can be regarded as an atavistic trait.

Since the one-humped camel (*Camelus dromedarius*) has for centuries been the chief transport animal throughout northern Africa and western Asia, it is probable that surra was gradually disseminated by caravans travelling across the Sahara, first to the Mediterranean coast, Egypt and Sudan, then into Arabia, Palestine, Asia Minor and other parts of Asia, including India. The close connexion between camels and surra is clearly shown in the accompanying map (Fig. 6), from which it is seen that their areas of distribution coincide almost everywhere except in those parts of eastern Asia which are occupied by the two-humped camel (*C. bactrianus*) and are free of surra. In addition to camels, *T. evansi* adapted itself to other domestic animals, producing races pathogenic to cattle and horses which are the chief hosts of this trypanosome in countries where camels are absent, e.g. Indo-China, Java, Philippines, Mauritius. Finally, *T. evansi* also found its way into Central and South America, where the disease affects mainly horses. Since horses were not indigenous to the New World, it is probable that the diseases murrina and desrengadera (= surra) were introduced there during the sixteenth century by the Spanish conquerors with their cavalry mounts (Hoare, 1950, 1965b), and the trypanosome became known as *T. hippicum.* The example of *T. evansi* shows how trypanosomes, whose geographical distribution in Africa was originally limited to regions occupied by their normal intermediate hosts were introduced through human agency with their mammalian hosts into countries far beyond their natural boundaries, where the parasites established themselves in local ungulates by adaptation to new insect-vectors. Furthermore, in South America this change of habitat involved also the acquisition of an unusual supplementary vector, represented by vampire bats (*Desmodus rotundus*), the role of which in the transmission of trypanosomes from a mammal of one order (Chiroptera) to mammals of another order (Ungulata) represents an exceptional and paradoxical phenomenon in parasitology (Hoare, 1965b).

The evolution of the subgenus *Trypanozoon* assumed a special form in *T. equiperdum.* This trypanosome is morphologically indistinguishable from *T. evansi,* but causes a distinct disease, dourine, in horses of southern Europe and North Africa, and is transmitted from stallion to mare during the sexual act, i.e. by direct contact between horses. This species has presumably originated from *T. evansi* by complete emancipation from the insect vector, whereby *T. equiperdum* was freed from the territorial restrictions imposed upon trypanosomes by the climatic and other ecological requirements of their insect-vectors, with the result that dourine spread far northwards into Europe (e.g. to Brittany) and into America (e.g. to Canada). In fact only the efficiency of the control measures has prevented wider dissemination of this trypanosome.

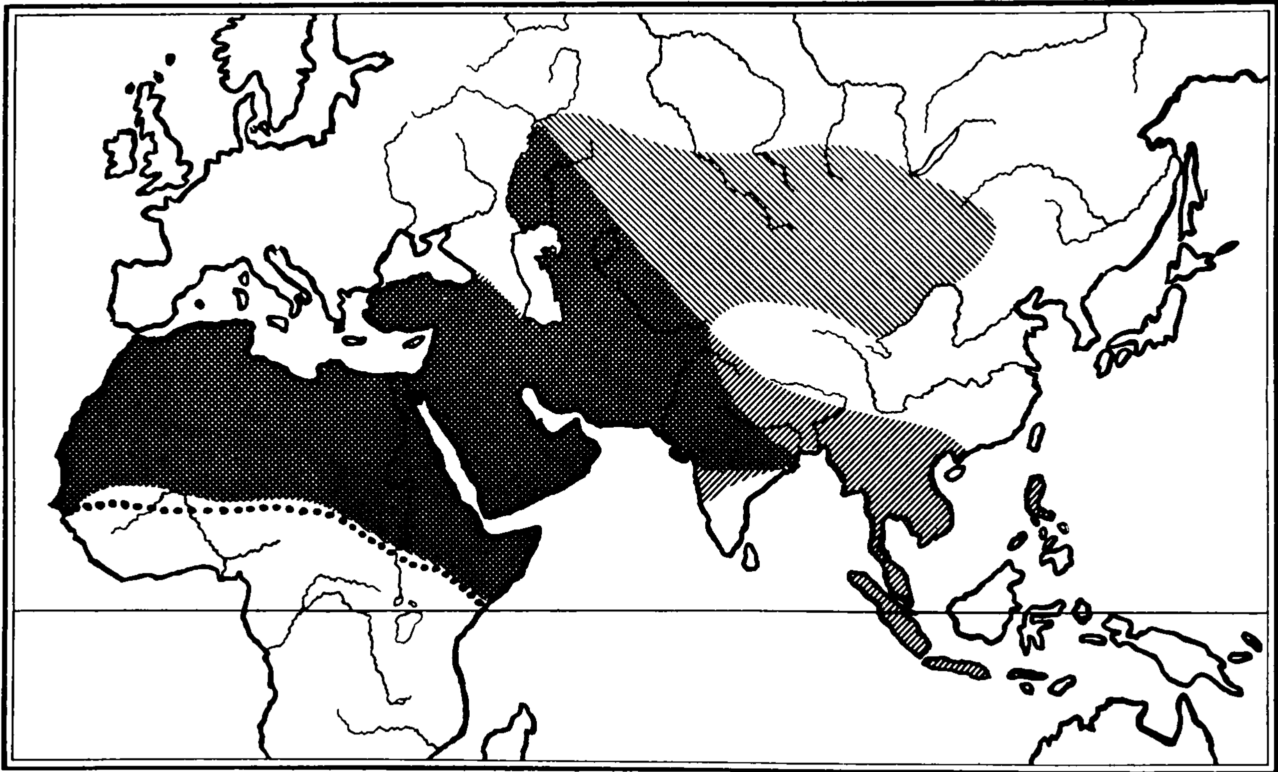


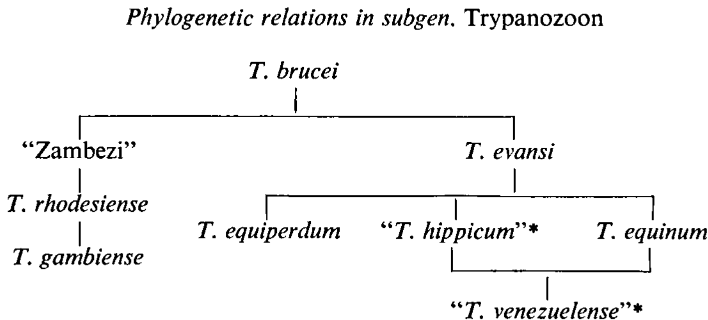
FIG. 6. Geographical distribution of surra (*T. evansi*) and camels in the Old World. \\\\\\\\, Range of camels; ///////, range of surra; • • • • • = northern boundary of tsetse zone. (From Hoare, 1957c.)

In addition to the features noted above, *T. evansi* and *T. equiperdum* differ significantly in the diseases produced by them in equine hosts, viz. surra and dourine, respectively. Although this nosological distinction is of the same order as that between *T. gambiense* and *T. rhodesiense*, the biological divergence between the two equine parasites is greater than that separating the human ones.

The phylogenetic relations of the *Evansi*-like trypanosomes find further support in the possession by *T. brucei*, *T. evansi* and *T. equiperdum* of common antigens, on account of which they cannot be differentiated by the complement fixation test (Robinson, 1926; Hornby, 1953, unpublished). In addition to *T. equiperdum*, *T. evansi* has probably given rise to *T. equinum*, the origin of which is discussed below (p. 81).

The postulated phylogenetic relations in trypanosomes of the subgenus *Trypanozoon* are represented in the following diagram.

Phylogenetic relations in subgen. Trypanozoon



* Species in quotes are invalid.

C. VARIATION IN *Duttonella*

A striking example of the emancipation of a trypanosome from its natural intermediate insect-host through substitution of the latter by another vector is provided by the parasite of African ungulates, *Trypanosoma* (*Duttonella*) *vivax*, whose normal range coincides with that of its tsetse vectors in Africa. This trypanosome occurs also outside Africa, in countries where these insects are absent, viz. in the West Indies, South America and Mauritius, into which it had been introduced in the last century with infected cattle imported from Africa and established itself in local bovines, among which it is transmitted by horse-flies (Tabanidae). These insects are mechanical vectors or inoculators of *T. vivax*, which has adapted itself to the new vectors so well that it has lost the power to develop cyclically in its original intermediate host, *Glossina* (cf. Hoare, 1957c). This replacement of vectors has thus enabled bovine trypanosomiasis caused by *T. vivax* to spread to distant lands outside Africa.

While the arguments regarding the descent of *T. evansi* from *T. brucei* discussed above are purely speculative, in the case of *T. vivax* the extension of an African species outside its natural range is beyond all reasonable doubt, for the non-African strains of this species (sometimes described as *T. viennei*) are morphologically indistinguishable from the African *T. vivax*. Here it must

be noted that other tsetse-borne trypanosomes were apparently unable to adapt themselves to mechanical vectors in this way, for, in spite of the fact that cases of sleeping sickness had been reported among African slaves, *T. gambiense* never succeeded in establishing itself in the New World (Sicé, 1937; Scott, 1943).

D. VARIATION IN *Schizotrypanum*

While the species of *Brucei*-like trypanosomes of the subgenus *Trypanozoon* considered above represent a closely knit assemblage of morphologically identical populations whose biological characteristics intergrade, the trypanosomes of the stercorarian subgenus *Schizotrypanum*, which are likewise indistinguishable morphologically, exhibit wider biological divergence in their host–parasite relations, host-range, vectors and geographical distribution. On this basis *Schizotrypanum* can be divided into two major groups. One, which is assigned to *Trypanosoma cruzi*, comprises strains or races confined to the New World, whose natural vectors are Reduviid Triatomine bugs and which are infective to a wide range of mammals. In the past it was believed that simian infections with this trypanosome occur naturally in Asia, but this assumption is completely unfounded (cf. Hoare, 1963). *T. cruzi* includes the causative agent of Chagas' disease in man, and the parasites of some American mammals (e.g. wood rats, opossums and armadillos) which are the proven reservoirs of human infection. However, the infectivity to man of other strains is unknown, and it is conceivable that they might bear the same relation to human strains of *T. cruzi* as *T. brucei* to the causative agents of sleeping sickness in Africa, viz. *T. gambiense* and/or *T. rhodesiense*. The second group is represented by the bat parasites, *T. vespertilionis* and/or *T. pipistrelli*, which have a cosmopolitan distribution. There is good evidence that some of the bat strains are rigidly restricted to their hosts, especially in the Old World, where bats are the only animals harbouring *Cruzi*-like trypanosomes, whereas in the New World some of the bat trypanosomes behave like *T. cruzi*.

Practically nothing is known about the vectors of bat trypanosomes but, since in Europe bats are commonly infected with *T. vespertilionis* and/or *T. pipistrelli* in the absence there of triatomine bugs, their vectors must be sought among other bloodsucking arthropods. Transmission by vectors other than triatomines is therefore another important biological character distinguishing the *Cruzi*-like trypanosomes of bats in the Old World. There are also some indications that the stages of development of these trypanosomes in the bats might differ from the pattern observed in *T. cruzi* (cf. Wenyon, 1926). Although there can be no doubt about the close affinity between trypanosomes of the subgenus *Schizotrypanum* in the Western and Eastern hemispheres, unfortunately there are no clues to their phylogenetic relations.

E. VARIATION IN *Herpetosoma*

Among the trypanosomes of the stercorarian subgenus *Herpetosoma* there are numerous species and strains whose adult blood forms are morphologically indistinguishable from those of the type-species *Trypanosoma lewisi*. The

great majority of these *Lewisi*-like trypanosomes are parasitic in rodents throughout the world, and they are transmitted by fleas in which they have a comparable pattern of development. Most of the rodent trypanosomes have been given an independent specific status on the basis of minor mensural or structural differences, host-restriction or merely because of their occurrence in a new mammalian host. However, a statistical analysis carried out by Davis (1952) in her comprehensive biometrical study of these trypanosomes demonstrated conclusively that their morphological and mensural characters do not provide reliable indications for the differentiation of species.

Nevertheless, in the few cases in which the life cycle of these trypanosomes has been more fully studied, it was shown that their stages and mode of reproduction were in fact dissimilar, thus providing both morphological and biological criteria for their separation (Hoare, 1936; Davis, 1952). On this basis it is possible to recognize the following six species: *T. lewisi* from rats, *T. musculi* (= *T. duttoni*) from house mice, *T. blanchardi* from dormice, *T. rabinowitschae* from hamsters, *T. zapi* from jumping mice, and *T. nabiasi* from rabbits. A common characteristic of the *Lewisi*-like trypanosomes is their rigid host-restriction, which is evident from the failure of attempts to infect heterologous animals, though these parasites are sometimes interchangeable between species of hosts belonging to the same genus (Galuzo and Novinskaja, 1961).

The similarity of the *Lewisi*-like trypanosomes leaves no doubt about their close affinity, but the views regarding their phylogenetic relations vary. Thus, Wenyon (1926) thought that they represented races of *T. lewisi* adapted to diverse hosts, while Davis (1952) suggested that *T. lewisi* and allied forms were all derived from a common generalized type of flagellate which had originated from fleas. Through the agency of these insects, the parasites became widely distributed until eventually the generalized form adapted itself physiologically to various mammals. Finally, it became host-restricted to particular mammals, while some of its descendants also underwent changes in the mode of reproduction in the mammalian hosts, which distinguishes some of the species known at present.

F. INFRASUBSPECIFIC VARIATION

In the foregoing account we have dealt with species of mammalian trypanosomes composed of morphologically identical populations, which differ in their hostal range, virulence (*Brucei*-like forms), host-restriction (*Lewisi*-like and *Cruzi*-like forms), types of disease produced by them (*T. evansi*, *T. equiperdum*), relation to vectors (*T. vivax*, *T. evansi*, *T. equiperdum*), geographical distribution, and other ecological features. It is conceivable that the biological divergence separating some of the subgenera and composite species is actually correlated with ultrastructural differences which are not detectable by conventional techniques, but might be revealed eventually by electron microscopy, thereby throwing light on the speciation of such groups. In the meantime (as will be shown below) some of these major groups can be regarded as species or subspecies. In addition, there are minor infrasubspecific trypanosome strains the differentiation of which is based on secondary

nosological, immunological and hostal characteristics. Such variants are comparable to "types" among bacteria but, since the connotation of this word in biology is so wide as to be confusing, the reviewer has adopted the term "deme" (cf. Gilmour and Heslop-Harrison, 1954; Hoare, 1955) to denote a distinct population (Greek *demos*) within a specified taxon, such as species. In order to indicate the nature of the differential criterion, the word "deme" may be combined with an appropriate prefix. As in the case of "types", the ultimate distinction between demes within a given group is determined by differences in antigenic constitution. However, since some demes have mixed characteristics (physiological, serological and/or hostal), their nature cannot always be rigidly defined.

1. *Plastodemes*

Apart from the differences noted above, infrasubspecific strains of trypanosomes differ also in the stability of the biological characters separating them from each other. Thus, the pathogenic *Brucei*-like trypanosomes, which have been studied more fully, are characterized by extreme antigenic lability which reflects the degree of their adaptation to the defence mechanisms of the mammalian host. It is well known that, when passaged through laboratory animals, *T. brucei* can rapidly adapt itself to successive antibodies elaborated by the host in the course of the infection, by producing a variety of new antigens, thereby giving rise to a series of "relapse strains" (Ritz, 1916; Brown, 1963; Gray, 1964). However, all substrains contain a basic antigen which is the only one transmitted cyclically through the tsetse fly (Gray, 1965). Since relapse strains represent populations, whose characters are labile and determined by environmentally induced antigenic variations, they are comparable to *Dauermodifikationen*, or transient modifications (Hoare, 1955), for which the term "plastodeme" was proposed (Gilmour and Heslop-Harrison, 1954).

2. *Serodemes*

Other immunologically distinct strains appear to be more stable. Some years ago much importance was attached—especially by the French school initiated by Laveran and Mesnil (cf. 1912)—to cross-immunity as a method for distinguishing strains among *Evansi*-like trypanosomes, with the result that a number of strains were separated from *T. evansi* as independent species (e.g. *T. soudanense*, *T. marocanum*, *T. annamense*, *T. aegyptum*, *T. hippicum*). However, the recognition of immunologically distinct infrasubspecific strains has discredited the diagnostic value of this method, and shown that these "species" are merely serodemes of *T. evansi*. Serodemes associated with various groups of mammalian hosts have been found in *T. cruzi* (see Nussenzweig *et al.*, 1963; Nussenzweig and Goble, 1966), and immunologically distinct strains occur also in *T. congolense* (see Laveran and Mesnil, 1912).

3. *Xenodemes*

T. evansi comprises also geographical strains differing in the degree of host-restriction. Thus, in Vietnam and Indonesia this parasite causes acute disease in horses and latent infection in cattle, whereas in Sudan and Somalia horses are refractory to infection and the disease in cattle is mild, but in

camels it runs an acute course; however, in North Africa both horses and camels suffer from surra. *T. evansi* is therefore represented by equine, bovine and cameline hostal strains or xenodemes (Hoare, 1943, 1955, 1956). It has been demonstrated that *T. cruzi* likewise comprises a number of xenodemes, represented by the causative agent of Chagas' disease and by diverse mammalian strains (Brener, 1965; Lambrecht, 1965).

As already mentioned before, another type of variation is observed in *T. rangeli* and allied species, in which there are diverse strains differing in the mode of cyclical development in their triatomine vectors (Hoare, 1967).

4. *Clinodemes*

A special kind of diversification occurs in *T. evansi*, *T. vivax* and *T. congolense*. Equine strains of *T. evansi* exhibit a gradation in the lengths of the parasites over the area of distribution of their hosts. Their mean lengths increase continually from the west (Morocco) to the east (Philippines), and this gradation appears to be correlated with the pathogenicity of the equine strains. Thus, in East Africa the disease in horses is symptomless, but farther to the east the virulence of the trypanosomes increases, the infection being subacute in the Middle East, becoming acute in Soviet Middle Asia and India, until in the extreme eastern part of the range of equine surra (Indo-China, Philippines) the course of the disease is extremely acute and lethal and epizootic outbreaks are common (Hoare, 1956).

As regards strains of *T. vivax*, they are represented in West Africa by smaller trypanosomes causing acute disease in ruminants, and in East Africa by larger trypanosomes causing chronic infection (Fairbairn, 1953). This type of variation corresponds to the concept of "clines" or "clinodemes", denoting the tendency for certain characters in a group of organisms (in our case trypanosome species) "to change gradually and continuously over large areas" (cf. Huxley, 1963; Gilmour and Heslop-Harrison, 1954).

5. *Nosodemes*

Among the demes considered above, those exhibiting also differences in virulence correspond to nosodemes. This category is represented by *T. congolense* which, according to Hornby (1952), "exists in the form of innumerable strains which vary greatly in their virulence, and the severity of the disease [in bovines] depends chiefly on the strain of parasite". This question was more fully investigated in Nigeria, where Godfrey (1960, 1961) demonstrated the occurrence in *T. congolense* of three main strains, in which differences in the mean lengths of the trypanosomes are correlated with differences in their virulence to and course of infection in mammalian hosts, as follows: (a) short typical *congolense* forms, with low pathogenicity and slight parasitaemia; (b) trypanosomes of medium size, with low pathogenicity but high parasitaemia, and (c) long *dimorphon*-type forms, highly pathogenic with high parasitaemia. These three strains thus showed a gradation from the shortest and least virulent type of trypanosomes to the longest and most virulent one. Similar strains of this species were detected also in other parts of Africa (Fairbairn, 1962). This type of variation bears some resemblance

to the clines in *T. evansi* and *T. vivax* considered above, but differs from them in the absence of continuity over a geographical range. Another example of nosodemes is provided by *T. cruzi*, which comprises a virulent strain causing the clinical forms of Chagas' disease in South America, and a non-pathogenic strain, which is probably associated with inapparent human infections in North America acquired from wild mammals (Kagan *et al.*, 1966).

G. SPECIATION BY MUTATION

In discussing the course of evolution of mammalian trypanosomes our arguments have so far been based chiefly on circumstantial evidence provided by their life cycles and host–parasite relations. However, these data give no

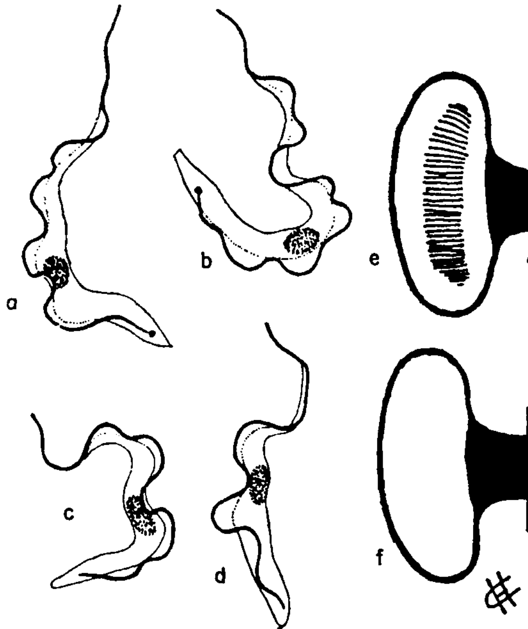


FIG. 7. *Evansi*-like trypanosomes (× 2 000). a and b, Normal strains of *T. evansi* (= "*T. hippicum*"); c and d, dyskinetoplastic strains of *T. evansi* and/or *T. equinum*; e and f, schematic ultrastructure of kinetoplast with portion of mitochondrial tube in normal trypanosomes (e = a, b) and in dyskinetoplastic trypanosomes (f = c, d).

information about the mechanism of the evolutionary process. We can now turn to the few cases where there are definite indications of the genetic factors which determine certain kinds of variation in trypanosomes. An example of these is provided by *T. evansi* and *T. equiperdum*, in which races and strains apparently devoid of a kinetoplast are known (Fig. 7).

The kinetoplast, which is probably homologous to the plastid in phytoflagellates (Hoare, 1940b), is an autonomous organoid which reproduces by bipartition when the trypanosome divides, so that each daughter-individual receives one of these organoids. In preparations of trypanosomes viewed by

light microscopy the kinetoplast appears as a dark-staining rounded or rod-shaped body, the ultrastructure of which was recently revealed by electron microscopy (Mühlpfordt, 1963, 1964). It is now known that the kinetoplast is invested in a membrane, while its centre is occupied by a dense mass containing DNA (Fig. 7, e). Although the kinetoplast is a constant organoid in *Trypanosoma*, in most species of mammalian trypanosomes some individuals —when viewed by light microscopy—appear to lack a kinetoplast (cf. Hoare and Bennett, 1937; Mühlpfordt, 1964). In addition to this individual variation there are two instances on record when in normal laboratory strains of *T. evansi* and *T. equiperdum* all the trypanosomes appeared to have suddenly lost their kinetoplasts. Similar so-called "akinetoplastic" strains can be produced artificially by injecting into infected animals various organic dyes (e.g. pyronine, acridine, oxazine or trypaflavine).

Futhermore, between 1934 and 1937 the reviewer discovered for the first time several "akinetoplastic" strains, which had arisen spontaneously in naturally infected camels in Sudan: out of three hundred camels examined in the course of 3 years such strains were isolated from five animals. One of these strains was studied in England, where it has been maintained in mice during the last 30 years (1967). In the course of this time over 100000 trypanosomes were examined but no kinetoplast could be detected in any of them (Hoare, 1954, 1959a). Since in the history of a micro-organism the millions of generations through which these trypanosomes have passed in the course of more than a quarter of a century amount to eternity, there can be no doubt that this strain is breeding true and the "akinetoplastic" condition is permanently fixed as a heritable character. It is also evident that, once the kinetoplast is apparently lost, its normal state is never restored, i.e. it does not arise *de novo*. Though a certain proportion of "akinetoplastic" variants occur in diverse species (e.g. *T. lewisi*, *T. vivax*, *T. congolense*, *T. simiae*), the only species in which totally "akinetoplastic" viable strains could be produced by treatment with dyes or had arisen spontaneously are those belonging to the subgenus *Trypanozoon* (see Hoare and Bennett, 1937; Mühlpfordt, 1963).

The genetic mechanism involved in the apparent disappearance of the kinetoplast is not clearly understood, but it is evident that this phenomenon has all the attributes of a mutation, for the "akinetoplastic" variants appear suddenly in a population of trypanosomes and breed true from the beginning, while the new character (apparent absence of a kinetoplast) becomes permanently and hereditarily fixed, giving rise to races which are seemingly devoid of this organoid. As already stated, this mutation is irreversible.

The true nature of the "akinetoplastic" state was first elucidated by Mühlpfordt (1963) in a comparative electron microscopic study of this organoid in normal and mutant strains of *Brucei*-like trypanosomes. He demonstrated that the kinetoplast is actually present in so-called "akinetoplastic" trypanosomes, but it differs from the normal organoid in the complete absence of the central DNA-containing mass (see Fig. 7, e, f). The disappearance of the kinetoplast in "akinetoplastic" forms is thus an illusion, due to changes in its ultrastructure which render it unstainable and therefore invisible by light microscopy. Since the aberrant trypanosomes actually possess a modified

kinetoplast, the term "akinetoplastic" should be replaced by the more appropriate term "dyskinetoplastic" (Trager and Rudzinska, 1964).

Although it is known that certain chemicals can produce ultrastructural changes in the kinetoplast similar to those observed under natural conditions, the primary cause of the abnormality in question remains obscure. As regards the perpetuation of the aberrant structure, there is some evidence that totally dyskinetoplastic strains may originate from normal ones containing mutant variants as the result of a more rapid rate of multiplication of the latter forms (Tobie, 1951; Hoare, 1954; Mühlpfordt, 1964). Furthermore, the genesis of such a mutant strain will be considerably accelerated through the chance inoculation by the vector of a single dyskinetoplastic variant.

We now turn to the survival value of the kinetoplast. Since dyskinetoplastic strains behave in their mammalian hosts like normal ones, the presence of an intact kinetoplast is not indispensable to the existence of trypanosomes. However, this structure seems to play some role in their life cycle. Thus spontaneous production of dyskinetoplastic strains was observed only in such species of *Trypanozoon* that have no intermediate host but are transmitted mechanically (*T. evansi* and *T. equiperdum*). But in those members of this subgenus which develop in tsetse flies (*T. brucei*, *T. gambiense* and *T. rhodesiense*) impairment of the kinetoplast takes the form of individual variation only. The significance of this difference was elucidated by Reichenow (1940). Working with a strain of *T. gambiense* containing 70% akinetoplastic forms, he produced circumstantial evidence that only normal trypanosomes are capable of developing in tsetse, whereas the aberrant forms are eliminated by the insect. Since in *T. evansi* the mutant variants are not affected by this adverse selection, they stand an equal chance of survival with normal forms, and may give rise to totally dyskinetoplastic strains. Hence the presence of an intact kinetoplast in some way ensures the digenetic life cycle of trypanosomes, while its absence is correlated with their adaptation to a monogenetic existence.

These facts provide circumstantial evidence of speciation in trypanosomes arising by mutation. In South America there is a form of equine trypanosomiasis, mal de caderas, caused by *T. equinum*, which is naturally dyskinetoplastic and indistinguishable from the dyskinetoplastic cameline strains of *T. evansi* in the Old World. This disease was apparently first introduced to Brazil with imported horses in the middle of the last century (Hoare, 1950, 1954). In the light of the foregoing observations it is conceivable that the original *T. equinum* was likewise a mutant strain of *T. evansi*, which spread in South America and has continued to breed true for the last 65 years since its discovery in 1901. Indeed, if one of the camels harbouring a dyskinetoplastic strain of *T. evansi* had been introduced into a country where susceptible mammals were present, it might similarly have given rise to a species identical with *T. equinum*. If this hypothesis is correct, *T. equinum* provides a rare example of the formation, under natural conditions, of a new species, the origin of which, by mutation from the parent species, can actually be traced.

In addition to the two *Evansi*-like species affecting domestic ungulates in South America, some authors recognize *T. venezuelense* as the causative agent

of desrengadera but, since both morphologically and geographically this parasite occupies an intermediate position between *T. evansi* (= *T. hippicum*) and *T. equinum*, it is conceivable that infections with *T. venezuelense* might be due to a mixture of the two last-named species (see scheme on p. 74) (Hoare, 1950, 1965b).

V. Taxonomic Treatment of Biological Variants

The classification of mammalian trypanosomes differing in morphological characters presents no special difficulties (cf. Hoare, 1966). However, as there is no uniformity in the choice of criteria for attributing an organism to any particular taxon, the decision is usually left to the author's individual judgment. On account of this, the categories of the species-group in the animal kingdom are not necessarily equivalent, though within the limits of a given genus-group they are as a rule commensurable, i.e. the degree of distinction between them is of the same order. In practice, allied groups are separated as species when the characters distinguishing them do not intergrade, so that there is a definite gap or discontinuity between them. But, if the characters of one group intergrade or are continuous with those of another, they are regarded as subspecies of the same species.

In the past zoological classification relied entirely on the degree of morphological divergence and made no provision for biological races. However, recent developments in genetics and systematics have considerably widened our concept of the methods of speciation and have shown that, since taxonomic differentiation is determined by isolation, there is no fundamental difference between geographical races of free-living organisms segregated spatially and biological races of parasites separated by differences in hostal environment. It can also be assumed that the genetical mechanism responsible for variation leading to biological divergence is of the same order as that producing morphological divergence. It is therefore evident that both biological and morphological attributes can be used as equivalent taxonomic criteria for classification in the conventional systematic categories (Hoare, 1955). Hence it follows that biological races are entitled to a distinctive taxonomic status, corresponding to that of morphological groups, but the differential criteria for determining their exact position in the system are more difficult to define objectively.

If the concept of biological differentiation were carried to its logical conclusion, certain species of mammalian trypanosomes, such as *Trypanosoma* (*Herpetosoma*) *lewisi*, *T.* (*Schizotrypanosoma*) *cruzi*, *T.* (*Duttonella*) *vivax*, *T.* (*Nannomonas*) *congolense* and *T.* (*Trypanozoon*) *brucei*, would have to be regarded as complexes of morphologically identical populations comparable to polytypic species (Huxley, 1963), which are differentiated on the basis of biological and/or geographical divergence. Taxonomic treatment on these lines would reduce many of the existing species to the infrasubspecific level of demes. However, the isolation imposed on biological variants of such species entitles them to a distinctive rank, while the formal recognition of the taxonomic status of pathogenic trypanosomes is dictated by their practical

importance in human and veterinary medicine. It has already been shown that the diverse races and strains of mammalian trypanosomes are not all on the same level of speciation, therefore they are not of equivalent status, and this fact should be taken into consideration when determining their place in the hierarchy. In the absence of morphological criteria, the systematic position of such groups can be assessed by the degree of discontinuity and stability of the biological characters separating them.

Among the groups of trypanosomes considered in this review, some have remained unaltered for such a long period of time that their status can be defined with some confidence. Thus, among the Stercoraria the representatives of the subgenus *Schizotrypanum*, *T. vespertilionis* and *T. cruzi*, are so widely separated in their biological characters, such as restriction to their mammalian and insect hosts, as well as in geographical distribution, that they can be regarded as valid species, though Dias (1936) assigned them to subspecies, viz. *T. vespertilionis vespertilionis* and *T. vespertilionis cruzi*. As already mentioned, *T. cruzi* comprises a number of immunologically distinct nosodemes and serodemes. In the subgenus *Herpetosoma*, the *Lewisi*-like trypanosomes are represented by several good species (*T. lewisi*, *T. musculi*, *T. blanchardi*, *T. rabinowitschae*, *T. zapi* and *T. nabiasi*), whereas the systematic position of allied forms differing mainly in host-restriction is uncertain: while some authors regard them as valid species (e.g. Krampitz, 1961), others (e.g. Davis, 1952) relegate them to infrasubspecific status. In the reviewer's opinion, it is preferable to retain distinctive names for those *Lewisi*-like trypanosomes which can be differentiated by some biological feature, for as our knowledge of their life cycle advances, it will be easier to re-group the named parasites than to separate the anonymous ones from a common pool. It is accordingly proposed to treat these trypanosomes as follows: (a) forms differing in their reproductive stages (*vide supra*) and forms from distinct genera of hosts can be regarded as valid species (e.g. *T. bandicotti*, *T. grosi*, *T. neotomae*); (b) forms with rigid host-restriction, as well as from different species of hosts belonging to the same genus (including those separated geographically) can be regarded as subspecies (e.g. *T. parkeri parkeri* and *T. parkeri marmoti*; *T. otospermophili otospermophili* and *T. otospermophili spermophili*; *T. microti microti* and *T. microti arvali*; *T. evotomys evotomys*, *T. evotomys kudickei* and *T. evotomys clethrionomi*).

In the case of the Salivaria the taxonomy of the polytypic subgenus *Trypanozoon* presents the greatest difficulty, because of the clash between a purely academic approach to the problem and the demands on the part of medical and veterinary workers for a practicable classification. If the principle of ecological divergence is strictly applied, most of the existing species of *Trypanozoon* could be regarded as biological races of one and the same species, viz. of the type-species *Trypanosoma brucei*. This interpretation is particularly applicable to the complex *T. brucei-rhodesiense-gambiense*, which exhibits a gradual transition from the purely enzootic *T. brucei* to the endemic *T. gambiense*. In fact, this view was expressed already more than 40 years ago by Chandler (1923) and Hoare (1925), who suggested that these three species were biological races of the same species, and this opinion has been recently

revived by Ormerod (1967). However, for reasons stated above, it is more convenient to distinguish trypanosomes of the *Brucei*-complex taxonomically. Since their biological characters intergrade, they are not entitled to full specific status but can be regarded as subspecies of the older species and designated as *T. brucei brucei* and *T. brucei gambiense*, as suggested by Hoare (1955, 1957b, 1966). As regards *T. rhodesiense*, it is probably merely a virulent nosodeme of *T. gambiense* (Hoare, 1948, 1962, 1965a), and therefore has no independent status. Although *ex hypothesi T. rhodesiense* had probably preceded *T. gambiense* in the course of evolution, the latter name must be retained on grounds of priority.

The position of other species of *Trypanozoon* is not so clear. Thus, the purist might be inclined to assign also *T. evansi* and *T. equiperdum* to *T. brucei* as subspecies (cf. Hoare, 1966) but this nomenclature is inconvenient for practical purposes. As a compromise solution, *T. evansi* and *T. equiperdum* can be treated either as two independent species or as subspecies, viz. *T. evansi evansi* and *T. evansi equiperdum*, both of which differ from *T. brucei* in the absence of intermediate hosts and of the corresponding stages of cyclical development, and from each other nosologically. As regards *T. equinum*, the origin of which by mutation from *T. evansi* was discussed above, it is undoubtedly a good species differing from the parent stock in a constant cytological character.

We now turn to the taxonomy of trypanosomes of the subgenus *Duttonella*. On morphological grounds, there is a case for uniting *T. uniforme* with *T. vivax* as a subspecies, viz. *T. vivax vivax* and *T. vivax uniforme* (Hoare and Broom, 1938). To these may be added *T. vivax viennei*, representing the South American race of *T. vivax* (sometimes designated as *T. viennei*), which differs from the parent African stock in the loss of its intermediate host (*Glossina*) and of the corresponding stages of development (cf. Hoare, 1957c).

Finally, in the case of the subgenus *Nannomonas*, it is justifiable to unite *T. dimorphon* and *T. simiae* with *T. congolense* as subspecies, *T. congolense congolense*, *T. congolense dimorphon* and *T. congolense simiae*, on the basis of intergradation of their morphological features, which—in the case of the first two subspecies—also exhibit character-gradients (clinodemes) correlated with differences in virulence (Hoare, 1959b, 1966).

In Section IV, F we have given examples of strains of mammalian trypanosomes differing in minor serological, clinical or hostal features, for which the deme terminology was proposed. In accordance with the International Code of Zoological Nomenclature (Art. 45, c) such infrasubspecific variants are excluded from the species-group and the provisions of the Code do not apply to them. Nevertheless, among such strains or demes can be distinguished assemblages differing in the stability of their characteristics. Thus the clinical variants or nosodemes represented by *T. rhodesiense* and *T. gambiense*, the immunologically distinct serodemes of *T. evansi* and *T. cruzi*, the hostal strains or xenodemes in *T. lewisi*, *T. rangeli* and *T. evansi*, as well as the character-gradients or clinodemes in *T. vivax*, *T. evansi* and *T. congolense*, are well-established constant variants.

On the other hand, plastodemes, represented by relapse strains of some

mammalian species (e.g. *T. brucei*), are unstable, and correspond to "morphae", a term used to denote transient modifications or *Dauermodifikationen* (cf. Hoare, 1943, 1955).

VI. Summary

This review has brought to light the multiformity of variation and the heterogeneity among mammalian trypanosomes, which have furnished abundant material for deductions regarding the evolution of these haemoflagellates. But, as was to be expected from a reconstruction of this process on the basis of existing forms, the genealogical tree is full of gaps.

Evidence of some of the phases of the evolutionary process is provided by certain "relict" species, while indications of incipient speciation are found among infrasubspecific strains or demes exhibiting minor biological divergence. The gradual transition from one morphological and/or biological type of trypanosomes to another, as well as the progressive complication of the life cycles and mode of transmission of these parasites, supply clues to their phylogenetic relations. These, in their turn, serve as a basis for the natural classification of the mammalian trypanosomes.

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Problems in the Cultivation of some Parasitic Protozoa

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I. Introduction 93
II. The Cultivation of Mammalian Trypanosomes 94
A. The Classification of Mammalian Trypanosomes 94
B. The Cultivation of Trypanosomes belonging to the Salivaria 95
C. The Cultivation of Trypanosomes belonging to the Stercoraria 110
D. The Cultivation of the Genus *Leishmania* and the Relationship of the Stage of Development of the Organism to the Type of Culture Medium and the Environmental Temperature 116
E. Discussion 119
III. The Cultivation of Malaria Parasites 121
A. Introduction 121
B. The Cultivation of the Erythrocytic Stage of the Life Cycle of the Malaria Parasite 121
C. The Cultivation of the Exoerythrocytic Stage of the Life Cycle of Malaria Parasites 125
D. The Cultivation of the Sporogonic Cycle of Malaria Parasites 127
E. Discussion 128
References 129

I. INTRODUCTION

Although MacNeal and Novy succeeded in cultivating *Trypanosoma lewisi* from the rat as early as 1903, and although some 9 years later *Plasmodium vivax*, *P. malariae* and *P. falciparum* were maintained in culture for up to four generations by Bass and Johns (1912), it is still not possible, in spite of a considerable amount of work in the intervening years, to maintain the blood-stream form of trypanosomes in culture, nor is it possible to cultivate the erythrocytic stage of the malaria parasite except for short periods of time.

Trypanosomiasis of animals is a disease of great economic importance in Africa since it makes animal husbandry impossible in enormous tracts of country where the population is chronically deficient in protein, and although trypanosomiasis of man in that continent has been largely reduced by the control of the insect vector and by the use of prophylactic drugs, outbreaks still occur. Chagas' disease in South America, caused by *Trypanosoma cruzi*, is of much greater importance than was formerly believed, and there is no really effective drug with which to treat it. Malaria eradication programmes

have been carried out successfully in many parts of the world, but it is still a disease of great importance in many of the less highly developed countries.

Little is known of the mechanisms by which trypanocidal drugs produce their inhibitory effects upon the parasite and, with the exception of proguanil and pyrimethamine which have been shown to interfere at different levels with folic acid metabolism in the malaria parasite, the mode of action of antimalarial drugs is not understood. Drug resistance is an important problem in trypanosomiasis of man and domestic animals in Africa where, according to Whiteside (1962), cattle trypanosomes readily become resistant to all existing drugs with the exception of berenil. Drug resistance also occurs in malaria parasites and the development, in South America and South-East Asia, of resistance to chloroquine, a drug used extensively in prophylaxis and treatment of the disease, has focused attention upon this problem.

As yet little is known of the changes which occur in trypanosomes when they become resistant to trypanocidal drugs and nothing is known of the changes associated with drug resistance in malaria parasites. But in addition to these important problems in human and veterinary medicine, the haemoflagellates and the plasmodia present the biologist with interesting problems in morphogenesis, since they exist in different forms in the vertebrate host and the invertebrate vector.

For the solution of all these problems, whether they be mechanisms of drug action, the development of drug resistance or the transformation of one morphological type into another with the accompanying physiological changes associated with change of host, a greater knowledge of the physiology and biochemistry of these organisms is necessary, and for this methods of cultivation of all the stages of the life cycle are essential. At present *in vitro* experiments upon blood-stream trypanosomes must be carried out upon suspensions obtained from experimentally induced infections in laboratory animals. Even under the best experimental conditions, the survival time is short, so that long-term metabolic experiments upon actively growing flagellates are not possible. In malaria the position is even less satisfactory because the plasmodia of human malaria cannot be transmitted to animals other than great apes, and species of plasmodia parasitic in monkeys, rodents and birds have to be used. Successful methods of cultivation should open up new fields of work in the study of both haemoflagellates and plasmodia.

In the following pages an attempt has been made to review some of the achievements and the difficulties encountered in work on the cultivation of haemoflagellates and plasmodia.

II. The Cultivation of Mammalian Trypanosomes

A. The Classification of Mammalian Trypanosomes

An understanding of the method of transmission of mammalian trypanosomes is of importance in assessing the problems encountered in their cultivation. With the exception of *Trypanosoma evansi* and *T. equinum* in which transmission is effected mechanically by the insect vector, and *T. equiperdum* in which transmission takes place by contact, the life cycle of

trypanosomes infecting mammals includes a cycle of development in the insect vector.

Mammalian trypanosomes have been divided into two groups, the division resting partly on differences in morphology and method of reproduction in the vertebrate host, and partly on their cycle of development in the insect vector. According to Hoare (1964, 1966), Stercoraria (formerly Section A, Hoare, 1957) includes trypanosomes in which development in the vector takes place in the posterior station, i.e. the hindgut, and transmission is contaminative. *T. cruzi*, *T. lewisi*, *T. theileri* and *T. conorhini* are placed in this group. The Salivaria (formerly Section B) includes trypanosomes in which development in the insect vector takes place in the anterior station, i.e. the salivary glands or proboscis, and transmission is inoculative. This section includes the following subgenera:

Duttonella Type species *Trypanosoma (Duttonella) vivax*. Monomorphic trypanosomes. Development in *Glossina* is limited to the proboscis.

Nannomonas Type species *Trypanosoma (Nannomonas) congolense*. Monomorphic trypanosomes. Development in *Glossina* takes place in midgut and proboscis.

Pycnomonas Type species *Trypanosoma (Pycnomonas) suis*. Monomorphic trypanosomes. Development in *Glossina* takes place in midgut and salivary glands.

Trypanozoon Type species *Trypanosoma (Trypanozoon) brucei*. This subgenus includes *T. gambiense* and *T. rhodesiense*. These three species once formed the *brucei* subgroup (Hoare, 1957) and for convenience will be referred to collectively by that term. Polymorphic trypanosomes occurring in the blood as slender, intermediate and stumpy forms. Development in *Glossina* takes place in the midgut and salivary glands.

Included in the subgenus are *Trypanosoma evansi* and *T. equinum*. Polymorphism is inconstant and there is no intermediate host, transmission being mechanical, by the bite of flies. Also included is *T. equiperdum*, which is transmitted by contact.

B. THE CULTIVATION OF TRYPANOSOMES BELONGING TO THE SALIVARIA

1. *Introduction*

Trypanosoma lewisi from the rat was the first trypanosome to be successfully cultivated. It was grown in the liquid which condensed on a nutrient agar slope to which defibrinated rabbit blood had been added (MacNeal and Novy, 1903). Whereas cultures of this species could be isolated from the rat relatively easily and maintained by subinoculation, only a few of the many attempts made to cultivate *T. brucei* were successful (Novy and MacNeal, 1904). A simplification of Novy and MacNeal's medium, without the addition of peptone or meat to the agar, was used by Nicolle (1908) for the isolation and cultivation of *Leishmania donovani*, and this medium, known as NNN (Novy-MacNeal-Nicolle) medium, consisting of a simple blood agar slope with the addition of 0·6% NaCl, has been used successfully not only for the cultivation of *Leishmania* but for *Trypanosoma cruzi* and certain non-pathogenic trypanosomes, but the cultivation of the pathogenic trypanosomes of the *brucei* subgroup has proved more difficult.

For work upon the metabolism of haemoflagellates and mode of drug action a method of continuous cultivation in a wholly liquid, defined medium (i.e. with constituents of known chemical composition) from which large numbers of organisms can easily be harvested, is desirable. Although some progress has been made, this goal has not yet been realized. A variety of media of varying degrees of complexity and containing blood, or serum and living cells, have been developed. The general principles involved in the cultivation of mammalian trypanosomes and the media most frequently used have been reviewed by Tobie (1964). It is therefore necessary to mention only the media used in relation to the problems discussed.

2. *Methods of Cultivation*

A strain of *Trypanosoma gambiense*, recently isolated from man, was cultivated by von Razgha (1929) in citrated human blood and Ringer's solution (0·6% NaCl), and a modification of this medium was used by Brutsaert and Henrard (1938) for the cultivation of *T. gambiense*, *T. brucei* and *T. congolense*, but these media have the disadvantage that they require human blood and that trypanosomes aggregate on the surface of the settled erythrocytes and are difficult to separate from them (Tobie *et al.*, 1950). Good growths of *T. gambiense* and *T. rhodesiense* have been obtained on a blood nutrient agar medium, also made with human blood, the citrated plasma being inactivated and the corpuscles washed before incorporation (Weinman, 1953). According to Weinman inactivation is important, since lysins which act upon that stage of the trypanosome life cycle that develops in culture occur in the blood of man and animals. The fact that the lytic activity was greatly reduced by heating to 56°C for 30 min suggested that it was dependent upon complement or some complement-like substance. As isolation cultures made with small inocula of infected blood succeeded more frequently than with relatively large inocula, and as infected blood from man and animals presumably contains the substance which will lyse the culture flagellates when they develop, Weinman (1960a) used polyvinyl sulphuric acid as an anticoagulant because of its high anticomplementary activity. This method allowed the use of much larger inocula than had been used previously, and positive cultures were obtained from patients whose blood had been found negative by other diagnostic methods. Experience with media containing human blood showed that the blood of different donors varied in suitability as a growth medium, blood from some donors producing a heavy growth whereas that from others produced little or none (Reichenow, 1937; Lwoff and Ceccaldi, 1939; Tobie *et al.*, 1950).

A diphasic medium consisting of a solid nutrient agar base incorporating inactivated rabbit blood and a liquid overlay consisting of Locke's solution containing 0·25% glucose has been used successfully for the long term cultivation of *T. gambiense* and *T. rhodesiense* (Tobie *et al.*, 1950). This medium has the advantage that human blood is not required for the cultivation of these two species though it is necessary for isolation and early subcultures of *T. congolense* (Tobie, 1958). A preliminary report on the cultivation of *T. rhodesiense* in large volumes of a wholly liquid medium consisting

of amino acids, vitamins and bases in a buffered physiological solution with the addition of lysed human blood, does not give details of the components (Pittam and Vickerman, 1962).

A new approach to the problem was made by Demarchi and Nicoli (1960); *T. gambiense* was grown in monolayer cultures of HeLa and HEP human epithelial cells in Lépine's medium containing casein hydrolysate and 5% calf serum. As the flagellates would not grow in Lépine's medium alone, it was concluded that the tissue cells provided necessary nutrients including haematin which is a growth factor for trypanosomes. It has been suggested that the ability of trypanosomes to grow with a particular cell line may depend upon the synthetic capacity of the tissue cells (Guttman and Wallace, 1964). Two strains of *T. gambiense* have been grown in tissue culture with KB human cells in a medium containing casein hydrolysate and serum, and some growth was obtained in cultures of chick fibroblasts (Fromentin, 1961).

Although it is thus possible to grow strains of certain pathogenic trypanosomes belonging to the Salivaria in culture medium indefinitely, these methods have a serious limitation; the organisms which grow are not the blood-stream trypanosomes but resemble the forms found in the midgut of the insect vectors.

3. *Effect of the Duration of Passage through Laboratory Animals by Syringe on the Capacity of Trypanosomes of the* brucei *Subgroup to Grow in Culture*

Trypanosomes of the *brucei* subgroup (i.e. *Trypanosoma gambiense, T. rhodesiense* and *T. brucei*) are polymorphic, occurring in the blood-stream of the mammalian host in three main types: slender forms with a long free flagellum, short stumpy forms with no free flagellum and intermediate forms with a free flagellum of intermediate length. In all three forms the kinetoplast is close to or not far removed from the posterior end of the body. It is well established that polymorphic trypanosomes, when maintained in the laboratory by syringe passage, become monomorphic, the only form present being long, slender forms (Bruce, 1914; Fairbairn and Culwick, 1947; Ashcroft, 1960).

In attempting to obtain cultures of trypanosomes of the *brucei* subgroup the period of time in which a strain has been maintained by syringe passage is an important factor in success or failure. Von Razgha (1929) obtained cultures of a strain of *T. gambiense* recently isolated from man, but only one out of five attempts succeeded with a strain which had been maintained in rats for 8 years. Attempts to cultivate a strain of *T. rhodesiense* maintained in laboratory animals for 17 years also failed, and only one out of twelve attempts succeeded with a second strain maintained in animals for 5 years. Reichenow (1932) found that whereas a strain of *T. gambiense* was readily cultivated in von Razgha's medium soon after its isolation from man, the capacity to grow in culture was lost after it had been maintained in laboratory animals for 3 years. Weinman (1953) confirmed these earlier observations; thus, whereas strains of *T. gambiense* and *T. rhodesiense* recently isolated from man and maintained in laboratory animals could readily be cultivated from the blood, after repeated syringe passage over periods varying from 16

months to 3 years they would no longer grow in media which had given successful cultures in earlier passages.

Evidence that it is not maintenance in an abnormal host which brings about this change was provided by Tobie *et al.* (1950); a strain of *T. rhodesiense* which had been maintained in rats by cyclical transmission (i.e. by tsetse fly) was successfully isolated and maintained in their diphasic medium some 13 years after it had been isolated from man, whereas it was not possible to establish cultures of an old laboratory strain which had been maintained by syringe passage.

On ingestion by the tsetse fly, the blood-stream trypanosomes pass into the midgut where they develop into forms in which the undulating membrane is less pronounced and the kinetoplast and the point of origin of the flagellum lie about half-way between the nucleus and the posterior end of the body. From the midgut they migrate to the proventriculus where they become elongated, and finally become established in the salivary glands. Here crithidial forms are produced in which the kinetoplast and the point of origin of the flagellum are anterior to the nucleus. The crithidia are attached to the walls of the glands. Infective metacyclic trypanosomes develop from the crithidial stage and lie freely in the lumen of the gland.

Duke (1923) found that strains of trypanosome maintained in animals by direct transmission (interrupted feeding or syringe passage) lost their ability to develop in the tsetse fly, or, if able to develop in the gut, failed to produce infections in the salivary glands. Duke's observations were confirmed by Murgatroyd and Yorke (1937). A strain of *T. brucei* isolated from *Glossina morsitans* and subsequently maintained by passage through guinea-pigs, partly by blood inoculation and partly by cyclical transmission through the fly, remained transmissible by the fly for at least 4 years before becoming non-transmissible. The immediate loss of infectivity to flies is not complete but proceeds by stages, the trypanosomes first losing their capacity to migrate from the proventriculus to the salivary glands and later becoming incapable of extending from the gut to the proventriculus. Von Razgha and Reichenow were of the opinion that the failure of syringe-passaged strains to infect flies and to grow in culture are related phenomena. As the stage of the trypanosome developmental cycle which grows in culture media corresponds to the gut stage (p. 97), it seems possible that strains which are no longer able to develop in the salivary glands and are thus non-transmissible, but which are capable of development in the midgut, might grow in culture.

The development, in recent years, of low temperature preservation of trypanosomes makes it possible to maintain strains without constant animal passage and thus provides suitable material for the study of problems of cultivation (Polge and Soltys, 1957; Walker and Ashwood-Smith, 1961).

Cultures of the monomorphic trypanosomes, *T. congolense*, which causes disease in African cattle, have been obtained from old strains maintained in the laboratory for 9 years (Reichenow, 1934) and 5 years (Tobie, 1958).

4. *Infectivity of Cultures*

a. Infectivity and the type of organism developing in the culture. Early

workers observed that cultures of *Trypanosoma lewisi* retained their infectivity to rats (MacNeal and Novy, 1903; Smedley, 1905), but although Novy and MacNeal (1904) and Behrens (1914) were able to infect mice with strains of *T. brucei* grown on blood agar, the experience of most workers has been that trypanosomes of the *brucei* subgroup rapidly lose their infectivity to mammals when grown in non-living culture media, and similar observations have been made upon *T. gambiense* and *T. rhodesiense* maintained on tissue culture (Demarchi and Nicoli, 1960; Fromentin, 1961).

In all early attempts to grow trypanosomes from mammalian hosts in culture, and, with certain exceptions, in later work upon the cultivation of these organisms including their cultivation in tissue culture (Demarchi and Nicoli, 1960), the optimum temperature for growth has been found to lie between 22° and 28°C—when the temperature is raised to 37°C the trypanosomes rapidly die. The reason for this was first suggested by some observations made by Gray and Tullock (1905) who, in attempting to grow *T. gambiense* in Novy and MacNeal's medium, found that the flagellates resembled certain forms which they had observed in the gut of the tsetse fly. Their observation was confirmed by Thomson and Sinton (1912) who found that flagellates occurring in culture were similar to those described by Bruce *et al.* (1911b) from the gut of *Glossina palpalis*.

The transformation in culture of the blood-stream form of trypanosome into forms resembling stages of development in the insect vector does not apply only to members of the Salivaria: trypanosomes classed in the Stercoraria are also transformed into flagellates similar to stages occurring in the insect vector. In culture *Trypanosoma lewisi* assumes a crithidial form, in which the kinetoplast and origin of the flagellum are situated in the middle of the body anterior to the nucleus; this corresponds to the crithidial stage occurring in the hindgut of the vector, the rat flea, *Ceratophyllus fasciatus*. Nor is this transformation limited to trypanosomes of warm-blooded hosts; the flagellates which developed in cultures of *Trypanosoma inopinatum* from *Rana esculenta* and *Trypanosoma granulosum* from the eel were similar to stages found in the leech (Ponselle, 1923a,b). Reichenow (1932) observed that the blood-stream forms of *T. gambiense* rapidly disappeared when inoculated into von Razgha's medium and incubated at room temperature, and were replaced by trypanosomes similar to those found in the midgut of *Glossina*; crithidial forms and metacyclic trypanosomes similar to the stages of development in the salivary glands of the tsetse fly failed to appear. The metacyclic trypanosome is the only stage of development in the tsetse fly which is capable of producing an infection when injected into a susceptible host (Bruce *et al.*, 1911a; Robertson, 1913), and metacyclic trypanosomes of *Trypanosoma rhodesiense* have been shown by Gordon and Willett (1958) to change into trypanosomes of the slender blood-stream type when incubated at 37°C in a solution of glucose in Ringer's solution and inactivated serum, though further culture was not achieved. It has been generally assumed that the absence of infective metacyclic trypanosomes explains why cultures of trypanosomes of the *brucei* subgroup are not infective to mammals. The opinion of Weinman (1953), however, is that forms indistinguishable from metacyclic

trypanosomes do occur in cultures of *T. rhodesiense* and *T. gambiense* so that the loss of infectivity cannot be attributed to their absence. Lehman (1961) observed trypanosomes which were indistinguishable from the metacyclic trypanosomes found in salivary glands, in cultures of recently isolated strains of *T. rhodesiense*, but the cultures did not produce infections in mice.

The results of experiments upon the infectivity of cultures of *T. congolense* are conflicting. This trypanosome differs from trypanosomes of the *brucei* subgroup in that it is monomorphic in the blood-stream of the mammalian host and completes its development in the proboscis of the fly, not in the salivary glands. Brutsaert and Henrard (1936) reported that they were able to infect two goats with a strain maintained in culture for 156 days but infective stages were not observed in cultures maintained in the diphasic medium of Tobie *et al.*, and a strain which had been maintained in culture for periods varying from 1 to 306 days failed to produce infections when injected into rats (Tobie, 1958).

Trager (1959a, b) made a new approach to the problem of *in vitro* development of the metacyclic trypanosomes with insect tissue culture. *T. vivax*, a monomorphic trypanosome which causes disease in domestic animals in Africa and Central and South America, differs from trypanosomes of the *brucei* subgroup in that its development in the fly is restricted to the proboscis. A recent attempt to grow it in a modification of Brutsaert and Henrard's (1938) medium and the medium of Tobie *et al.* failed, although good growths of *T. congolense* and *T. rhodesiense* were obtained in the latter medium (Hawking, 1962). It was suggested by Trager (1959b) that the reason why *T. vivax* had not been grown previously in culture might be related to the absence of a midgut stage in its development, as this is the stage which develops in the media normally used.

The trypanosomes were isolated and maintained in cultures of the alimentary tract and salivary glands from late pupae and newly emerged adults of *Glossina palpalis* in a complex medium containing sheep serum and pupal extract. Initial cultures were only successful when incubated at 30–32°C and when the inoculum was obtained from a sheep with a chronic infection of long standing; attempts to isolate strains from the blood of recently infected sheep with heavy infections failed. No growth occurred in the absence of living tissue. Crithidial forms and trypanosomes similar in appearance to the metacyclic forms observed in the proboscis of the fly developed in the cultures but they were not infective to sheep. When the cultures were incubated overnight at 38°C some of the trypanosomes survived and two of several sheep inoculated with the cultures subsequently showed trypanosomes in their blood. One of the sheep, in which trypanosomes were observed, had been born and reared in the stock farm at Vom in Northern Nigeria, where the experiments were carried out. Since this is a tsetse-free area, it was known that this sheep had never previously been exposed to infection.

The conditions required to induce infectivity in the cultures could not be precisely defined, but Trager was of the opinion that the presence of living fly tissue, a complex medium containing pupal extract and a short exposure to 38°C might all play some part in inducing the change.

A strain of *T. brucei* maintained in rats, but which was still infective to tsetse flies, was grown in tsetse fly tissue culture at 28°C (Trager, 1959b). The flagellates were mostly midgut and proventricular forms, but a few crithidia were seen. Although forms resembling metacyclic trypanosomes developed after the culture had been exposed to 38°C for 19 h, they were not infective to rats. The trypanosomes had been only 5 days in the gut of the fly and 21 days in culture prior to inoculation into rats, and it was suggested that a total of 26 days might not have been sufficient to permit the development of infective metacyclic trypanosomes. In tsetse flies the rate of the completion of the cycle of development is variable even at a given temperature (Buxton, 1955), but the factors determining it are not known.

A strain of *T. congolense* was also grown in tsetse fly tissue culture, but although forms resembling metacyclic trypanosomes developed in the cultures they did not produce infections in sheep. The effect of increased temperature was not tested on this strain.

Nicoli and Vattier (1964) obtained cultures of a strain of *T. rhodesiense*, which had been maintained in rats for ten to twenty passages since its isolation from man, in tissue cultures from pupae of *Glossina fuscipes quanzensis*. The cultures were incubated at 24–28°C. Metacyclic trypanosomes were not observed and the cultures were not infective to rats. The effect of increased temperature was not studied, however.

b. Attempts to induce infectivity in cultures of trypanosomes of the brucei *subgroup.* Attempts have been made to induce infectivity in cultures of mammalian trypanosomes by the addition of substances to the medium. The importance of a factor in the medium on the transformation of crithidia into trypanosomes has been demonstrated in cultures of *Trypanosoma mega* from *Bufo regularis*. When this organism was maintained in a serum-free medium consisting of inorganic salts, glucose, "Bacto tryptose Difco", "Oxoid" liver infusion and haemin (Boné and Steinert, 1956), it grew in the crithidial form, but when the organisms were inoculated into a medium containing serum some of the crithidia were transformed into trypanosomes. The competence of the culture to transform increased towards the end of the exponential growth phase, but not all the crithidia were capable of transforming (Steinert and Boné, 1956; Steinert, 1958a). Steinert (1958b) concluded that in *Trypanosoma mega* the transformation of the crithidial to the trypanosome stage is controlled by at least two factors—a factor in serum, identified as urea, and an endogenous factor in the flagellate which determines its sensitivity to the exogenous factor.

Since the infective metacyclic trypanosomes of the *brucei* subgroup are produced in the salivary glands, a greater knowledge of the conditions to which the flagellates are exposed in the glands should prove useful in identifying the factors bringing about the transformation of the proventricular trypanosomes into crithidial forms and crithidial forms into metacyclic trypanosomes. These stages of development are bathed in saliva and this secretion must therefore contain the nutrients essential for their growth and transformation. That substances are used up during the development of the trypanosomes is indicated by the observation that, whereas saliva of uninfected

tsetse flies stains reddish to pink with Giemsa stain, this characteristic is lost as infection in the glands develops. An attempt has been made to analyse the constituents of the saliva of *Glossina palpalis* by histochemical methods, and to determine the changes occurring in it as the infection develops (Fairbairn and Williamson, 1956). Positive reactions were obtained for protein and mucoprotein (containing a neutral mucopolysaccharide moiety) or both, glycolipid (containing unsaturated bonds) and ionized calcium. The loss of Giemsa staining of the saliva was accompanied by the disappearance of the positive reactions for these substances. Analysis by paper chromatography led to the identification of a number of amino acids, inositol and possibly arabinose. The carbohydrate analysis demonstrated the absence of all the common hexoses (Williamson, 1956).

The results of experiments by Weinman, and Geigy and his colleagues attempting to produce infective metacyclic trypanosomes in cultures of the *brucei* subgroup demonstrate the complexity of the problem. Cultures of *Trypanosoma rhodesiense* and *T. gambiense* recently isolated from man and maintained in Weinman's blood agar medium to which had been added trehalose, a disaccharide present in significant concentrations in the intestine of *Glossina morsitans* and *G. brevipalpis*, proved to be infective to mice whereas control cultures, maintained in the same medium without trehalose, were not (Weinman, 1957; Geigy *et al.*, 1959; Weinman, 1960b). However, as strains which had responded favourably to trehalose suddenly ceased to respond, it seemed improbable that it was the only factor involved in inducing infectivity. Moreover, Lehman (1961) was unable to induce infectivity to mice in cultures of two recently isolated strains of *Trypanosoma rhodesiense* by the addition of trehalose, and doubt on the part played by this sugar was also cast by the work of Bowman *et al.* (1960) who were unable to induce infectivity to rats in *T. gambiense* and *T. rhodesiense* grown in media containing it. Their strains, however, differed from those of Weinman in that they were old laboratory strains. The flagellates were unable to utilize the sugar, and, moreover, the trehalose was hydrolysed to glucose by trehalase, an enzyme present in the blood-containing media.

In a few instances cultures of *T. rhodesiense* and *T. brucei* proved to be infective to mice when grown on Weinman's medium to which inositol had been repeatedly added, but the results were sporadic and the incubation period in the mice very prolonged (Geigy and Kauffmann, 1964). In a further series of experiments with recently isolated strains the surprising observation was made that untreated control cultures proved to be infective to mice. In one series fifteen out of sixty untreated cultures proved to be infective. Further investigations showed that the age of the culture was an important factor in determining infectivity, the optimal period being about the 18th day of incubation; but since infectivity was completely lacking in some of the series it was obvious that other factors were involved. Since the only known variable in the medium was the source of the human blood, the suitability of different donors was investigated and it was found that whereas the blood of some donors produced a proportion of infective cultures that of others produced none, or only did so after prolonged storage at low temperature.

Of the 803 cultures constituting thirty-six separate series, 10% were infective (Amrein *et al.*, 1965). This is a high rate compared with that of natural infections of the *brucei* subgroup in the salivary glands of tsetse flies, which, according to Gordon and Willett (1958), is only about 0·25%. It is to be hoped that these interesting observations will be followed up and the active factors in the blood determined.

5. *Relationship of Culture forms to Blood-stream Forms of Trypanosomes of the* brucei *Subgroup*

a. The physiology of culture and blood-stream forms. The change from a warm-blooded host to life in the insect vector involves changes in the environment of the flagellate which presumably include a change in oxygen tension and a more varied and, generally, lower temperature. As the tsetse fly will survive and the parasite develop in it at temperatures varying between 26° and 37°C, and as in nature the fly must occasionally experience environmental temperatures only a little below blood heat (Gordon and Willett, 1958), it seems unlikely that temperature is the only factor involved in the conversion either of metacyclic trypanosomes into blood-stream forms or of blood-stream trypanosomes into the midgut form in the fly.

Comparative studies of the physiology of culture forms and blood-stream forms of the *brucei* subgroup show that differences occur in their respiration and metabolism. In the experiments upon which this work is based the blood-stream trypanosomes were old laboratory strains which had been maintained by syringe.

It has long been known that consumption of sugar is extremely high in the blood-stream form of African pathogenic trypanosomes (von Brand, 1951). The culture forms consume much less glucose and oxygen than the blood-stream forms, even allowing for the difference in environmental temperature (von Brand *et al.*, 1955). Respiration of blood-stream trypanosomes of the *brucei* subgroup is insensitive to cyanide and no cytochrome pigments have been detected in them (von Brand and Johnson, 1947; Ryley, 1956; Fulton and Spooner, 1959). Under aerobic conditions intact blood-stream trypanosomes degrade glucose or glycerol only as far as pyruvic acid and the respiratory quotient (R.Q., i.e. the ratio of the volume of carbon dioxide evolved to the volume of oxygen consumed) is extremely low. Under anaerobic conditions glycerol and pyruvate accumulate in the presence of glucose. Moulder (1950) pointed out that in blood-stream trypanosomes of this group the cytochrome system must have been replaced by a new respiratory enzyme system capable of being oxidized as fast as or faster than the cytochrome system. Evidence has been produced that the major part of the respiration of these forms is effected by a L-α-glycerophosphate-oxidase-L-α-glycerophosphate dehydrogenase system which is insensitive to cyanide (Grant and Sargent, 1960, 1961; Grant *et al.*, 1961).

In the culture forms a well-developed cytochrome spectrum has been observed, and respiration is very sensitive to cyanide (von Brand *et al.*, 1955;

Fulton and Spooner, 1959; Ryley, 1961, 1962). Under aerobic conditions glucose and glycerol are oxidized, with a high R.Q., mainly to carbon dioxide; under anaerobic conditions they are converted into succinic and acetic acids and carbon dioxide is assimilated. Unlike the blood-stream forms they are able to utilize Krebs cycle intermediates.

It has been suggested by Grant and his co-workers (Grant *et al.*, 1961) that the change from the mammalian host to the insect vector, and presumably to the culture form, might involve an adaptive respiratory change similar to that described in *Pseudomonas fluorescens*. When grown at high oxygen tensions only traces of cytochrome pigment were detected in this organism, whereas at low oxygen tensions the amount of cytochrome pigment was relatively large (Lenhoff *et al.*, 1956).

As yet nothing is known of the physiology of the crithidial and metacyclic trypanosomes which develop in the salivary glands. Unless some method can be devised by which these stages can be grown in pure culture, histochemical techniques offer the only mode of attack on this problem.

b. The relationship of the ultrastructure and physiology of trypanosomes of the brucei *subgroup to their ability to grow in culture.* The study, in recent years, of the ultrastructure of blood-stream trypanosomes and culture forms of the *brucei* subgroup has thrown some light upon the problem of why old monomorphic laboratory strains fail to establish themselves in culture media or in the tsetse fly. It has long been known that the kinetoplast gives a positive reaction for DNA. Electron microscopy has revealed this organelle as a disk-shaped mitochondrion surrounded by a double membrane from which cristae may project into the lumen, the mitochondrial matrix being continuous with a tubular canal which extends anteriorly through the body of the flagellate (Steinert, 1960; Baker *et al.*, 1961; Mühlpfordt and Bayer, 1961). Cristae are poorly developed or absent from the mitochondrial tube of old laboratory strains (Mühlpfordt and Bayer, 1961; Vickerman, 1962), whereas in culture forms the chondriome is much more complex consisting of one or more sinuous tubes which arise from the kinetoplast and ramify thoughout the anterior end of the flagellate and a similarly convoluted but posteriorly directed mitochondrion which also arises from the kinetoplast. Cristae are much more conspicuous in the mitochondria of culture forms than in those of monomorphic blood-stream trypanosomes of old laboratory strains maintained by syringe passage (Vickerman, 1962).

The mitochondria in cells of higher animals are known to be the site of complex enzymatic systems necessary for aerobic respiration, including the Krebs citric acid cycle and the cytochrome system. The fact that Krebs cycle enzymes and cytochrome pigments have not been found in monomorphic trypanosomes of old laboratory strains of the *brucei* subgroup but were present in culture forms suggests that the chondriome is inactive in the monomorphic trypanosomes whereas it is active in culture forms (Vickerman, 1965).

Steinert (1960, 1964) applied the NAD-diaphorase reaction, which is an indicator of mitochondrial activity, to the culture (i.e. crithidial) forms of *Trypanosoma mega* and found that it coloured an elongated structure which

was similar in shape and position to the mitochondrion as revealed by electron microscopy. Using this reaction, Vickerman (1965) has found that whereas old laboratory strains of *T. brucei* showed no activity in the mitochondrion, intense activity was demonstrated in the mitochondria of intermediate and short stumpy forms in recently isolated polymorphic strains, though slender forms resembled monomorphic forms in showing no activity. It has been suggested by Vickerman that the reason why polymorphic trypanosomes of the *brucei* subgroup are able to establish themselves in the midgut of the fly (and by analogy in culture media) is that they are preadapted to the mode of respiration required in their new environment. Although the changes which occur when polymorphic trypanosomes are incubated in culture media have not been described, the changes which occur when tsetse flies are fed upon a mammalian host with a polymorphic infection have been described by Wijers and Willett (1960). When *Glossina palpalis* was fed on monkeys infected with polymorphic strains of *Trypanosoma gambiense* the incidence of short stumpy forms in the blood meal and the rate of infection in the fly were directly related. When the fate of the blood meal in the gut was followed, a series of transitional stages between the stumpy forms and the gut forms were observed. Transitional stages between intermediate forms and gut forms were occasionally seen, but transitional stages between long slender forms and gut forms were never observed, though the long slender forms could change in the gut of the fly into stumpy forms which might later become gut forms.

If the analogy between the ability of trypanosomes to infect susceptible flies and to grow in culture is a true one, then polymorphic strains with a high proportion of stumpy forms, in which the mitochondria are active, should prove the most successful for establishing cultures, which agrees with previous experience that cultures can be obtained from newly isolated strains but not from old strains maintained by syringe passage. But, by analogy, the possibility cannot be excluded that long slender forms, in which mitochondrial activity cannot be demonstrated, may develop in culture media provided that they can make the necessary respiratory switch which presumably they make if they change into short stumpy forms in the gut of the fly. This might account for the occasional successful culture reported from old laboratory strains.

Since in comparative studies of the physiology of culture and blood-stream trypanosomes of the *brucei* subgroup the blood-stream trypanosomes were old laboratory strains, work upon the physiology of recently isolated polymorphic strains seems desirable. More than half a century ago David Bruce (1914) remarked that it was "as dangerous to classify trypanosomes from old laboratory strains, as it would be to attempt to give the natural history of the blue rock pigeon from a study of our tame varieties". Evidence from recent work upon ultrastructure suggests that it would be dangerous also to assume that the physiology of old monomorphic strains is similar to that of recently isolated polymorphic strains.

Unlike the blood-stream forms of the monomorphic strains of polymorphic trypanosomes, the monomorphic species *T. vivax* and *T. congolense* have well-developed cristae and gave a positive diaphorase reaction indicating mitochondrial activity (Vickerman, 1965).

6. *Relationship of the Ultrastructure of Akinetoplastic Trypanosomes to the Problem of their Cultivation*

Although the presence of a kinetoplast close to the origin of the flagellum is a characteristic of all members of the Trypanosomatidae, it is well known that this organelle is not essential to the survival of trypanosomes in the blood-stream of the vertebrate host. Akinetoplastic trypanosomes may be present in varying proportions in strains of *Trypanosoma vivax*, *T. congolense*, *T. evansi* and trypanosomes of the *brucei* subgroup; *T. equinum*, which causes the disease of mal de cadaras in horses in South America, is totally lacking in a kinetoplast. Moreover akinetoplastic trypanosomes may be produced by the *in vivo* treatment of kinetoplastic strains with certain dyestuffs. The problem has been reviewed by Hoare (1954).

T. equinum, and *T. evansi*, which may produce akinetoplastic infections in camels, have lost the cycle of development in the fly and are transmitted mechanically by biting fly. Working on the analogy of the relationship between infectivity of trypanosomes to tsetse flies and their ability to develop in culture, Reichenow (1940) investigated the ability of akinetoplastic trypanosomes to grow in culture. A freshly isolated strain of *T. gambiense* which could readily be grown in culture and which was infective to *Glossina* was treated with trypaflavine *in vivo*. Although it was not possible to produce a completely akinetoplastic strain, 70% of trypanosomes in the treated strain were akinetoplastic; but in spite of the high proportion of akinetoplastic trypanosomes in the inoculum, all the flagellates which developed in the culture had a kinetoplast. He therefore concluded that akinetoplastic trypanosomes were incapable of developing in culture, and, by analogy, in *Glossina*. Attempts made by Mühlpfordt (1963) to grow akinetoplastic strains in culture also failed, and there is no report of the cultivation of these forms by other workers.

Electron microscopy has shown that the kinetoplast is present in so-called akinetoplastic strains, but differs from that of normal strains in that the dense central mass which gives a positive Feulgen reaction for DNA and stains with the conventional stains used in light microscopy, is absent (Mühlpfordt, 1963; Vickerman, 1963, 1965). Akinetoplastic trypanosomes were found to retain a "membraneous ghost" of a mitochondrion but no indication of enzymatic activity could be detected by the NAD diaphorase reaction (Vickerman, 1963, 1965). These observations therefore suggest that the inability of akinetoplastic trypanosomes to grow in culture is due to their lack of a functional mitochondrion, and the resulting inability to switch to the type of respiration which that environment demands.

7. *Electric Charge upon Trypanosomes of the* brucei *Subgroup at Different Stages of the Life Cycle*

Many years ago the electric charge carried by different stages of development of trypanosomes of the *brucei* subgroup was determined by a microcataphoretic technique, and also by a simple "salt concentration" test in which positively charged trypanosomes adhered to the red cells of the test system whereas negatively charged trypanosomes remained free. It was

observed that blood-stream trypanosomes of old laboratory strains of *T. brucei*, *T. gambiense* and *T. rhodesiense* might be either positively or negatively charged, in some cases negatively and positively charged trypanosomes being present in the blood of an animal simultaneously (Broom *et al.*, 1936).

When tsetse flies were fed upon animals infected with a strain of *T. brucei* which was positively charged in the blood, the trypanosomes retained the charge for the first 12 h, but thereafter negatively charged trypanosomes appeared and increased in number until after 72 h only negatively charged trypanosomes could be found in the gut. On the other hand, trypanosomes negatively charged in the blood retained the negative charge in the gut of the fly (Broom and Brown, 1937). A strain of *T. rhodesiense* which had carried a negative charge in the blood-stream remained negatively charged over a period of 3 months when grown in culture (Brown *et al.*, 1936).

Whereas the charge on gut and culture forms was consistently negative, a change in charge took place between the midgut and salivary glands, since the developmental stages in the glands were consistently positively charged irrespective of the charge originally carried by the the blood forms (Broom and Brown, 1939).

More recently, the electrophoretic mobility of culture and blood-stream stages of *T. rhodesiense* has been studied by the method described by Bangham *et al.* (1962), and it was confirmed that the culture forms carried a negative charge. The blood-stream trypanosomes were usually found to carry a small negative charge. The observation that the addition of uranyl ions caused a charge-reversal with the culture forms was interpreted as indicating that the surface of the organism is phosphatide in character; but since these ions did not appear to have any effect upon the electrophoretic mobility of blood-stream trypanosomes, it was suggested that their surface differs from that of the culture forms and might be of a polysaccharide or polypeptide nature. It was also suggested that the difference in electrophoretic behaviour might be associated with a difference in response to drugs, since the trypanocidal compounds prothidium, quinapyramine and homidium decreased the negative electrophoretic mobility of culture forms whereas it had no effect upon blood-stream forms (Hollingshead *et al.*, 1963).

8. *The Problem of the* in vitro *Study of Drugs upon Trypanosomes*

a. The in vitro *action of drugs upon blood-stream and culture trypanosomes.* The preliminary screening of new synthetic compounds for activity against trypanosomes is usually carried out in experimentally induced infections in mice, or, less frequently, in rats; but although such problems as the relationship of the activity of the drug upon the trypanosome to its toxicity in the mouse, the concentration of the drug in the blood-stream required to produce an inhibitory action upon the development of infection, and the ability of the drug to produce drug-resistant strains can be studied by *in vivo* methods, *in vitro* methods are required for a study of the mode of action of the drug.

Although the blood-stream trypanosomes cannot be maintained in culture, a method for studying the *in vitro* action of trypanocidal compounds upon blood-stream trypanosomes was devised by Yorke and his co-workers as

early as 1929 (Yorke *et al.*, 1929) and has been described in detail by Hawking (1963a). A suspension of a known number of trypanosomes, obtained from infected blood, is added to a fluid medium consisting of serum and Ringer's solution containing 0·2% glucose to which the drug is added to give the required concentration. The suspensions are incubated at 37°C and counts of living trypanosomes in a known volume of the medium are made at intervals and compared with counts made from control suspensions which are similar except for the absence of the drug. In tests carried out by this method the organisms are not in a state of active growth, division does not occur and the period of their survival does not exceed 24 h. The test, therefore, has a very limited application. The action of drugs which are trypanostatic and act by depressing the growth rate of the organisms cannot be studied by this method. For the study of such drugs a suitable culture method which allows the organisms to multiply freely, is required.

Since important physiological differences have been found between blood-stream trypanosomes of old laboratory strains and the "insect" forms which grow in culture media, the important question arises whether a study of the action of drugs upon culture forms would yield information of value in relation to the blood-stream trypanosomes. As yet little has been done upon this problem.

Hawking (1963b) has studied the action of trivalent tryparsamide, berenil, quinapyramine (antrycide), the phenanthridinium compounds homidium, prothidium and *iso*metamidium, and suramin upon cultures of *T. rhodesiense* and *T. congolense*. The tests were carried out upon strains growing at 26°C in Brutsaert and Henrard's (1938) medium and in Tobie *et al.*'s medium (1950), no difference being found between results in the two media. The compounds tested fall into two groups according to their chemotherapeutic action; trivalent tryparsamide and berenil are directly trypanocidal killing blood-stream trypanosomes rapidly both *in vitro* and *in vivo*, whereas the remaining compounds are not trypanocidal *in vitro* and act *in vivo* only after an interval of 1–3 days. The 24-h *in vitro* test upon blood-stream trypanosomes therefore is not applicable to drugs of the latter group. Homidium, prothidium *iso*metamidium and suramin, in concentrations very much greater than the calculated minimum effective concentration in mice, were completely inactive upon the culture forms of both species when incubated at 26°C for 4 days, but quinapyramine had a slight trypanostatic action. The minimum concentration of berenil required to kill culture forms of *T. rhodesiense* at 26°C in 4 days was about 0·1 mg per ml, and to kill blood forms at 26°C in 1 day, about 0·01 mg per ml. In the tests upon trivalent tryparsamide a very marked difference was observed between the response of the culture forms and the blood-stream trypanosomes. At 26°C the minimum concentration of trivalent tryparsamide required to kill culture forms of this species in 4 days was approximately 0·01 mg per ml whereas the minimum concentration required to kill blood forms in 1 day at 26°C was about 0·00025 mg per ml and about 0·000016 mg per ml at 37°C. Since both media used in the culture tests contained particulate matter in the form of blood cells, part of the drug may have been absorbed on the solid constituents so that the actual concentration

bathing the organisms may have differed significantly from the estimated concentration. Until a wholly liquid medium has been developed for the cultivation of the "insect" forms, it is not possible to estimate their *in vitro* sensitivity to drugs accurately.

b. The use of cultures of the trypanosomid flagellate Crithidia oncopelti *in studies of drug action and drug resistance.* The difficulties encountered in attempting to cultivate the pathogenic trypanosomes has directed attention to the trypanosomid flagellates of the genus *Crithidia* as experimental tools for metabolic studies and studies on the mode of action of trypanocidal drugs. *Crithidia fasciculata* (*Herpetomonas culicidarum*) isolated from the gut of the mosquito by Noguchi and Tilden in 1926, was first grown in a complex, defined medium by Cowperthwaite *et al.* (1953). Subsequently, *Crithidia* (*Strigomonas*) *oncopelti*, a flagellate parasitic in the gut of Hemiptera and in latex plants, and isolated in axenic culture, i.e. without other living organisms, from the gut of the insect by Noguchi and Tilden, has been grown in a simple, chemically defined medium by Newton (1956, 1957a). This medium contained methionine, thiamine, nicotinamide, *p*-aminobenzoic acid, glucose, inorganic salts and trace metals. It had been observed by Lwoff (1937) that *C. oncopelti* did not require haematin when grown on peptone medium, and this observation has been confirmed by Newton in the defined medium.

C. oncopelti has proved sensitive to homidium (ethidium) bromide (Newton 1957b), quinapyramine (antrycide) (Newton, 1958), prothidium and pentamidine (Newton, 1964), compounds which are used in the treatment of animal or human trypanosomiasis. In logarithmically growing cultures of *C. oncopelti* in a peptone glucose medium concentrations of 20–200 μg per ml homidium bromide lead to a progressive decrease in growth rate (Newton, 1957b) and a concentration as low as 3 μg per ml of quinapyramine resulted in an immediate change from logarithmic to linear growth (Newton, 1964). The results of experimental work upon *C. oncopelti* suggest that quinapyramine does not act primarily on the synthesis of DNA or DNA-primed RNA, but that it reduces the growth rate by inactivating cytoplasmic ribosomes. The inhibitory effect of quinapyramine upon *C. oncopelti* by depressing the growth rate is in agreement with what is known of its action upon trypanosome infections in the mammalian host. It has been shown that an effective dose of the compound may take 2–4 days to clear the infection from the blood of the mouse and that the trypanosomes continue to multiply for 24–72 h before they begin to diminish in numbers and finally disappear (Ormerod, 1951; Hawking and Sen, 1960). The experiments of Sen *et al.* (1955), in which the clearance of *Trypanosoma evansi* infections in splenectomized rats treated with quinapyramine was much slower than in intact rats treated with the drug, suggest that the defence mechanisms of the host play a part in overcoming the infection. The results of the experiments with *Crithidia oncopelti* agree with the hypothesis that quinapyramine is not trypanocidal, but acts by reducing the growth rate of the trypanosomes to a level sufficiently low to enable the defence mechanisms of the host to overcome the infection (Newton, 1966).

C. oncopelti has also proved to be a suitable organism for the study of drug resistance. Although attempts to produce a strain resistant to homidium

failed, a quinapyramine-resistant strain with a 100-fold enhancement of resistance was developed by serial subinoculation in the presence of increasing concentrations of the drug. The quinapyramine-resistant strain was cross-resistant to the phenanthridinium compounds, prothidium and homidium, which is in agreement with Whiteside's (1962) observations upon quinapyramine-resistant strains of *Trypanosoma congolense* and *T. vivax* (Newton, 1964). A strain has also been made resistant to pentamidine and proved to be resistant to stilbamidine and propamidine and showed some resistance to quinapyramine and homidium (Wallis, 1966).

It has been observed that trypanosomes resistant to arsenical drugs, acriflavine and stilbamidine bound less of these compounds than normal strains, and it has been suggested that resistance to these compounds is due to a decreased permeability to these compounds. The quinapyramine-resistant strain of *Crithidia oncopelti*, however, proved to be permeable to that compound and in this it resembles a quinapyramine-resistant strain of *Trypanosoma equiperdum* (Ormerod, 1952).

It has been emphasized by Newton (1964) that results obtained from experiments upon drug action using *Crithidia oncopelti* as a test organism must be confirmed by studies on blood-stream forms of pathogenic trypanosomes before any definite conclusions can be reached. It is obvious, however, that work upon problems of metabolism and drug action can be carried out upon this easily cultivated flagellate which will only be possible with pathogenic trypanosomes when methods for their cultivation equally suitable to those at present available for *C. oncopelti* have been developed. In the meantime, the correlation so far obtained between the results upon drug action and drug resistance in *C. oncopelti* and the blood-stream stage of the pathogenic trypanosomes indicates that information obtained from *C. oncopelti* may serve as a useful guide to the solution of these problems in pathogenic trypanosomes.

C. THE CULTIVATION OF TRYPANOSOMES BELONGING TO THE STERCORARIA

1. *Cultivation of* Trypanosoma cruzi *and the Effect of Temperature upon the Type of Organism which Develops*

Trypanosoma cruzi is the cause of Chagas' disease, a form of trypanosomiasis of man which occurs in South and Central America and in Mexico. It differs from trypanosomes of the Salivaria in that it does not multiply in the blood-stream but invades various tissues of the body, particularly the myocardium, where it assumes a leishmanial form and multiplies by binary fission. After repeated fission it changes into the crithidial form and finally into the trypanosome form and passes from the tissues into the blood-stream.

T. cruzi has proved easier to isolate and maintain in culture than trypanosomes of the Salivaria. It can be grown successfully upon NNN medium. Reichenow (1934) obtained good cultures of a strain, which had previously been maintained for 9 years in laboratory animals, in von Razgha's blood-Ringer medium with the addition of glucose. In culture the flagellates resemble the stages of development occurring in the gut of the vectors, the triatomid

bugs. Although the predominating forms occurring in culture were crithidial, Reichenow observed that trypanosomes similar to those occurring in the hindgut of the vector were also present, and the cultures were infective to laboratory animals. Cultures of *T. cruzi* appear to keep their infectivity to susceptible mammals indefinitely, since a strain which had been maintained in NNN medium for nearly 13 years produced infections in mice (Packchanian and Sweets, 1947).

Amongst a variety of media which have proved successful for the growth of *Trypanosoma cruzi*, a medium consisting of peptone, sodium chloride and glucose (Little and Subbarow, 1945) and a partially defined medium (Little and Oleson, 1951) have given good growths, but in both the addition of erythrocytes was essential. Like trypanosomes of the Salivaria, *T. cruzi* requires haematin. More recently, it has been maintained on a partially defined medium free from particulate matter consisting of inorganic salts, casein hydrolysate, crystalline serum albumin, haematin, a series of growth factors and Tween 80, though it was found later that albumin was not essential. Growth compared favourably with that on undefined media (Citri and Grossowicz, 1955a).

When strains of micro-organisms are isolated and maintained in culture, the problem arises whether growth on a particular medium is due to the selection of variants of less exacting nutritional requirements than the original population; thus certain strains of a species may grow well on the medium whereas others fail to grow or grow poorly. In order to discover whether such a selection had taken place when the strain of *T. cruzi* was maintained on the partially defined medium, Citri and Grossowicz inoculated washed trypanosomes of the parent strain, which had never previously been grown on a blood- or serum-free medium, into the partially defined medium when growth was equal to that of the strain which had been maintained in this medium for over 2 years; there was thus no evidence that selection had taken place. Nevertheless, Boné and Parent (1963) failed to obtain a satisfactory growth of their strain of *T. cruzi* on this medium which evidently will not support the growth of all strains of this species equally well.

Taking as a basis a heat sterilizable medium containing tryptose, liver infusion and haemin, which had maintained growth of *T. mega* from *Bufo regularis* but would not support the growth of mammalian trypanosomes, Boné and Parent found that the addition of calf serum or egg yolk produced a medium in which *Trypanosoma cruzi* would grow. A simplified modification of this medium was developed in which serum or egg yolk was replaced by sodium stearate, and liver extract by thiamine and folic acid, leaving tryptose as the only undefined constituent. A strain of *T. cruzi* was maintained on this medium for 18 months. Neither Citri and Grossowicz nor Boné and Parent comment on whether the type of flagellate which developed was exclusively crithidial, or whether the cultures were infective to susceptible mammals.

A medium consisting of liver infusion, tryptose, inactivated calf serum, and rabbit or calf haemoglobin solution in place of the haemin used by Boné and Parent, has recently been found by Fernandes and Castellani (1966) to give excellent cultures of *T. cruzi*. At the optimum temperature of 28°C the

generation time was 14 h, and a yield of about 6 g fresh weight per litre was obtained after incubation for 4 days. The liver infusion and tryptose could be replaced by lactalbumin hydrolysate, but growth was not so heavy in this medium, the generation time being 24 h.

It has been observed (Camargo, 1964) that the transformation of crithidial forms into metacyclic trypanosomes *in vitro* occurs at the end of the exponential growth phase of the culture. If crithidia were maintained in a state of constant exponential growth by frequent subculture, transformation did not occur. Moreover, if cultures in which metacyclic trypanosomes had begun to develop were subinoculated into fresh growth medium, the development of metacyclic trypanosomes ceased, whereas if subinoculation was made into an "incomplete" medium not capable of sustaining the growth of the organisms, transformation of the crithidial forms into metacyclic trypanosomes continued. Camargo suggested that the depletion of one or more factors in the medium is required for the transformation of crithidial forms into metacyclic trypanosomes. Fernandes and Castellani (1966) also observed that in their strain a small but variable proportion of metacyclic forms began to appear after the exponential growth phase had levelled out.

The intracellular leishmanial stage of *T. cruzi* has been grown in tissue culture and this work has been reviewed by Pipkin (1960). It is therefore sufficient to mention that success was first achieved by Kofoid *et al.* (1935) who inoculated embryonic heart cells of rat and mouse, grown at 37°C in rat plasma–rat embryo extract medium, with cultures of the crithidial stage which had been maintained on a blood agar medium. Leishmanial forms developed in the cells and trypanosomes similar to those found in the circulating blood appeared in the cultures. Although leishmanial forms developed when cultures made with tissue implants of rat embryo were inoculated with trypanosomes in infected mouse blood (Hawking, 1946), Neva *et al.* (1961) found this method less satisfactory than inocula of flagellates from NNN cultures.

A strain of *T. cruzi* was maintained for more than 3 years in chick embryo cells in hanging-drop cultures by the Carrel technique. When cultures of chick embryo cells infected with *T. cruzi* from NNN cultures were incubated at 38° or 39°C, the intracellular leishmanial forms multiplied by binary fission until they filled the cell and only then were they transformed into crithidia and finally into short broad trypanosomes which were liberated when the cell ruptured. The transformation into the crithidial forms began almost simultaneously in all the leishmanial forms in the cell. Development was similar to that which occurs in the mammalian host and the strain retained its infectivity to mice over the 3-year period of cultivation (Meyer and de Oliveira, 1948).

The relation of temperature to the type of parasite produced has been studied in primary cell cultures grown from tissue fragments in plasma clots, or on trypsinized cell suspensions. Human and bovine embryonic tissue and embryonic tissue from chick and rat were used. The culture fluid consisted of bovine embryonic fluids, Hanks' balanced salt solution, beef embryo extract and inactivated horse serum. The survival of flagellates from cultures of

T. cruzi grown on NNN medium, when incubated in tissue culture at 38°C, was poor and did not appear to differ significantly from that obtained in the liquid medium alone at this temperature. The optimal temperature for the infection of cells with the flagellates and the development of the leishmanial stage, surprisingly, was 33°C. At this temperature the leishmanial forms multiplied and developed into trypanosomes. At 38°C the proportion of cells infected was lower than at 33°C, and although multiplication of the leishmanial stages occurred, only rarely did trypanosomes develop in the cells. At 25° and 28°C, although the flagellates introduced with the inoculum multiplied actively in the fluid phase, few entered the cells and leishmanial stages were rare. When cultures grown at 33°C were incubated at 38°C, the intracellular trypanosomes disappeared and were replaced by leishmanial forms within 3–4 days, whereas when cultures grown at 38°C and containing a high proportion of leishmanial forms were incubated at 33°C, trypanosomes developed in the cells. Since few intracellular trypanosomes developed at 38°C, these strains evidently differed from that of Meyer and de Oliveira in which crithidia and trypanosomes developed intracellularly at that temperature when the multiplying leishmania had filled the cell. Strains of *T. cruzi* were also found to differ in their infectivity to tissue culture cells (Neva *et al.*, 1961).

Metacyclic trypanosomes form only a small proportion of the total population of flagellates present in NNN cultures. There is, as yet, no direct proof that this is the only form present in cultures which is capable of infecting tissue culture cells; such a proof would require the separation of crithidial from metacyclic forms, or comparative single cell inoculation of crithidial flagellates and metacyclic trypanosomes. Some indirect evidence is provided from experiments by Neva and his colleagues who found that older NNN cultures produced a higher rate of infection in tissue culture cells than younger cultures, an observation which must be assessed in the light of that of Camargo that metacyclic trypanosomes develop after the end of the exponential growth phase of a culture; but observations upon the infectivity of cultures of *T. avium* to canaries, which suggested that "pre-metacyclic" crithidia as well as metacyclic trypanosomes may be infective, are also of relevance to this problem (p. 115) (Baker, 1966).

In commenting on the low optimal temperature for the development of intracellular leishmanial stages, Neva and his colleagues point out that the body temperature of some of the common hosts of *T. cruzi*, such as the armadillo and the opossum, is in the range of 32–35°C.

The effect of temperature upon changes in the morphology of *T. cruzi* when growing in tissue culture has also been studied by Trejos *et al.* (1963). A line of "L" cells grown in Eagle's medium was inoculated with a strain of *T. cruzi* maintained on NNN medium, the flagellates in the inoculum being predominantly crithidial. At 37°C, when cells filled with leishmanial forms ruptured, stout trypanosomes similar to those occurring most frequently in the blood-stream were released in addition to unchanged leishmanial forms; but at 26°C leishmanial forms and intracellular stout trypanosomes were rare, and when infected cells ruptured slender trypanosomes, similar in

appearance to metacyclic trypanosomes and to forms occasionally seen in the blood-stream of the mammalian host, were liberated. At this temperature crithidia introduced in the inoculum multiplied in the fluid phase but after the 6th day their numbers began to decrease and the metacyclic type of trypanosome appeared and gradually predominated.

2. *Methods of Testing Drugs upon* Trypanosoma cruzi *Growing in Tissue Culture*

Attempts have been made to establish a satisfactory method of testing drugs upon *T. cruzi* in tissue culture. Since the reproductive phase of this organism in the mammalian host is intracellular, in order to eradicate the infection the action of drugs must be directed against this stage of the life cycle. Lock (1950) used the technique described by Hawking (1946) in which cultures of explants from the hearts of embryo mice, in plasma clots, grown in a rat serum-chick embryo extract in Tyrode's solution in Carrel flasks, were infected with *T. cruzi.* After incubation at 37°C for 10 days, drugs were added in the required concentration and allowed to remain for 48 hours after which the medium was replaced by drug-free medium. Explants were withdrawn at intervals of 1 or 2 days and examined for living flagellates in the fluid medium, or for intracellular stages after fixation and staining with Giemsa's stain.

Recently, an attempt has been made to develop a more standard method for the determination of drug action (Bayles *et al.*, 1966). Monolayer cultures of known numbers of trypsinized chick embryo cells were grown on cover-slips at 37°C for 2 days in a growth medium consisting of Morgan, Morton and Parker's No. 99 synthetic medium supplemented with calf serum. The inocula of known numbers of flagellates were obtained from NNN cultures, the proportion of metacyclic trypanosomes to crithidial forms being determined, Before the flagellates were introduced into the tissue culture preparations, the preparations were supplemented with 2% sterile overlay from NNN cultures, which was essential to growth and presumably supplied haematin. The infected tissue cultures were incubated at 33°C. At this temperature multiplication of tissue cells was effectively suspended, as verified by inspection for mitosis, without the viability of the cells being affected. Viable extracellular flagellates persisted throughout the period of observation and intracellular growth proceeded at a relatively steady rate. Criteria of growth of *T. cruzi* was assessed in terms of the numbers of extracellular flagellates, the proportion of tissue cells infected, and the rate of intracellular growth as determined by the proportion of tissue cells having more than ten intracellular parasites. In tests upon the *in vitro* action of drugs upon *T. cruzi*, the drugs were added to the cultures 2 or 3 days after infection and the effects of the drug assessed in terms of the above criteria 3 days later.

3. *Factors Affecting the Type of Organism Produced in Cultures of* Trypanosoma theileri *and* T. conorhini

Observations of interest in relation to the changes in morphology occurring in *Trypanosoma cruzi* in culture have been made upon two non-pathogenic members of the Stercoraria. *T. theileri*, which occurs in cattle and is generally

presumed to be non-pathogenic, can, like other members of the Stercoraria, readily be cultivated at 25° or 28°C in the crithidial and metacyclic forms, but trypanosomes similar to those occurring in the blood-stream do not develop and the cultures usually die out if maintained above 30–31°C. A strain was isolated in culture by Ristic and Trager (1958) from the blood of cows that showed a lowered milk production, and maintained in a medium consisting of blood lysate, glucose and whole blood at 37°C. Whereas the crithidial stage is normally not viable in cultures maintained at this temperature, it predominated in the lysate medium, but trypanosomes of the blood-stream type were also numerous. The strain was successfully carried through six subinoculations.

T. conorhini was first observed in the gut of *Triatoma rubrofasciata* and later proved to be infective to rats and mice, though its natural host is not known. Reproductive stages have not been seen in the blood-stream, though trypanosomes undergoing division have been observed in the peritoneal cavity of animals which had received an intraperitoneal inoculation with cultures containing metacyclic trypanosomes (Deane and Kirchner, 1963). It was maintained by Johnson (1947) on a medium consisting of a blood agar base overlaid with Locke's solution and the cultures were infective to rats. When cultures, maintained in this medium or in NNN, were incubated at 25° or 28°C, the predominating form for the first few days was crithidial but, as in *T. cruzi*, metacyclic trypanosomes developed as the culture aged. Although Johnson found that the cultures died out when maintained at 37°C, Desowitz (1963) has observed that whereas only crithidial forms and metacyclic trypanosomes developed in NNN cultures inoculated with gut contents of *Triatoma rubrofasciatus* if incubated at 25°C, blood-stream trypanosomes appeared if the temperature was raised to 37°C, though dividing forms were never seen and attempts to subcultivate the trypanosomes failed.

Similar observations were made by Deane and Kirchner (1963) when tissue cultures of monkey heart cells or HeLa cells growing in a medium consisting of Hank's balanced salt solution, lacto-albumin hydrolysate and calf serum were inoculated with flagellates grown on NNN medium. Unlike *Trypanosoma cruzi* no intracellular stage developed, but large trypanosomes similar to blood-stream forms developed in the liquid phase, or in the liquid medium in the absence of living tissue, in cultures incubated at 37°C but not at 28°C. However, survival was short and although this medium was enriched with haematin and glucose, subinoculations were not successful. Blood-stream trypanosomes, when inoculated into this medium and incubated at 25° and 28°C, began to change into crithidia within 3 or 4 h, but at 37°C no change took place. Both Desowitz and Deane and Kirchner concluded that temperature is an important factor in the conversion of the blood-stream trypanosomes into crithidia, though it could not be the only one since Deane and Kirchner found that this conversion did not take place unless whole blood or washed corpuscles were present.

Some observations recently made upon the cultivation of *T. avium*, a parasite of birds, are of interest to the general problem of cultivation of trypanosomes (Baker, 1966). This trypanosome, which was isolated from the

blood of *Corvus frugilegus*, was readily cultivated in the diphasic medium of Tobie *et al.*, and in a blood nutrient agar medium (4 N), at 28°C. The forms developing in culture resembled those seen in the insect vector, *Ornithomyia acicularia*, in which the cycle is similar to that of the Stercoraria. Small trypanosomes resembling metacyclic trypanosomes developed in the cultures but were more numerous and occurred more regularly in the 4 N medium than in Tobie's medium. Canaries inoculated with cultures grown in 4 N medium regularly produced heavy infections and, since the proportion of metacyclic trypanosomes to the total number of flagellates was small, it was suggested that "pre-metacyclic" crithidia as well as metacyclic trypanosomes might be infective. Canaries inoculated with cultures grown on NNN medium also became infected, but the infections were so light that they could only be demonstrated by subinoculation of blood into 4 N medium. Attempts to induce metacyclic trypanosomes to develop into trypanosomes of the blood-stream type by raising the temperature to blood heat, as in *Trypanosoma conorhini*, met with only slight success. At 40–41°C a few large trypanosomes which differed slightly from blood-stream forms appeared in some of the cultures, but their presence was very sporadic. Although, as in *T. conorhini*, temperature appears to be a factor concerned with the transformation of crithidia into blood-stream trypanosomes, as in *T. conorhini* it is not the only factor since, very rarely, trypanosomes of the blood-stream type developed at 28°C. In neither species would trypanosomes of the blood-stream type grow in media which supported the growth of the crithidial forms.

D. THE CULTIVATION OF THE GENUS *Leishmania* AND THE RELATIONSHIP OF THE STAGE OF DEVELOPMENT OF THE ORGANISM TO THE TYPE OF CULTURE MEDIUM AND THE ENVIRONMENTAL TEMPERATURE

The genus *Leishmania*, species of which cause oriental sore, kala-azar and a muco-cutaneous disease in man in South America, occurs in only two forms: the intracellular leishmanial stage in the vertebrate host and the leptomonad flagellate in the insect vector. In the cells of the vertebrate host the parasites, known as Leishman-Donovan (L.D.) bodies, are present in the form of small, round or oval bodies which contain a single nucleus and a kinetoplast. When the sandfly, *Phlebotomus*, bites an infected human being or other host, the L.D. bodies are ingested by the fly and pass into the midgut where they are transformed into leptomonads. In the leptomonad the kinetoplast and the point of origin of the flagellum lie at the anterior end of the elongated body, anterior to the nucleus. After a period of active multiplication, the leptomonads move forward into the pharynx and the buccal cavity of the fly, and infection takes place when an infected fly bites a susceptible host.

It was first observed by Rogers (1904) that flagellates developed when citrated blood from patients infected with *Leishmania donovani* was incubated at 22°C, and it was later found that the leptomonad stage could readily be cultivated on NNN medium (Nicolle, 1908); and semisolid, diphasic and wholly liquid media have since been successfully developed for its cultivation (e.g. Salle and Schmidt 1928; Senekjie, 1943; Chang, 1947; Fulton and

Joyner, 1949; Jadin and Pierreux, 1960; Lemma and Schiller, 1964). These media all contain blood or haemoglobin solution and serum. An analysis of the factors provided by the whole blood showed that, in a basal medium consisting of peptone agar solution, serum and haematin were essential to growth of *L. tropica*, *L. donovani*, *L. brasiliensis*, *L. ceramodactyli* and *L. agamae*, but that certain strains of *L. tropica*, *L. donovani* and *L. brasiliensis* required ascorbic acid in addition (Lwoff, 1939, 1951). Adler (1934), however, was able to maintain rich cultures of *L. tropica*, *L. donovani* and *L. tarentola* (from *Tarentola mauritanica*) on a Locke-serum-agar medium in which haemoglobin was present only as a contaminant of the serum in a concentration less than 0·0025%, which suggests that the haemoglobin requirement of these strains was very small.

Whereas the partially defined medium developed by Citri and Grossowicz (1955a) for the cultivation of *Trypanosoma cruzi* supported the growth of the leptomonad stage of thirty-one different strains of *Leishmania tropica*, strains of *L. donovani*, *L. brasiliensis* and *L. agamae* failed to grow on it (Citri and Grossowicz, 1955b); and although the leptomonads of *L. tarentola* from the gecko have been grown continuously on a defined medium consisting of glucose, haemin, seventeen amino acids, purines, pyrimidines, a mixture of the B vitamins and inorganic salts, this medium would not support the growth of *L. donovani* (see Trager, 1957a).

The range of temperature over which cultures of the leptomonads have been grown has varied from 19° to 32°C, but the cultures usually died if incubated at 36° or 37°C.

Cultures of the leptomonad stage of *Leishmania* are known to retain their infectivity to the vertebrate host for long periods; thus strains of *L. tropica* have proved infective to man 11 years after isolation (Nicolle, 1925) and to laboratory animals 9 years after isolation (Adler, 1934).

When cultures of leptomonads of *L. tropica* were inoculated on to a serum-agar or blood-agar slope containing pieces of surviving guinea-pig tissue, leishmanial forms developed in the midst of or within the tissue cells, whilst leptomonads were present in the liquid surrounding the tissue. This transformation of flagellates into leishmanial forms was observed at temperatures ranging from 22° to 37°C, but was most extensive at 37°C. At 37°C the leptomonads died out if surviving tissue were not present in the culture (Weinman, 1939). Living tissue was also found to have a favourable effect upon the survival at 37°C of leptomonads of *L. donovani* (Hawking, 1948). When tissue cultures from explants of infected hamster spleen were incubated at 37°C for 16 days or longer, the leishmania were liberated from the cells and formed masses of extracellular flagellates; moreover, although some of the flagellates penetrated into the cells and developed into leishmania when leptomonads from cultures maintained at 25°C were incubated at 37°C in tissue cultures made from explants of normal guinea-pig spleen, others multiplied as leptomonads in the fluid medium. Since leptomonad cultures maintained in a medium devoid of living cells die out at 37°C, it must be assumed that the metabolizing tissue cells produce metabolites necessary for the survival and growth of the leptomonads at the higher temperature.

Attempts have been made to cultivate the intracellular stage of *L. donovani* in media without living cells since such cultures would be better suited to metabolic study. Suspensions of leishmania from the spleen of infected hamsters were incubated at 37°C in a medium consisting of human erythrocyte extract and serum (Trager, 1953). Rounded aflagellate "intermediate" stages developed which multiplied, but their survival time was short. Motile leptomonads were also present in some of the cultures and survived for at least 4 days. Trager suggested that the organisms are more exacting in their nutritional requirements when grown at 37°C than are the leptomonads when grown at 28°C. Evidence for this had been found in strains of *Pasteurella pestis* growing in a defined medium; additional nutrients were required when the strains were grown at 37°C to those required for growth at 32°C (Hills and Spurr, 1952).

The transformation at 34°C of leptomonads into leishmanial forms and their subsequent growth in a cell-free medium, has been described by Lemma and Schiller (1964) in strains of *Leishmania donovani*, *L. tropica* and *L. brasiliensis*. Transformation was brought about by gradual acclimatization of the strains to higher temperatures, in a medium consisting of a nutrient-blood-agar base overlaid with Hanks' balanced salt solution. After periods of growth at 28° and 32°C, the leptomonads were transformed into leishmanial forms when the temperature was raised to 34°C. The leishmanial forms multiplied at this temperature and were similar in appearance to those occurring in the liver of infected hamsters. When cultures of *L. donovani*, which were growing in the leishmanial form at 34°C, were incubated for periods of 4 days at 24° and 34°C alternately, the leishmanial forms transformed to leptomonads within 48 h at 24°C and multiplied in that form, but transformed back to the leishmanial form when the temperature was raised to 34°C. It was suggested that increasing temperature acted as a selective agent under which only variants capable of living at the higher temperature and transforming into leishmanial forms survived. It is obvious, however, that temperature is not the only factor determining morphological type in *Leishmania* since in Weinman's and Hawking's experiments leptomonads were able to multiply in this form at 37°C in the presence of living tissue.

Leishmania enrietti from the guinea-pig, when grown in tissue culture had a lower optimal temperature than *L. donovani;* the cells multiplied when incubated at 32° or 34°C, but began to degenerate when the temperature was raised to 37°C (da Castro and Pinto, 1960). Earlier workers had observed that this parasite multiplies actively in the cooler, more superficial parts of the body of the host where the temperature is below 36°C. Greenblatt and Glaser (1965) have studied the effect of temperature on the morphology, activity and physiology of the leptomonad stage of this species. When cultures maintained in a diphasic medium consisting of a blood agar base with a modified Locke's solution as overlay were subjected to temperatures above 30°C, there was a decline in viability, and between 35° and 37°C morphological changes including loss of the flagella and formation of lipid inclusion bodies occurred, followed by death. With increasing temperatures there was an increasing leakage of nucleic acid bases and amino acids from the flagellates.

E. DISCUSSION

In reviewing the advances made and the difficulties encountered in the cultivation of haemoflagellates, certain points emerge for general consideration. As yet no method has been developed for the cultivation of the blood-stream trypanosomes of any species belonging to the Salivaria; the flagellates which develop in cultures inoculated with blood from animals infected with these trypanosomes are similar in appearance to those occurring in the insect vector. Although in trypanosomes belonging to the Stercoraria the stages which develop in media devoid of living tissue are also, in general, similar to those occurring in the insect vectors, blood-stream trypanosomes have been described from cultures of *Trypanosoma theileri* and *T. conorhini* (and also from those of the avian trypanosome, *T. avium*), but only in a strain of *T. theileri* was subinoculation successful. It has been pointed out by Hutner and Provasoli (1955) that many protists require suitable supplements to the medium if grown at higher temperatures, and the inability of culture forms of mammalian trypanosomes to transform into blood-stream trypanosomes, or to survive if they do transform, when the temperature is raised to 37°C may be due, in part at least, to a deficiency of essential metabolites. The fact that the leptomonads of *L. tropica* and *L. donovani* can survive at 37°C in tissue culture whereas they die if maintained in non-living media suggests that the metabolizing tissues provide nutrients essential for their growth at the higher temperature.

Whereas infective metacyclic trypanosomes usually develop in cultures of species belonging to the Stercoraria, and strains maintained *in vitro* for long periods retain their infectivity to susceptible animals, the general experience with trypanosomes belonging to the Salivaria has been that cultures are not infective, and it has been assumed that this is because infective metacyclic trypanosomes similar to those occurring in the salivary glands or the proboscis do not develop in culture. However, the observations of Weinman and Lehman that trypanosomes resembling metacyclic trypanosomes do develop in cultures of the *brucei* subgroup, and the findings of Amrein and his colleagues that such cultures may be infective, call for a reconsideration of this problem.

It is well established that the length of time during which strains of the polymorphic trypanosomes of the *brucei* subgroup have been maintained by syringe passage adversely affects the chances of their successful isolation in culture, old "laboratory" strains being incapable of establishing themselves in culture. Evidence from earlier work upon the infectivity to tsetse flies of trypanosomes of the *brucei* subgroup maintained in this way has shown that strains which could no longer develop in the salivary glands might still produce heavy infections in the gut of the fly. In establishing themselves in the salivary glands, the gut forms undergo a change in morphology and, according to Broom and Brown, a change in electric charge. Some change may therefore occur in strains maintained by syringe passage which makes

them incapable of transforming into crithidia and metacyclic trypanosomes before that change occurs which renders them incapable of developing in the gut of the fly or in culture medium and which a study of their ultrastructure suggests may be a loss of functional mitochondria. Thus in some strains failure to produce infective metacyclic trypanosomes in culture might be due to an intrinsic change in the organism and not to the lack of essential metabolites in the medium or the physical conditions of the culture. It is of interest that the cultures in which Lehman observed metacyclic trypanosomes and those which Amrein and his colleagues found to be infective were from strains recently isolated from the natural hosts.

There is evidence that the age of the culture and the development of metacyclic trypanosomes are related; thus in cultures of *T. cruzi* and *T. conorhini* the proportion of metacyclic trypanosomes was found to increase as the culture aged, and in the experiments described by Amrein and his colleagues cultures of recently isolated strains of the *brucei* subgroup gave the highest percentage of infections when inoculated into mice around the 18th day of incubation. It has been suggested that in *T. cruzi* the depletion of certain nutrients in the medium provoked the development of the metacyclic stage, a suggestion which opens up an interesting field of investigation.

Whereas cultures of *T. cruzi* and *T. conorhini* and of the strains of the *T. brucei* subgroup studied by Amrein and his colleagues were infective when maintained at "room temperature", cultures of *T. vivax* grown in tissue culture at 28°C and containing forms resembling metacyclic trypanosomes were not infective until incubated overnight at 38°C. It is possible that the sojourn at the higher temperature merely speeded up the metabolic processes involved in the production of infective metacyclic trypanosomes, but unfortunately the infectivity of the cultures was not tested after a longer period of incubation at 28°C.

Temperature appears to be an important factor in determining the stage of the life cycle which will develop *in vitro*; thus when *T. cruzi* was grown in tissue culture there was evidence that it determined whether the organisms would develop intracellularly in the leishmanial stage or as crithidia and metacyclic trypanosomes in the fluid phase, though the response to either higher or lower temperatures was not complete. Moreover, in *T. theileri*, *T. conorhini* and *T. avium* increase in temperature to 37°C led to the development of trypanosomes of the blood-stream type, though it was not the only factor involved; thus in *T. conorhini* the change to the blood-stream form did not take place in the absence of whole blood or washed red cells.

The solution of many of the interesting problems encountered in the cultivation of the haemoflagellates awaits methods of cultivation in chemically defined media, but although some progress has been made in the development of such media for the cultivation of the "insect" forms of *Trypanosoma cruzi* and *Leishmania*, the fact that strains within species have been found to differ in their growth requirements adds to the difficulty of the problem. Such observations raise the question whether in dealing with parasitic protozoa with very exacting nutritional requirements, the more defined the medium, the more restricted may be the organisms which will grow in it.

III. The Cultivation of Malaria Parasites

A. Introduction

In 1911 Bass reported the successful cultivation of *Plasmodium vivax*, *P. malariae* and *P. falciparum*, the claim resting upon the subculture of these parasites in citrated blood and their survival for over 2 weeks. The method was described in greater detail by Bass and Johns (1912) who succeeded in maintaining up to three generations in defibrinated blood from which the leucocytes had been removed and to which a small volume of a 50% solution of glucose had been added. The optimum temperature for cultivation was 41°C. No further progress was made in this problem for some 30 years when, as a result of the important part which malaria played in the Second World War, a more satisfactory method of cultivation was sought.

The life cycle of the malaria parasite differs from that of trypanosomes because, in the vertebrate host, except for short periods which do not involve growth, the parasite is intracellular in erythrocytes and (in human, monkey and rodent (*P. berghei*) malaria) in the parenchymal cells of the liver, or (in bird malaria) in cells of the reticulo-endothelial system. The sporozoites are free in the blood-stream only during the short period between their introduction into the vertebrate host and their penetration into cells other than erythrocytes. Merozoites are liberated into the plasma when exoerythrocytic or erythrocytic schizonts segment and the cells containing them rupture, but no growth occurs until they have penetrated into other cells.

In the mosquito the development of the gametes, the fertilization of the macrogamete and the formation of the ookinete takes place in the lumen of the stomach, but later the ookinete enters the mucosal cells (Garnham *et al.*, 1962). When the oocyst has completed its development on the stomach wall, it ruptures and the sporozoites are liberated into the haemocoele and finally penetrate into the cells of the salivary glands; but since sporozoites liberated from oocysts growing in culture were infective (Ball and Chao, 1961), presumably no further development takes place either in the haemocoele or in the salivary glands.

The cultivation of malaria parasites therefore presents several different problems: the cultivation of the exoerythrocytic parasites depends upon the development of suitable tissue culture; the cultivation of erythrocytic parasites involves the development of media in which the parasites can grow and multiply in erythrocytes, or a medium which contains the nutrients and physical conditions available to the parasite in the erythrocyte and in which it can grow extracellularly; and finally, the mosquito stage demands cultures of mosquito stomach tissue or a non-cellular medium providing the nutrient and physical conditions which the oocysts would require for extracellular development.

B. The Cultivation of the Erythrocytic Stage of the Life Cycle of the Malaria Parasite

Since the parasites of human malaria cannot be maintained in laboratory animals attempts have been made to develop methods for the cultivation of

species of *Plasmodium* which can easily be maintained, and which produce the heavy infections necessary for such preliminary studies, with the ultimate objective of applying successful techniques to the cultivation of the plasmodia causing malaria in man. Two species of *Plasmodium*, one from birds and the other from monkeys, have been most frequently used in studies of culture methods. *Plasmodium lophurae*, first described by Coggeshall (1938) from the blood of a fireback pheasant from Borneo, produces heavy infections in the blood of ducks and young chickens, and *P. knowlesi* produces heavy infections in monkeys (*Macaca mulatta*).

As the malaria parasite grows in the erythrocyte, granules of pigment appear in the cytoplasm and form into clumps. Such pigment does not appear in exoerythrocytic parasites which grow in cells which do not contain haemoglobin. It has long been assumed that this pigment, haemozoin, a complex of haematin and a nitrogenous moiety (Deegan and Maegraith, 1956a, b), was formed as a product of digestion of haemoglobin by the parasite, and evidence that the parasite can hydrolyse globin and utilize the products for the synthesis of its own protein was presented by Fulton and Grant (1956), who found that some 80% of the methionine in the protein of erythrocytic parasites of *P. knowlesi* was derived from erythrocyte globin. Whether the intact haemoglobin molecule could pass through the plasma membrane was, however, not known. As a result of the study of the ultrastructure of malaria parasites it has been shown that the contents of the erythrocyte are incorporated by phagotrophy into food vacuoles in the parasite and that digestion, with the accumulation of haemozoin, takes place, in some species within these vacuoles, in others in small vesicles pinched off from them (Rudzinska and Trager, 1957, 1959; Rudzinska *et al.*, 1965). Evidence has recently been put forward that these food vacuoles are not formed by random invaginations of the plasma membrane, but that a functional cytostome is present in all stages of development in the erythrocyte, but no evidence of feeding by phagotrophy was found in exoerythrocytic stages (Aikawa *et al.*, 1966).

In attempts to cultivate the erythrocytic phase of the life cycle of malaria parasites either within erythrocytes or extracellularly, the viability of the parasites has been assessed by their appearance in fresh or stained material, by increase in parasite numbers and in the proportion of small trophozoites in the erythrocytes, and by the infectivity of the cultures to susceptible animals.

In preliminary experiments upon the cultivation of *P. lophurae* in erythrocytes (Trager, 1941) an attempt was made to develop a balanced salt solution similar in electrolyte composition to the contents of red blood corpuscles. This solution had a higher potassium and phosphate content and a lower pH than Tyrode's or Locke's solutions which approximate to the composition of serum. Survival of the parasites, when parasitized erythrocytes together with normal erythrocytes were suspended in the balanced salt solution and incubated at 39·5–42°C, was favoured by the addition of a concentrated extract from normal chicken red cells, plasma or serum, chick embryo extract, glucose or glycogen and glutathione. Whereas high oxygen tension was detrimental to survival of the parasites, exposure to air, either by maintaining the

culture as a shallow layer or by exposing it to a current of air, was beneficial. A disadvantage in dealing with the parasite-erythrocyte complex was the impossibility of determining whether a particular factor had a direct effect upon the parasite or whether its effect was primarily upon the erythrocyte. In the best cultures more than 40% of the parasites appeared to be alive on the 3rd day after which the numbers decreased to 0·05% by the 6th day. The addition of calcium pantothenate, together with gentle agitation on a rocking machine, improved survival further though not beyond a 2-week period (Trager, 1943).

Since the erythrocytes of monkeys, unlike those of birds, are non-nucleated, it was thought that monkey malaria parasites might be more suitable for biochemical experiments than those of birds. Attempts were therefore made to grow *P. knowlesi* in erythrocytes in culture. Preliminary experiments had shown that the addition of glucose to suspensions of parasitized red cells increased their respiration and that the glucose was converted to lactate at a greater rate than it was utilized. A supply of glucose and an efficient buffering system therefore appeared to be essential to any culture method (Geiman *et al.*, 1946). Such a system requires either a considerable dilution of the blood with a buffered isotonic nutrient medium or the continual addition of glucose and the removal of lactic acid from the blood by dialysis against a suitable medium. Two techniques were devised on these principles: the rocker-dilution technique which consisted of diluting parasitized blood with normal blood in a nutrient medium, and the rocker-perfusion technique in which the parasitized blood was separated from the nutrient medium by a cellophane membrane (Geiman *et al.*, 1946). With this latter method metabolic products were removed by diffusion as they were formed and nutrients were supplied across the membrane. Nutritional deficiencies in the nutrient medium were more rapidly obvious with this technique than with the dilution method, since growth factors present in the blood were more rapidly diluted out if they were absent from the diluting medium.

A complex nutrient medium based partly on preliminary respiratory and glycolytic studies and partly on the known nutritional requirements of other organisms was used. It contained inorganic salts, sodium acetate, glucose, glycerol and ribose, amino acids, vitamins, choline, and purines and pyrimidines. The composition of the inorganic salt solution was based on analyses of normal blood plasma. This medium is frequently referred to as the Harvard medium. The addition of whole blood or plasma was necessary for successive subculture and good multiplication, and under these conditions parasites of the 6th and 7th generations were infective to monkeys. Trager's observations upon the importance of aeration and the adverse effect of a high concentration of oxygen was confirmed. Attempts to determine which nutrients in this complex medium were essential to growth were impeded by the presence of undefined nutrients in the red cells and plasma.

A strain of *P. lophurae* in suspensions of duck erythrocytes was maintained for 8 days, and was still infective at the end of this period, when subcultured at 2-day intervals in the Harvard medium with the addition of the B vitamin, pyridoxamine (Trager, 1947). The addition of glutathione also favoured

growth and multiplication of this parasite in suspensions of chicken red cells in the Harvard medium (McGhee and Trager, 1950).

Attempts to cultivate the avian malaria parasites *P. cathemerium*, *P. hexamerium* and *P. relictum* in the Harvard medium by the rocker perfusion technique gave less good results than with *P. knowlesi*, though some growth in primary cultures and limited survival in first generation subcultures were obtained with *P. hexamerium* (Manwell and Glenn, 1953). The addition of vitamin B12, folic acid and a porcine liver concentrate rich in coenzyme A to the Harvard medium produced an increase in growth and reproduction of this latter parasite over a 72-h period (Glenn and Manwell, 1956).

Since in experiments on the cultivation of malaria parasites within red blood corpuscles the intracellular state of the parasite made it impossible to analyse the direct effect of individual compounds or groups of compounds upon the parasite, Trager has studied the conditions necessary for extracellular survival and growth in *P. lophurae*. The basal medium consisted of a concentrated preparation of duck erythrocyte extract in a complex nutrient medium with a high potassium content. The parasites were liberated from the red cells into the medium by a specific anti-duck-erythrocyte serum which acted in the presence of complement provided by guinea-pig serum. The antiserum was produced in rabbits by the injection of washed duck erythrocytes. The addition of gelatin, yeast adenylic acid and cozymase (nicotinamide-adenine-dinucleotide, NAD), adenosine triphosphate and sodium pyruvate favoured the survival and growth of the parasites over a period of 3–5 days (Trager, 1950). It has been suggested that the incorporation of gelatin in the medium may promote phagotrophy, since this might be expected to take place more easily in a viscous than in a non-viscous medium (Trager, 1957b).

Concentrates rich in coenzyme A and l-malic acid also favoured the survival of *P. lophurae* (Trager, 1952), and malate was shown to be of importance in the extracellular survival of *P. gallinaceum* in a medium containing erythrocyte extract. The fact that coenzyme A favoured extracellular survival whereas pantothenate itself appeared to have no effect, although it had previously been shown to have a favourable effect upon the *in vitro* survival of the parasites in intact red cells (Trager, 1943), suggested that *P. lophurae* required the complete coenzyme but was unable to synthesize it (Trager, 1954).

Whereas it had previously been found that *p*-aminobenzoic acid (Geiman *et al.*, 1946) and folinic acid (Glenn and Manwell, 1956) favoured the *in vitro* growth and survival of malaria parasites, folic acid when added, even in relatively high concentrations, had no apparent effect upon cultures of *P. lophurae* maintained extracellularly, though cultures to which folinic acid had been added had a better survival rate on the 4th day of incubation. However, a higher rate of invasion of erythrocytes by young trophozoites was observed on the 2nd day of incubation in cultures of *P. falciparum* maintained intracellularly in a modification of the Harvard medium to which a high concentration of folic acid had been added than in similar cultures containing a low concentration of this vitamin (Trager, 1958). It is suggested that whereas *p*-aminobenzoic acid and folic acid can improve growth and

survival of parasites within the erythrocytes, the parasite itself requires folinic acid. The relationship of folic and folinic acids to the parasite erythrocyte complex and the parasite alone therefore resembles that of pantothenate and coenzyme A.

Since in these experiments upon the extracellular cultivation of *P. lophurae* frequent changes of the medium were necessary, the lower portion of the Erlenmeyer flasks in which the cultures were maintained was coated with a thin layer of plasma clot made from duck plasma and chick embryo extract. Many of the parasites adhered to this clot and changes of the medium could thus be carried out with the minimum damage to or loss of the parasites (Trager, 1964). A mechanical medium changer was also devised (Trager and Jernberg, 1961).

C. THE CULTIVATION OF THE EXOERYTHROCYTIC STAGE OF THE LIFE CYCLE OF MALARIA PARASITES

The history of the cultivation of the exoerythrocytic stage of the life cycle of malaria parasites was reviewed by Pipkin and Jensen (1958), and the methods of cultivation in current use, more especially in his own department where the cultivation of plasmodia in tissue culture has been studied for a number of years, have recently been described by Huff (1964). It is therefore sufficient to outline the main advances made and some of the difficulties encountered.

The exoerythrocytic stage of malaria parasites was first successfully cultivated by Hawking in 1944. Tissue from 20-day-old chicks which had been infected 8 days previously by the intravenous injection of sporozoites of *Plasmodium gallinaceum*, and which was heavily infected with exoerythrocytic parasites, was implanted in roller tubes in a mixture of plasma and chick embryo extract, the fluid phase consisting of Tyrode's solution and chick embryo extract with a few drops of 0·1% phenol red as indicator. Since the tissue was fixed to the side of the tube in the clot its removal for sampling was not easy, and implantation in thin plasma clots on coverslips in Carrel flasks was later found preferable. In some cultures 5–100 units per ml of penicillin and up to 100 μg per ml of streptomycin were added to help prevent contamination. The cultures were incubated at 37°C and the fluid in the flasks was changed at intervals of 3–4 days, or more frequently if the medium became acid. Living parasites were demonstrated by direct observation, by observation of fixed and stained preparations or by inoculation of the tissue into young chicks. The tissues proved to be infective for periods up to 89 days, those which produced the best results being spleen, bone marrow and leucocytes (buffy coat layer of blood) (Hawking, 1945, 1951).

The infections produced in young chicks by the inoculation of exoerythrocytic parasites growing in tissue culture resembled those produced by the inoculation of sporozoites rather than infected blood (Hawking, 1945), being characterized by the immediate development of exoerythrocytic infections; indeed in a strain maintained by Lewert (1950) the birds died of the exoerythrocytic infection before erythrocytic parasites developed in the blood. However, not all strains maintained in tissue culture were equally virulent; thus a strain maintained in tissue culture of embryonic brain and heart muscle for

4 years produced only slight blood infections and no demonstrable exoerythrocytic infections when inoculated into young chicks (Meyer and Musacchio, 1958).

Whereas Hawking made explants from the organs of infected chicks, Zuckerman (1946) was of the opinion that spleen from 9- to 10-day-old chick embryos, which had been inoculated with exoerythrocytic parasites on to the chorioallantoic membrane, was superior to spleen from infected chicks, since it was a far richer source of exoerythrocytic parasites, and the embryonic tissues grew more successfully in tissue culture than those of chicks. The use of infected chick embryos has been adopted by other workers, and Huff and his co-workers (Huff *et al.*, 1960; Huff, 1964) have maintained strains of *P. gallinaceum* and *P. fallax*, likewise a parasite of birds, by continuous passage through chick embryos as a source of material for experiments upon the cultivation of these plasmodia in tissue culture.

De Oliveira and Meyer (1955) and Meyer and Musacchio (1958) maintained a strain of *P. gallinaceum* in tissue cultures of embryonic brain and heart muscle, uninterruptedly, for more than 4 years, the strain being grown alternately in plasma clots in roller tubes, the supernatant fluid being chick serum with Tyrode's or Ringer's solution and dilute chick embryo extract, and in hanging drops. When the tissues showed a good growth of parasites fresh, uninfected tissue was added, and parasites passed into the fresh tissue. When, however, fresh uninfected chicken blood was added to the tissue cultures, no parasites penetrated into the erythrocytes although unpigmented parasites were observed lying free in the medium.

Attempts by several workers to infect clean tissue culture with sporozoites were unsuccessful, one of the difficulties being that salivary glands of mosquitoes are frequently infected with bacteria and yeasts. The first successful experiments were made by Dubin *et al.* (1949, 1950). Cultures of chick embryo spleen in 20% chicken serum in Tyrode's solution, incubated at 37°C, were infected with sporozoites of *P. gallinaceum* from the salivary glands of *Aëdes aegypti*, penicillin and streptomycin being added to prevent bacterial contamination, and the reaction of the medium being controlled by a continuous flow of 2% CO_2. Pre-erythrocytic stages developed in the macrophages and could be maintained for up to 7 days. Attempts to infect cultures of human liver and bone marrow with sporozoites of *Plasmodium vivax* were unsuccessful.

Since one of the advantages from growing exoerythrocytic parasites in tissue culture is that stages of development of the parasites can be studied both in fixed and stained material and in the living state by phase contrast microscopy and cinemicrography, it is important that the growth should take place in as thin a layer of tissue as possible and that the culture vessels should be of a kind to allow observation of the living tissue *in situ* and its removal for fixation. Cultures of exoerythrocytic stages of *P. gallinaceum* and *P. fallax* have been maintained in monolayer cultures made by the trypsinization of infected chick embryo livers. The dispersed cells were maintained in a nutrient medium consisting of 15–20% chicken serum and 5% chick embryo ultrafiltrate in 75–80% Earle's balanced salt solution, the reaction of the

medium being adjusted by a flow of 5% CO_2 and 95% air. Penicillin (50 units per ml) and streptomycin (50 μg per ml) were added to prevent contamination (Huff *et al.*, 1960).

A variety of culture vessels have been designed in which the tissue is grown on detachable coverslips fixed to open "windows" in the culture vessels. In the prototype used by Zuckerman (1945) the tissue culture was grown on a coverslip cemented on to the base of a bottomless Carrel flask. The tissue was fixed *in situ* and the coverslip later dissolved off for further treatment. More sophisticated vessels such as that designed by Pipkin and Mack (1958) have been described by or referred to by Huff (1964). The development of a technique whereby the explants are completely covered by sheets of sterile dialysis cellophane (Rose, 1963), and are thus separated from the nutrients in the flask, have enabled Huff and his colleagues to maintain primary cultures of exoerythrocytic parasites of avian malaria up to a maximum of 7 months, and to infect normal embryonic tissue cultures more easily with sporozoites of *P. gallinaceum*.

Although work upon the cultivation of exoerythrocytic stages of avian malaria parasites has met with some success, attempts to cultivate exoerythrocytic parasites from man and monkeys have so far failed.

D. THE CULTIVATION OF THE SPOROGONIC CYCLE OF MALARIA PARASITES

In the genus *Plasmodium*, as in the Trypanosomatidae, one of the interesting problems is the physiological adaptation which the change from the vertebrate host to invertebrate vector, or vice versa, must involve. Almost nothing is known of the physiology of the malaria parasite in the mosquito. With the objective of providing a method by which this lack of knowledge might be remedied Ball, alone or with Chao, has been attempting to develop a method of cultivating those stages of the life cycle which occur in the mosquito. It is essential in the earlier stages of such work, whilst fundamental problems of cultivation are being solved, that the parasite and vector should be easily maintained in the laboratory, and Ball chose *Plasmodium relictum*, maintained in canaries, and *Culex tarsalis* as the subject of his study since the rate of infection in this mosquito is very high.

The results of the experiments and the methods used have been reviewed by Ball (1964), Ball and Chao (1963), Chao and Ball (1964). Essentially, the work has consisted of the development of methods for maintaining infected mosquito stomachs and isolated oocysts in culture. It was found that when mosquitoes were starved for 24–48 h before dissection, the bacterial and yeast populations of the gut were reduced and a high proportion of bacteriologically sterile cultures were obtained; but streptomycin and amphotericin B were used in routine cultures. Standard media for the cultivation of vertebrate and insect tissues were not suitable for cultivating stages of the sporogonic cycle of the parasite or the insect tissue. A variety of media were used but the most successful consisted of a basic salt solution buffered with phosphates and bicarbonates, glucose, amino acids, B vitamins, purines and pyrimidines, chicken serum and chick embryo extract, but more recently the

Na/K ratio has been lowered, nucleosides were substituted for the purine and pyrimidine bases, the adult chicken serum was replaced by sera from 2-week-old chicks and organic acids of the Krebs cycle were added. A 95% air–5% CO_2 mixture was most satisfactory for the mosquito tissue and for all the stages of development of the parasite except the older oocysts and sporozoites which did better in an atmosphere of air alone. Early stages of the sporogonic cycle developed best at pH 7 and later stages at pH 8. The cultures were incubated at temperatures ranging from 18° to 31°C, the rate of development of the parasite increasing with the rise in temperature.

In early experiments (Ball, 1948) in which infected stomachs bearing visible oocysts were removed from the mosquito 7–12 days after it had taken an infective meal, the oocysts could be maintained in culture on the stomach wall for 7–20 days, but no development was seen. Although it is not yet possible to maintain the complete cycle of development from the zygote in one stage, it has been possible as a result of improvements in technique to obtain the development of infective sporozoites in culture from zygotes by a series of stages, each stage lasting for about 5 days.

Although exflagellation began almost at once when blood containing gametocytes was mixed with culture media, no zygotes developed, but they developed readily when stomachs, isolated from the mosquito immediately after the blood meal, were maintained in suitable culture media (Ball and Chao, 1960). Under such conditions oocysts developed and were observed on the stomach wall by the 4th day. When stomachs containing oocysts which had reached a considerable state of development but were still immature, were dissected out of mosquitoes and transferred to suitable media, the oocysts continued to develop and sporozoites, which proved infective to canaries, were liberated into the medium (Ball and Chao, 1961). These experiments disproved previous suggestions that the infectivity of sporozoites was dependent upon a sojourn in the salivary glands.

It had been shown by Weathersby (1952, 1954) that when stages of *P. gallinaceum*, from gametocytes to sporozoites, were injected into the haemocoele of *Aëdes aegypti*, development continued and sporozoites appeared in the salivary glands. Since it had thus been demonstrated that the stomach wall was not essential for the development of mature oocysts, Ball and Chao (1957) attempted to cultivate isolated oocysts of *Plasmodium relictum*. The oocysts, dissected aseptically from the stomach wall, were maintained in hanging drops or in Carrel flasks, but although the oocysts continued to increase in size for a period of 5–8 days, in no case did sporozoites develop.

Since it was not possible to maintain cultures of any part of the sporogonic cycle for more than an average of 5 days, it must be concluded that some factors essential to growth and development were lacking from the media.

E. DISCUSSION

From a survey of the literature on the cultivation of the erythrocytic stages of malaria parasites it can be concluded that, although some multiplication of these stages in some species of avian malaria and in *Plasmodium knowlesi* of monkeys has been obtained in culture, survival has been very limited,

and even under the best conditions could be counted only in days. Moreover, the culture media have been complex, including such undefined constituents as whole blood or plasma. When maintained extracellularly, the period of growth and survival of *P. lophurae* was also limited to a few days and the medium was complex containing duck erythrocyte extract. It is obvious, therefore, that the goal of continuous cultivation in a defined medium is still far distant.

Although a strain of the exoerythrocytic stage of *P. gallinaceum* has been maintained for more than 4 years in tissue culture of embryonic brain and heart muscle, cultures of exoerythrocytic stages of mammalian malaria parasites have not yet been obtained. However, the recent description of Yoeli and Most (1965) of the development of pre-erythrocytic stages of *P. berghei* in the parenchymal cells of the liver of small mammals, including white mice, which had been experimentally infected with sporozoites from *Anopheles quadrimaculatus* and *A. stephensi* suggests a source of material for experiments upon the cultivation of the exoerythrocytic stages of mammalian malaria parasites which should be more easily maintained in laboratories than monkey malaria. Huff (1964) pointed out, however, that in *P. falciparum* only a few generations of these stages, at most, occur in the liver, *in vivo*, and that therefore the probability of establishing continuous cultures seems less than with avian malaria. However, the problem of the nature of the factors, whether intrinsic or extrinsic, which may limit development is itself a fascinating one.

Although it is possible to maintain different stages of the sporogonic cycle of *Plasmodium relictum* in the mosquito's stomach *in vitro*, it has not yet been possible to obtain the complete cycle of development in a single culture. The recent description of the establishment of a line of cells from *Aëdes aegypti* in tissue culture (Grace, 1966) suggests that new techniques may soon be available with which to approach this problem. Grace was not able to determine the tissues from which the cells had developed, but the work of Weathersby (1954) has shown that, *in vivo*, the sporogonic development of *Plasmodium gallinaceum* is not limited to the walls of the mosquito's stomach but will take place, experimentally, in a variety of the insect's tissues. Such a cell line, therefore, might support the development of the zygotes into the young oocyst.

In spite of technical advances and the progress already made it is obvious that the problems involved in the cultivation of all stages of malaria parasites are very great.

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Malaria in Mammals Excluding Man

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I. Introduction ... 139
II. Taxonomic Problems and New Species ... 140
A. Taxonomic Problems ... 140
B. New Species ... 142
III. Life Cycles ... 150
A. Rodent Parasites ... 150
B. Simian Parasites ... 153
C. Relapses ... 155
IV. Host Susceptibility and the Zoonosis Problem ... 156
A. Zoonoses ... 156
B. Other Host Susceptibilities ... 159
V. Ultrastructure ... 160
A. Erythrocytic Stages ... 160
B. Exflagellation ... 166
C. Ookinete ... 172
D. Oocyst ... 174
E. Sporozoites ... 176
VI. Pathogenesis ... 181
VII. Immunity and Modern Serological Reactions ... 184
A. Introduction ... 184
B. Serological Measurement of Immunity ... 185
C. Cross Immunity Reactions ... 189
D. Passive Immunity ... 190
E. Malaria Antigens ... 192
References ... 195

I. Introduction

When Huff (1963) wrote his contribution on avian malaria for *Advances in Parasitology*, practical interest in malaria research seemed inevitably to be declining and many malariologists were transferring their interest to schistosomiasis or virology. Huff rightly pointed out the danger of abandoning interest in malaria, which would leave us "unprepared for combating an unexpected return of this great scourge"; but at that time most people believed that eradication of the human disease was nearing achievement and, although rumours of resistance of mosquitoes to insecticides and of parasites to drugs were current, few people foresaw then the sudden halt of progress which lay ahead. In 1963 it was realized that the anti-malarial measures so

successful in Europe, the United States and India, were ineffectual in Africa and other parts of the tropics, where, in quite large areas, malaria cases had become incurable with the new synthetic drugs. *Plasmodium falciparum* had become resistant to drugs and treatment with the old-fashioned remedy quinine was necessary.

In these circumstances a great burst of activity in malaria research took place and many advances in knowledge of all aspects of the subject were made. These have been described in various monographs (including Russell *et al.*, 1963; Garnham, 1966), but the subject is in such a state of flux today, particularly in the experimental study of primate and rodent malaria, that in some details even the most recent works are already out of date.

This contribution is devoted to malaria parasites of mammals other than man, but in two respects human malaria must be considered. First, two species of simian malaria are now known to infect man in nature and eight species, accidentally or deliberately, in the laboratory. Second, the ultrastructure of malaria parasites has been studied intensively during the last few years and comparisons must concern human and non-human parasites, and several important papers concern both types. The scope of this review is limited to members of the genus *Plasmodium sensu strictu*, and various haemoproteids, once, or even today, placed in this genus have not been considered, e.g. such important forms as *Hepatocystis* (= *Plasmodium*) *kochi*; *Polychromophilus* (= *Plasmodium*) *murinus* and *Plasmodium* (*incertae sedis*) *brodeni*. The first two of these parasites are of particular interest in that their exoerythrocytic development in the liver of monkeys and bats respectively foreshadowed the type of schizogony which was later to be discovered in the true malaria parasites of primates, while the remarkable sporogonic cycles of *H. kochi* in *Culicoides* spp. and of *Po. murinus* in nycteribiid flies were shown to differ fundamentally from the type of development in *Plasmodium* (see Garnham, 1947; Mer and Goldblum, 1947). The general subject of exoerythrocytic schizogony of malaria parasites has been discussed in a number of reviews, and notably in the monograph by Bray (1957); only researches on the tissue phase after that date are therefore mentioned here.

It is proposed to discuss the advances made during recent years in the following topics: new species and questions of taxonomy; life cycles; vertebrate host susceptibility and the problem of the zoonoses; ultrastructure; pathogenesis; immunity and serology.

During the last decade much research has been concerned with chemotherapy and the drug resistance of parasites; but, although some of this work was done on animals, it was directed mainly towards the alleviation of human malaria and it has no place here. The closely related and fundamental question of the biochemistry of the parasite in all stages of its life cycle is also largely outside the scope of the present review.

II. Taxonomic Problems and New Species

A. Taxonomic Problems

The malaria parasites of mammals were classified by Garnham (1964) into three subgenera of *Plasmodium*, viz. *Plasmodium*, *Laverania* and *Vinckeia*, the

first two including the species found in primates above the level of the lemuroids and the third including the species occurring in lemurs and lower groups. The major difference between the subgenera *Plasmodium* and *Laverania* is the shape of the gametocytes, round in the former and crescentic in the latter, while distribution of their pigment granules is also distinctive—dispersed in *Plasmodium* and perinuclear in *Laverania*. The characters of the subgenus *Vinckeia* are less clear-cut, but important criteria are the rapidity of primary exoerythrocytic schizogony (about 48 h instead of at least 5 days), the small size of the erythrocytic schizont, which usually has eight or fewer merozoites, and the absence of obvious stippling in the infected erythrocyte. No criterion is absolute, but the sum offers a practical guide, aided by the absence of the subgenus in primates.

The designation of organisms at the specific or infraspecific level presents a greater problem in at least two directions. First, many of the earlier descriptions of rare parasites were limited, and the material on which they were based was often small and may no longer exist; type specimens are usually missing, making re-examination or direct comparison with new examples impossible. In such circumstances, there has been a tendency to give new names to parasites found in the same host, because their appearance does not correspond exactly with the original description. Examples of this procedure may be found in the malaria parasites of gibbons in the East Indies and of lemurs in Madagascar, and these will be discussed. Certain criteria which are dependent upon good staining methods may not have been elicited in the original observations, and the presence or absence of stippling, for instance, should not be taken as absolute; periodicity is important for identification but may not have been determined with accuracy; the presence of the same parasite in a different vertebrate host often entails changes in its morphology. All these points must be taken into consideration before a new species is created.

Second, the question of subspecies and strains must be given careful thought. The situation today is the same as in 1892 when Grassi and Feletti were faced with the problem of subspecies, varieties and polymorphism in the human and avian malaria parasites. In certain tropical or subtropical regions, the combination of parasite, vertebrate host and mosquito vector is exposed to constant stress from the surrounding environment; mutations are liable to arise, and all stages in the process of speciation ensue. The difficulty here is to recognize their extent—do they constitute new species, subspecies or strains (= demes), the last at present still lacking taxonomic status? The creation of subspecies of Protozoa is generally disliked, but the writer considers this solution desirable when full specific status cannot be designated; such a course leaves open transfer of the organism to specific status, or its relegation to the level of a strain when more information eventually becomes available.

Speciation of avian malaria parasites in the *relictum* group was discussed by Corradetti and Neri (1956) and Garnham (1965b). The migrations of birds and the intermingling of various potential vertebrate and invertebrate hosts render their malaria parasites prone to change, and there is little doubt

that new fixed characters are produced which gradually assume taxonomic proportions. Similar examples are to be found in the *cynomolgi* group of simian parasites in the Orient, and in the *berghei* group of rodent parasites in tropical Africa; these are discussed briefly below.

B. NEW SPECIES

1. *Recently Described Species in Gibbons* (*P. youngi*, *P. jefferyi* and *P. eylesi*)

The first malaria parasite to be identified in gibbons was *P. hylobati* Rodhain, 1941. Unfortunately the species was described from a single specimen of *Hylobates moloch* and blood films were taken only twice, but other specimens were found later and the original description of Rodhain was slightly amended by Garnham (1966). A closely allied species found in *H. lar* and named *P. youngi* by Eyles *et al.* (1964) differs from *P. hylobati* in its amoeboidicity, the larger size of the schizont and the production of stippling in the infected erythrocyte. *P. jefferyi* Warren, Coatney and Skinner, 1966 is also found in *H. lar* and this differs from the preceding two gibbon species in the virtual absence of schizonts in the peripheral blood and minimal stippling of the host's blood corpuscles. *P. eylesi* Warren, Bennett, Sandosham and Coatney, 1965 is the clearest defined of parasites of the white-handed gibbon, because it provokes great enlargement of the host erythrocytes, which become heavily stippled, and it produces thirty-four or more merozoites. *P. hylobati* and *P. eylesi* represent the extremes of a series and are unquestionably valid species, but the other two parasites might be regarded as subspecies of *P. hylobati*, although their true systematic position cannot be established until full details of their respective life cycles are obtained. The chief diagnostic characters are shown in Table I.

2. *The "Cynomolgi" Complex*

P. cynomolgi cynomolgi Mayer, 1907 was described from a specimen of *Macaca irus* from Java. Since that date many strains have been isolated and the complete life cycle of this common parasite of Asian monkeys has been elucidated. The identity of the subspecies *P. cynomolgi cyclopis* Inoki *et al.*, 1941 remains dubious, because in spite of intensive surveys in Taiwan, its type locality, the parasite has never been encountered again. *P. cynomolgi bastianellii* Garnham, 1959 from *Macaca irus* in Pahang has been much studied in the laboratory and in induced infections in man; it is easily distinguished from *P. c. cynomolgi* during the sporogonic and exoerythrocytic stages (see Table II). The status of *P. c. bastianellii* has been raised by Bray (1963) to specific level, and was lowered by certain North American workers (e.g. Schmidt *et al.*, 1961) to a strain (*P. cynomolgi* B strain). *P. cynomolgi ceylonensis* Dissanaike *et al.*, 1965 occurs in *Macaca sinica* in Ceylon and probably in South India, and minor morphological differences from the other members of the complex are demonstrable at all stages of the life cycle (Table II). Intensive recent work on monkey malaria in Malaya, Cambodia and Ceylon has revealed the existence of a number of other strains or subspecies of *P. cynomolgi*, which are without valid names and a precise status at present.

TABLE I

Important characters of subspecies and strains of Plasmodium cynomolgi

Subspecies or strains	Locality	Vertebrate host	Suscepti-bility of rhesus	Invertebrate host	Duration (days) of sporogony at 27°C	Size of oocysts (μ)	Duration of EE schizogony (days)	Character of EE schizogony	Suscepti-bility of *A. maculatus* (%)
cynomolgi	Malaya	*M. irus*	High	*A. hackeri* *A. balabacensis*	10	65	8	Large vacuoles	14
bastianellii	Pahang	*M. irus*	High	?	9·5	55	7	No vacuoles	34
ceylonensis	Ceylon S. India	*M. sinica* *M. radiata*	High	? *A. elegans*	8	75	8½	Rather small	?
Cambodian	Cambodia	*M. irus*	High	*A. balabacensis*	7·5	45	7	Irregular	60
Gombek	Kuala Lumpur	?	High	*A. balabacensis*	9·5	50	?	?	13
Berok	Perak	*A. nemestrina*	High	?	8	45	?	?	62
Langur (Ceylon)	Ceylon	*P. entellus*	Low	?	7·5	80	7½	Rather small (30 μ)	?
Langur (Malaya)	Malaya	*P. cristatus*	Low or nil	?	?	?	?		?

TABLE II

Differential characters of P. knowlesi, P. coatneyi *and* P. fragile

Species	Susceptibility of rhesus	Susceptibility of man	Periodicity	Blood forms			
				Stages	Red cells	Merozoites	Pigment
P. knowlesi	Always fatal	High	Quotidian	All forms	Mature	10	Normal
P. coatneyi	Often fatal	Nil	Tertian	Only rings	Reticulocyte	20	Rice grains
P. fragile	Often fatal	Nil	Tertian	All forms	Mature	12	Heavy in rings

Bennett *et al.* (1966a–c) analysed in an important series of papers the susceptibility of five strains and subspecies of *P. cynomolgi* in various Malaysian mosquitoes; they showed that sporogony of a single strain pursued an identical course in *Anopheles maculatus* and *A. kochi* and to a slighter extent in *A. vagus* and *Mansonia uniformis* but the individual strains differed from one another in ways which have a potential taxonomic value. These parasites can be distinguished from one another, not only by their behaviour in different species of *Anopheles*, but also by the nature of exoerythrocytic schizogony and by the response of the rhesus monkey to infection (Table II). The last character is of particular interest because, both in Malaya and Ceylon, *P. cynomolgi*-like parasites have been isolated from leaf monkeys (*Presbytis* spp.) which either fail to infect the rhesus or produce only the mildest infections. Repeated passage enhances the virulence of the parasites, however, which may make them come to resemble one or other of the known subspecies. These findings indicate that the correct taxonomic treatment of these parasites is in doubt, and cannot perhaps be settled until more information becomes available, e.g. the identification of the natural invertebrate hosts.

3. *Other Oriental Malaria Parasites of Monkeys*

Three closely allied parasites were described from macaques in Malaya, India and Ceylon. *P. coatneyi* Eyles *et al.*, 1962 and *P. knowlesi edesoni* Garnham, 1963 were described from *Macaca irus* in Malaya, though the first isolations of the former were made from sporozoites from wild *Anopheles hackeri*, and *P. fragile* Dissanaike *et al.*, 1965 from *M. sinica* in Ceylon. The Malayan parasites cause severe infections in *M. mulatta*, which are frequently fatal, and the parasitaemia of both is largely confined to rings in the peripheral blood, with schizogony occurring at midnight in the internal organs. There are, however, minor morphological differences between them; moreover, the subspecies *edesoni* has a quotidian, and *P. coatneyi* a tertian periodicity, *P. fragile* occurs in *M. sinica* in Ceylon, and in *M. radiata* in Southern India and produces virulent tertian infections on subinoculation into rhesus monkeys. All three parasites distort or destroy the erythrocytes, as reflected in the name of *P. fragile*, but the latter may be distinguished from *P. coatneyi* by the character of the pigment and the numbers of merozoites (Table II).

Two other tertian parasites occur in Malaya and Ceylon respectively: *P. fieldi* Eyles *et al.*, 1962 and *P. simiovale* Dissanaike *et al.*, 1965, which appear to have affinities with one another and possibly with the human species *P. ovale*. The nuclei are unusually large, Schüffner's stippling is very heavy, and some oval distortion of the infected erythrocytes is apparent. *P. fieldi* gives rise to characteristic eosinophilic masses in the erythrocyte and *P. simiovale* to prominent, *matutinum*-like vacuoles in the cytoplasm of the erythrocytic stages. Another tertian parasite, resembling the foregoing, is *P. simium* da Fonseca, 1951, described from howler monkeys in southern Brazil; it has great interest because Deane *et al.* (1966a) showed that its presence in other monkeys near São Paulo is responsible for human infections (see p. 158).

4. *Quartan Malaria Parasites of Oriental Monkeys*

P. inui Halbastädter and von Prowazek, 1907 is widely distributed in Asia and has been much studied. A closely allied species found by Shortt *et al.* (1961) in *M. radiata* in Hyderabad was provisionally called *P. osmaniae* and later given the replacement name *P. shortti* by Bray (1963). Eyles (1963) demonstrated its quartan periodicity and, finding no real differences between it and *P. inui*, reduced it to a subspecies of *P. inui*. However, it causes a greater degree of erythrocytic enlargement and, more important, the sporogonic cycle lasts only 9–10 days, instead of twice this period in *P. inui*, the exoerythrocytic schizogony occupies 9–10 days instead of 11 and the schizonts of *P. shortti* are larger.

5. *Rodent Malaria Parasites (Subgenus* Vinckeia)

The discovery by Vincke and Lips in 1948 of *P. berghei* (Vincke and Lips, 1950), released a most useful tool for malaria research, and more than 500 papers have already been written about this organism. *P. vinckei* Rodhain, 1952 was isolated from sporozoites of *A. dureni*, caught in the same gallery forests of the Katanga, and was found to differ morphologically and biologically from *P. berghei.* Both parasites are capable of infecting *Thamnomys surdaster* and mice, but *P. vinckei* infects rats and hamsters only after adaptation by long passage. The full life cycle of *P. berghei* is discussed below. For a long time, these two parasites were believed to constitute, with *A. dureni* and *Thamnomys surdaster*, a fine example of a closed biocenose in a highly specialized environment. This is still true, but the situation is not unique for Landau and Chabaud (1965), Landau (1965), Landau and Killick-Kendrick (1966) and Adam *et al.* (1966) have disclosed that other biocenoses, involving other species of parasites, rodents and mosquitoes, exist elsewhere in tropical Africa. Thus, *P. berghei yoelii* and *P. chabaudi* were found in numerous samples of *Thamnomys rutilans* at Bukoko in the Central African Republic, and a similar pair of counterparts of *P. berghei* and *P. vinckei* near Brazzaville. In both these localities, *A. dureni* is absent, the altitude is lower and the forest is different, and it is likely that some speciation has occurred in the changed environment; it is possible that the key to the situation will be found in the existence of a highly specialized anopheline host, for other species of the subgenus *Vinckeia* are always accompanied by their own particular vectors. The French workers refer to such examples of parasitism as *vicariance* or co-evolution of host and parasite. The condition is thus more widespread than was originally thought and extends at least to Nigeria, where Bruce-Chwatt and Gibson (1955) discovered a parasite resembling *P. vinckei* in a specimen of *Praomys jacksoni.* On the eastern side of tropical Africa, malaria seems to be absent in *Thamnomys* and other forest rodents (Pringle, personal communication) though unidentified sporozoites have frequently been found in the sylvatic mosquito, *A. machardyi*, in the vicinity of the Malaria Institute of East Africa at Amani.

P. berghei berghei has been isolated on many occasions from *A. dureni* in the Katanga and *P. berghei yoelii* from *Thamnomys rutilans* in the Central

African Republic. The strains of each subspecies are uniform in their major characters, though some appear to undergo sporogony in mosquitoes with greater facility than others. An interesting difference in the strains of *P. berghei yoelii* has, however, been reported recently by Warhurst and Killick-Kendrick (1967). The usual strains of this parasite are fully susceptible to chloroquine, but one strain was found to be naturally resistant to the drug, 50 mg/kg failing to clear the blood of parasites. It is easy to make *P. berghei* and other species of malaria parasites resistant to drugs by inadequate dosage, but this is the first occasion on which a natural mutant has been noted, and it is certain that the character could not have arisen from previous exposure to the drug. Natural resistance should prove a most useful "marker" for studying the genetics of this species.

Tables III and IV provide a key for differentiating the rodent parasites at all stages of their life cycles.

6. *Other Species of* Vinckeia

Until 1951 only two other species of *Vinckeia* had been described, viz. *P. cephalophi*, Bruce, Harvey, Hamilton and Bruce, 1913 in duikers in Malawi (= Nyasaland), and *P. bubalis* Sheather, 1919 in domestic buffaloes in India. Since that date ten more species have been identified and their characters are very briefly summarized below.

P. girardi Bück, Coudurier and Quesnel, 1952 is found in lemurs in Madagascar, usually only after splenectomy. The trophozoites show an unusual peripheral distribution of the pigment. This feature is absent in the larger *P. lemuris* Huff and Hoogstraal, 1963 described from a single blood film of the black lemur. It exhibits gametocytes of extraordinary size. A further malaria parasite *sensu latu* also occurs, viz. *P.* (*incertae sedis*) *foleyi*, Bück, Coudurier and Quesnel, 1952 which exhibits gametocytes only in the blood-stream. Clearly, in this group of parasites of primitive primates in secluded conditions interesting examples of speciation are flowering.

P. sandoshami Dunn, Eyles and Yap, 1963 is one of the oddest species of *Plasmodium*; it is found in the colugo of the Malaysian jungle, exhibits six sausage-shaped merozoites in the schizont, and excretes a pink-staining debris into the erythrocyte.

P. brucei Garnham, 1966 occurs in duikers in the Congo, Tanzania, Malawi and probably elsewhere in tropical Africa. The pigment takes the form of golden crystalline bars, smaller than those present in *P. cephalophi* but equally distinctive.

P. traguli Garnham and Edeson, 1962 is the smallest malaria parasite and occurs in the mouse deer of Malaya and Ceylon. The natural invertebrate host is *A. umbrosus*, and another host is probably *Mansonia crassipes* according to Warren *et al.* (1964). It might be noted that Warren *et al.* (1962) had already shown that *P. cynomolgi bastianellii* (and later *P. falciparum*) was able to develop in *Mansonia*, though transmission was never effected.

P. atheruri van den Berghe *et al.*, 1958 is associated with *A. smithii vanthieli* in a closed biocenose near Lake Kivu in the Congo, the association

TABLE III

Differential characters of malaria parasites of Muridae

	Distribution			Sporogony		Primary exoerythrocytic schizogony		
Parasite	Geographical	Natural invertebrate hosts	Natural vertebrate hosts	Effect of temperature on sporogony	Size of mature oocyst (μ)	Time to maturity (h)	Usual size of schizonts at maturity	Approx. no. of merozoites in mature schizonts
P. b. berghei	Katanga	*A. dureni*	*Thamnomys surdaster; Praomys jacksoni; Leggada bella*	At 24°C and above sporozoites not infective	34–40	48	26 μ	4 000
P. b. yoelii	Central African Republic	?	*Thamnomys rutilans*	At 24–26°C sporozoites infective	60–80	43	37 μ	8 000
P. vinckei	Katanga	*A. dureni*	Probably *Thamnomys surdaster*	?	?	?	?	?
P. chabaudi	Central African Republic	?	*Thamnomys rutilans*	At 24–26°C sporozoites infective	60–80	53	37 μ	20 000

Table IV

Differential characters of malaria parasites of Muridae

	Blood forms						
Parasite	Rings with double chromatin dots	Usual no. of merozoites in mature schizont in mice	Size of gametocyte in mice (μ)*	Polyparasitism common early in infection	Preference for immature r.b.c.'s	Stippling of r.b.c.	Infective to rats and hamsters
P. b. berghei	Uncommon	6–10	8	Yes	Marked	None	Yes
P. b. yoelii	Uncommon	8–16	8	Yes	Marked	None	Yes
P. vinckei	Common	Seldom more than 8	6	No	None	None	No†
P. chabaudi	Common	8–12	6	No	None	"Blue pearl" stippling sometimes seen; a dark mauve or red colour common in cells with old trophozoites	No†

* In general, the blood forms of *P. b. berghei* and *P. b. yoelii* are all larger than those of *P. vinckei* and *P. chabaudi*.
† Strains infective to rats and hamsters can, however, be selected by syringe-passage in the laboratory.

including the vertebrate host, the brush-tailed porcupine. The mature schizont possesses only four merozoites.

P. voltaicum van der Kaay, 1964 has a rather similar ecology to the last-named species because the vertebrate host is *A. smithii rageaui* and infection in the roussette fruit bat is limited to a small region in Ghana. The blood infection is usually confined to ring forms and, for this reason, the organism was suspected at first of being a piroplasm.

P. roussetti van Riel, L'Hoest and L'Hoest, 1951 occurs in another species of fruit bat (*R. leachi*) far to the east in the caves of Mont Hoyo near Stanleyville. Little is known about this species, but the vector is likely to be *A. faini* or some other specialized cave-dwelling anopheline. The parasite is said to be congenitally transmitted to the offspring.

P. anomaluri Pringle, 1960 was found in the scaly-tailed flying squirrel in the Eastern Usambaras in Tanzania. The ring forms are exceptionally large and the gametocytes (but not the asexual forms) produce considerable hypertrophy and distortion of the host cell.

P. booliati Sandosham, Yap and Omar, 1965 is also found in a flying squirrel (of a different family) but in Malaya. It is a small parasite, and the merozoites are limited to four to eight in number.

III. Life Cycles

A. Rodent Parasites

The most important discovery in recent years relating to the life cycles of mammalian species of *Plasmodium* was the elucidation by Yoeli of the sporogonic and exoerythrocytic stages of *P. berghei*. Mystery had surrounded the tissue stages of this parasite from the time of its discovery; van den Berghe *et al.* (1950) described unpigmented forms in histiocytes in the liver and bone marrow, but these reports were generally discounted as being of no significance; another false trail was the suggestion by the writer that the exoerythrocytic stages might well be found to follow an avian pattern, particularly as Vincke and Peeters (1953) had shown that the brevity of the prepatent period (as short as 48 h) was more like that of the bird parasites than of the primates. Wolcott (1957) had also pointed out that the two dot-like chromosomes of *P. berghei* resembled those of avian and saurian malaria parasites.

Then, in a series of patient researches including a visit to the remote forests of Katanga, Yoeli succeeded, first, in obtaining prolific sporogony of the parasite in laboratory bred mosquitoes (Yoeli and Most, 1960) and, second, in demonstrating the exoerythrocytic stages in the liver of mice and other rodents inoculated with sporozoites (Yoeli and Most, 1965).

Yoeli had noticed that in the biotope in the Congo, the ambient temperature varied between 14° and 21°C, a much lower range than that usually prevailing in insectories. He accordingly kept his mosquitoes at the natural temperature and at once sporogonic development of *P. berghei* progressed further; normal oocysts matured in the midgut and numerous sporozoites invaded the salivary glands. The infection was readily transmitted by mosquito bite, or by the intravenous inoculation of sporozoites, and the shortness

of the prepatent period was verified. A large supply of viable sporozoites thus became readily available, and this was the essential prerequisite for the next stage of the investigation. The precise effect of temperature was later determined by Vanderberg and Yoeli (1966) by exposing infected mosquitoes, for varying periods, to temperatures ranging between 14° and 28°C. At 14°C oocysts alone developed, while a temperature of 28°C was fatal; the optimum temperature was found to be 21°C when 32 000 sporozoites per mosquito (*A. stephensi*) were produced. High temperatures were shown to have the most deleterious effect on the earliest stages of sporogony; later in the cycle, oocysts were able to withstand a temperature of 28°C for as long as 3 days.

Yoeli had made many attempts to identify tissue stages of *P. berghei* in rodents inoculated with sporozoites and, finally, he was able to elucidate the cycle in the liver of infected rats, mice and hamsters. Exoerythrocytic schizogony was found to occur in the parenchyma cells, with the production of large bodies, maturing at 48 h after the introduction of sporozoites. Yoeli and Most (1965) described the earliest forms at 28 h as spherical bodies with five nuclei, which quickly assume a peripheral position. By 30 h Garnham *et al.* (1966) found that the schizont is 9 μ long and is characterized by the possession of large nuclei, containing four to six irregular masses, which may be chromosomes. The cytoplasm later becomes studded with dense basophilic spheres, and vacuoles and clefts are often present. On maturity, at 50 h, the schizont reaches 28 μ in diameter and contains about 4 000 minute merozoites, which tend to assume a palisade arrangement on the border of the schizont although there is only slight nuclear patterning. Up to the present, secondary exoerythrocytic schizogony has not been detected in this species of parasite. Although the same strain (NK 65) of *P. berghei* was used in New York and London, the dimensions of the tissue forms were found to differ, the American workers giving much higher figures (e.g. mature schizonts with an average diameter of 40 μ instead of 28 μ).

The life cycle of *P. berghei yoelii* was studied in detail by Landau and Killick-Kendrick (1966) and was shown to differ markedly from that of *P. berghei berghei*. Sporogony of the subspecies occurs readily at a temperature of 24–26°C and oocysts grow to twice the size (80 μ). At all stages of development, the exoerythrocytic schizonts of the subspecies are larger (reaching 37 μ instead of 28 μ), while patterning of the nuclei is much more distinctive with the production of clear-cut pseudocytomeres. Successive inoculations of blood from sporozoite-infected animals show that the earliest schizonts mature at 43 h. Double the number of merozoites (8 000) are produced by *P. berghei yoelii.*

The *berghei*-like parasite, isolated by Adam *et al.* (1966) from *T. rutilans* in gallery forest 15 km from Brazzaville, has not yet been fully investigated, but the size of its oocyst (60 μ) suggests that this "vicariant" lies intermediate between *P. berghei berghei* and *P. berghei yoelii.*

The life cycle of *P. chabaudi* is now fully known, except that the natural vector has not been identified; in fact, none of the invertebrate hosts of the newly described rodent parasites are known. It is remarkable that, whereas in Katanga the sporogonic stages of the parasite have been repeatedly seen

in mosquitoes (*A. dureni*) and the parasite is rare in the vertebrate host, in the Central African Republic and in Congo-Brazzaville, the situation is reversed —parasites are common in the rodents, but have not yet been found in mosquitoes in nature.

Sporogony of *P. chabaudi* in *A. stephensi* occurs readily at a temperature of 24–26°C, but lasts 2 days longer than in *P. berghei yoelii* infections. The growth of the exoerythrocytic stage is also slower, the schizonts first maturing at 53 h; however, more merozoites (18 000) are produced.

Landau and Killick-Kendrick (1966) describe what appear to be secondary exoerythrocytic stages of the parasite in *T. rutilans*, caught in the Central African Republic. In one instance, the animal had been kept for 8 months in captivity. Such schizonts possess a conspicuous limiting membrane (0·5 μ thick), and have rather sparse and poorly staining nuclei often lying remote from the borders of the parasite. Marked hypertrophy of the nucleus of the host cell is an interesting feature, in contrast to the reported absence of enlargement of this body in the experimental primary infections. Only one other instance of the natural occurrence of an exoerythrocytic schizont of a species of *Plasmodium* has been recorded: the tissue stages of *P. traguli* were found after a prolonged search by Garnham and Edeson (1962) in the livers of the Malayan mouse-deer, a finding which was later confirmed by Sandosham *et al.* (1962). The precise nature of the schizonts is still doubtful; they are certainly tissue forms of the respective malaria parasites, but whether they represent latent stages of a primary infection or the product of repeated secondary exoerythrocytic schizogony cannot be decided at present.

The life cycle of *P. vinckei* may well be found to resemble that of *P. chabaudi*, as the blood stages of the two organisms are very much alike. The former species has been isolated only on a single occasion, and its maintenance by repeated blood passage since 1952 quickly produced a monstrous efflorescence of asexual growth which permanently swamped the production of gametocytes. Bafort *et al.* (1966), however, succeeded in restoring some degree of normality to the old strain by changing the host and keeping the parasites at −79°C for 12 or more months; after such treatment, gametocytes reappeared and flickering exflagellation of the males was observed. Nevertheless, success stopped here and fertilization and sporogonic development of the parasite in the mosquito still remained impossible.

All the rodent parasites are peculiarly liable to degeneration when they are maintained under abnormal conditions, and if they are to be preserved for malaria research it is essential that one of two procedures should be adopted. A supply of the original parasite should be kept at a low temperature for use when required, or better a rigid routine should be devised which allows the continuous production of healthy sporozoites. It is often not realized that successful transmission by mosquito bite does not necessarily mean that the sporozoites are all healthy; often less than 1% may be viable and the remainder may be dead. The few living sporozoites will lead to an infection (perhaps after a delayed prepatent period if only few healthy sporozoites are present), but exoerythrocytic schizonts will be undetectable because of their sparsity in the liver. The only guide to the viability of a sporozoite is to

see the product, viz. an exoerythrocytic schizont in the liver, and theoretically each sporozoite should become transformed into such a schizont. Of course this never happens, because a varying proportion of sporozoites becomes lost *en route*; probably less than half reach their destination, yet in small animals like rodents it is relatively easy to obtain livers rich in tissue forms. Owing to the excessive vulnerability of the sporozoites of these species, it is essential that all experiments should be accompanied by controls to determine the extent of liver invasion as an indication of the degree of viability of the sporozoite inoculum. Many failures in experimental work on these parasites, even today, are due to the lack of appreciation of these hazards.

In order to set up a suitable routine for the maintenance of healthy strains of rodent malaria parasites, Wery (1967) worked out the following method, which, if carefully followed, may be expected to guarantee the production of five to forty or more exoerythrocytic schizonts in single sections of liver about 20 mm^2 in surface area.

(1) A suspension of sporozoites in 199 medium is prepared by rapid dissection of salivary glands of infected mosquitoes (*A. stephensi*) and is injected into the caudal vein of a white mouse.

(2) On the second day of the parasitaemia, arising from step (1), blood is taken and inoculated into other white mice.

(3) Mosquitoes (*A. stephensi*) are fed on mice from step (2) 4 or 5 days after inoculation; the animals which exhibit the best gametocytes are selected.

(4) The mosquitoes are maintained at a temperature of 23–24°C. Sporozoites appear in the glands about the 11th day and are most "infective" between the 13th and 15th days, when they are used as shown in step (1) above.

Nussenzweig *et al.* (1966a) attempted to standardize infections in rodents by inoculating known numbers of sporozoites into the peritoneal cavity, and found that 25 000 were able to produce a uniform infection in young rats, hamsters and *Thamnomys*, but not in mice. Their criteria, however, did not include any estimation of the numbers of exoerythrocytic schizonts, and without this information no true idea of uniformity can be obtained.

B. SIMIAN PARASITES

Experimental transmission of simian malaria parasites has been particularly successful in the hands of North American workers in recent years. Teams were sent to Malaya, where not only were several natural vectors discovered but sporogony was effected in a variety of local *Anopheles* under experimental conditions. Thus *A. maculatus*, *A. sundaicus*, *A. philippinensis*, *A. vagus*, *A. letifer* and *A. kochi* were infected with *P. knowlesi* in the laboratory and *A. hackeri* and *A. pujutensis* were found to harbour the parasite in nature. Thirty-five species of *Anopheles* are reported to be hosts in varying degrees of susceptibility to *P. cynomolgi* and its subspecies *bastianellii* (Warren *et al.*, 1963), while *A. balabacensis introlatus* and *A. hackeri* were shown to be the

natural vectors in Malaya by Eyles *et al.* (1963) and *A. elegans* in India by Choudhury *et al.* (1963). Wharton *et al.* (1962) isolated *P. inui* from *A. hackeri* and *A. leucosphyrus*, and Cheong *et al.* (1965) from *A. balabacensis balabacensis* in Malaya; Eyles *et al.* (1962) found *P. coatneyi* in *A. hackeri*, and Eyles (1963) showed that sporozoites from wild-caught *A. hackeri* and *A. balabacensis* were those of *P. fieldi*. Strains of these parasites were taken to the United States, where detailed studies of their life cycles were carried out. The usual laboratory species of *Anopheles* were first used for transmission, but in several instances were found to be unsuitable; accordingly attempts were made to breed the natural hosts of the resistant parasites, and by the technique of artificial mating (Baker *et al.*, 1962) colonies of *A. maculatus*, *A. balabacensis* and other species have been established.

Attempts to discover the natural vectors of simian malaria parasites elsewhere in the tropics have been largely unsuccessful. Investigations on a large scale in Taiwan so far have had negative results, while Dissanaike (1965) hitherto has failed to incriminate the mosquito hosts of the four species of monkey malaria parasites occurring in Ceylon. No natural vectors of the gibbon parasites have been discovered, though *P. eylesi* was found by Warren *et al.* (1965) to develop readily in laboratory-bred specimens of *A. kochi*, *A. maculatus* and *A. sundaicus*. The invertebrate hosts of the malaria parasites of chimpanzees and gorillas are completely unknown, while the few trials, using laboratory-bred mosquitoes by Rodhain (1955) and Bray (1958), were largely a failure in that viable sporozoites were never obtained.

Less attention has been paid to the two New World species of simian *Plasmodium*, *P. brasilianum* and *P. simium*, but research by Deane *et al.* (1966a) is in active progress in the State of São Paulo, Brazil. Sporozoites have been found in the salivary glands of *A. cruzi* (2·4% infection rate), captured in the canopy of forests near the city of São Paulo, but these failed to infect splenectomized squirrel monkeys.

The exoerythrocytic cycle of the following simian malaria parasites has been discovered in recent years.

1. *P. brasilianum*

Exoerythrocytic schizogony of a Colombian strain of this parasite was first described by Garnham *et al.* (1963a) and, strangely enough, was found to resemble that of the human quartan species, *P. malariae*, rather than the simian parasite, *P. inui*. Thus the schizont is relatively large, contains curious pink or orange vacuoles, takes over 12 days to become mature and causes gross enlargement of the host cell nucleus. All these characters are shared by *P. malariae* as recently described by Lupasçu *et al.* (1967). Later, American workers found a similar picture in a Panamanian strain of *P. brasilianum*.

The remarkable resemblance of this New World quartan parasite of monkeys to the human form is not only evident in the tissue stage but in the immunological response of the host, for *P. brasilianum* proves to be a much better antigen than the Old World *P. inui* for fluorescent antibody tests in human malaria. The question therefore arises as to whether *P. brasilianum* was perhaps originally the human *P. malariae*, brought to the New World

at some remote date where it spread into the monkey population—in other words, the situation represents a "reversed zoonosis".

2. *P. inui*

The tissue stages of this quartan parasite of oriental macaques were described in detail by Garnham (1951); further observations were made by Mohiuddin (1957) and Sezen (1958).

3. *Subspecies of P. cynomolgi*

The subspecies of this tertian parasite are most easily differentiated by the character of their exoerythrocytic cycles; *P. c. bastianellii* is smaller and matures earlier than *P. c. cynomolgi*, while *P. c. ceylonensis*, although also smaller, has a slower rate of development (Dissanaike *et al.*, 1965).

4. *P. fieldi*

Held *et al.* (1967) found exoerythrocytic schizonts of this species in monkeys inoculated with sporozoites. At 8 days the schizonts were 24–34 μ in size, and at 9 days cytoplasmic clumps were prominent. At maturity the schizonts of *P. fieldi* attain a size of about 40 μ, and the prepatent period is 9 days.

C. RELAPSES

Probably the most important gap in our knowledge of the life cycle of mammalian malaria parasites is a complete explanation of the relapse phenomenon, a feature which nevertheless has been recognized in the human disease from the earliest times. The classical theory of the Italian workers (e.g. as explained by Bignami, 1913) assumes that parasites persist in small numbers in the blood for years and are reawakened at intervals to produce relapses; Corradetti (1965) continues to uphold this theory, which nevertheless fails to explain certain phenomena. If the theory were true, parasites should be demonstrable in the intervals between relapses by subinoculation into new individuals of large amounts of blood, but this often meets with no success; moreover, provocative measures usually fail in an attempt to drive the parasite from the internal sinuses (Bruce-Chwatt, 1963b). This theory moreover offers no explanation of the profound differences which exist between blood- and sporozoite-induced infections; the former are easily eradicated by the administration of quinine and are unaccompanied by long-term relapses, the latter cannot be cured by ordinary schizontocides and late relapses are common.

After the demonstration of the occurrence of exoerythrocytic schizogony in the liver of primates, another explanation for the relapse phenomenon was forthcoming, and the discovery by Shortt and Garnham (1948) of secondary forms in monkeys, 3½ months after sporozoite-induced infections and just before a relapse, appeared to provide further confirmation that the relapse is due to the persistence of tissue forms in the liver and their reactivation by an unknown stimulus. Fairley (1949) was one of the first to cast doubt on

the complete validity of this theory, which does not explain the mechanism of delayed primary infections, the suppression for months or years of the parasite by another species, or prolonged latency induced by drugs; nor in general does it account for the sudden and sometimes regular onset of a relapse, which has been inadequately ascribed to the collapse of immunity.

Fresh data have recently become available in support of both theories of the causation of relapses. Brown and Brown (1966) were able to demonstrate "relapse variants" of blood infections of *P. knowlesi*; they assumed that the monkeys became immune to the (drug-treated) primary infection, but a variant of the strain persisted and eventually gave rise to a relapse. This process was indefinitely repeated, with the production of a succession of variants, each giving rise to specific agglutinins, by which they could be distinguished. This work also indicated that some members of a population of parasites manage to survive in the presence of antibodies, and possibly the phenomenon of premunition in primate malaria depends upon the successive multiplication of a series of variants, just as in trypanosomiasis (Lourie and O'Connor, 1937) or in relapsing fever.

Histological evidence for an exoerythrocytic origin of relapses is comparatively poor. The regular occurrence of secondary exoerythrocytic schizogony has never been demonstrated, and Bray *et al.* (1963) noted a rapid decline in the production of tissue forms of *P. ovale* in chimpanzees; moreover, satellite schizonts have never been observed in the vicinity of a burst parent form. Accordingly, this theory has been modified (Garnham, 1967) by assuming that a dormant stage of the original parasites exists in the liver, which at a given moment starts growing again and a relapse or a delayed primary infection ensues. Such dormant stages have possibly been seen by Landau and Chabaud (1965) in malaria-infected *Thamnomys rutilans*, kept in captivity for 8 months, and by Garnham (1967), both in old sporozoite-induced infections of a Ceylon strain of *P. cynomolgi* and in aberrant infections of the same parasite. A characteristic of all these retarded forms is some thickening of the lining membrane of the schizont; the small size and the slow rate of growth, and the crinkled or convoluted border. The nuclei may show peculiar abnormalities, or more often are unchanged in appearance. It has long been known that the "old" sporozoites, at the end of the season, are liable to give rise to delayed infections (as in the Dutch strain of *P. vivax*) and possible relapses in general are due to the persistence of such abnormal forms. The question might be settled by the deliberate exposure of sporozoites to adverse conditions such as radiation or prolonged storage and the subsequent tracing of their development in the monkey's liver.

IV. Host Susceptibility and the Zoonosis Problem

A. Zoonoses

Until recent years, the view was widely held that the malaria parasites of monkeys and of the anthropoid apes, with few exceptions, were unable to infect man. Only the blood forms of *P. knowlesi* were known to be readily transmissible to man; the sharing of *P. malariae* by chimpanzee and man had

also been recognized (Rodhain, 1948). But ideas on the relative insusceptibility of man to monkey malaria had to be profoundly changed, when Eyles *et al.* (1960) reported the sudden occurrence in the United States of laboratory infections in man of *P. cynomolgi bastianellii*. This organism had been isolated by Garnham in 1957 (see Garnham, 1959), and had been transmitted by frequent mosquito and blood passage in the laboratory. Three years later it was taken to the United States, where at first it betrayed no unusual character. Then early in 1960, the late Dr Don Eyles suddenly developed a fever with a tertian periodicity; his blood films showed a vivax-type of parasite, which proved to be capable of infecting rhesus monkeys. Eyles had been engaged on large scale experiments involving the transmission of *P. c. bastianellii* by mosquitoes, and he (and another member of the staff) must have been accidentally bitten by infected specimens.

Further laboratory-acquired cases arose in other workers in the United States, and in November of the same year two men in my own laboratory in London also acquired accidental infections. It was quickly shown by the North American observers and by Schneider (1961) in Paris that *P. cynomolgi bastianellii* could easily be transmitted to volunteers or general paralytics by mosquito bite. The significance of these occurrences was not lost on the workers at Bethesda, and arrangements were made for Dr Eyles to take a team to Malaya to investigate the extent of transmission of monkey malaria to man in nature, and the problem of animal species of *Plasmodium* in general. A splendid harvest of the latter was reaped, during which the premature death of the eminent leader unfortunately occurred, although no isolations of simian parasites from man were made (but see below, p. 158).

The potential danger of malaria as a zoonosis was fully discussed by Eyles and his colleagues in 1962. The subject has also been considered *in extenso* by Hoare (1965), Bray (1963) and Bruce-Chwatt (1967).

The susceptibility of man to many monkey parasites has been tested, chiefly by mosquito transmission, and the following species proved to be infective (asterisks indicate blood transmission):

P. cynomolgi bastianellii: Eyles *et al.* (1960)
P. cynomolgi cynomolgi, M. Strain: Schmidt *et al.* (1961)
P. cynomolgi, Cambodian strain: Bennett and Warren (1965); Coatney *et al.* (1961)
P. inui: Dasgupta (1938).*
P. shortti: Coatney *et al.* (1966)
P. brasilianum: Contacos *et al.* (1963)
P. eylesi: Warren *et al.* (1965)*
P. knowlesi: Ciuca *et al.* (1955)*; Chin *et al.* (1965).

Experimental infections in man are usually light and evanescent at the commencement of a series, but later the virulence is enhanced and viable gametocytes may be produced, which are capable of infecting mosquitoes. At first only about 10% of the volunteers exhibited patent infections, and this poor reaction of man in the first passage is probably the reason why the early attempts to infect man failed—not enough volunteers were tried, and probably

too few mosquitoes were used. Moreover, the prepatent period may be greatly prolonged and the parasitaemia, when it does arise, is invariably very low; such infections could easily have been missed in the past. The severity of symptoms, including high fever and enlargement of the spleen and liver, is quite out of proportion to the low parasitaemia, particularly in the *P. cynomolgi* forms of the disease.

The existence of natural infections in man of simian malaria parasites may be demonstrated either by observing the typical morphology in a thin blood film or, better, by subinoculating blood into rhesus monkeys. Nearly 1 200 natives of the Malayan jungle were tested by the latter method by the North American workers (Warren *et al.*, in prep.) without incriminating a single example of a zoonosis. Parasites with unusual characters had previously been observed in patients in Malaya and also in Thailand, Ceylon, Brazil and elsewhere, but their suspected simian origin had never been confirmed.

At last, in 1965, Chin *et al.* uncovered a true zoonosis in a North American surveyor, who had spent a little time in forests in Malaya, became ill in Bangkok, was worse on repatriation to California and was finally diagnosed as a case of suspected *P. falciparum* malaria in Maryland. His blood was inoculated into rhesus monkeys, which died of typical *P. knowlesi* malaria. A second example was described by Deane *et al.* (1966a) in the following circumstances in Brazil. An entomologist was engaged in mosquito surveys in a small forest near São Paulo, where a focus of simian malaria was known to be present in the howler monkeys. He became ill with a tertian fever which spontaneously disappeared after a week; his blood films showed scanty malaria parasites, resembling *P. simium*, and a sample of his blood gave rise to a typical infection when inoculated into a splenectomized *Saimiri* monkey. The interpretation of such positive subinoculations may be difficult, for Deane *et al.* (1966b) showed that splenectomized *Saimiri* monkeys are susceptible to *P. vivax*, with a peak parasitaemia of over 6 000 per mm^3, the parasites persisting for over 4 months.

An assessment of the danger of simian malaria as a zoonosis is now possible and the following facts should be taken into consideration.

(1) Two examples of a zoonosis have been found—one in Malaya and the other in Brazil.

(2) Accidental infections in the laboratory of *P. cynomolgi bastianellii* are fairly common.

(3) Deliberate transmission of at least six simian species of *Plasmodium* to man has been achieved by mosquito bite, and repeated transmission of some species from man to man by mosquitoes has taken place.

(4) Certain sylvatic species of *Anopheles* are known to bite man in nature, as readily as their ordinary simian hosts; *A. cruzi* acts in this way in Brazil, and Cheong *et al.* (1965) emphasize the danger of a zoonosis constituted by the presence of infections of *P. cynomolgi* and *P. inui* in *A. balabacensis balabacensis* in Perlis, for this mosquito bites both man and monkey.

(5) A small human population visits or lives in tropical forests in the Orient, West Africa and Latin America. As in the cycle of yellow fever,

such people could become infected in the forest and introduce the simian parasite into urban or village communities.

The foregoing circumstances appear to be disturbing from the point of view of malaria eradication campaigns, but a few further facts may now be regarded.

(6) Only a minority of sylvatic vectors of monkey malaria feed on man as avidly as on the animal.

(7) Parasitaemia of a simian species in its first passage in man is low and terminates quickly without the production of gametocytes, so there is not much chance of secondary cases becoming established.

(8) Monkey species of *Plasmodium* are often unable to develop in domestic *Anopheles*, e.g. *P. knowlesi*, *P. reichenowi* or *P. schwetzi.*

(9) The progress of malaria eradication campaigns, e.g. in jungle regions of India and Taiwan, has proceeded smoothly and without interruption by unexplained infections of a possible simian source.

(10) Deliberate searches in enzootic regions for simian malaria in man have given negative results.

It is possible to conclude from the above considerations that monkey malaria is unlikely to interfere seriously with malaria eradication, and probably no alteration in the strategy of campaigns is necessary. Nevertheless, a potential danger exists of which malariologists should remain aware.

B. OTHER HOST SUSCEPTIBILITIES

The same wide spectrum of host susceptibilities is seen amongst the species of primates in general, where an interesting range of variations in response to a parasite may be observed. Thus, *P. c. bastianellii* exhibits a high parasitaemia (10%) in *Macaca mulatta*, a modest parasitaemia (1%) in *M. irus*, the natural host, 0·01% in Caucasian man and a total resistance in the erythrocytic stages in Negroes and New World monkeys. On the other hand, the livers of New World monkeys are fully susceptible to the exoerythrocytic stages. A somewhat similar gradation of response may be seen in *P. knowlesi* malaria, to which *M. mulatta* and baboons are extremely and fatally susceptible, to which *M. irus* and other African monkeys are moderately susceptible, to which man can become adapted and show heavy infections, and to which New World monkeys are in general rather feebly receptive.

The rodent malaria parasites have also been found to infect a large variety of animals. Cox (1964) relates the susceptibility to the phylogeny of the host: the severest infections occur in the Murinae, and mild or occult infections in squirrels and bats; splenectomy usually breaks down the natural resistance and even Old and New World monkeys may become infected with *P. berghei* after several passages (Wellde *et al.*, 1966).

Interesting examples of the susceptibility of splenectomized marmosets and gibbons, and even of baby mice, to the human species of *Plasmodium* have been described recently by Porter and Young (1966), Ward and Cadigan (1966) and Weinman *et al.* (1966), but this subject is outside the scope of this review.

V. Ultrastructure

When Huff (1963) reviewed avian malaria in this series, the study of the fine structure of the parasites had not proceeded very far, but it was evident that new developments would lead to great advances in our knowledge of the morphology, physiology, taxonomy and life cycle of the organisms. A brief description is now given of the fine structure of the erythrocytic and sporogonic stages of the mammalian species of *Plasmodium*, excluding the human or avian except where comparison is essential. The interpretation and functions of some organelles are still doubtful, and will remain so until cytochemical techniques have been allied to electron microscopy of this group of organisms. Pitelka (1963) and Rudzinska and Vickerman (1968) have produced useful monographs on the fine structure of protozoa.

A. Erythrocytic Stages

The various species of mammalian *Plasmodium* show minor differences in ultrastructure, but the main features are similar. Rudzinska and Trager (1959) first demonstrated the existence of phagotrophy in the trophozoites of *P. berghei* (Figs. 1–3). The host cell cytoplasm is engulfed by the invagination of

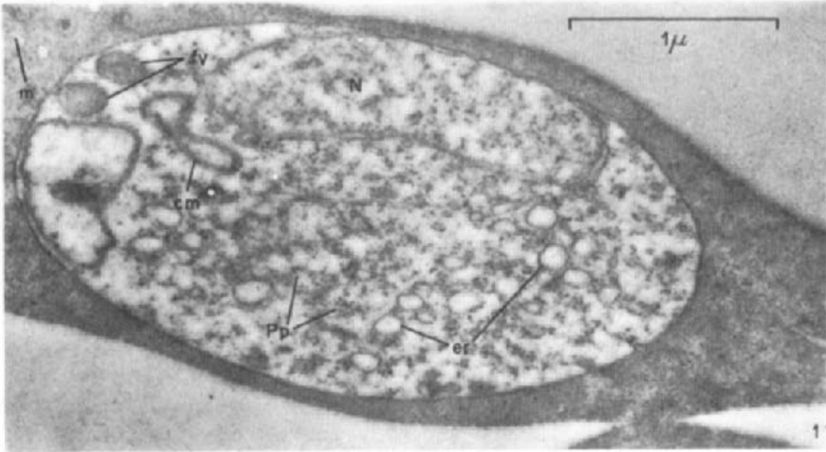


Fig. 1. Sections through *P. berghei*. The elongate nucleus (*N*) is enveloped in two membranes. Endoplasmic reticulum (*er*). Palade's particles (*Pp*.) Two small food vacuoles (*fv*). Concentric double-membraned structure at *cm*. The body is covered by a double limiting membrane *m*, Host cell mitochondrion. (From Rudzinska *et al*., 1965.)

the plasma membrane, until a portion is pinched off within the parasite as a food vacuole which is enclosed by a double membrane. Digestion then proceeds, not in the vacuole itself as in the avian species or in *P. falciparum*, but in minute vesicles which appear in all parts of the cytoplasm of the parasite and accumulate in one area during the later stages of schizogony. Malaria pigment is the metabolic residue within small vesicles in this species, the particles being almost invisible by light microscopy until schizogony is

completed. The first food vacuole is large and it corresponds to the vacuole of the ring stage. The engulfment of submicroscopic amounts of host cell cytoplasm is known as pinocytosis (almost synonymous with phagotrophy) and it entails the gradual incorporation of the host cell cytoplasm in the parasite.

In *P. gonderi*, *P. schwetzi* and *P. coatneyi* (Fig. 4) a similar phenomenon was observed by Rudzinska *et al.* (1965) and in *P. knowlesi* by Fletcher and Maegraith (1962). In these parasites of primates the food vacuole itself is devoid of pigment and the granules are found as before scattered in vesicles

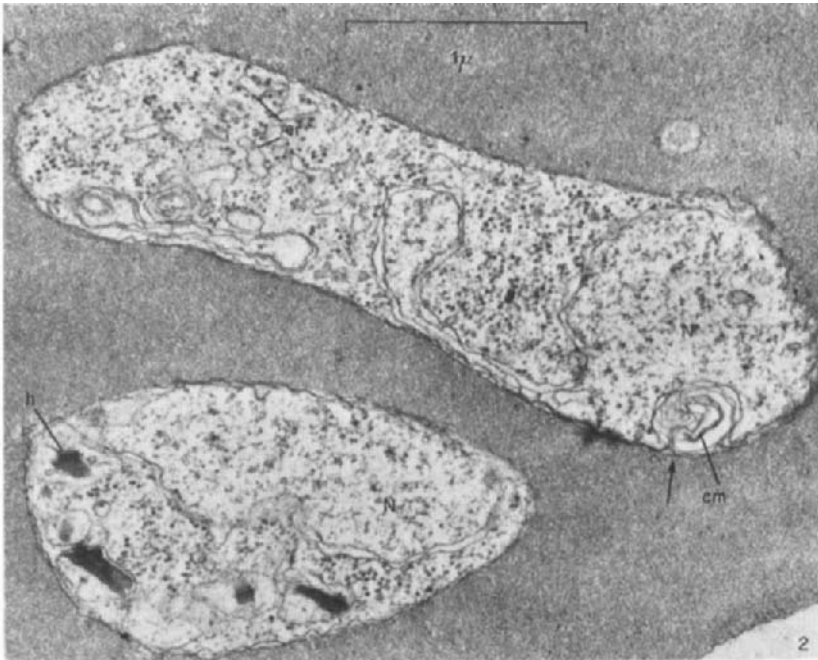
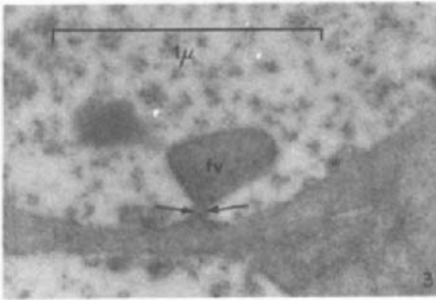
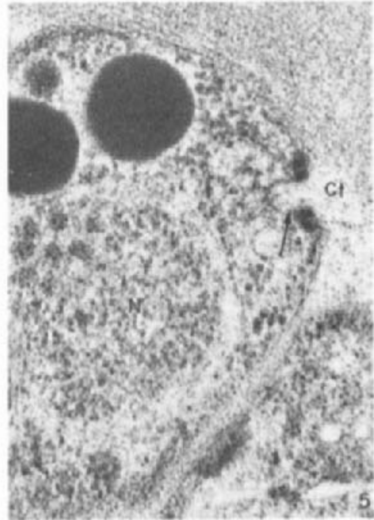


FIG. 2. Electron micrograph of two organisms of *P. coatneyi* in an erythrocyte of the rhesus monkey. The parasite is surrounded by two membranes. *N*, Nucleus: *er*, endoplasmic reticulum; *cm*, concentric structure; *h*, haemozoin. (From Rudzinska *et al.*, 1965.)

throughout the cytoplasm. Aikawa *et al.* (1966b) claim that in *P. knowlesi* and *P. cynomolgi* crystalline pigment occurs in the food vacuole itself (Fig. 8) and they note that exposure of their preparations to alkalies dissolves the pigment, leaving clear spaces of characteristic shape. In a strain of *P. berghei* rendered resistant to chloroquine, Peters *et al.* (1965), found that pigment was no longer produced, its absence being confirmed by electron microscopy.

The malaria pigment of *P. vinckei* is much coarser than that of *P. berghei*, and Cox and Vickerman (1966) described as a product of pinocytosis crystals up to 450 mμ long and in peripheral vesicles, and also a large food vacuole that indents the nucleus. It was suggested that the vacuole is a means of increasing





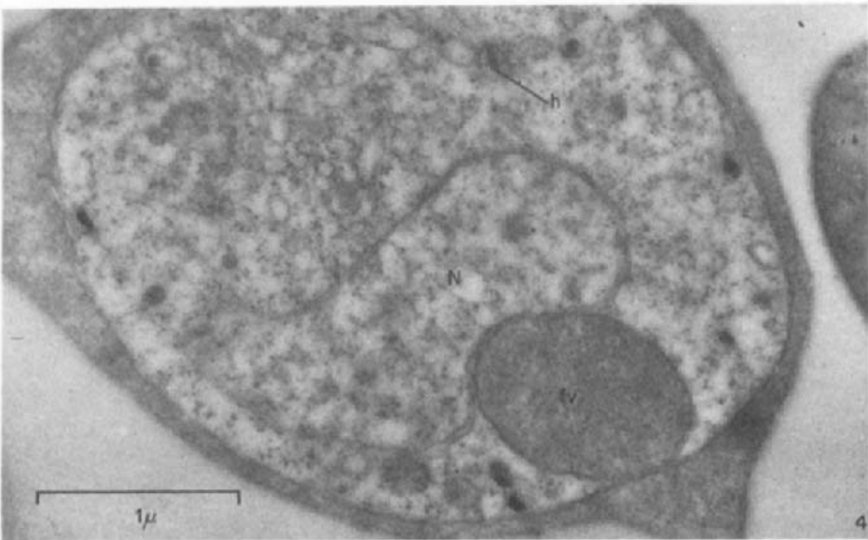
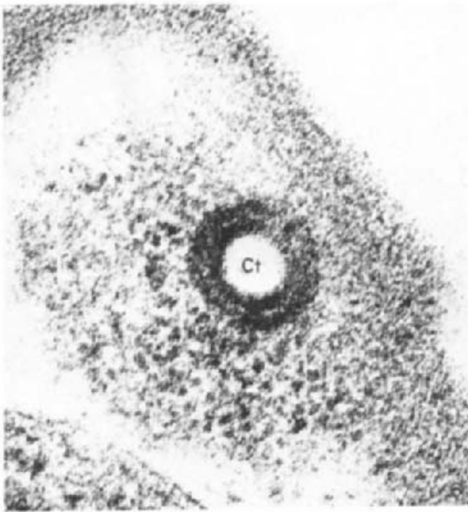
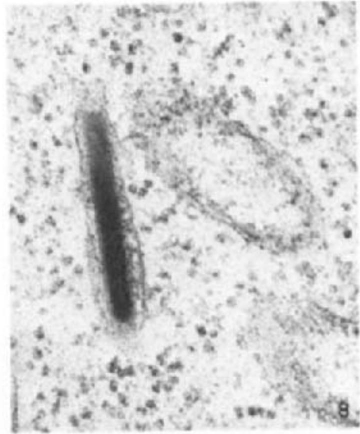


FIG. 3. Section through *P. berghei* representing a step in the indentation of the invaginating plasma membrane at arrows. *fv*, Food vacuole. (From Rudzinska *et al.*, 1965.)

FIG. 4. Section through *P. berghei*. A food vacuole (*fv*) lies in close proximity to the double plasma membrane and the nucleus (*N*). *h*, Haemozoin. (From Rudzinska *et al.*, 1965.)

FIG. 5. An erythrocytic merozoite of *P. knowlesi*. The longitudinal section of a cytostome (*Ct*) shows that the sides of the cytostome are bounded by two dark segments and the base is formed by a single membrane. *N*, Nucleus. × 95 000. (From Aikawa *et al.*, 1966b.)





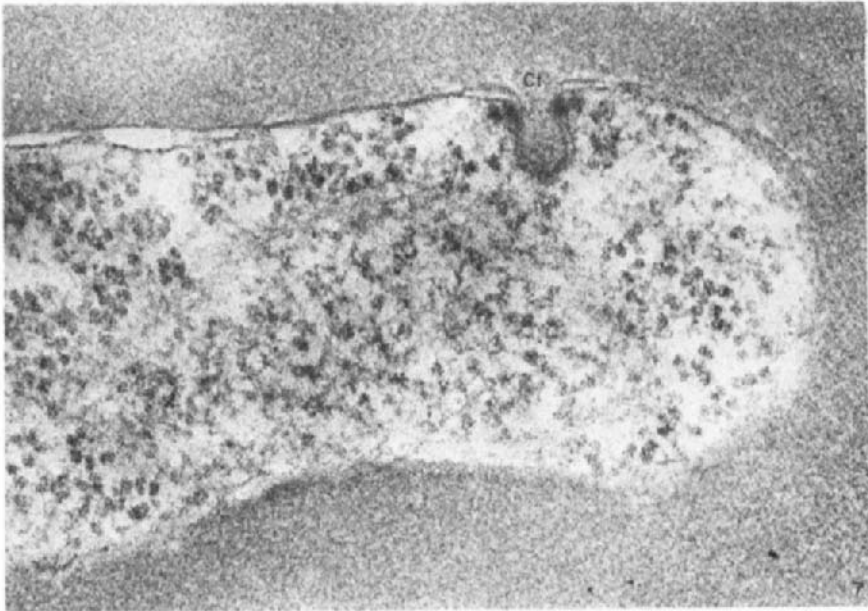


FIG. 6. Face view of the cytostome (*Ct*) of the erythrocytic merozoite of *P. gallinaceum*. × 95 000.

FIG. 7. An erythrocytic trophozoite of *P. cynomolgi* shows a cytostome (*Ct*) which is ingesting host cell cytoplasm. × 90 000.

FIG. 8. A malarial pigment particle within a food vacuole of *P. cynomolgi*. × 86 000. (All from Aikawa *et al.*, 1966b.)

the surface area of the parasite to provide greater opportunity for pinocytosis.

The above interpretation of the feeding process of erythrocytic stages of *Plasmodium* has been challenged by Aikawa *et al.* (1966a,b), who described a pore or cytostome (Figs. 5–7) on the surface of the parasite, exactly corresponding to the "micropyle" first seen by Garnham *et al.* (1961) in the sporozoites of *P. falciparum*. The relationship of this organelle in the various stages of development of *Plasmodium* is discussed below, but in the erythrocytic merozoite, trophozoite and immature schizont, there seems to be no doubt that the cytostome is the site of feeding by the parasite. Host cell cytoplasm bulges into the orifice, the basal membrane expands inwards and a food vacuole is gradually engulfed, surrounded by two double membranes. Cox

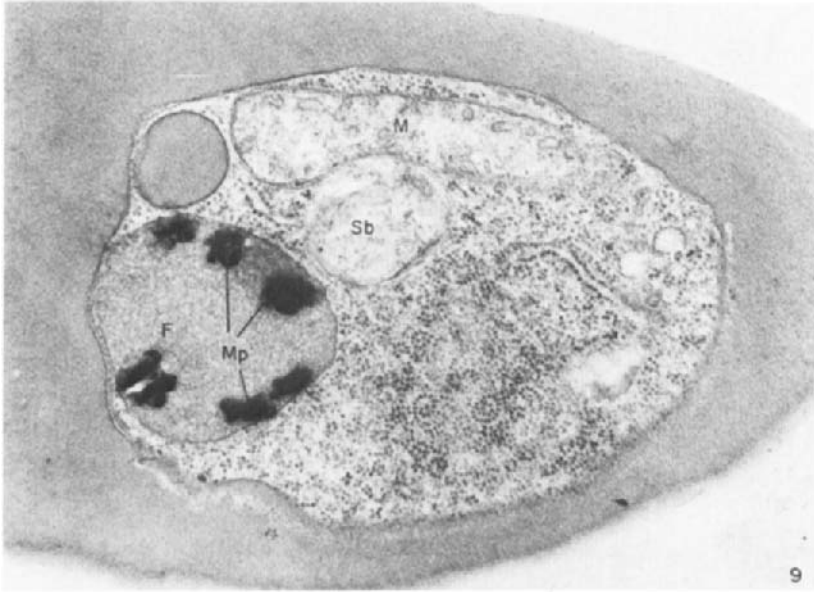


FIG. 9. A trophozoite of *P. gallinaceum* shows a large food vacuole (*F*). *Mp*, Malaria pigment; *Sb*, spherical body; *M*, mitochondria. (From Aikawa *et al.*, 1966b.)

and Vickerman (1966) tried to reconcile these opposing views by suggesting that the avian parasites and *P. falciparum* absorb food through a single cytostome, while *P. berghei* and *P. vinckei* utilize pinocytosis. Other species are supposed to lie between these two extremes utilizing both methods of food uptake. The problem remains unresolved, but possibly the multiple pinocytotic vesicles on the surface of *P. berghei* and other species represent small cytostomes, while a single large cytostome suffices for the avian parasites. Aikawa *et al.* (1966b) drew attention to the great difference in size of this structure in the various species of *Plasmodium*. Ladda *et al.* (1966) also describe in the trophozoites of *P. berghei* and *P. falciparum* a micropyle that is always associated with engulfed host cell cytoplasm.

The presence or absence of mitochondria is an important characteristic of the different stages of *Plasmodium* spp. The erythrocytic forms of *P. berghei* lack mitochondria, but possess structures composed of two to six double membranes arranged concentrically, usually near the periphery of the organism and possibly continuous with the limiting plasma membrane. Rudzinska and Trager (1959) consider that these bodies have a secretory function and may be assumed to represent mitochondria. Mitochondria were not detected by Fletcher and Maegraith (1962) in *P. knowlesi* trophozoites, although they saw contours with double membranes. These were more fully illustrated by Aikawa *et al.* (1966b) (Fig. 9). In the growing stages of the

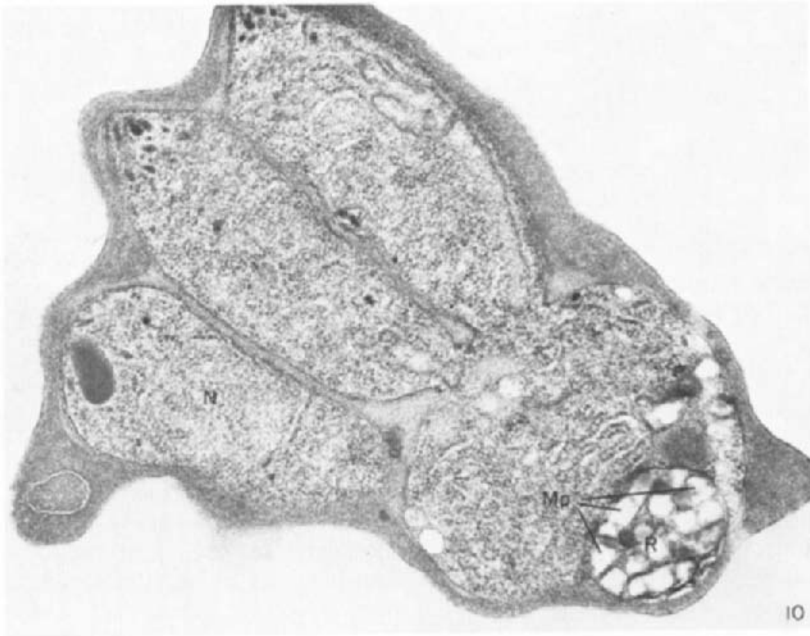


FIG. 10. A schizont of *P. knowlesi* with budding new merozoites. *Mp*, Malarial pigment particles; *R*, residual body; *N*, a nucleus. × 23 000. (From Aikawa *et al.*, 1966b.)

mammalian parasite in the blood fully formed mitochondria may be absent, whereas in the avian parasites these organelles are abundant.

Most workers studied the feeding mechanism of the blood stages of the malaria parasite but little information is available about other details of ultrastructure. The nucleus shows nothing of special interest until schizogony begins. The formation of merozoites in *P. knowlesi* was described by Aikawa *et al.* (1966), who also showed the presence of numerous ribosomes, endoplasmic reticulum and a paired organelle leading to the conoid. The merozoites, particularly of three avian species, best show these structures, including the termination of the microtubules (i.e. peripheral fibrils) in the conoid. The

description is likely, as a whole, to be applicable also to mammalian species (Fig. 10). In a few instances during the schizogony of avian malaria parasites (*P. fallax*, *P. lophurae*, *P. cathemerium* and *P. elongatum*; Aikawa 1966; Aikawa *et al.*, 1967), the formation of a spindle has been observed. The fibres are 180 Å diam. and appear to arise from a centriolar plaque on the edge of the nuclear membrane, which persists throughout division. Small granules (250 Å in size) which may represent chromosomes are strung on the fibres, but their number is greater than the figures given by Wolcott (1954) or Bano (1959).

Hepler *et al.* (1966) compared the fine structure of the merozoites of exoerythrocytic schizonts of several species of avian malaria parasites, and noted the presence of a non-functional cytostome (micropyle), paired organelle, conoid and other structures. These observations were made on preparations in tissue culture, which unfortunately is not yet applicable to the study of mammalian species. Garnham *et al.* (1967b) demonstrated these stages in excessively heavy sporozoite infections of *P. berghei yoelii*. The exoerythrocytic schizonts were so numerous in the liver that individual mature schizonts could be recognized under low magnification, and were then photographed under the electron microscope. A micropyle and conoid were detected, while the paired organelle was a constant feature (Figs. 11 and 12).

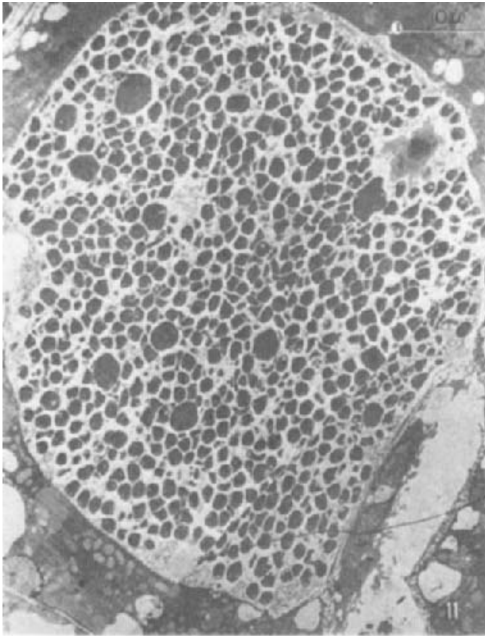
Few observations have been made specifically on the gametocytes of the primate parasites, except in relation to exflagellation of the male which is described below.

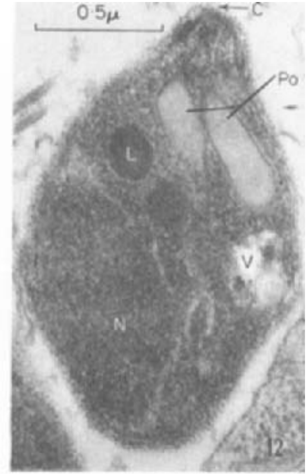
B. EXFLAGELLATION

The process of maturation of the microgametocyte with the production of gametes obviously constitutes a fundamental aspect of the life cycle of the malaria parasite, and the determination of the fine structure of the stages concerned was thought to be of basic importance. The techniques involved in cutting thin sections of the exflagellating body presented many difficulties, both in regard to the rapidity of the phenomenon and the relative scarcity of the forms. Eventually, after four years' work, Garnham *et al.* (1967a) obtained preparations of these stages, in which many structures were visible. Raffaele (1939) had regarded a free "flagellum" as an essential part of the microgamete in six species of *Plasmodium*, but it was uncertain if this structure was a true flagellum. The observations of Cheissin and Snigerevskaya (1965) and of Scholtyseck and Spiecker (1964) on the microgametes of allied sporozoan parasites of the genus *Eimeria*, confirmed that at least two typical flagella were present. The observations of the British authors relate to various malaria parasites, including *P. berghei* and *P. cynomolgi bastianellii*, and all showed the same picture. Shadow cast replicas (Fig. 13) of microgametes indicated no free flagellum, while sections demonstrated that the microgamete itself (Figs. 14 and 15) is really a nucleated flagellum with 9+2 fibrils (double, and accompanied by radial spokes) and an adjacent elongated nucleus, surrounded by a common flagellar pellicle, probably derived from the surface membrane of the microgametocyte. The microgamete is more comparable to a simplified spermatozoon than a flagellated protozoon such as *Bodo* or *Trypanosome*

(see Fig. 18). Under the coverslip, using light microscopy, the flagellar bundle easily becomes herniated from the body of the microgamete and may then simulate as a free flagellum.

Maturation of the microgametocyte is a complex and enigmatic process (Fig. 19), which is completed in about 8–12 min in the mammalian species.





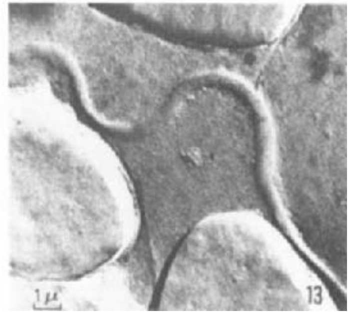


FIG. 11. Exoerythrocytic schizont of *P. berghei yoelii* showing merozoites. Some free merozoites in an adjacent sinusoid.

FIG. 12. Merozoites of exoerythrocytic schizont of *P. berghei yoelii*. *Po*, Paired organelle; *N*, nucleus; *C*, conoid; *V*, vacuole of unknown origin; *L*, lysosome.

FIG. 13. Shadow cast replica of microgamete of *Leucocytozoon marchouxi*.

(Figs. 11 and 12 from Garnham *et al.*, 1967b; Fig. 13 from Garnham *et al.*, 1967a.)

The nucleus divides endomitotically three times, giving rise to eight nuclei which are arranged around the original nuclear membrane. A centriole (called a karyosome by some authors) lies adjacent to (and sometimes apparently inside) the nucleus. During nuclear division this body is believed to divide three times, or possibly replication occurs and eight centrioles are formed. Each centriole migrates to the vicinity of a nucleus and becomes a

basal body: 9+2 fibrils grow out and by the violence of the process draw out the nucleus, and the pellicular covering, to give rise to the lashing microgametes, which remain attached for a few minutes to the parent body.

FIG. 14. Gametocyte with numerous sections of developing flagella in its cytoplasm. (Figs. 14 and 15 from Garnham *et al.*, 1967a.)

FIG. 15. Exflagellating gametocyte showing incorporation of nuclear material (*NG*) into base of developing gamete, and a single dense body (*DB*) close beside the nucleus.

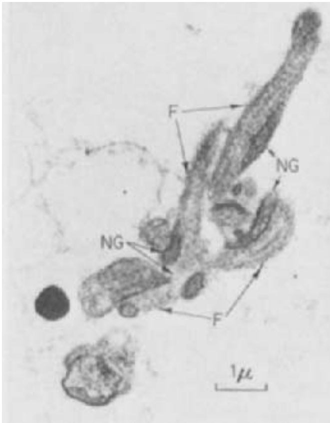


FIG. 16. Oblique section through several entwined microgametes showing nucleus (*NG*) and flagellum (*F*).

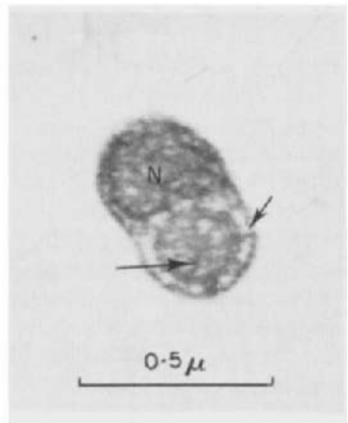


FIG. 17. Transverse sections of microgametes showing (arrows) radial spokes of flagellum, external and internal respectively. (Figs. 16 and 17 from Garnham *et al.*, 1967a.)

Hitherto no convincing evidence of a basal body with 9+0 fibrils has been obtained; nor is the origin of the pellicle of the gamete certain, for it may be derived from endoplasmic reticulum and not the plasma membrane. The source of energy of the hyperactive gamete remains unknown; mitochondria have not been identified with certainty, but tubular oval bodies have occasionally been observed near the protrusions of the emerging gametes.

The surface layer of the maturing microgametocyte appears to consist of two unit membranes, and not three, as have been described by Duncan *et al.* (1959) and Aikawa (1966) in the gametocytes of avian species.

It is of interest from the point of view of evolution to compare the fine structure of the gametes of *Eimeria* and *Plasmodium*. The former possess two

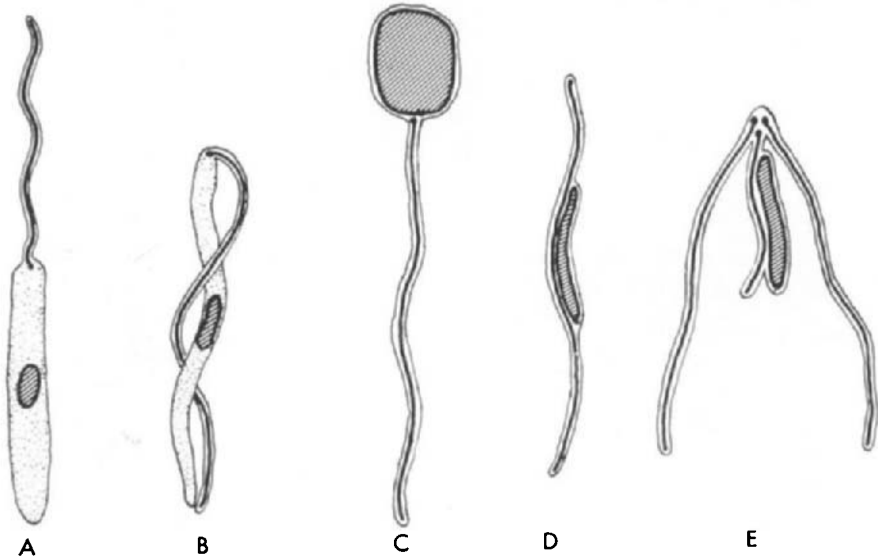


FIG. 18. Diagrammatic representation (not to scale) of relationship between nucleus and flagellum in (A) a simple mastigophoran, (B) Raffaele's (1939) conception of a malarial microgamete, (C) a mammalian sperm, (D) a malarial microgamete as described in the present paper, and (E) a microgamete of *Eimeria perforans* (after Scholtyseck, 1965). Mitochondria are omitted from all the diagrams. (From Garnham *et al.*, 1967a.)

free flagella and one reflexed flagellum incorporated in the body of the microgamete. All three flagella arise from basal bodies at the base of the gamete, and nearby is a prominent mitochondrion. It seems clear that, in the course of evolution of the more complex haemosporidian parasite, the two free flagella have been suppressed; probably the male gamete in the blood of the insect's gut is less likely to meet with obstacles than the coccidial microgametes in the lumen of the vertebrate intestine—the latter therefore require both a more elaborate motive power and to be in greater numbers (a single coccidian microgametocyte producing hundreds of gametes instead of the eight in *Plasmodium*). It may be noted that the length of the plasmodial microgamete is many times greater than that of the eimeriad.

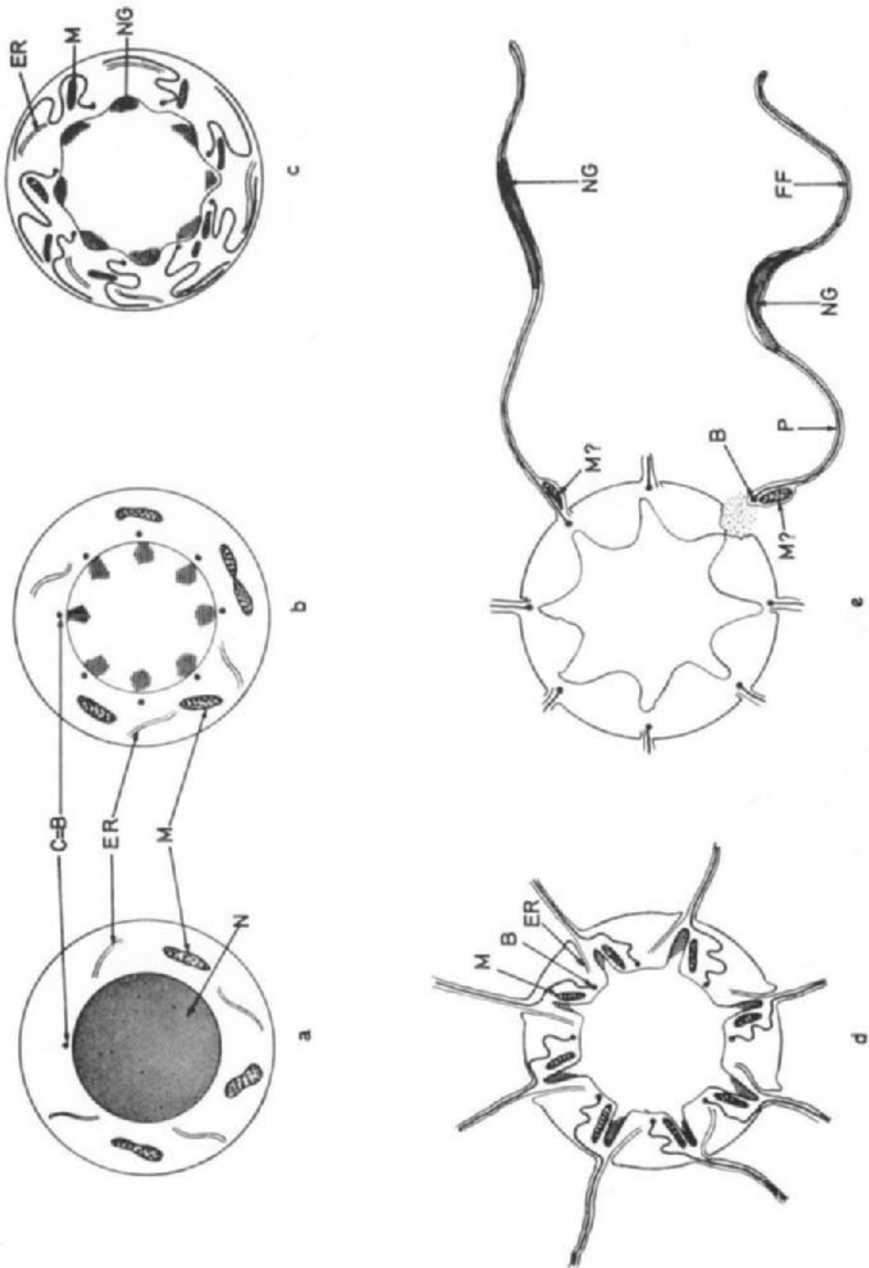


FIG. 19.

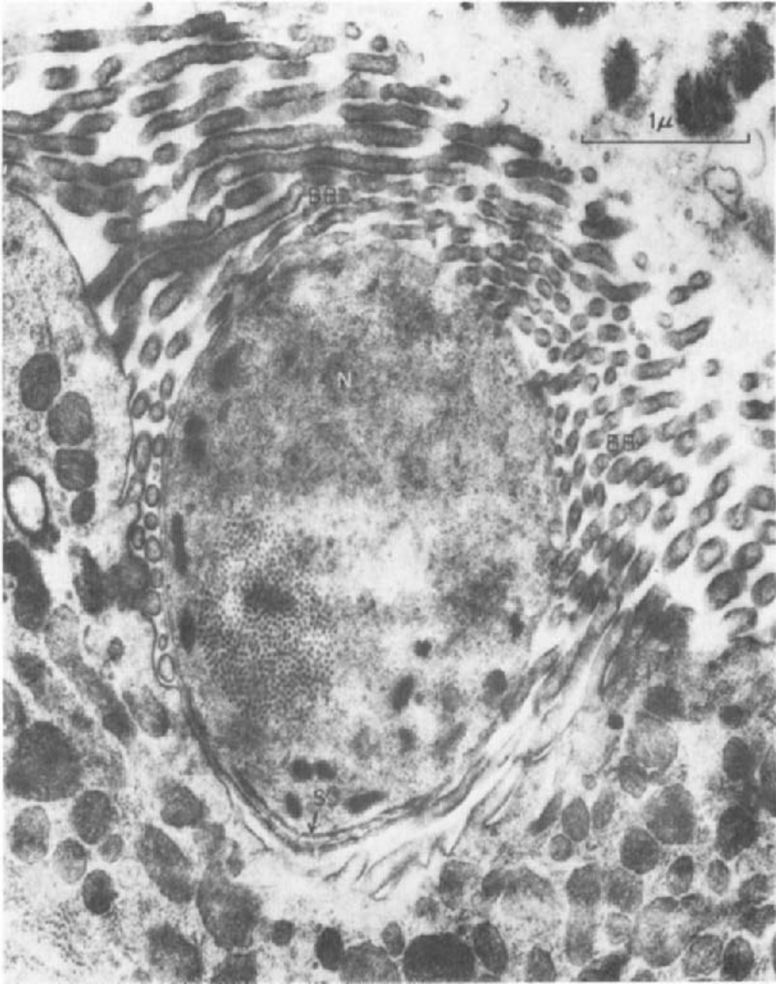


FIG. 20. Penetration of ookinete of mosquito gut cell. *N*, Nucleus of ookinete; *S*, anterior slit; *BB*, brush border. *P. c. bastianellii.*

FIG. 19. Diagrammatic representation of the process of exflagellation (pigment is omitted). (a) Microgametocyte with centriole and single nucleus. (b) Endomitosis and centriolar multiplication occurring. (c) Formation of flagellar fibrils from the eight daughter centrioles (basal bodies). (d) Association of nuclei and mitochondria near point of emergence of fibril complexes, with endoplasmic reticulum possibly contributing to the pellicle of each gamete. (e) Fully formed microgametes breaking away from the remains of the gametocyte. *B*, basal body; and *C*, centriole (homologous structures); *ER*, endoplasmic reticulum; *FF*, flagellar fibrils; *M*, mitochondrion; *N*, nucleus of microgametocyte; *NG*, nucleus of microgamete; *P*, pellicle of microgamete. (From Garnham *et al.*, 1967a).

C. OOKINETE

Controversial views on the mode of penetration of the mosquito midgut by ookinetes have been expressed in recent years, and it was expected that the application of new techniques like cinéphotomicrography and electron microscopy would clarify the nature of the process. Howard (1962) concluded from a study of *P. gallinaceum* that ookinetes possess no innate motility and are largely degenerative forms of the parasite which are rapidly excreted by the mosquito; the true zygotes were thought to become passively enclosed by the expanding mucosal cells of the midgut during digestion of the blood meal, and were thus squeezed through to the outer surface of the wall, where they were transformed into oocysts. This theory is disproved by many direct observations (Garnham, 1965c), during which the ookinete may be seen twisting and turning, and moving in one direction like a gregarine, while the detailed description by Freyvogel (1966), based on a ciné film, gives a permanent record of the movements of the ookinete. Freyvogel noted that the simian parasite, *P. c. bastianellii*, moved in a straight line and the rodent parasite, *P. berghei*, took a more serpentine course; both species travelled at about the same speed, less than 10 μ per min.

Then there are two theories regarding the route that the ookinete takes in order to reach the outer wall of the midgut, the intercellular and intracellular. Stohler (1957) thought that the ookinete of *P. gallinaceum* travels between the epithelial cells. But Garnham *et al.* (1962) found that the ookinete of *P. cynomolgi bastianellii* evaded or penetrated the peritrophic membrane, pushed aside the brush border of the epithelium, and liquefied the membrane of the latter in order to enter the cell (Fig. 20). The ookinete crosses the epithelial cell, reaches the basement membrane and finally rounds off externally as an oocyst. This last step was not seen in the simian parasite but was described in *P. berghei* by Vanderberg and Yoeli (1966).

The ookinete possesses some remarkable features (Figs. 21 and 22). It has a pellicle consisting of two unit membranes anteriorly thickened into a crescentic slit. Secretory structures such as paired organelles or convoluted rods were not observed leading to this extremity, but possibly not enough material had been examined. Immediately below the pellicle lies a row of hollow peripheral fibrils or microtubules; these number about sixty-five and are presumably the elements responsible for the slow locomotion of the parasite. Apart from the nucleus, pigment and prominent mitochondria, the ookinete also contains several large objects called "crystalloids", which may correspond to the prominent white, circular vacuoles so often seen by light microscopy. Under the electron microscope these bodies resemble fingerprints, are about 1·5 μ diam. and are packed with irregular spheres, 35 mμ in size; they bear some resemblance to virus particles, but their constant presence in *Plasmodium* and in various mosquitoes suggests that they are characteristic of the ookinete. The crystalloids possibly represent the material used in the formation of cyst wall; certainly they are present only in the ookinete of *Plasmodium* and are not seen in the macrogametocyte or in the oocyst. The subpellicular fibrils may represent the third surface layer which is said to exist in gametocytes, but is absent in other stages.

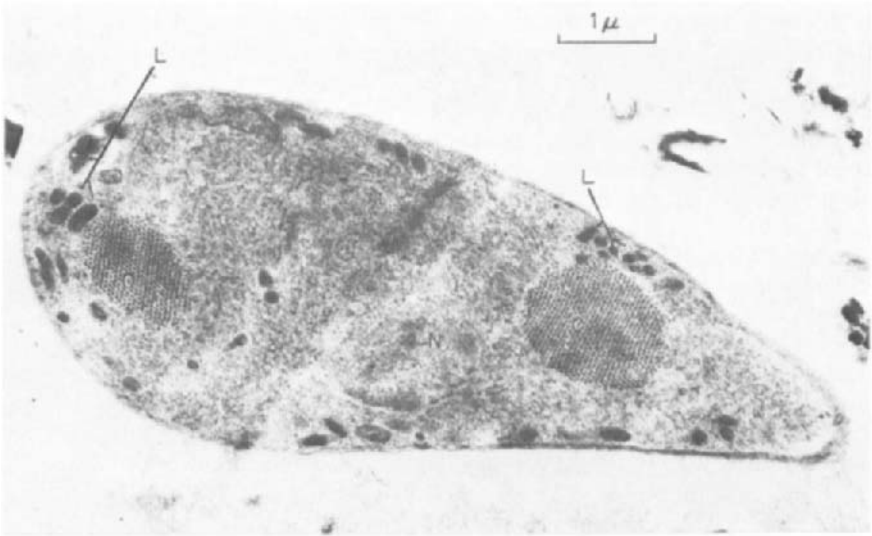


FIG. 21. Ookinete of *P. c. bastianellii* showing crystalloids (*C*) lysosomes and (*L*). *N*, Nucleus. (From Garnham *et al.*, 1962.)

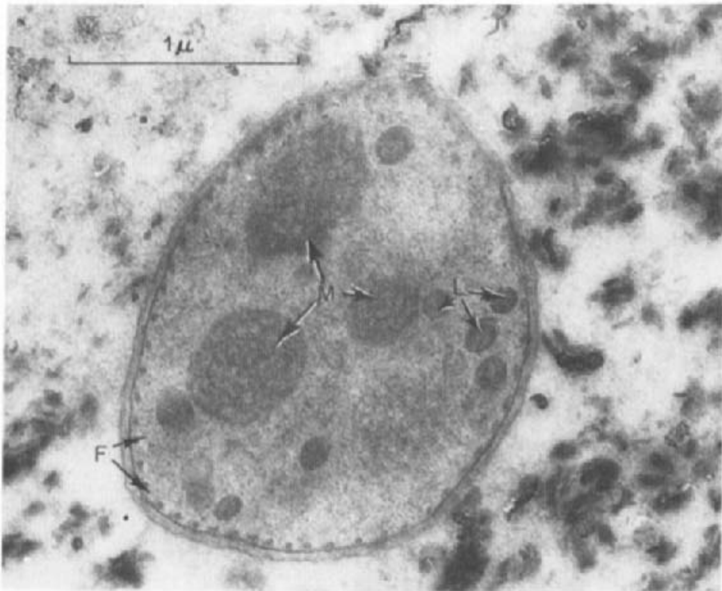


FIG. 22. Transverse sections of ookinete of *P. gallinaceum* showing the surrounding envelope, peripheral fibrils (*F*), mitochondria (*M*), lysosomes and the nucleus (*N*). (From Garnham *et al.*, 1962.)

(It is not easy to find a reasonable explanation for the arrest of the ookinete of *Plasmodium* on the external border of the mosquito's gut. It is true that the oocysts of *Leucocytozoon* and *Haemoproteus* are also halted in this situation, but in another genus of the suborder Haemosporidiidea—*Hepatocystis*—the ookinete quickly leaves the vicinity of the midgut of *Culicoides*, in which insect the sporogony of *Hepatocystis* is known to occur, and proceeds to various parts of the body, particularly to the thoracic muscles and to the ganglia of the head. Likewise, in the haemogregarines, the zygote enters the haemocoelomic cavity of the invertebrate host and grows into an oocyst, either in the cavity (as in *Hepatozoon*) or, through a more complicated cycle (in *Karyolysus*) to enter the ovary of the tick, developing in the form of sporokinetes. It must be noted that the sporogonic stages of *Plasmodium* have the inherent capacity to grow if they are artificially placed in the haemocoelome of a suitable species of mosquito, as was demonstrated in the ingenious experiments of Weathersby (1952). Of course, ectopic growth has long been known in mammals, the ova of which will develop as easily on the surface of the kidney, for instance, as in the endometrium of the uterus.)

D. OOCYST (Figs. 23–25)

The ultrastructure of the oocyst of *P. berghei* was observed by Vanderberg

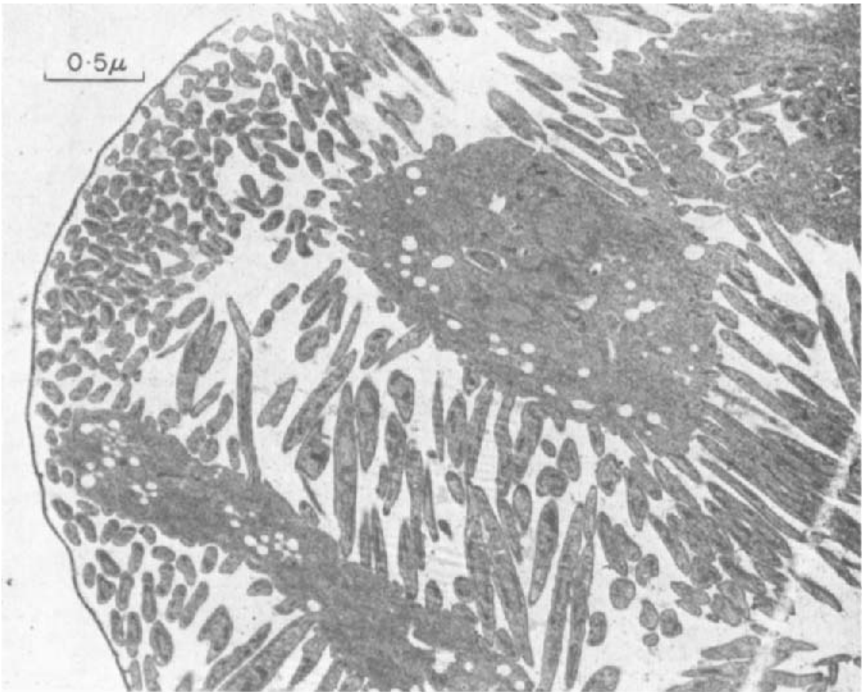


FIG. 23. Oocyst *P. berghei*. Thin section. (Courtesy of R. G. Bird.)

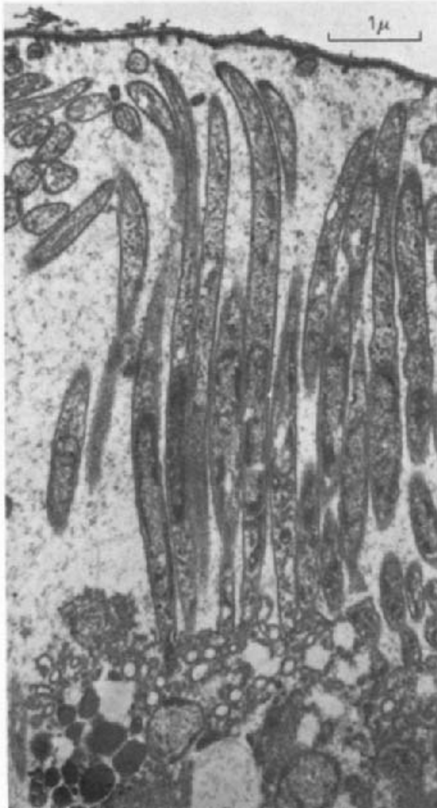


FIG. 24. Sporozoites in longitudinal section, *P. berghei* oocyst.

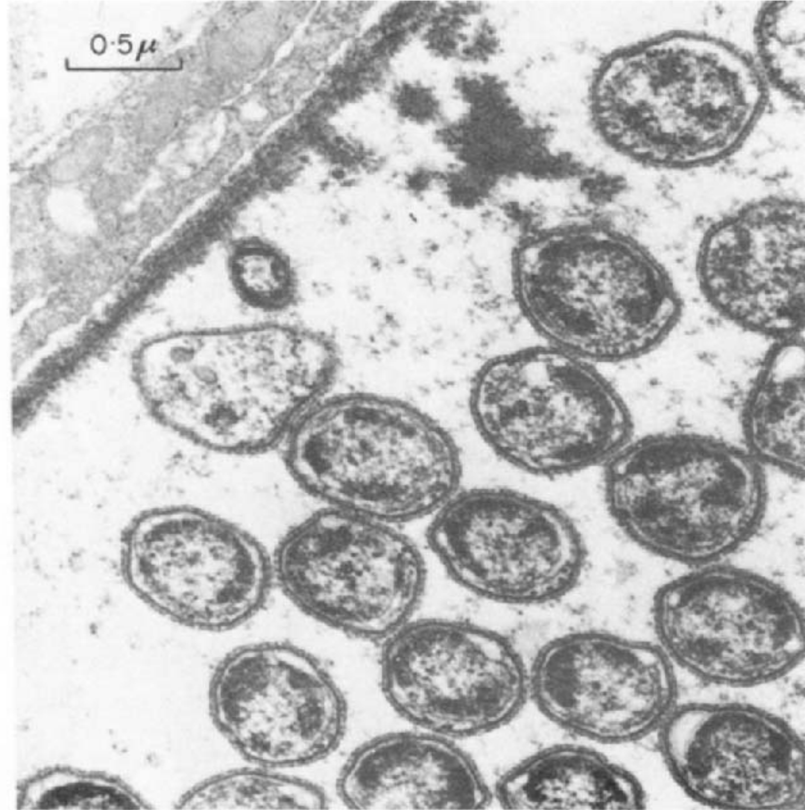


FIG. 25. Sporozoites in transverse section, *P. berghei* oocyst. (Both by courtesy of R. G. Bird.)

and Yoeli (1966), who found that the sporozoites budded off a residual body; hollow subpellicular fibrils were first noted in the incipient bud, while other organelles were present at the distal end of the protrusion. A more complete study of oocyst development was made by Terzakis *et al.* (1966) in relation to the avian parasite, *P. gallinaceum*.

E. SPOROZOITES

The ultrastructure of the sporozoites of mammalian and avian species of *Plasmodium* is of interest to the student of malaria and to the taxonomist, who is confronted with similarities between this group and many other sporozoans. There are two extreme points of view; the Russian idea (Cheissin, 1965) is that the possession of identical organelles with a particular morphology merely indicates that the organisms share similar environments, and that functions and morphological features become adapted along the same lines, though they may be phylogenetically unrelated. The other view, to which the present writer adheres, is that such ultrastructures are as indicative of taxonomic position as any other morphological features, such as pseudopodia or flagella seen under the light microscope. A compromise between these outlooks is perhaps possible, if the theories of Manton (1965) are considered. In a consideration of the phyletic implications of ultrastructure in plants, Manton concluded that there is no *a priori* guidance as to the relative importance of the characters, and that the details of fine structure must be studied in conjunction with general morphology, life history and biochemistry before the systematic position can be correctly interpreted.

Garnham *et al.* (1963b) studied the fine structure of the sporozoites of six species of *Plasmodium*, including *P. cynomolgi bastianellii* and *P. brasilianum*, and noted only minor differences between them. The sporozoite was found to have an unusually thick pellicle (25–30 mμ) composed of at least three double membranes, the innermost lying adjacent to the subpellicular fibrils or microtubules (Fig. 26). The pellicle has an interrupted pattern, probably due to the "chicken wire" arrangement of its layers. The anterior extremity is occupied by two or three apical rings in which the fibrils terminate; the features of their posterior attachment, if any, is unknown but they extend to the nucleus and probably beyond. The fibrils possess a denticulate, or periodic, hollow structure, and are thought to be concerned in sporozoite locomotion. The sporozoite normally moves almost imperceptibly, and this does not allow of its transport either from the midgut to the salivary glands of the mosquito or from the skin to the liver of the mammalian host. More active movements have been reported, however, and Yoeli (1964) described the graceful eel-like gliding movements of sporozoites of *P. berghei*. Other species are said to have a jerky motion with sharp right angle turns. Such sporozoites seem to be *in extremis*, and probably active movements signify

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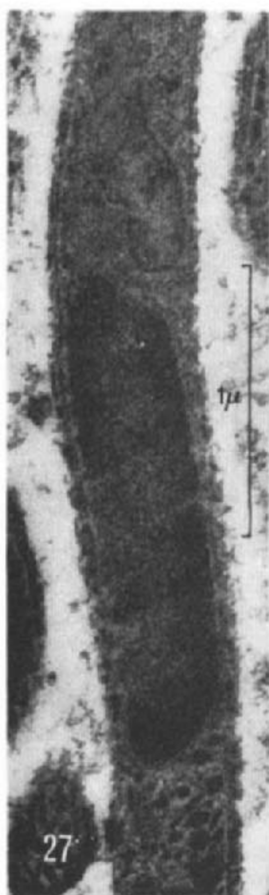
FIG. 26. *P. vivax* sporozoite in transverse section.

FIG. 27. *P. c. bastianellii* sporozoite in longitudinal section.

FIG. 28. Longitudinal sections showing pellicle (*P*), paired organelle (*Po*) and peripheral fibrils (*F*). *P. gallinaceum* sporozoite.

(Figs. 26 and 27 from Garnham *et al.*, 1963b; Fig. 28 from Garnham *et al.*, 1961.)

26
0·5μ


1μ
27

an unsuitable external medium and not vitality. When such movements are noted in material prepared for inoculation into a new animal, the sporozoites are usually found to be non-viable and the experiment fails.

The number of subpellicular fibrils varies from species to species, and may be from ten to fifteen, the number seemingly constant for each organism and the arrangement of the fibrils characteristic. A single fibril lies in the middle one-third of the circumference of the sporozoite, and the remainder are distributed regularly around the other two-thirds. Probably this asymmetrical pattern is responsible for the bow shape of the sporozoite.

The interior of the sporozoite is occupied by a large elongated nucleus, several mitochondria with typically protozoal arrangement of membranes, endoplasm with ribosomes and other electron dense bodies, and various "glandular" structures which discharge into the apical cup through narrow "ductules". The largest is the so-called "paired organelle"—two lobes,

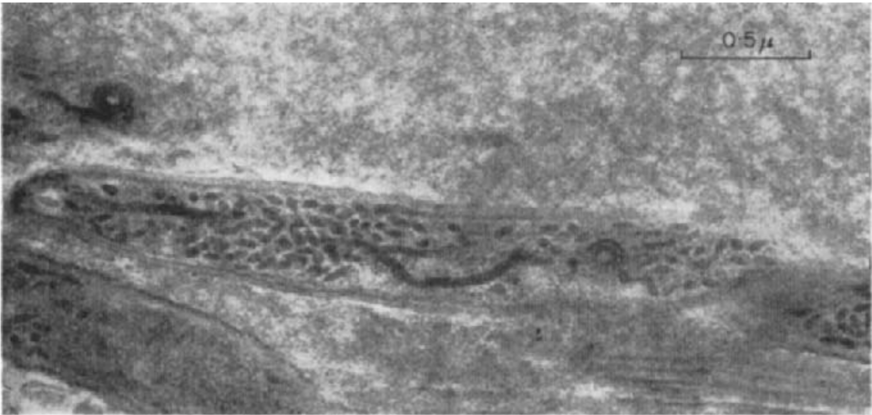


FIG. 29. *P. vivax* sporozoite. Longitudinal section. (From Garnham *et al.*, 1963b.)

except in the sporozoites of *P. brasilianum*, which has three, stretching from the anterior end to the nucleus (Fig. 28). The paired organelle of the merozoites of *P. elongatum* is of quite a different shape from that of the sporozoite stages of other malarial parasites, being squat or like a tear-drop instead of elongate or club-shaped. The paired organelle changes its form during asexual growth of the parasite (Aikawa *et al.*, 1967); it first appears during schizogony as a not very dense spherical body enclosed by a membrane, but in the merozoite it becomes longer and denser, and in the trophozoite it becomes dedifferentiated along with other structures such as the conoid.

In addition to the paired organelle, there are other "opaque" structures, numerous oval bodies seen in thin sections, densely packed and sometimes uniting (Fig. 29). These have been interpreted as mitochondria, lysosomes or convoluted rods or tubules and, like the paired organelle, they are believed to be secretory, producing proteolytic enzymes which dissolve the host cell membranes through which the sporozoites must pass.

The organelle most in dispute is the micropyle (Fig. 30), first described by Garnham *et al.* (1961) in the sporozoites of *P. falciparum* and then observed in other species and stages of *Plasmodium*, *Toxoplasma*, haemogregarines and other sporozoans. The micropyle is a deep circular pit in the pellicle of the sporozoite, sealed at its base by a thin membrane. The sides of the pit have a double wall, thicker and denser than the membrane of the pellicles from which it appears to arise. The dimensions of the micropyle are shown in Table V. Only a single micropyle is normally present; we have rarely found two. Lying just anterior to the nucleus, to the snout of which it sometimes appears to be connected by a thread, its position is constant. In surface view, the structure is striking, taking the form of two concentric rings.

The first interpretation of the micropyle was that it represents the point of emergence of a "sporoplasm" when the sporozoite reaches its final location in the vertebrate host. This theory has never been confirmed directly, but

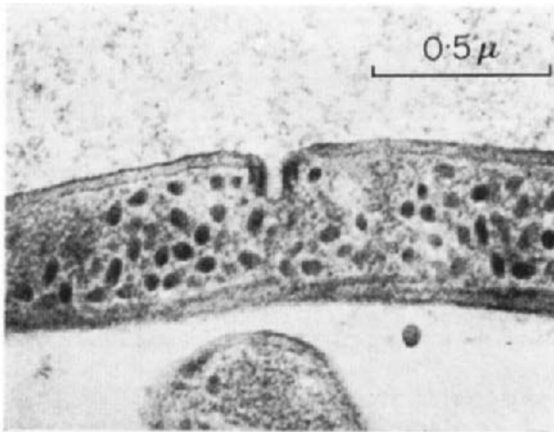


FIG. 30. *P. vivax* sporozoite. Longitudinal section. (From Garnham *et al.*, 1963b.)

Garnham (1966) described strange changes in the sporozoites of *P. chabaudi*, the large round nucleus and other contents apparently escaping, leaving behind attenuated sporozoite remains. What seemed to be empty husks were described by Emmel *et al.* (1942), and such objects have been seen near 1- or 2-day-old primary exoerythrocytic schizonts of *P. cynomolgi* in liver cells.

The theory seems to be invalidated by the occurrence of a "micropyle" in the asexual stages of other parasites, e.g. in *Eimeria intestinalis* (see Cheissin and Snigerevskaya, 1965), other coccidial parasites such as *Coelotropha durchoni* (see Vivier and Hennere, 1965) and *Eucoccidium* dinophili (see Bardele, 1966), and in both cystic and proliferative stages of *Toxoplasma*, *Sarcocystis* and M organism. The Russian workers believed the organelle to be a kind of cytostome, and this opinion was upheld by Aikawa *et al.* (1966b) in the merozoites of *Plasmodium* (see p. 164). The writer has, however, pointed out that a mouth can both ingest and expel, and, except in the

erythrocytic stages of *Plasmodium*, there is no evidence that the cytostome is the site of absorption of food. Aikawa *et al.* (1966) pointed out that at other stages its function is dormant, and that the diameter of the quiescent cytostome of exoerythrocytic merozoites is half that of the fully functioning cytostome of the erythrocytic stage. The American workers also noted that the size of the cytostome differs greatly in the avian and primate species of *Plasmodium*, measuring up to 190 mμ diam. in the former, and 50–80 mμ diam. in *P. cynomolgi* and *P. knowlesi*. In the sporozoite stage of the respective groups of parasites, the dimensions of the micropyle are approximately the same throughout (see Table V).

TABLE V
Dimensions of micropyle (cytostome) in different species and stages of Plasmodium
Based on Aikawa *et al.* (1966) and Garnham *et al.* (1962).

Species	Stage	Depth (mμ)	Internal diameter (mμ)
P. falciparum	Blood	—	—
	Sporozoite	60	80
P. vivax	Blood	—	—
	Sporozoite	100	60
P. ovale	Blood	—	—
	Sporozoite	70	50
P. c. bastianellii	Blood	—	—
	Sporozoite	100	60
P. knowlesi	Blood	70	65
	Sporozoite	—	—
P. gallinaceum	Blood	180	140
	Sporozoite	—	—
P. cathemerium	Blood	—	190
	Sporozoite	—	—
P. elongatum	Blood	—	185
	EE merozoite	—	185

The cytostome of *P. elongatum* was described by Aikawa *et al.* (1967) and found to be functional, although the process of ingestion of the "bolus" differs slightly in erythrocytic and exoerythrocytic stages, in both of which it measures 185 mμ diam. Secondary exoerythrocytic schizogony of this species, however, takes place in primitive cells of the haemopoietic system, instead of in endothelial cells or fibroblasts, where the substrate is more solid. The site of development of the sporozoite of *P. elongatum* is unknown, but it may well be in the latter cells, and, if so, the cytostome at this stage is likely to be dormant.

VI. PATHOGENESIS

The pathological features of malaria have been recognized from the earliest days; the chief historical landmarks are probably (1) the description by Hippocrates of splenomegaly, (2) the discovery of pigment in the internal organs of people dying of malaria (Lancisi in 1717, Schütz in 1848 and, most specifically, Meckel in 1847), and (3) the account of the general pathological features in fatal cases of the human disease by Marchiafava and Bignami (1892). The discovery of malaria parasites in birds, monkeys, and finally in rodents led to detailed studies of the pathology of experimental infections, but, strangely enough, little work was devoted specifically to the subject and only the monographs by Maegraith (1948) and Al-Dabagh (1965) on the pathology of mammalian and avian malaria respectively are available.

The pathology of malaria was once believed to be much the same whatever the species of parasite or host in the animal kingdom; but research on the non-human forms gradually revealed the diversity of the morbid processes. Garnham (1965a) compared the pathological features of the different types of infections and pointed out, not only the profound differences between the pathological effects of avian and primate species (the former often killing the host by the proliferation of its exoerythrocytic stages in the endothelial cells of the cerebral capillaries, and the latter usually exerting a lethal action by blockage of the capillaries by the erythrocytic stages), but the special response of individual species within a group. Thus, in avian malaria, *P. gallinaceum* kills chicks by gross infiltration of the brain by exoerythrocytic schizonts; *P. elongatum* has a totally different action, killing canaries and other passerine birds by the destruction of the bone-marrow; while *P. juxtanucleare* produces a chronic though equally fatal disease in chickens by causing a slowly developing encephalitis or myocarditis. Similar variations are observed in the primate parasites. *P. knowlesi* kills rhesus monkeys, according to Tella and Maegraith (1965a,b), by toxins which induce shock and complete failure of the circulation; on the other hand, *P. coatneyi* and *P. fragile* give rise to a gross anaemia in monkeys, the erythrocytes sinking to fewer than 2×10^6 of blood and the bone-marrow exhibiting excessive changes—such animals usually die after 2 or 3 weeks, or if they survive the blood picture remains unchanged for many months.

Zuckerman (1957, 1958) studied for many years the mechanism of blood loss, and showed that in rodent malaria, the corpuscles were destroyed not only by the parasites, but even more by an autoantibody which becomes deposited on the envelopes of the erythrocytes, rendering the latter susceptible to immediate phagocytosis by the lymphoid-macrophage system. Angus *et al.* (1967) suggest that haemolysis of non-infected cells may be caused by the presence, in the blood of monkeys dying of *knowlesi* malaria, of oleic and other non-esterified fatty acids. Zuckerman (1960) showed that a similar picture of blood loss and replacement exists in monkey malaria, due to *P. cynomolgi*, *P. gonderi* and *P. knowlesi*, in which the excessive anaemia is again due to the production of autoantibodies against the host's erythrocytes which have become altered by the presence of the parasite.

Amongst many other examples of the diversity of malarial pathology is the well-known, but still unexplained action of *P. malariae* on the kidney of man, whereby a fatal condition, quartan nephrosis, supervenes, due to a glomerulo-nephritis, accompanied by sclerosis and secondary tubular degeneration (Gilles and Hendrickse, 1963). *P. malariae* is a common parasite of the chimpanzee in which, however, no renal lesions have been reported.

Undoubtedly the most interesting recent work on the pathogenesis of mammalian malaria emanates from the Liverpool School of Tropical Medicine, and stems from the textbook by Maegraith (1948) on the pathology of malaria and blackwater fever. It was not the first time that a physiologist has undertaken fruitful research on malaria, for an equally notable example occurred in 1884 when Danilewsky, physiologist at the University of Kharkov, took up the study of the blood parasites of birds, lizards and other wild animals of the Ukraine. Maegraith deliberately by-passed the morbid anatomical features of the disease and concentrated instead on the morbid physiology. His experience of fatal cases of malaria in West Africa during the Second World War had convinced him that a condition of acute circulatory failure was as important a factor in causing death as blockage of capillaries. About the same time, too, Knisely *et al.* (1945) had vividly shown in ciné films the "sludging effect" on the circulation which takes place in monkey malaria: at a certain stage of a *P. knowlesi* infection, a sudden change occurs in the physical condition of the plasma—a fluffy or glassy precipitate of fibrin is formed, and the sludge of infected and uninfected cells halts the circulation.

More recently, Devakul *et al.* (1966) undertook experiments in Thailand in which they administered radioactive (iodine-125) fibrinogen to patients with cerebral malaria; the rapid disappearance of this substance from the plasma led these workers to conclude that fibrin was being formed in large amounts, and was causing intravascular coagulation. It is well known that quinine may abolish the parasitaemia in such conditions, but the subject dies, either as a result of irreversible damage to vital centres in the brain, or, as these results suggest, to the failure of the drug to circulate in the cerebral vessels blocked by the clotted blood. If the latter explanation is true, then thromobolytic and anticoagulant therapy might well affect the outcome, as in fact has already been confirmed (Devakul and Maegraith, 1959) in monkeys in the last stages of *P. knowlesi* infections.

Maegraith's original theory is largely based on the changes in the vascular system of both a general and local nature. In algid malaria, the syndrome is one of shock as demonstrated by the profound collapse, falling blood pressure and the reduction of the circulatory blood volume. The endothelial linings of the smaller blood vessels in certain organs are damaged, a progressive loss of plasma takes place, and the surrounding tissues become waterlogged and anoxic. The initiating factors for these phenomena were thought to be non-specific substances, rather than a definite malarial toxin and certainly not mechanical blockage of capillaries. The action is probably felt both on vasomotor centres in the brain and on the sympathetic nervous supply to the organs.

The above conclusions were based on observations of serious or fatal infections in man, supplemented by a study of experimental malaria in birds and monkeys. They raised many questions, of which the most important were the nature of the "toxin" which produced the effects, the mechanism of its action (including the study of both the blood flow through the affected organs and the ultrastructural damage to the cells) and the chemical changes in the blood. Research on the biochemistry and physiology of rodents and monkeys suffering from malaria was then started on a large scale. The goal is near, if not quite reached.

It was clearly important to establish base lines for this essentially biochemical problem and in a series of papers, which are not yet completed, Tella and Maegraith (1965a,b, 1966) demonstrated some, at least, of the important factors in monkeys and rodents before and after exposure to malaria infections (*P. knowlesi* and *P. berghei*). The effect on the erythrocytes was assessed by reticulocyte and red blood cell counts, packed cell volume, and osmotic fragility, and the experiments were carefully controlled by maintaining the animals under standard conditions of housing, age, sex and diet, as well as "stress", which Kretschmar (1965) has shown to have a profound importance. As parasitaemia rose, the erythrocyte mass fell, and a slight increase in osmotic fragility occurred.

The Liverpool workers then proceeded to measure the changes in the serum protein levels as a result of infection with these parasites; the total protein was not greatly affected, but the α_2 albumen and globulin fractions were reversed in *P. berghei*, but not in *P. knowlesi* infections. The γ-globulin was greatly increased in the mice, but the infections in the monkeys were too rapidly fatal for the effect to become apparent in these animals. Other biochemical components of the serum of infected animals have been investigated by Sadun *et al.* (1966), using new microtechniques, with special reference to the enzymes (transaminases and alkaline phosphatase). In splenectomized chimpanzees, infected with *P. falciparum* and mice infected with *P. berghei*, a dramatic increase in both SGP and SGO transaminase occurred early in the infection; the alkaline phosphatase was not significantly affected. In general, a lowering of albumen and an increase in γ-globulin was observed, and liver function tests remained disturbed until some time after the infection had ended. The depression of the fasting glucose level in malaria has, of course, long been known.

In rhesus monkeys suffering from *P. knowlesi* malaria, bradykininogen was estimated in the plasma by Tella and Maegraith (1962), and the level was shown to fall, particularly during the last 2 days of the infection, which kills the animals on the 5th day. This indicates that towards the end, when parasitaemia has climbed to a peak, large quantities of the polypeptide, bradykinin, must be released into the circulation. This substance is known to increase capillary permeability and Tella and Maegraith (1966) point out that circulatory failure in malaria is due at least in part to this phenomenon. Intravascular haemolysis occurs in most monkeys infected with *P. knowlesi*, and is followed by haemoglobinuria, the well-known terminal event of this disease. If the infection is partially suppressed by drugs, the monkey survives, but only to

succumb weeks later with a severe haemolytic anaemia; this condition probably has a different pathogenesis than the former and appears to be due to permanent interruption of haemopoiesis in the bone marrow (Garnham, 1966).

The effect of the "toxin" on the circulation of the blood in the liver and kidney of experimental animals was very clearly demonstrated by the ingenious experiments of Maegraith (1966), using X-ray angiography. Presumably as the result of excessive stimulation of sympathetic nerve endings by circulating toxins, the lumen of the centro-lobular vessels in the liver becomes reduced, the blood flow diminishes, relative haemostasis occurs and the neighbouring cells degenerate and become necrotic. Subsequently, the Liverpool workers described the successive change in the mitochondria of the parenchyma cells in the last stages of fatal malaria infections. About the 5th day, respiratory activity of the mitochondria is depressed, and oxidative phosphorylation is inhibited. These changes were first observed by Riley and Maegraith (1961) *in vitro* by the exposure of mitochondria isolated from normal cells to the serum of animals in the late stages of *P. berghei* and *P. knowlesi* infections. Later, Fletcher *et al.* (1967) demonstrated the nature of the damage to the mitochondria by electron microscopy. The membranes become progressively damaged and the mitochondria swell up and lose much of their internal structure. The vacuolated appearance of the cytoplasm of the parenchyma cell may be thus due to disintegrating mitochondria.

VII. Immunity and Modern Serological Reactions

A. Introduction

There are several reasons why the subject of immunity in malaria has again sprung into prominence, of which perhaps the most important relates to problems in the eradication of the human disease. If complete immunity could be established, either naturally or by vaccination, the present methods of eradication, which rely mainly on insecticides, might be abandoned in favour of immunization campaigns, which have proved so successful against smallpox, poliomyelitis, yellow fever and other infectious diseases. In spite of innumerable studies on natural immunity many facets of the problem remain unknown, and experimental research on immunity in mammalian malaria is being conducted on a wide scale today.

The direction of research on this problem has changed from observations on the cellular response to infection (culminating in the work of Taliaferro and Mulligan (1937) and briefly reviewed in general by Huff (1963)) to the detailed examination of humoral factors concerned in immunity. The application of new serological reactions has greatly aided the latter research, and the fundamental importance of the cell has tended to become forgotten. It is only necessary to look at the hyperactivity of the Kupffer cells of the liver and the red pulp of the spleen in fatal cases of the disease, or at the profound transformations undergone by the lymphoid-macrophage cells in the blood spaces of a chronically infected placenta, to realize that these material phenomena must be at least as important as the esoteric γ-globulins. The

latter emanate from the cells, yet this aspect is ignored, because the research is either too difficult or the subject is thought to be too well known (in other infections) to warrant further study. Sooner or later, we shall undoubtedly have to come back to it.

In the meantime, a large volume of work is being undertaken on humoral immunity in malaria, in relation to the following points: the progress and decline of immunity as measured by various serological reactions and by cross immunity; the protective power of immune globulin; the site and nature of the antigens which provoke the antibodies concerned in the above.

B. SEROLOGICAL MEASUREMENT OF IMMUNITY

It is a far cry today from the buffer precipitation test of Wolff (1939) or Henry's melanoflocculation reaction (Trensz and Raab, 1966); these tests were not specific for malaria and merely indicated the altered protein composition of the serum, but they still retain a use. Trensz and Raab (1966) showed that the melano-reaction could be used quantitatively for the estimation of IgM, and that this simple test is valuable for screening large numbers of blood samples in transfusion centres against malaria or viral hepatitis.

Specific tests for mammalian malaria include the complement fixation test (CFT), several types of agglutination reactions including indirect haemagglutination, the fluorescent antibody test (FAT), both direct and indirect, and double diffusion in agar gel. Most of these reactions indicate the incidence of antibodies and their respective curves follow approximately similar courses; some are only group specific, and others are highly specific even to the level of the strain (agglutination).

1. *Complement Fixation Reaction*

This reaction was first used in simian malaria by Eaton (1938) when he was able to show that an infection of *P. knowlesi*, in partially treated rhesus monkeys, was accompanied by the production of antibodies which declined before and increased after a relapse. The CFT was later adapted to human malaria by Rein *et al.* (1949) and a simplified technique was devised by Pautrizel and Nien (1953) for use in rodent malaria. The latter workers demonstrated a rise in titre on the 12th day of infection in the serum of rats infected with *P. berghei*; the peak was reached during the 4th week, after which the titre gradually dropped to near zero in 3 months. A challenge with the homologous strain resulted in an immediate rise in titre to above the previous maximum.

Still further refinements of the CFT were made by d'Antonio *et al.* (1966), by preparing antigen from parasites disintegrated in a French pressure cell. *P. knowlesi*, *P. falciparum* and *P. vivax* infections were compared and cross reactions were entirely absent between *P. knowlesi* and *P. falciparum*, though the antigen prepared from the former, reacted strongly with *P. vivax* antiserum. These results suggest that an analysis of the complement fixing antibodies produced by different species of parasite may provide a further method for specific differentiation and taxonomic relationship. The pattern of serological activity followed closely the results of Pautrizel and Nien.

2. *Agglutination Tests*

Simple agglutination methods in malaria were tried in the past without much success (Eaton, 1938), although Mulligan *et al.* (1940) succeeded in obtaining high agglutination titres of homologous sera against sporozoites and even effected active immunization of fowls against *P. gallinaceum*. Richards (1966) similarly noted the protective power of a sporozoite vaccine of *P. gallinaceum*, and found that it was active against challenge by sporozoites but not by trophozoites.

Agglutination techniques have been revived recently in the interesting work of Brown and Brown (1965) on the relapse variants of *P. knowlesi* (see p. 156). These workers separated erythrocytes containing schizonts or trophozoites from heavily parasitized blood, 1 week after infection with a particular stabilate, and added suspensions of the material in normal saline to wells containing dilutions of the antiserum. The mixtures were incubated at 20–22°C for 3 h or more and were then examined for agglutination, which occurred in titres up to 1:30 000 of schizonts but not of trophozoites.

The haemagglutination test has been developed in recent years by Stein and Desowitz (1964) and modified by Bray and El-Nahal (1966). It is based on the tanning of sheep's erythrocytes (best used in a non-formalized state) and their subsequent coating with antigen, prepared from malaria parasites; the sensitized erythrocyte suspension behaves like antigen and agglutinates with the homologous immune serum in titres as high as 1:25 000. The preparation of the reagents for this test presents many difficulties. The length of storage of the antigen and antisera affects the results, and, as Desowitz stated, factors of a subtle nature can interfere with the performance of the test; moreover, control sera may react in dilutions of up to 1:500. The haemagglutination reaction certainly offers promise for the future, but it is unsuitable at present for routine purposes. It is, however, particularly useful for determining antigenic relationships, which Desowitz *et al.* (1966) summarized as follows.

(1) Non-specific cross-reactions may occur at low titres of the immune serum.
(2) *P. cynomolgi* antiserum cross-reacts with *P. vivax* antigen.
(3) *P. coatneyi* cross-reacts with *P. falciparum*.
(4) *P. coatneyi* cross-reacts with *P. berghei* and less with *P. cynomolgi* or *P. vivax*.
(5) *P. gonderi* and *P. inui* antisera at high dilutions cross-react with *P. cynomolgi* but other simian species show a lower degree of cross-reactions.

Desowitz and his co-workers also showed that the parasite rate and serological positivity ran parallel in members of a population exposed to hyperendemic malaria in New Guinea.

3. *Fluorescent Antibody Test*

Fluorescent immunology techniques for the study of mammalian malaria have been applied on the widest scale, in spite of the fact that complicated apparatus is needed, that the test itself requires special reagents and its

interpretation is subjective. With the introduction of the indirect or "sandwich" technique, quantitative studies became possible, and the course of production of fluorescent antibodies could be precisely ascertained. Although the usual antigen is contained in a film containing blood stages of the parasite, fractions of the latter may be used, while sporozoites and other sporogonic stages and even the tissue forms have been found to react; moreover, the antiserum can be replaced by immune γ-globulin. The reaction is only group-specific and various simian parasites, such as *P. cynomolgi bastianellii* and *P. fieldi*, may be conveniently used for the diagnosis of human infections.

Briefly, the FAT is performed by overlayering the malaria blood films with unlabelled human immune globulin (test serum) when conjugation takes place; the film is then exposed to fluorescein-labelled rabbit or goat anti-human globulin—the dye becomes attached to the conjugate, and, in a positive reaction, fluorescence of the parasites in the film will ensue. Dilutions of the test serum are made and the titre is determined. Other dyes besides fluorescein have been tried, but with inferior results.

The technique of the FAT is continually undergoing modification, and Sodermann and Jeffery (1966) draw attention to the various fallacies in its interpretation and difficulties in its application. They note that the intensity of fluorescence depends upon the actual size of the parasite; a comparatively low fluorescence is exhibited by rings and a high by schizonts; they point out the different end points of the reaction as presented by antigen slides containing various stages of parasites.

The various species of malaria parasite may be grouped according to their spectrum of cross immunity, as has been shown by Voller (1962) and El-Nahal (1967b). Four classes can be distinguished as follows.

(1) The primate parasites including the four human species, *P. cynomolgi*, *P. gonderi*, *P. coatneyi*, *P. inui*, *P. shortti* and *P. knowlesi*, produce antisera which react strongly with *P. cynomolgi bastianellii* films and differ only in one or twofold dilutions of the respective sera.

(2) The four rodent parasites comprise a second group in which cross-reactions occur. *P. berghei berghei* and *P. berghei yoelii* showing identical titres and *P. chabaudi* and *P. vinckei* producing practically the same reactions with each other; all four parasites, however, cross-react strongly.

(3) Avian parasites of the subgenus *Haemoeba* form a third group; *P.* (*H.*) *relictum* and *P.* (*H.*) *gallinaceum* give nearly identical reactions to the FAT.

(4) Avian parasites of the subgenus *Novyella* (e.g. *P.* (*N.*) *juxtanucleare* and *P.* (*N.*) *rouxi*) comprise a fourth group, which show cross immune fluorescence.

The cross-reactions are strictly limited to the members of the above groups and the test is almost entirely negative between the four groups. It is interesting, however, to note that the monkey parasite, *Hepatocystis kochi*, reacts in a low dilution to *P. c. bastianellii* antiserum, although the two parasites belong to different families (Haemoproteidae and Plasmodiidae respectively).

Collins *et al.* (1966) examined in detail the fluorescent antibody cross-reactions between the following nine species of primate parasites: *P. inui*, *P. shortti*, *P. brasilianum*, *P. fieldi*, *P. cynomolgi*, *P. coatneyi*, *P. gonderi*, *P. fragile*, *P. knowlesi* and *P. jefferyi*. Their technique was slightly different, but hardly enough to account for a considerable discrepancy in the levels of cross-immunity, which were often reduced to 10% or less in the heterologous species. *P. fieldi*, however, gave a high level of cross-reactivity with almost all the examples, and this resembles its behaviour towards the human species also. *P. fieldi*, which incidentally produces such a feeble parasitaemia in monkeys, must nevertheless contain a large quantity of an antigen, common to the group.

Immunofluorescence is confined to certain constituents of the parasite. The nucleus and the malaria pigment never react and fluorescence is strongest in the surface membrane and cytoplasm. In mammalian malaria, the lesions in the infected erythrocytes, as represented by stippling after Romanowsky staining, fluoresce vividly. The reaction is even seen in infections such as those of *P. berghei*, where stippling is otherwise invisible (Voller, 1964). Fluorescence on the envelope of the red blood cell is thought by Tobie and Coatney (1961) to be due to the deposition of antigen, presumably cast off by the growing parasite. The phenomenon is entirely absent in nucleated erythrocytes invaded by avian plasmodia. Stippling is otherwise demonstrated only by Romanowsky staining, and it is possible that the application of other methods, involving the use of other reagents, leads to the solution or denaturation of the antigen on the erythrocyte envelope.

The FAT has been applied to all stages of the parasite with some conflicting results, though, in general, most workers have been able to demonstrate fluorescence throughout the life cycle and, curiously enough, with antisera derived from the blood stages of the parasite. Ingram and Carver (1963) demonstrated by the FAT the earliest exoerythrocytic schizonts (24 h old) of *P. cynomolgi bastianellii* in the livers of monkeys which had been inoculated with sporozoites, and they also showed fluorescence in the later stages. Using the indirect test, other workers (Voller, 1962; El-Nahal, 1967a) have been unable to repeat this observation with the same species of parasite, though striking reactions, in titres up to 1:80, can be obtained in the exoerythrocytic stages of other organisms, e.g. *P. malariae*, *P. berghei* and *P. gallinaceum*. The reaction usually only occurs with homologous antisera. Corradetti *et al.* (1964) applied the technique to sporozoites and showed that the reaction was highly specific, using an antiserum prepared from rabbits immunized with sporozoites. Recently, El-Nahal (1967b) has extended this technique to the earlier sporogonic stages of a number of species, and demonstrated fluorescence in ookinetes and sporozoites, but heterologous sera to neighbouring species (e.g. *P. berghei* and *P. chabaudi*) were found to react in the test.

The study of the rise and fall of immunity as shown by the FAT is extremely valuable, and the results have been presented in great detail by Tobie *et al.* (1966) in experimental malaria under different conditions. The serum was never found to exhibit antibodies until parasites had appeared in the blood; only the blood stages are apparently capable of provoking an immune

response, though sporozoites in a non-susceptible animal (e.g. rabbit) are known to provoke a positive response. In general, the FAT antibodies wax and wane during the course of an experimental infection in much the same way as in other serological reactions.

4. *Gel Diffusion*

In non-human malaria, the Ouchterlony double diffusion technique for the detection of precipitating antibodies has been applied more for antigenic analysis than for the study of immunity, as titration of antiserum is usually impracticable. Moreover, Mahoney *et al.* (1966) found that immuno-diffusion tests were negative in sera obtained from monkeys with active infections of *P. knowlesi*, though positive in animals artificially immunized with parasite fractions and Freund's adjuvant. The techniques employed by Spira and Zuckerman (1966) and Banki and Bucci (1964) were confined to artificial immunization of rabbits with simian and rodent parasites. Nevertheless, McGregor *et al.* (1966) showed that circulating antibodies to *P. falciparum* could be measured by gel-diffusion in naturally immune Gambian adults. These workers employed antigen samples prepared from two sources—trophozoites in heavily infected blood and schizonts from malarial placentas; the latter proved to be much more reactive, though possibly this was because a greater quantity of parasitic material was available in the schizonts. Incidentally, these results in the Gambia showed no correlation between FAT titres and precipitation, and it is suggested that different antibodies are concerned.

The pattern of the appearance of antiplasmodial precipitins was studied by Guberman and Zuckerman (1966) in rats infected with *P. berghei*, by a double diffusion in agar technique. The antibodies were found at the end of the 1st week of patency and persisted for 10 weeks, though they were absent on some occasions during this time, while the intensity of the lines showed certain variations.

C. CROSS-IMMUNITY REACTIONS

The subject of sterile immunity and premunition has been discussed in great detail in the past (see Garnham *et al.*, 1963c), and few fundamental advances have been made in recent years except, perhaps, the blurring of the boundaries between these two so-called types. Yet for the practical measurement of immunity it is necessary to be conversant with the phenomena, in order to use the cross-immunity test for the identification of strains or closely related species of *Plasmodium*. This method has long been used in malaria research and, as a whole, the challenge of a mammal, immune to one species, by another parasite is followed by a normal infection of the latter, indicating an absence of cross-immunity between the two organisms.

In simian malaria as a whole cross-immunity does not exist, even between strains; e.g. Mulligan and Sinton (1933) found that five strains of *P. knowlesi* from Malaya only conferred partial protection on each other, some animals immune to one strain dying when challenged with another. The various

strains or subspecies of *P. cynomolgi* are also immunologically distinct (see above, p. 142).

Voller *et al.* (1966), however, showed that some species of *Plasmodium* of monkeys appeared to possess a wider spectrum of antigens, which enabled them to produce antibodies effective against other species. *P. knowlesi, P. coatneyi* and *P. fragile* are examples of parasites which thus confer a certain degree of cross-immunity, although repeated challenges over many months may be necessary before this stage is reached. Ciuca *et al.* (1955) pointed out that people who had previously suffered from malaria, and particularly from the form due to *P. malariae*, were often immune to *P. knowlesi*. Fractionation studies on the protein constituents of the different parasites will undoubtedly throw more light on this problem.

The rodent parasites were shown by Cox and Voller (1966) and others to fall into two immunological groups, *P. berghei berghei* and *P. berghei yoelii* on the one hand and *P. vinckei* and *P. chabaudi* on the other. There is cross-immunity between the members of each group, but immunity conferred by one group completely fails to protect against parasites of the other. There is a slight immunological difference between the members of a pair; *P. berghei* immunity is completely protective against *P. b. yoelii*, whereas the immunity conferred by *P. b. yoelii* is not so absolute. A similar distinction between the two subspecies is demonstrable by the fluorescent antibody test (see above, p. 186). It may be noted that Bray and El-Nahal (1966) found that antisera to *P. vinckei* and *P. chabaudi* contained few antibodies, as shown by the fluorescent antibody or haemagglutination tests, and considered that these species provoke a poor immunological response.

D. PASSIVE IMMUNITY

The protective power of immune blood in cases of malaria can be demonstrated experimentally by deliberate inoculation, and observed in nature in infants of immune mothers following the transmission of maternal antibodies.

The early attempts to treat infections with immune serum either failed entirely, or had only a partial effect, and on low parasitaemias. The outlook has entirely changed in the last few years as a result of the experiments of Cohen and McGregor (1963), who used immune γ-globulin (IgG) for the treatment of *P. falciparum* malaria in infants living in the same region from which the antisera had been obtained. Not only did these workers employ the purified constituent of the serum, containing the antiparasitic antibodies, but they gave enormous doses (2·5 g). Probably the earlier workers had never used a sufficient quantity of the antibodies to effect a cure.

It was generally considered that only the homologous antiserum was likely to have any protective power, but McGregor and Carrington (1963) showed that immune IgG, prepared from West African adults, was effective in terminating parasitaemia (*P. falciparum*) in East African infants, where a different strain or strains of the parasite were thought to exist. Sadun *et al.* (1966), on the other hand, observed that splenectomized chimpanzees were not protected from a South East strain of *P. falciparum* by the administration of West African immune γ-globulin; though such γ-globulin had a marked

prophylactic and suppressive effect on blood-induced *P. falciparum* infections derived from the same geographical area (Nigeria). The use of splenectomized apes is invaluable for the study of human species of malaria parasites, but it is important to bear in mind the fact that the removal of the spleen interferes profoundly with the immune status of the animal, and that the interpretation of the results of experiments involving immunity on such animals requires much consideration.

The production of IgG in non-human malaria is only now being undertaken on a large scale, but the results of immunization by repeated challenge on monkeys have already shown that large quantities of the substance can be collected in *P. knowlesi* infections, and that these have an effect similar to that observed in *P. falciparum* infections.

The small size of rodents makes them generally unsuitable for similar studies, as it is difficult to collect enough immune serum for the extraction of IgG. Attempts at immunoprophylaxis or suppression of *P. berghei* infections have, however, been made by injecting the crude antiserum. Fabiani and Fulchiron (1953) inoculated young rats with serum taken from animals in various states of immunity; the serum was found to confer no protection if it had been obtained from rats in the early stages of infection, but it had an effect from about the 10th day of patency. Briggs *et al.* (1966) found that immune serum was capable of temporarily inhibiting the multiplication of *P. berghei* in mice, if given early in the infection, but ultimately all the mice died.

Congenital transmission of immunity in malaria is now thought to be due to the conveyance of antibodies from the mother to the offspring either via the placental circulation pre-natally or through the milk post-natally. The mechanism varies according to the species of mammal and the structure of the placenta; primates, including man, deriving protection largely by the placental route, and rodents through the milk. The subject is discussed by Covell (1950) in relation to human malaria and by Bruce-Chwatt (1963a) in general. The latter puts forward the hypothesis that not only do antiparasitic antibodies pass through the placenta but that other substances are also concerned which sensitize and cause proliferation of the lymphoid macrophage cells of the spleen of the foetus *in utero*; the infant is born with a spleen 50% larger than the newborn of non-immune mothers, and is better equipped to combat the infection that it will shortly meet.

P. berghei in rats provides an excellent model for studying these phenomena, and many workers including Bruce-Chwatt (1963a), Terry (1956) and Sergiev and Demina (1957) have clearly shown that baby rats, suckled by an immune mother and infected with a small number of parasites, developed low parasitaemias and survived the disease, whereas baby rats, suckled by a non-immune mother, developed heavy infections from which they all died.

Adler and Foner (1965) carried out similar experiments with *P. vinckei*, using female mice which had been repeatedly challenged first with an avirulent strain of the parasite, then with a virulent strain and finally given *p*-aminobenzoic acid to enhance the parasitaemia. The immunized mice were mated and the progeny was inoculated with the virulent strain of *P. vinckei*, half being nursed by immune and half by normal mothers. The mortality was

33% in the former and 100% in the latter; the parasitaemia, however, was about equally high in both, but death was prevented in the immune babies, presumably because antitoxins were secreted in the milk. This work suggests, therefore, that antigenically specific toxins exist in rodent malaria, the evidence for which is otherwise very scanty.

E. MALARIA ANTIGENS

Probably the time is still not ripe to review this aspect of malariology; active research is being undertaken in many centres, but the situation remains much the same as in 1964 when Zuckerman stated that the work was in an embryonic stage. Techniques have, however, been much improved, and the problem itself has been clearly defined.

The methods for the purification and characterization of antigens have progressed from precipitation in gel, to paper and cellulose acetate membrane chromatography, to starch gel, and finally to polyacrylamide gel disk electrophoresis. Analytical ultracentrifugation with sucrose density gradients is another approach which has not yielded very satisfactory results. It is most important in all this work to free the parasite extracts of host cell contaminants, and today good techniques are available. Chavin (1966) recommended the use of rivanol fractionation which leaves most of the haemoglobin in solution; ten to fifteen different proteins of the parasite (*P. berghei*) were then demonstrable in polyacrylamide gel.

Spira and Zuckerman (1966) demonstrated twelve to sixteen protein bands in three simian species of *Plasmodium*, three rodent species and one avian (*P. gallinaceum*); major and minor components were visible and several of the former were shared by the simian group, but others were specific and even such closely related species as *P. vinckei* and *P. chabaudi* or *P. cynomolgi bastianellii* and *P. c. ceylonensis* were shown not to have identical patterns.

The objective of antigenic analysis is ultimately to determine which fraction is capable of producing an antiparasitic and an antitoxic response. Once antigens can be identified and produced in a large enough quantity, they can be tested against the homologous immune γ-globulin to see if the curative effect of the latter is neutralized. An equally important aspect of the problem is the site of production of the antigen, and hitherto this question has only been directed to the blood forms of the parasite. It is desirable that antigenic analysis should be made of the following stages of a number of species.

Blood stages:	Ring forms
	Large trophozoites (uninucleate)
	Immature schizont
	Mature schizont
	Gametocytes
	Stippled envelope of the erythrocyte
Sporogonic stages:	Oocysts
	Sporozoites
Tissue stages:	Primary exoerythrocytic schizonts
	Secondary exoerythrocytic schizonts
	Merozoites

The discovery of the therapeutic activity of immune γ-globulin had obvious implications for malaria eradication or control, and it was quickly asked if there were possibilities for the production of this substance on a commercial scale, or, alternatively, if a vaccine might become available. Two factors appeared to make both these approaches impracticable: (1) no good method yet exists, in spite of much research, for the culture of the parasite, and (2) the probable existence of numerous strains, or of relapse variants, would entail the production of a polyvalent vaccine.

Flickering attempts at immunization against malaria parasites, human, simian and avian, have been made in the past and have briefly been referred to above (p. 184). The inoculation of animals with non-virulent strains of a parasite usually confers immunity against virulent strains of the same species, e.g. *P. vinckei* (see Adler and Foner, 1965). Nussenzweig *et al.* (1966b) showed that even a related species—the non-lethal *P. chabaudi*—is able to protect mice against the lethal *P. vinckei*, and that the resistance is apparent within 24 h of the inoculation of the former parasite, so that no mice died. Active immunization with dead rodent malaria parasites has also been successfully achieved; Zuckerman *et al.* (1965) fragmented freed parasites in a Hughes press at −20°C and inoculated the product subcutaneously into young rats which were then challenged with living *P. berghei*. One to five immunizing injections were made, and three were shown to have an optimum effect in reducing the parasitaemia by one-quarter (7% as compared with 30% in the uninoculated) and the mortality by one-third (5% as compared with 15%).

The whole subject of immunity in malaria is in a state of flux today and it is important to keep the following two principal objectives clearly in mind: the identification and origin of the antibodies available for combating the infection and its results, and the antigenic differences between stages of a parasite and between strains, subspecies and species. The malaria parasite in the course of its life cycle undergoes so many transformations and is exposed to such different environments that its susceptibility to the immune response of the host is naturally diverse. The effect on the different stages is summarized below.

1. *Gametocytes*

There is much evidence that circulatory gametocytes are damaged by exposure to antibodies in the plasma of the mammalian host, and, particularly at the time of the crisis of the primary infections, their viability is greatly diminished. Hawking *et al.* (1966) have recently described in detail the steep rise in infectivity of the gametocytes of *P. cynomolgi* to a maximum on the 9th day, followed by an abrupt fall to zero on the 10th day (crisis) in spite of the presence of numerous gametocytes, and infectivity subsequently remained low. The gametocytes of different species of *Plasmodium* vary greatly in their susceptibility to immunity or other extrinsic factors; *P. berghei* is easily affected and the precautions necessary to avoid deleterious consequences in experimental work are discussed on p. 152. At the other extreme, the crescents of *Laverania* spp. circulate apparently unharmed for months in the presence

of large quantities of immune γ-globulin. It is possible that alterations in the chemical composition of the blood may affect the viability of gametocytes, e.g. the depletion of glucose which occurs in the final stages of many infections might be responsible, or other biochemical changes likewise.

2. *Gametes*

Provided that gametocytes are able to reach maturity, the next step in the life cycle—the production of gametes—can take place, even in adverse conditions. Immune serum was shown by Bishop and McConnachie (1956) to have no inhibiting effect whatsoever upon exflagellation of microgametocytes (of *P. gallinaceum*); fertilization followed normally and sporogony continued with the formation of oocysts.

3. *Sporogonic Stages*

The mosquito appears to be unable to develop any immunity either to the growing oocyst or to the sporozoites, however many times sporogony has run its course in the insect. When the sporozoite reaches the vertebrate host, it appears to be unaffected by immunity, however highly developed, either in the subcutaneous tissues of the skin into which it first penetrates, or in the circulation where it travels for 30–60 min before entering the liver.

4. *Exoerythrocytic Stages*

The tissue stages of mammalian parasites are equally unaffected by immunity, at least until they rupture at maturity. Then the merozoites become exposed to a violent onslaught by various phagocytic cells. In chronic infections, there is a little evidence (Garnham, 1967) that exoerythrocytic schizonts may react to the danger of such an attack, by developing a thickened wall and by the retreat of the nuclei from the periphery; rarely phagocytes may be found around such forms while they are still immature.

5. *Asexual Stages in the Blood*

Probably the mature schizonts, and particularly the rupturing merozoites are the stages in the blood most susceptible to immunity. However, spleen or placental smears of semi-immune animals often exhibit phagocytosis or erythrocytes, containing parasites in all stages of development. It seems likely that growth of the organism is interrupted after nuclear division begins, when a greater range of metabolites is required by the immature schizont and the plasma has become depleted of these substances, more as a result of the toxic process than of immunity itself.

A useful summary of the problems involved in immunity in malaria is provided by the W.H.O. technical report on immunology of parasitic diseases (1965), and a detailed consideration of the whole question by Singer and Jackson (1967).

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The Biology of the Acanthocephala

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I. Introduction 205
II. General Morphology 206
A. External Features 206
B. Internal Anatomy 207
III. Development 209
A. Reproduction 209
B. Embryology 210
C. The Role of the Acanthor 214
D. Development within Intermediate Hosts 215
E. Development within the Definitive Host 220
IV. Histology and Cytology 222
A. General Histology 222
B. Histochemistry 222
C. Ultrastructure 224
V. Biochemistry 228
A. Composition 228
B. Assimilation 229
C. Intermediary Metabolism 229
D. Respiration 231
VI. Host–Parasite Interaction 232
A. The Arthropod Host 232
B. The Vertebrate Host 233
VII. Practical Importance for Man 235
A. Human Medicine 235
B. Veterinary Medicine 235
C. Parasites of Wild Birds and Fishes 236
VIII. Conclusion 236
References 236

I. INTRODUCTION

The earliest descriptions of Acanthocephala by Redi and Leeuwenhoek take us back to the close of the seventeenth century, and many eminent parasitologists have studied the biology of the group since then. However, they

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remain outside the field of interest of many parasitologists. Undoubtedly their minor importance in human and veterinary medicine has contributed to this state of affairs. Moreover, the group is a small one, with 600–650 described species (Crompton, 1964a), which shows little variation in either morphology or life history, features which it has been suggested have made them uninteresting to many parasitologists. However, in recent years, they have shared with other helminths the interests of a much wider range of biologists than in the past. The major stimulus to include Acanthocephala in these studies has come from an appreciation of the advantages of a comparative approach to biological problems.

This review will cover studies in their embryology, post-embryonic development, histology, cytology, histochemistry, biochemistry, host–parasite interactions and importance in human affairs. It will seldom be profitable for this purpose to go back further than Meyer's comprehensive treatise on the Acanthocephala in "Bronn's Klassen und Ordnungen des Tierreichs" (Meyer, 1932–33). It will not deal with taxonomy, host ranges or geographical distribution, all of which have been the subject of several recent comprehensive works (Petrochenko, 1956, 1958; Golvan, 1957, 1959a, 1960 a–d, 1961 a–c, 1962; Golvan and Houin, 1963, 1964; Yamaguti, 1963). The collection, preservation and mounting of Acanthocephala for taxonomic work has been discussed by Van Cleave (1953).

Morphology will be discussed only so far as is necessary to give a background to the fields reviewed. Fuller treatments have been given by Hyman (1951), Golvan (1958) and Baer (1961). Little fresh information with a bearing on the phylogeny of the group has come to light in recent years and the subject, which has been very thoroughly reviewed by Golvan (1958), will not be taken up again here. Nor will epidemiology be dealt with because the matter has received too little attention to make it worth while at this time.

It may be helpful at this point to summarize briefly the life cycle, anticipating a more detailed discussion. The adults, which are bisexual, are parasites of the alimentary canal of vertebrates. Reproduction is entirely sexual and, following copulation and fertilization, which is internal, the female releases shelled acanthors into the host's intestine. The acanthors, passed in the host's faeces, infect arthropods, in which they develop through a larval form, acanthella, to the cystacanth stage. The cystacanth infects the definitive host when ingested, though paratenic hosts in which the cystacanth penetrates the tissues but does not develop, are interpolated in many life cycles.

II. General Morphology

A. External Features

The body is worm-like, flaccid and sometimes shows superficial annulations. It may be white, slightly yellow or orange. The anterior end terminates in a proboscis, armed with recurved hooks, which becomes deeply embedded in the gut wall of the definitive host. Most species are not much more than 1 cm in length, but some are larger, up to 70 cm.

The proboscis usually shows radial symmetry, with its hooks arranged in longitudinal rows or a spiral pattern (Van Cleave, 1941), but this symmetry may be distorted. The adaptive significance of variations in the proboscis have been discussed by Van Cleave (1951a). Hooks and spines (smaller and without "roots") may be present on other parts of the body, usually anteriorly or around the genital orifice. The proboscis is joined to the trunk by a more or less obvious neck, but the neck may carry armoured inflations, obscuring the distinction, which is important in taxonomy (Van Cleave, 1947a).

Posteriorly the female carries a terminal gonopore and the male a copulatory bursa, which may be withdrawn from view, with a centrally located intromittent organ or penis.

B. INTERNAL ANATOMY

The internal anatomy is illustrated diagrammatically in Fig. 1. The proboscis, neck, proboscis receptacle, with its associated nervous system and muscles,

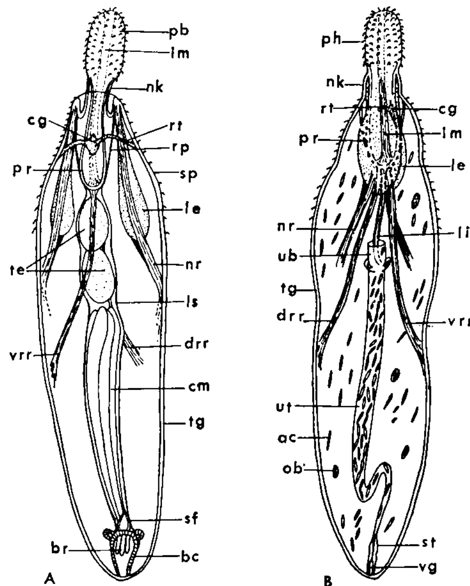


FIG. 1. Diagrammatic representation of acanthocephalan morphology, based on *Polymorphus*. A, Male, from ventral surface; B, female, from right side. *ac*, Acanthor; *bc*, copulatory bursa; *br*, bursal rays; *cg*, cerebral ganglion; *cm*, cement glands; *drr*, dorsal receptacle retractor; *im*, invertor muscle; *le*, lemniscus; *li*, ligament; *ls*, ligament sac; *nk*, neck; *nr*, neck retractor; *ob*, ovarian ball; *pb*, proboscis; *ph*, proboscis hooks; *pr*, proboscis receptacle; *rp*, receptacle protrusor; *rt*, retinacula; *sf*, Saefftigen's pouch; *sp*, spines; *st*, sphincter muscle; *te*, testes; *tg*, tegument; *ub*, uterine bell; *ut*, uterus; *vg*, vagina; *vrr*, ventral receptacle retractor.

and the lemnisci form a functional unit, the presoma. The rest of the body comprises the trunk, or metasoma, and contains the reproductive organs. There is no trace of any alimentary canal at any stage in the life cycle.

1. *The Presoma*

The proboscis and neck enclose a cavity, which extends backward into the body cavity of the metasoma as a pocket, the proboscis receptacle, and into which it can in most species be inverted by the action of the invertor muscles which extend through the cavity. Dorsal and ventral retractor muscles arising from the body wall of the metasoma are inserted at the base of the receptacle, or, in some species, pass through foramina in its wall to insert at the apex of the proboscis, replacing the invertors. In the latter species, their contraction withdraws the proboscis into the trunk with only the neck becoming inverted.

The lemnisci are two lateral protrusions from the body wall at the base of the neck into the body cavity concerned with the movement of fluid when the proboscis is active (Crompton, 1963; King, 1965). They are partly enclosed by the neck retractor muscles running from the metasoma to the neck. The wall of the receptacle is itself muscular and additional muscles, receptacle protrusors, connect it to the body wall.

2. *Body Wall and Body Cavity*

The body is covered by a leathery tegument, described more fully in Section IV, which contains a system of fluid-filled channels, the lacunar canal system. In the metasoma the tegument is lined by outer circular and inner longitudinal muscle layers embedded in a collagenous connective tissue. The cavity enclosed by the body wall of the metasoma lacks a definite epithelial lining and is termed the pseudocoel.

3. *Reproductive Organs*

A strand of connective tissue, the ligament, extends from the base of the receptacle to the hind end of the body, separating two thin-walled sacs, the ligament sacs, which may be either dorsal and ventral or lateral (in different orders). In some, only the dorsal sac develops in the male, and, in others, both rupture in the female during development.

In the male the testes, lying one behind the other, develop from the ligament, which also supports their ducts and the accessory sexual organs. The ducts join to form a common sperm duct which discharges by way of the penis at the centre of the copulatory bursa. A number of cement glands surround the ligament posterior to the testes and discharge their contents by the same route. Their form and number varies and is used in taxonomy (Van Cleave, 1949). Saefftigen's pouch, a muscular fluid reservoir, forces fluid into the bursal tissues when the bursa, which is also supported by rays of connective tissue, is everted for copulation.

In the female, the ovary, which develops from the ligament, breaks up during development releasing ovarian balls into the ligament sacs (discussed in Section III). A complex sorting apparatus, the uterine bell, which separates fully embryonated eggs, the acanthors, from immature ones, develops from the ligament. It passes the fully formed acanthors to a thin-walled uterus, from which they are extruded through a sphincter muscle into a short vagina and out through the gonopore. The immature acanthors are returned to the body cavity.

4. *Excretory Organs*

In some Acanthocephala paired protonephridia project from the ligament sacs into the posterior of the pseudocoel. Their ducts open into the common genital duct.

5. *Nervous System*

A cerebral ganglion is present within the proboscis receptacle and, in the male, a second paired ganglion lies at the base of the penis. Nerves run from the cerebral ganglion to the muscles of the presoma, to the apical sense organ at the tip of the proboscis and, where present in some species, to paired lateral papillae on the neck. Two larger nerves, sheathed in muscle fibres, the retinaculae, cross the pseudocoel from the receptacle to the body wall and continue to the posterior of the metasoma in the longitudinal muscle layer. In the female, branches from these nerves run to the genital tract. In the male, nerves from the genital ganglion run into the genital tract, its associated musculature and the sensory papillae located on the bursa and at the base of the penis. The nervous system is discussed more fully by Bullock and Horridge (1965) in their comprehensive treatment of invertebrate nervous systems.

6. *Orientation*

Though clearly bilaterally symmetrical in their internal anatomy, Acanthocephala show no obvious criteria for determining which surfaces are homologous with the dorsal, ventral and lateral surfaces of other animals. By convention, the aspect of the proboscis on which the cerebral ganglion lies is taken as ventral.

III. Development

A. Reproduction

1. *The Gonads*

The ovarian rudiment breaks up into ovarian balls early in development, and these float freely in the ligament sacs, or, where these rupture, in the pseudocoel. This may occur in the acanthella, as in *Moniliformis dubius* (see Moore, 1946a), *Macracanthorhynchus hirudinaceus* (see Kates, 1943) and *M. ingens* (see Moore, 1946b), the cystacanth as in *Prosthorhynchus formosus* (see Schmidt and Olsen, 1964) or only after infecting the definitive host as in *Leptorhynchoides thecatus* (see DeGiusti, 1949).

The ovarian ball has a syncytial core, with many nuclei, surrounded by developing oocytes. Fertilization is internal, and following copulation spermatozoa can be found clustered around it. Spermatozoa penetrate the ovarian ball in which fertilization and the maturation of the oocytes occur, with the formation of two polar bodies (Meyer, 1928; Nicholas and Hynes, 1963a, b). The ovarian ball is probably bounded by a thin membrane through which the fertilized oocyte is extruded to complete its embryonic development in the body cavity of the female worm.

The testes, which become apparent in the dorsal ligament sac of the acanthella, remain cellular. The spermatozoa, which are thread-like in form, are passed by ducts, which may be expanded to form vesicular seminales, to the penis.

2. *Gametogenesis*

Robinson (1964) has studied gametogenesis in *M. hirudinaceus* and confirmed the suggestion of Jones and Ward (1950) that sex determination is of the XX,XY kind, with the male the heterogametic sex. The chromosomes, which are very small, are most easily studied in the developing oocyte.

A diploid number of six had previously been recorded for *M. hirudinaceus* by Kaiser (1913) and Kilian (1932) though Meyer (1928) thought it varied from four to seven. It is also six in *Hamanniella microcephala* (see Kilian, 1932) and six or twelve in *Polymorphus minutus* (see Nicholas and Hynes, 1963a, b). Hamann (1891) figures twelve in *Acanthocephalus ranae* (= *Echinorhynchus haeruca*) and von Voss (1910) found ten in *Pomphorhynchus laevis* (= *P. proteus*).

3. *Copulation*

Nicholas and Hynes (1958) believed that *P. minutus*, in the duck, moved about in the gut up to the time of copulation, but that afterwards the female remained in one place, so that a dense fibrous capsule could form in the gut wall around the proboscis, while the male soon disappeared from the gut. For copulation to occur the male and female must be attached close together in the gut. During copulation the everted male bursa is sealed around the female gonopore by the secretions of the male cement glands. The secretion persists as a hard brown copulation cap for a time after the worms have separated, but must be shed before acanthors can be passed. Nicholas and Hynes (1958) frequently found misplaced copulation caps on other parts of the female body and even on male worms.

B. EMBRYOLOGY

After copulation the body cavity of female worms soon becomes filled with developing embryos, acanthors and lesser numbers of ovarian balls. Females which have failed to copulate contain only ovarian balls.

The embryonic development of three species, one from each major taxonomic group, has been studied, i.e. *M. hirudinaceus* (Archiacanthocephala) by Meyer (1928, 1936, 1937, 1938a, b), *Neoechinorhynchus rutili* (Eoacanthocephala) by Meyer (1931b) and *P. minutus* (Palaeacanthocephala) by Nicholas and Hynes (1963a, b), the first of these in great detail. A short summary in English of the first two has been given by Hyman (1951).

Early in the development of the embryo cell boundaries disappear, though mitosis continues and produces a mass of tiny nuclei in a syncytial embryo, so that it is difficult to follow the process. The small size of the embryo, which shows little growth, and the hard shell in which it becomes enclosed, add further difficulties. Embryonic development, which is illustrated in Figs. 2 and 3 by drawings of *M. dubius*, a species in which the process has not been

described in detail but in which it resembles that of *M. hirudinaceus*, can be divided into three phases: (1) cleavage, (2) the formation of the inner nuclear mass and (3) the formation of the acanthor organs.

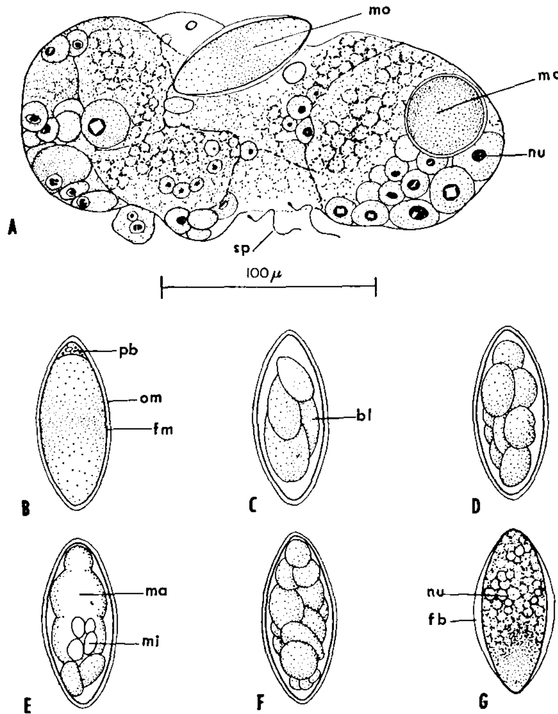


FIG. 2. Early embryonic stages of *Moniliformis dubius*. A, Reconstruction of ovarian ball from stained and phase contrast preparations; B–G, successive stages in development of the fertilized oocyte as seen in phase contrast preparations. *bl*, Blastomere; *fb*, fibrillar coat beginning to form; *fm*, fertilization membrane; *ma*, macromere; *mi*, micromere; *mo*, mature oocyte; *nu*, nucleus; *om*, outer acanthor membrane; *pb*, polar body; *sp*, spermatozoa.

1. *Cleavage*

The fertilized ovum, spindle shaped and with two polar bodies at one pole, is enclosed within a thin membrane. The first two cleavages are slightly unequal, one of the four macromeres produced being distinctly larger than the others. Subsequent divisions of the macromeres are highly unequal and cleavage follows a spiral pattern, distorted by the spindle shape of the egg, leading to the formation of several generations of micromeres.

Typical spiral cleavage is most closely approached in *M. hirudinaceus*, but with the axis of development lying at an angle to the long axis of the egg. Continued asymmetrical division of the four primary macromeres into macromeres and micromeres, and the subsequent division of the micromeres, builds up four quadrants of cells. At the thirty-four cell stage, two tiers of four macromeres lie obliquely across the egg between eight micromeres at the future

anterior end of the worm (still marked by two polar bodies) and eighteen micromeres at the posterior. The largest macromere, D, has produced two additional micromeres posteriorly. At this stage cell boundaries begin to disappear, though nuclear division continues.

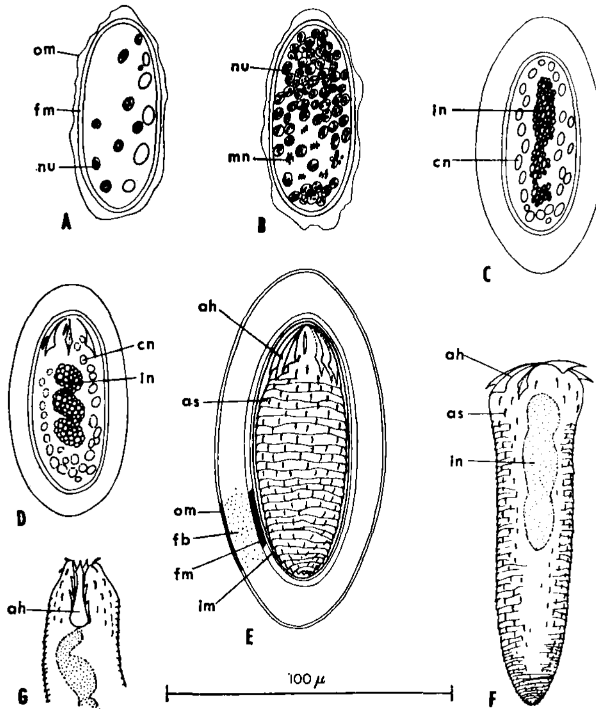


FIG. 3. A–D, Later stages in the embryonic development of *Maniliformis dubius* as seen in preparations from the female body cavity stained by Feulgen's technique for nuclei; E–F, the acanthor of *M. dubius* in phase contrast, E before and G and F after hatching; G, shows the hooks retracted. *ah*, Acanthor hooks; *as*, acanthor spines; *cn*, cortical nucleus; *fb*, fibrillar coat; *fm*, fertilization membrane; *im*, inner membrane; *in*, inner nuclear mass; *mn*, metaphase nucleus; *nu*, nucleus; *om*, outer membrane.

In *P. minutus* the axis of development is more distorted, so that, although the polar bodies still lie at what is probably the future anterior end, the four primary macromeres lie one behind the other along the length of the egg and several generations of micromeres come to lie laterally. Here too, cell boundaries begin to disappear at about the thirty-six cell stage, the largest D macromere, lying third from the anterior, persisting longest. In *N. rutili* the first two cleavages give rise to a D macromere which is much larger than the others. Cell boundaries begin to disappear at the twelve cell stage, after the D macromere has cut off two micromeres anteriorly and the remaining macromeres six posteriorly.

2. *Formation of the Inner Nuclear Mass*

There now follows a process characteristic of the Acanthocephala, which corresponds to gastrulation in other animals. The nuclei, while continuing to divide, become smaller and draw closer together producing a dense core of tiny nuclei, the inner nuclear mass (also termed the central nuclear mass, the embryonalkern and entoblast). In the course of larval development these nuclei will separate again and will be associated with the development of organ systems, no trace of which exists in the embryo. In *M. hirudinaceus*, the core develops initially from three groups: the rudiments of (1) the cerebral ganglion, (2) the ligament and (3) the gonads and musculature. Later groups associated anteriorly with the proboscis and posteriorly with the urinogenital system are added. In *P. minutus* and *N. rutili* the inner nuclear mass shows no distinguishable groupings.

Not all the embryonic nuclei become condensed and drawn together. In *P. minutus* the uncondensed nuclei, about twenty-four, become the nuclei of the future larval tegument, i.e. the cortical nuclei. In *N. rutili* they come from the micromeres derived from the D macromere, which come to enclose those derived from the remaining macromeres that form the inner nuclear mass. In this species, they disintegrate in the late embryo and the tegumentary nuclei of the acanthella are derived from a nucleus that separates from the inner nuclear mass. In *M. hirudinaceus*, the cortical nuclei are more numerous, some coming from uncondensed embryonic nuclei and some from the periphery of the inner nuclear mass, where condensation is less extreme, so that the distinction between the two kinds of nuclei is not so sharp as in the other species.

3. *Acanthor Organs*

As the embryo completes its development within the body of the female worm it becomes enclosed in a hard shell. There are probably always four membranes surrounding the fully developed acanthor, but there are differences in which of these becomes the major component of the shell (West, 1964).

The thin outermost membrane appears first as a loose fitting membrane around the oocyte within the ovarian ball. A thicker hyaline membrane develops after fertilization, the fertilization membrane, and in Acanthocephala of aquatic hosts it becomes the major component of the shell, often becoming drawn out at both ends to give the shell a spindle shape. A third membrane, the fibrillar coat, develops between the fertilization membrane and the oocyte membrane as the embryo completes development. It is poorly developed in parasites of aquatic animals, but forms the major component of the elliptical shell of those of terrestrial animals. Often a groove, the raphe, forms a line of weakness in the shell. In *N. emydis* this membrane, instead of enclosing the embryo, encloses a large vacuole between the fertilization and outer membranes (Hopp, 1954). Burnham (1957) has made some observations on the membranes with the electron microscope.

The anterior end of the acanthor is usually armed with six or eight large blade-like hooks which radiate outwards from the anterior tip. In *M. dubius*, when the acanthor hatches, the three hooks spring out from two groups of

pockets in which they have lain recessed (King, 1965). Smaller spines may cover the whole surface of the acanthor or may replace the hooks at the anterior end. In *N. cylindratus* and *N. rutili* both are lacking and the anterior end is marked by an invagination (Ward, 1940; Hopp, 1954). Schmidt and Olsen (1964) have proposed naming the armoured anterior end the aclid organ in preference to the term rostellum that has sometimes been applied to it.

Meyer (1932–33) has described the musculature of *M. hirudinaceus* acanthors and Moore (1946a, b) and King (1965) that of *Moniliformis*. A sheet of fine fibrils lies just beneath the surface membrane and a band of muscle fibrils encircles the base of the aclid organ. Two retractor muscles run from the anterior tip to the body surface one-third of the distance along the body.

C. THE ROLE OF THE ACANTHOR

1. *Dissemination and survival*

The acanthor is a resting stage which is passed in the host's faeces and undergoes no further development unless ingested by an appropriate intermediate host. It may survive for prolonged periods in the external environment. The acanthors of *M. hirudinaceus* can tolerate sub-zero temperatures and drying. In the soil they may remain infective to beetle larvae for up to 3½ years (Spindler and Kates, 1940; Kates, 1942). Acanthors of *Leptorhynchoides thecatus* which infect aquatic isopods remain infective for at least 9 months in water at 4°C (DeGiusti, 1949), while those of *P. magnus* (= *P. minutus*?) which infect aquatic amphipods survive at least 6 months under environmental conditions (Petrochenko, 1956). Hynes and Nicholas (1963) found freezing the acanthors of *P. minutus* reduced their infectivity for amphipods. They studied the dispersal of the parasite along a river system but concluded that the movement of the infected shrimps was more important than the passive movement of the acanthor.

2. *Hatching of the Acanthor*

The physiological mechanism of hatching in the gut of the intermediate host is not known, though the acanthors of some species can be hatched *in vitro*. Crompton (1964a) thought that chewing by the amphipod's mouth-parts and grinding in the gastric mill were important in releasing *P. minutus* from its shell. The acanthors of *M. dubius* can be hatched in 0·25 M $NaHCO_3$ (Edmonds, personal communication) and this technique has been used by others (Nicholas and Grigg, 1965; King, 1965). Apparently pH, CO_2 and tonicity all affect hatching and Edmonds found evidence of a chitinase (chitin forming part of the shell).

The acanthor of *M. dubius* when hatched in this way (shown in Fig. 3) can crawl on glass surfaces by waves of contraction which pass down the body. The aclid organ is repeatedly retracted, forming a pocket at the anterior end, and released, so that the hooks sweep forward and outward, an action likely to aid in penetrating the gut of the intermediate host. The cytoplasm of the interior is quite fluid and the inner nuclear mass moves backwards and forwards with the action of the aclid organ to which it is attached. DeGiusti (1949)

studied the movement of *L. thecatus* acanthors in the host's gut and reported that the aclid pocket grasps the host's epithelial cells.

The acanthors of several species from terrestrial hosts, e.g. *Macracanthorhynchus hirudinaceus*, *M. ingens*, *Mediorhynchus grandis* and *Hamoniella tortuosa*, can be hatched by drying, followed by immersion in water, but only when taken directly from the body cavity of the female and not from the host's faeces (Manter, 1928; Moore, 1942).

D. DEVELOPMENT WITHIN INTERMEDIATE HOSTS

With the exception of *Centrorhynchus ptyasus* which develops in the peritoneum of snakes (Gupta, 1950), the first intermediate hosts are arthropods. The developing stages, which are transparent, are easily found in the haemocoel, and as long ago as 1864 Greef reported on experimental infections of freshwater shrimps with *P. minutus*. Since then many workers have studied larval development (see Table I).

Van Cleave (1935, 1946, 1947b) proposed the names acanthor and acanthella. He used acanthor for both the infective stage and the early stages in the intermediate host. Acanthella was used for later stages, following what he considered a metamorphosis, and to include the second infective stage. Some workers have restricted the term to the second infective stage, using pre-acanthella for the earlier stages, but it would be better to use the term cystacanth for the second infective stage and retain acanthella for the developing stages. This nomenclature has been followed in most recent studies.

1. *Acanthor Development*

The acanthor, after hatching in the gut of an appropriate intermediate host, works its way through the gut wall, a process which may take several days, and comes to lie under the serosal connective tissue. Here it grows considerably in size, becoming globular in shape, and eventually breaks free to lie in the haemocoel. It becomes invested in haemocytes and encapsulated, a process discussed in Section VI.

In some species, e.g. *L. thecatus* (see DeGiusti, 1949) and *P. formosus* (see Schmidt and Olsen, 1964), growth leads to a shift in the anterior posterior axis so that the parasite bulges out from one side. The bulge enlarges until it represents virtually the whole parasite, which remains for a time attached by a stalk to the original acanthor body, still embedded in the host's gut. Eventually the stalk breaks releasing the parasite into the haemocoel.

As the acanthor enlarges, the cortical nuclei become much larger and vesicular. The nuclei of the inner nuclear mass also enlarge, beginning to separate into groups which can be recognized as the precursors of organ systems of the adult worm. The first to become recognizable are those of (1) the proboscis, (2) the cerebral ganglion and (3) the gonads, ligament and musculature. This and later stages in development are illustrated in Fig. 4 by drawings of *M. dubius*. A split may appear between the cytoplasm and the inner nuclear mass, and this may be apparent even earlier in the acanthor as in *Neoechinorhynchus* (see Ward, 1940; Hopp, 1954), but it is not the direct precursor of the pseudocoel.

2. *Acanthella*

The acanthella stage is marked by the rapid transformation of the undifferentiated acanthor to a stage in which all the adult organs have appeared and the parasite resembles a miniature version of the adult. While continuing to increase rapidly in size it becomes elongated and worm-like in form. This elongation is most marked in the region which will give rise to the proboscis

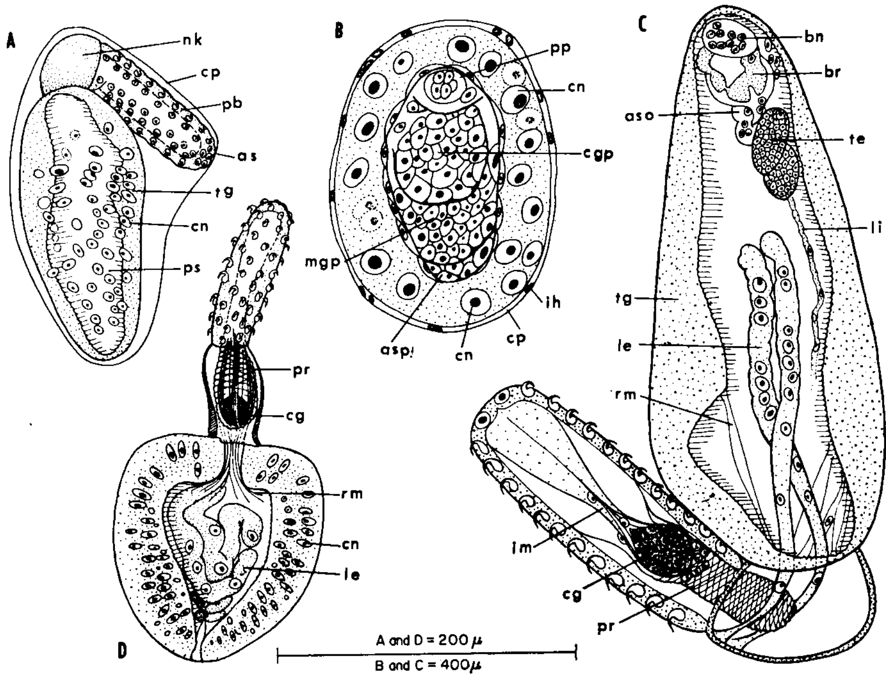


FIG. 4. Developmental stages of *Moniliformis dubius* from the cockroach as seen in whole mounts stained with haematoxylin. A and C, The same late acanthella in different optical sections; B, an early acanthella stage; D, cystacanth with the proboscis forcibly everted. The capsule is not shown in C and D. *as*, Apical sense organ; *aso*, accessory sexual organs; *asp*, primordium of accessory sexual organs; *bn*, bursa nuclei; *br*, bursa; *cg*, cerebral ganglion; *cgp*, cerebral ganglion primordium; *cn*, cortical nucleus; *cp*, capsule; *ih*, insect haemocyte; *im*, invertor muscle; *le*, lemniscus; *li*, ligament; *mgp*, muscles, gonads and ligament sacs primordium; *nk*, neck; *pb*, proboscis; *pp*, proboscis primordium; *pr*, proboscis receptacle; *ps*, pseudocoel; *rm*, retractor muscles; *te*, testis; *tg*, tegument.

and neck of the adult, i.e. in the region anterior to the ring of lemniscal nuclei (see below). As it does so, the proboscis primordium, the most anterior group of nuclei in the inner nuclear mass, gives rise to the proboscis.

The proboscis primordium expands to form a fluid-filled sac which will become the cavity of the proboscis and the proboscis receptacle. Groups of nuclei within it become associated with the formation of the apical sense organ, the proboscis armature and the intrinsic musculature of the proboscis. Other groups of nuclei lying adjacent to the sac become associated with the extrinsic musculature and the muscular wall of the receptacle.

K

TABLE I
Description of larval development

Species	Intermediate hosts	Authors
Neoechinorhynchus cylindratus	Ostracods	Ward, 1940
Neoechinorhynchus emydis	Ostracods	Hopp, 1954
Neoechinorhynchus rutili	Ostracods	Merritt and Pratt, 1964
Polymorphus minutus (= *Echinorhynchus polymorphus*)	*Gammarus* (Amphipod)	Hamann, 1891 Hynes and Nicholas, 1957 Crompton, 1964a, b
*Polymorphus magnus**	Gammarus (Amphipod)	Petrochenko, 1956
Acanthocephalus lucii	*Asellus* (Isopoda)	Leuckart, 1876†
Leptorynchoides thecatus	*Hyalella* (Amphipoda)	DeGiusti, 1949
Prosthorhynchus formosus	Terrestrial Isopoda	Schmidt and Olsen, 1964
Centrorhynchus ptyasus	*Ptyas mucosa* (Reptilia)	Gupta, 1950
Macracanthorhynchus hirudinaceus (*Echinorhynchus gigas*)	Larval Coleoptera	Kaiser, 1891–92† Meyer, 1932–33, 1938a Kates, 1943
Macracanthorhynchus ingens	Larval Coleoptera	Moore, 1946b
Moniliformis dubius	*Periplaneta* (Dictyoptera)	Moore, 1946a Yamaguti and Miyata, 1942† King, 1965
Mediorhynchus grandis	Orthoptera Coleoptera	Moore, 1962
Moniliformis clarki	*Ceuthophilus* (Orthoptera)	Crook and Grundman, 1964

* According to Bezubik (1957) a synonym for *P. minutus*.

† Publication not available to reviewer.

The first indication of the proboscis hooks comes from fine strands which appear on the inner surface of the proboscis sac. Their subsequent development differs in different orders. In the Archiacanthocephala, where the proboscis develops initially in an everted position, the hooks make their appearance at regular intervals along these strands, growing outwards through the overlying cortical tissues to erupt through the surface. In the Palaeacanthocephala, and probably also the Eoacanthocephala, it begins to develop in the inverted position. It everts as development proceeds, turning the strands inside out as it does so. The hooks develop from regularly spaced crenellations which appear along their length in the process. In either case, the fully formed proboscis is finally re-inverted into the receptacle, which, together with the neck, is also withdrawn by muscular contraction into the metasoma.

The ganglion primordium, containing many small nuclei, becomes enclosed within the receptacle. Nerve fibres grow out from the ganglion, two main nerves to the body wall passing through the receptacle sheath, and becoming enclosed by muscle fibres, the retinaculae, where they cross the body cavity.

Behind the receptacle and ganglion lie the primordia of the metasoma musculature, the ligament, the gonads and, at the posterior end, the accessory sexual organs. The musculature of the body wall develops from cells which are delaminated from the inner nuclear mass and which move outwards to lie against the inner surface of the cortex. In some species, lateral bands of these cells are recognizable and in *M. dubius*, at least, they do not complete their migration to line the body wall or their differentiation into muscle cells until post-cystacanth development (King, 1965). The cavity left by their delamination is the pseudocoel. A central strand of nuclei forms the ligament, the membraneous ligament sacs, which partly fill the pseudocoel, and the gonads. In the male two compact groups of nuclei remain in the ligament forming the testes, while in the females a more diffuse group forms the ovary.

The posterior primordium forms, in the male, the cement glands, Saefftigen's pouch and the bursa, and, in the female, the uterine bell, the uterus and the vagina.

The cortex differentiates in the late acanthella into a tegument essentially like that of the adult. It becomes granular and opaque and both fibres and lipid deposits appear. In parasites of Crustacea carotenoids are taken up from the host turning it orange. The epicuticle forms a thin layer over its outer surface. The vesicular cortical nuclei become very large and may increase in number, presumably by recruitment from the inner nuclear mass. They may number from six to ninety or more in the fully formed acanthella. In *Neoechinorhynchus*, where they number from six to nine, they remain vesicular in the adult, but, in others, they become dendritic or fragment amitotically (Van Cleave, 1928).

An anterior cortical nucleus adds material to the apical sense organ, and the posterior nucleus contributes to the bursa in the male and the terminal gonoduct in the female. A ring of cortical nuclei, numbering three to twelve in different species, marks the boundary between the metasoma and neck. Paired lateral inpushings of the cortex into the pseudocoel at this juncture form the lemnisci which incorporate these nuclei.

A complex network of channels, the lacunar canal system, develops in the cortex. In the metasoma, two, or more rarely a single principal canal, forms, often in association with a row of cortical nuclei. The system is also present in the presoma.

The development of the acanthella has been described in a number of species and it is clear that there is a good deal of variation in the process. Only a very general account of the process has been attempted here, but several important aspects of development which remain obscure should be noted. Some of the adult tissues are syncytial, for example the tegument, while others, such as the nervous system, become cellular but it is not clear when cellularity develops. Some workers have concluded that the inner nuclear mass becomes cellular at an early stage (Ward, 1940; Merritt and Pratt, 1964), while others have described organ formation in terms of nuclei, implying a syncytial condition. Nuclear division has been reported in the developing presoma musculature (Hynes and Nicholas, 1957), the ganglion (Schmidt and Olsen, 1964) and the metasoma musculature (King, 1965). However, mitosis has not been reported and it seems desirable to distinguish between mitosis, amitotic fragmentation and the recruitment of additional nuclei from the inner nuclear mass more carefully. Indeed, the relationship between the nuclei and the differentiation of the organ systems would repay investigation at the ultrastructural level. It is also uncertain how far differential growth can account for the rearrangements that take place during development, or whether protoplasmic streaming or cell motility are involved. In this respect, the experiments of King (1965) with X-irradiated developing larvae of *M. dubius* are of interest.

3. *Cystacanth*

The cystacanth is an infective resting stage that marks the completion of development within the intermediate host. In most species the proboscis and neck are inverted by muscular contraction completely filling the pseudocoel. In some, such as *P. minutus*, the anterior and posterior parts of the metasoma are also inverted, producing a hard oval body. Others are flattened laterally, like *M. dubius*, while in *M. hirudinaceus* the proboscis, though withdrawn into the metasoma, is not inverted.

As the acanthella completes its development, fluid accumulates between the parasite and the capsule in which it has been enclosed by the host's reaction. The oval cystacanth, with the proboscis inverted, remains dormant in this thin-walled capsule until ingested by another host.

4. *Paratenic Hosts*

When ingested by a potential definitive host the cystacanth is activated, as described in the next section, and becomes attached to the gut wall, but in certain other hosts it may work its way right through the gut and remain viable and infective in the deeper tissues. Such hosts may represent a blind-end in the life cycle, or serve as possible or essential paratenic hosts, depending on the feeding habits of the definitive host. So far as is known no further development takes place in the paratenic host, which when obligatory for the

completion of the life cycle is generally thought to be so only in the ecological and not the physiological sense. However, Ward (1940) thought that a fish paratenic host was physiologically necessary for the completion of the life cycle of *N. cylindratus*, in which the definitive host is also a fish and the intermediate host an ostracod. She did not observe any morphological development in the parasites which invade the liver of the paratenic host.

E. DEVELOPMENT WITHIN THE DEFINITIVE HOST

1. *Activation of the Cystacanth*

The cystacanth swells in distilled water, which may lead to evagination of the proboscis with consequent rupture of the capsule. The capsule can also be digested with pepsin in HCl (Crompton, 1964b; King, 1965; Graff and Kitzman, 1965), but this does not activate the cystacanth and is not a necessary preliminary for infection of the rat by *M. dubius*.

King (1965) found that in *M. dubius* contraction of the muscles surrounding the lemnisci was stimulated by bicarbonate solutions (with 0·25 M optimal) and that, so long as the tonicity lay within certain limits, this was followed by the eversion of the proboscis. Graff and Kitzman (1965), however, reported that bile salts were essential in this species for the eversion and movement of the proboscis. They found that an alkaline pH and CO_2 stimulated cystacanths that had been activated by bile salts in physiological saline, but that O_2 was inhibitory. They also demonstrated that the surgical transfer of the bile-ducts to open beyond the region normally occupied by *M. dubius* in the intestine of the rat prevented infection, but did not lead to the loss of already established worms. Pre-activated cystacanths were infective for unoperated rats.

Crompton (1964a) thought that the formation of the epicuticle in *P. minutus* rendered the cystacanth resistant to digestion by pepsin in HCl, unlike the acanthella, and also that bile salts might be important for activation in this species.

2. *Completion of the Life Cycle in the Definitive Host*

The activated cystacanth becomes attached to the wall of the alimentary canal of its host by its proboscis. In some species the mature adult is very little larger than the cystacanth, while in others it shows considerable growth. Some observations on growth, maturation and survival are summarized in Table II.

In addition to spermatogenesis and oogenesis other cellular changes may occur. In *M. dubius* the metasoma musculature continues to differentiate, involving both the migration of cells and the differentiation of myofibrils (King, 1965), and the fibres in the tegument increase in density (Nicholas and Mercer, 1965). The lacunar canal system may also undergo further development in some species.

3. In vitro *Culture*

Many of the biochemical experiments discussed in Section V have been made on worms maintained *in vitro* for short periods of time, but Acanthocephala will survive for many days *in vitro* in simple physiological solutions

TABLE II

Some data on the maturation of Acanthocephala in the definitive host

Species	Final host	Length (mm) when fully formed			Pre-patent period	Survival in host	References*
		Cystacanth (proboscis invaginated)	Male	Female			
Leptorhynchoides thecatus	Rock bass	1·9–2·4	8	12	8 weeks	Less than 1 year	DeGiusti, 1949
Metechinorhynchus truttae (= *Echinorhynchus truttae*)	Trout	3·3	8–10	15–20	9–10 weeks	Less than 14 weeks	Awachie, 1963
Polymorphus magnus (= *P. minutus*?)	Duck	2·8–3·0	11–12	13–16	27–30 days	Several months	Antipin, 1956
Moniliformis dubius	Rat	1·0–1·3	120	300	5–6 weeks	Several months	Burlingame and Chandler, 1941
Macracanthorhynchus hirudinaceus	Pig	3·6–4·5	70–150	350–680	70–100 days	10–23 months	Antipin, 1956

* Not all information in the table is drawn from cited references.

(Gettier, 1942; Van Cleave and Ross, 1944; Dunagan, 1963, 1964). Gettier and Van Cleave and Ross studied osmoregulation in *N. emydis*, and a 0·80–0·85% NaCl solution seemed best for this parasite. Jensen (1952) reported the development of the cystacanth of *Pomphorhynchus bulbocolli* to the adult form *in vitro* in a complex medium, but Harms (1965) was unable to obtain such development in *Octospinifer macilentis*. Nicholas and Grigg (1965) were also unable to report development in *M. dubius*. In *M. dubius* acanthor development soon ceased in the adult female *in vitro*.

IV. Histology and Cytology

A. General Histology

Early histological studies were published by Hamann (1891) and Kaiser (1913), followed later by Meyer (1931a, c). Kilian (1932) published a detailed and well-illustrated study of the tegument, musculature, protonephridia and sexual organs of several species of Archiacanthocephala. Harada (1931) described the nervous system of *Bolbosoma*, also in considerable detail. Meyer's monograph (1932–33) summarized the knowledge at that time and added further histological studies on some of the organ systems. Van Cleave (1949) made a comparative study of the cement glands and Van Cleave and Bullock (1950) described the presoma of *Neoechinorhynchus emydis* Baer. (1961) and Crompton (1963) have made more recent studies, concentrating on the tegument.

The acanthocephalan body, excluding the gonads, contains a few large nuclei, which can often be seen in whole mounts. A good method for staining whole amounts with haematoxylin has been given by Chubb (1962). Van Cleave (1914) studied the number and location of the nuclei in four species of *Neoechinorhynchus* (= *Eorhynchus*) and concluded that the number and position was fixed within a species and showed little variation between related species. He considered that nuclei represented cells and concluded that, though many tissues were syncytial, the body was made up of a small number of cells in fixed positions, i.e. that it was eutelic.

Later studies of development have confirmed Van Cleave's view that eutely is characteristic of the Acanthocephala, the significance of which he discussed again later (1932).

Van Cleave (1928, 1932, 1951b) studied the amitotic subdivision of the tegumentary nuclei, which occurs in some species, and found parallels between their development (vesicular, through dendritic to fragmented) and a series taken from adult forms. He suggested this was an example of ontogeny recapitulating a phylogenetic process.

B. Histochemistry

1. *Food Reserves*

The distribution of glycogen has been studied in the adults of a number of species (von Brand, 1939a, b, 1940; Bullock, 1949b; Haley and Bullock, 1952; Crompton, 1965). Glycogen is concentrated in the non-contractile parts of the muscles, the deeper layers of the tegument and the anterior part of the

proboscis, but is also present in other tissues. Some of the glycogen is rather water soluble (von Brand, 1939a; Crompton, 1965) and difficulties in fixation may have accounted for von Brand (1939a) and Bullock (1949b) finding glycogen in the lacunar canals, though von Brand thought the canals were concerned with glycogen transport. Crompton (1965), using an improved method of fixation on *Polymorphus minutus*, confirmed von Brand's conclusion that the glycogen present in the tegument was more soluble than that in the muscles, suggesting differences in polymerization.

As acanthors complete development, glycogen accumulates (von Brand, 1939a, 1940; Crompton, 1965) and may be utilized in chitin synthesis. Miller (1943) found that in *Macracanthorhynchus hirudinaceus* glycogen rapidly disappeared from the acanthor on hatching and remained low in the early acanthella stages, but accumulated in the late acanthella, appearing first in the tegument. Crompton (1964a) found similar changes in developing *P. minutus* larvae. Other polysaccharides are present in the acanthor (von Brand, 1940) and cement glands (Haley and Bullock, 1952), possibly galactogen (von Brand and Saurwein, 1942).

Lipids accumulate in considerable quantities in the tegument in the form of large numbers of droplets which stain with lipophyllic dyes. Von Brand (1939a) found lipid heavily concentrated in the tegument of *M. hirudinaceus* but the ovarian balls also contained considerable amounts. Bullock (1949b) found fats widely distributed in the tissues of several other species, with heaviest concentrations in the body-wall musculature. In the tegument it was concentrated around the lacunar canals, but not within them, and there was more in the proboscis than in the trunk. The ovarian balls and early embryonic stages contained a lot of fat, but this disappeared progressively as the acanthor completed its development. Phospholipid and cholesterol were concentrated in the tegument. Haley and Bullock (1952) found lipid in the cement glands of two species.

2. *Enzymes*

Bullock (1949a) found lipase and alkaline phosphatase concentrated in the tegument of several species. Later in a study of twenty-three species (Bullock, 1958), he found that where alkaline phosphatase was demonstrated it was concentrated in two layers of the tegument (striped and inner radial), but that in some, including all eight Neoechinorhynchidae, it could not be detected. Larval forms sometimes differed from adults in this respect. Crompton (1963) and Crompton and Lee (1963) found alkaline phosphatase in *P. minutus* in the tegument and cement glands. Non-specific esterase was present in the metasoma tegument, lemnisci, testes and cerebral ganglion, though the latter may have been due to cholinesterase. Crompton observed interesting localizations of these enzymes within the tegument. Acid phosphatase has been localized in nuclei and, rather erratically, in the cytoplasm of the lemnisci, main nerves and muscles of several Neoechinorhynchidae (Bullock, 1960).

Crompton (1965) studied the localization of ten dehydrogenases in *P. minutus*, viz:

Alcohol	Malate	Succinate
Glutamate (NAD)	Malate (decarboxylating)	Isocitrate (NAD)
Glutamate (NADP)	Lactate	Isocitrate (NADP)
Glycerophosphate		

Bryant and Nicholas (1966) studied the localization of succinic dehydrogenase and NADH oxidase in *Moniliformis dubius*, and they agreed with Crompton (1965) that these enzymes are localized in mitochondrial particles. Crompton drew attention to the close parallel between concentration of these particles and of glycogen.

3. *Structural Materials*

Chitin has been found in the acanthor membranes of several species, but not elsewhere (von Brand, 1940; Monné and Hönig, 1954; Monné, 1964). In *P. minutus* protein was found in all four membranes, with evidence, from the action of solvents that break -SS- bonds and from tests for SH groups, of keratin-like properties in all except the thin outer membrane. Chitin is a major component of the fertilization membrane and probably present in lesser amounts in the fibrillar coat and innermost membrane. The shell gave evidence of being lightly tanned. No lipid layer was detected.

Crompton (1963) tested many histochemical reactions on the tegument of *P. minutus* and concluded that a lipoprotein, rich in NH_2 groups and -S—S bonds, was an important structural component. Monné (1959) found some evidence of tanning in the tegument of this genus and Haley and Bullock (1952) have reported a basic protein rich in -SH groups in the cement glands of two other species. Crompton (1963) identified the thin epicuticle which coats the outer surface of the tegument as acid mucopolysaccharide.

The musculature is embedded in a connective tissue which shows evidence of polysaccharide (PAS-positive, diastase stable) and stains like collagen in *Moniliformis* (Mallory's triple and Van Giesen's stains). The nature of the connective tissue will be discussed again later.

C. ULTRASTRUCTURE

1. *The Tegument*

Crompton and Lee (1965) and Nicholas and Mercer (1965) have described the ultrastructure of the teguments of *P. minutus* and *M. dubius*, respectively, while shorter notes on the same subject have been published by Edmonds and Dixon (1966) and Nicholas and Hynes (1963b) on *M. dubius*, by Crompton and Lee (1963) on *P. minutus* and by Rothman and Rosario (1961) on *M. hirudinaceus*.

Crompton and Lee used terms derived from light microscopy to describe the successive layers of the complex tegument of *P. minutus*, while Nicholas and Mercer used roman numerals for *M. dubius* because they felt this was less confusing. Both terminologies are given in the following simplified account of the tegument of these two worms, as revealed by electron microscopy of sections from osmium fixed tissue stained with uranium and lead. The tegument is illustrated diagrammatically in Fig. 5 and by electron photomicrographs in

Figs. 6 and 7. The surface is covered by a thin secreted layer (epicuticle; I), which in *M. dubius* is formed from randomly orientated fibrils but was not clearly present in *P. minutus*. The syncytial tegument is bounded by an outer

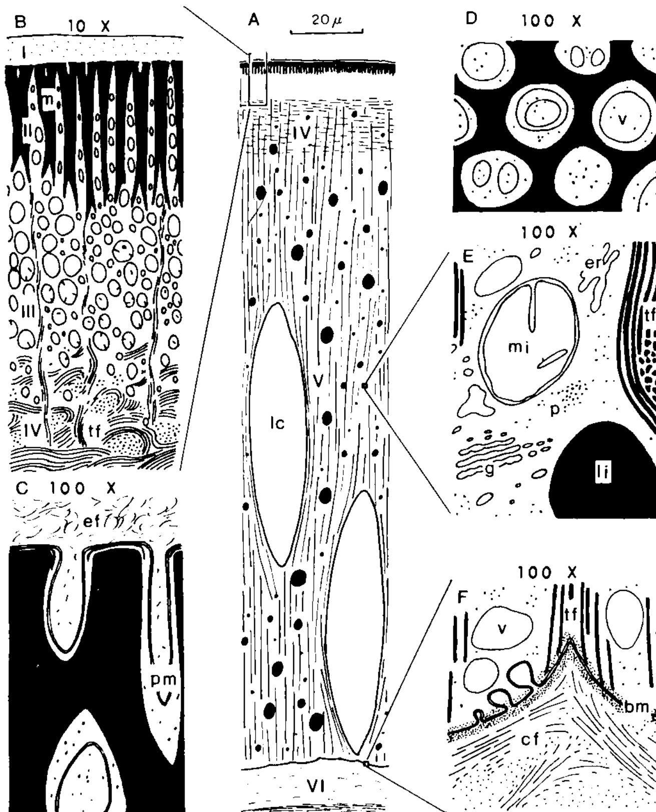
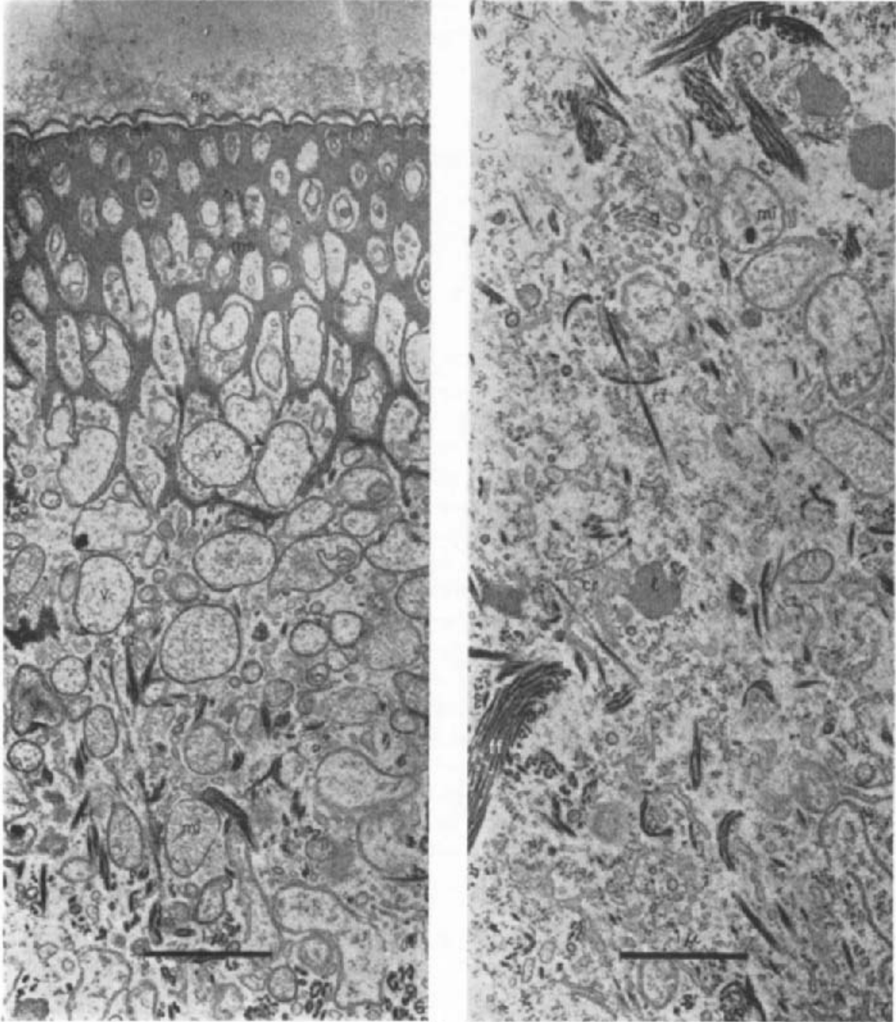


FIG. 5. Diagrammatic representation of the tegument of *M. dubius* as seen in araldite sections of osmium fixed tissue stained with lead and uranium. A is drawn from light microscopy and B–F from electron microscopy; A is a transverse section of the tegument, B is a tenfold and C, E and F are 100-fold enlargements of parts of A; D shows a tangential section through layer II at the same magnification. I, Epicuticle; II, meshwork or striped layer; III, vesicular layer; IV, felt layer; V, radial layer; VI, subtegumentary connective tissue; *bm*, basement membrane; *cf*, connective tissue fibrils; *ef*, epicuticle fibrils; *er*, endoplasmic reticulum; *g*, Golgi cluster; *lc*, lacunar canal; *li*, lipid; *m*, meshwork; *mi*, mitochondrion; *p*, ribosome and/or glycogen particles; *pm*, plasma membrane; *tf*, tegumentary fibres; *v*, vesicle. Relative dimensions are not accurate and apart from bounding plasma membranes, no attempt has been made to represent cell membranes as multi-layered structures.

plasma membrane, which separates it from the epicuticle, and an inner plasma membrane, overlaying a diffuse dense zone so that it resembles a typical basement membrane (VI), which separates it from the subtegumentary connective tissues.



FIGS. 6 and 7. Electron micrographs of transverse sections of the tegument of *Moniliformis dubius* (osmium tetroxide fixation and uranyl acetate staining). Fig. 6. Superficial layers; meshwork of striped layer, cut somewhat tangentially. Fig. 7. Deeper layers. *ep*, Epicuticle; *cr*, cytoplasmic (endoplasmic) reticulum; *ff*, feltwork of tegumentary fibres; *g*, Golgi cluster; *l*, lipid inclusion; *mi*, mitochondrion; *ms*, meshwork; *rf*, radial tegumentary fibres; *v*, vesicles. (From Nicholas and Mercer, 1965.)

In *P. minutus* the outer plasma membrane is backed by a thicker layer of low density to give a triple layered structure, termed by Crompton and Lee the cuticle. In *M. dubius* a third thin dense line appears beneath the double-contoured plasma membrane, separated by an irregular space, but it is quite possible that these differences are the result of differences in the methods of preparation.

The tegument is supported by a complex framework. Directly beneath the surface membranes this takes the form of a meshwork (striped layer; II) which, in transverse of longitudinal sections, appears as a homogeneous layer penetrated by closely spaced narrow extensions of the underlying cytoplasm that reach the surface membranes. In *M. dubius*, because of its moniliform shape, many of these sections strike the surface tangentially, showing a honeycomb-like structure, with cytoplasm in the interstices. Where the cytoplasm reaches the surface the plasma membrane is frequently drawn down into it as though pinocytotic vacuoles were forming, a hypothesis supported by observations of Edmonds and Dixon (1966) on the uptake of carbon and thorium dioxide particles by *M. dubius*, or alternatively, as though secretion was being released. There was evidence of secretion in the anterior trunk region of *P. minutus*, and possibly the epicuticle is formed in this way. Vesicles are common in these cytoplasmic "pores", perhaps of pinocytotic origin, and vesicles form a distinct layer (III) at the base of the meshwork.

The underlying felt (IV) and radial (V) layers, the latter providing most of the thickness of the tegument, are so named because of the arrangement of the many fibres that complete the structural framework. In *M. dubius* it was found that their number increased as the animal grew, until, in the felt layer, they largely excluded other cytoplasmic elements. Some were observed to cross the vesicular zone (III) to become continuous with the meshwork, and those in the radial layer appeared to originate from the basement membrane.

Mitochondria are concentrated in the vesicular zone (III) and radial layer (V), but are difficult to recognize because of the poorly developed cristae. Supporting evidence for their nature has come from histochemistry and biochemistry (Crompton, 1965; Bryant and Nicholas, 1966). Numerous vesicles are also present, but a typical smooth-membraned endoplasmic reticulum is poorly developed and rough membranes are not apparent. Golgi clusters were found in *M. dubius*. Dense particles are common, but both groups of workers found difficulty in distinguishing between glycogen and free ribosomes. Lipid droplets are common. The lacunar canals, bounded by a thin membrane and with homogeneous contents of low density, were common in *P. minutus* but were unrecognized in *M. dubius*. The basement plasma membrane is thrown into folds, suggesting vacuole formation on the tegumentary side, which in *P. minutus* often contained lipid. Nuclear structure has not been described.

Only the metasoma of *M. dubius* was investigated, but the presoma of *P. minutus* was also seen. Dense material in and beneath the "pores" in the neck may indicate the uptake of lipid. The pores become less numerous and the felt and radial layers taper away in the proboscis.

2. *Hooks and Spines*

The presoma spines of *P. minutus* have a core of tegumentary fibres and possess both "cuticle" and attenuated striped layers (II). The proboscis hooks are extensions of the subtegumentary tissues consisting of extracellular material surrounding a cone of cytoplasm which contains fibres and myofilaments.

3. *Development of the Tegument*

The development of the tegument has not been studied with the electron microscope, but epicuticle, meshwork, outer plasma membrane and tegumentary fibres are all well developed in the cystacanth of *M. dubius* (Nicholas, unpublished). The continuity of the meshwork and fibres is very obvious at this stage.

4. *Subtegumentary Musculature and Connective Tissue*

The body wall muscles have been briefly described by Crompton and Lee (1965) in *P. minutus*. Nicholas and Mercer (1965) studied the subtegumentary connective tissue of *M. dubius*. It consists of many fibres, much finer than the tegumentary fibres, set in a homogeneous matrix of low density. No sharp bands were observed in the fibres, but they were digested by *Clostridium histolyticum* collagenase (Knock-Light; at pH 7·6) in tissue blocks treated before fixation (Nicholas, unpublished) and it is concluded that they are collagenous, resembling tropocollagen.

V. Biochemistry

A. Composition

1. *Carbohydrate Reserves*

Glycogen is the principal carbohydrate reserve (von Brand, 1939a, 1940; von Brand and Saurwein, 1942; Laurie, 1959; Graff and Allen, 1963). Galactogen may be present in *Macracanthorhynchus hirudinaceus* (see von Brand and Saurwein, 1942), and trehalose has been found in *Moniliformis dubius* where, together with glycogen, it formed virtually all the carbohydrate reserves of worms taken from fasted rats (Laurie, 1959). The significance of differences between the sexes in glycogen deposition remains doubtful (von Brand, 1940; Read and Rothman, 1958; Laurie, 1959; Graff and Allen, 1963). Read and Rothman (1958) found a diurnal fluctuation in the polysaccharide reserves of *M. dubius* in the rat, which fell sharply when the host was starved. This fall was arrested when the host was fed on starch. With rats fed on a carbohydrate-free diet, the parasite stopped growing, but resumed growth after several weeks when the rats were returned to a normal diet. Glycogen is utilized under *in vitro* conditions by *Neoechynorhynchus* (see Dunagan, 1963, 1964) and *M. hirudinaceus* (see Ward, 1952), in the latter more rapidly under anaerobic than aerobic conditions.

2. *Lipids*

Histochemical studies, already reviewed, have shown that Acanthocephala accumulate considerable amounts of lipid, and crude characterization has been attempted by Bullock (1949b) and von Brand (1939a). Some Acanthocephala contain an orange pigment that is probably a carotenoid (Van Cleave and Rausch, 1950; Crompton, 1964a) originating from plant material in the host's food.

Beames and Fisher (1964) have reported a sophisticated chemical analysis of the lipids from *M. dubius* and *M. hirudinaceus.* Males contained more lipid than females, per unit weight, and *M. dubius* contained more fat (glycerides) than *M. hirudinaceus,* on the same basis, though both contained similar quantities of unsaponifiable lipid and phospholipid. Esterified fatty acids, from the neutral lipid fraction, from C_{10} to C_{21}, were detected in both and some unsaturated C_{22} was found in *M. dubius.* More than 70% was C_{18} and 80% was unsaturated in both. Cholesterol formed a large part of the unsaponifiable fraction and lecithins, inositol, cephalins and plasmalogens were found in the phospholipid fraction.

3. *Proteins*

Analyses have been made of total and protein nitrogen in *M. hirudinaceus* (see von Brand, 1939a) and *M. dubius* (see Laurie, 1959).

4. *Inorganic Ions*

Spectrographic analyses of *M. hirudinaceus* have shown Na, Mg, Ca, Mn, Cu, Fe, Al, PO_4, SO_4, CO_3 and Cl (von Brand, 1939a; von Brand and Saurwein, 1942).

B. ASSIMILATION

Methionine can be taken up against a concentration gradient by *M. dubius* and *M. hirudinaceus in vitro* (Rothman and Fisher, 1964). Its uptake is inhibited by certain other neutral amino acids and this inhibition is reciprocal. The degree of inhibition of methionine uptake can be predicted from a general equation for the interaction of amino acids proposed by Read and Simmons (1963) from more extensive work on cestodes (Read and Simmons, 1963; Read *et al.*, 1963). Edmonds (1965) similarly showed the inhibition of leucine uptake by *M. dubius* by other neutral amino acids *in vitro.*

Edmonds (1965) compared the uptake of ^{32}P by *M. dubius* in rats dosed orally with $Na_2{}^{32}PO_4$ with rats dosed intraperitoneally. It seems that the parasite derives its P from the gut contents rather than from the mucosa. Similar results were obtained with ^{14}C-leucine. Edmonds and Dixon (1966) observed the uptake of C and ThO_2 particles by *M. dubius* electron microscopically.

C. INTERMEDIARY METABOLISM

1. *Carbohydrate Synthesis*

Laurie (1959) found that glucose, fructose, mannose and maltose stimulate glycogen synthesis by *M. dubius in vitro* in worms from fasted rats. Trehalose neither stimulated glycogen synthesis nor was its endogenous level significantly altered by any of these sugars. Fisher (1964) demonstrated the synthesis of trehalose, using ^{14}C-U-glucose, by tissue minces of *M. dubius* and *M. hirudinaceus* in a reaction stimulated by ATP and UDPG. Graff (1964) studied the uptake of ^{14}C-U-glucose by intact *M. dubius in vitro* and its incorporation in glycogen, which was greatly accelerated in worms from fasted

rats. CO_2 does not stimulate glycogen synthesis by this worm *in vitro* under anaerobic conditions (Kilejan, 1963).

2. *Glycolysis*

There is some evidence for the operation of the Meyerhof-Embden scheme of glycolysis in *M. dubius*. Read (1961) notes, without further details, the presence of several of the enzymes involved, i.e. aldolase, triose phosphate dehydrogenase and lactic dehydrogenase. Laurie (1959) found lactic acid production was stimulated by several different sugars *in vitro* under aerobic conditions, and Graff (1964) reported the labelling of lactate and probable glycolytic intermediaries when worms were incubated in ^{14}C-U-glucose under similar conditions. Bryant and Nicholas (1965) identified several radioactive spots in chromatograms prepared from tissue preparations of *M. dubius* incubated in ^{14}C-U-glucose as glycolytic intermediaries.

3. *Citric Acid Cycle*

Graff (1964) and Bryant and Nicholas (1965), in the experiments referred to above, studied the labelling of citric acid cycle acids, and amino acids derived from them, chromatographically, with very similar results. They found alanine, aspartate, malate, fumarate, succinate, lactate and serine (Graff, only) labelled, but, surprisingly, citrate and aconitate were not found at all and glutamate, though present in considerable amounts, was not labelled. Aspartate probably indicates the transamination of unstable oxalo-acetate, alanine of pyruvate, serine of phosphoenolpyruvate and lactate the reduction of pyruvate. These results suggest an incomplete and reversed citric acid cycle. Pyruvate or phosphoenolpyruvate, formed by glycolysis from glucose, may be carboxylated to form malate, which is then reduced, via fumarate, to succinate. Bryant and Nicholas (1965) also studied the metabolism of ^{14}C-labelled acetate and succinate by tissue preparations and obtained results consistent with this hypothesis, though the labelling of glutamate with succinate suggests a limited capacity to oxidize this substrate. Further support comes from Graff's (1965) observations on the labelling of malate, fumarate, succinate, lactate, aspartate, alanine, serine, pyruvate and oxaloacetate when *M. dubius* was incubated *in vitro* in $Na^{14}CO_3$. α-Ketoglutarate was not labelled. No labelling was detected when 1-^{14}C-propionate was used (a precursor of succinate via methylmalonyl-CoA).

4. *Excretory Products*

M. dubius excretes organic acids under aerobic and anaerobic conditions *in vitro*. The excretion of unidentified acids is stimulated by glucose, galactose, fructose, mannose and maltose anaerobically (Laurie, 1957). Lactic and probably acetic and formic acids are excreted aerobically (Laurie, 1959), the proportion excreted as lactic acid being considerably increased by exogenous glucose, fructose, mannose and maltose. Nitrogenous excretion has not been studied.

D. RESPIRATION

M. dubius will take up O_2 *in vitro* and this uptake can be stimulated by some exogenous sugars (Laurie, 1959; Bryant and Nicholas, 1966), but it seems probable that respiration is adapted to an anaerobic or semi-anaerobic environment.

Bryant and Nicholas (1966) studied the oxidation of succinate and NADH by intact and various tissue preparations from *M. dubius*. They made use of redox dyes and enzyme inhibitors to isolate steps in the electron transport system, using manometric, spectrophotometric and histochemical techniques. Their conclusions are summarized in Fig. 8. Both substrates lead to the reduction of cytochrome *b*, but two alternative pathways are postulated for the reoxidation of this compound, a major one independent of cytochrome *c* and cytochrome oxidase, and a minor one including them. A similar scheme has been suggested for *Ascaris* muscle (Kikuchi *et al.*, 1959; Kikuchi and Ban, 1961).

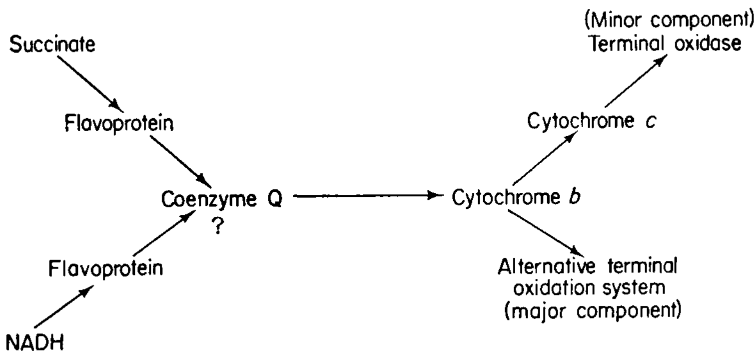


FIG. 8. The electron transport system of *M. dubius* according to Bryant and Nicholas (1966).

The fixation of CO_2 with the accumulation of succinate, which it has been suggested above occurs in *M. dubius*, has been reported from other parasitic worms, e.g. *Heterakis* (see Fairbairn, 1954), *Ascaris* (see Saz and Vidrine, 1959), *Trichuris* (see Kmetec and Bueding, 1965), *Echinococcus* (see Agosin and Repetto, 1965) and *Hymenolepis* (see Prescott and Campbell, 1965; Scheibel and Saz, 1966). Probably in *M. dubius*, as in these worms, pyruvate, formed by the Meyerhof-Embden scheme of glycolysis, is carboxylated by malic enzyme to form malate, which is then metabolized to fumarate and succinate, though alternative pathways, such as the carboxylation of phosphoenolpyruvate to form oxaloacetate, may be important.

The reduction of fumarate to succinate may be important in providing a mechanism for the re-oxidation of NADH under anaerobic conditions, thus maintaining glycolysis and the synthesis of ATP. It may possibly serve, as in the metabolism of *Ascaris* where it has been shown that the transfer of electrons from NADH to fumarate is linked with the anaerobic formation

of ATP (Chin and Bueding, 1954; Seidman and Entner, 1961), as an additional source of respiratory energy. It may be significant in this respect that the electron transport system of *M. dubius* shows similarities with that of *Ascaris* rather than that of higher animals and that both *M. dubius* and *Polymorphus minutus* (Section IV), like *Ascaris* (Kmetec *et al.*, 1962) and certain other helminths, have mitochondria with very poorly developed cristae.

VI. Host–Parasite Interaction

A. The Arthropod Host

1. *Specificity and Pathogenicity*

Some Acanthocephala can infect a wide range of intermediate hosts. *Macracanthorhynchus hirudinaceus*, for example, has been recorded from many different insects (Golvan, 1962), but not all serve as equally good hosts. Miller (1943) found that many of the acanthellae developing in the larva of *Popilla japonica* died prematurely, while in *Autoserica castanea*, another beetle, they normally completed development. In contrast, three subspecific strains have been found in *Polymorphus minutus* in Great Britain, each adapted to only one of the three commonly occurring species of *Gammarus* (Amphipoda) (Hynes and Nicholas, 1958). Specificity is not complete and it is possible, depending on the cross infection attempted, to obtain normal cystacanths in one species of shrimp with parasites derived from another.

A surprisingly large number of parasites may complete development in a single host, e.g. over thirty *P. minutus* in *G. pulex* and 300 *M. hirudinaceus* in *P. japonica*, but the host can be killed by exposure to too many acanthors and there is no evidence of acquired immunity. At sublethal levels, heavy infection retards development and may lead to stunting of the parasite (Crompton, 1964a). The female of *Gammarus* is sterilized by the development of a cystacanth of *P. minutus*, but is not affected in the same way by cystacanths of *Metechinorhynchus* (= *Echinorhynchus*) *truttae* (see Hynes, 1955; Pflugfelder, 1956; Hynes and Nicholas, 1958).

2. *Encapsulation*

The larval stages of Acanthocephala become encapsulated in the haemocoel by a cellular reaction of the arthropod host. Crompton (1964b) has reviewed scattered observations on the capsule in the literature and studied its formation by *Gammarus* around *P. minutus*. It is formed initially by a bulging out and pinching off of the serosal lining of the host's gut, which becomes invested by the host's haemocytes in the process. It continues to grow as the parasite grows from material, rich in neutral mucopolysaccharide, contributed by the haemocytes, which continue to invest it after it has become free in the haemocoel. If pricked (with a needle) they repair the damage. At a later stage, acid mucopolysaccharide forms an inner layer, probably from the fluid which accumulates within the capsule as the acanthella completes its development and is probably secreted by it.

Mercer and Nicholas (1967) have studied the capsule surrounding *Moniliformis dubius* in *Periplaneta americana* with the electron microscope. In the

acanthella stage it was formed from a mass of adherent vesicles, which, they believed, were formed from protoplasmic filaments thrown off the surface of the surrounding haemocytes. In the cystacanth stage the vesicles become separated and an amorphous material, possibly of parasite origin, was deposited between them.

Crompton (1964a) ingeniously transferred developing larvae of *P. minutus* from the haemocoel of one shrimp to that of another and found that the capsule was essential for the survival of the parasite. It seems that the capsule, which is permeable to small molecules, prevents an osmotic loss of fluid by the parasite, perhaps by retaining large molecules secreted by it.

Crompton (1964a) also studied the encapsulation by *Gammarus* of experimentally introduced foreign objects. The capsule formed around living *P. minutus* resembles the connective tissue laid down by the haemocytes around the internal organs of the shrimp and in the plugging of superficial wounds. Perhaps its formation is stimulated in the first instance by the acanthor stretching the serosal tissue. It differs from the capsule laid down by haemocytes around glass or wax objects, dead or damaged natural parasites, mosquito eggs, unnatural parasites or damaged *Gammarus* tissue. In these cases the haemocytes form a laminated structure in which melanin granules are deposited between disintegrated cells and droplets of acid mucopolysaccharide appear. Hynes and Nicholas (1958), however, thought melanin was deposited within parasites that died in the shrimp and not in the capsule.

3. *Resistance to Infection*

P. minutus of *G. pulex* origin cannot complete development in *G. duebeni* (Hynes and Nicholas, 1958; Crompton, 1964a). Acanthors fed and "hatched" in *G. pulex* can be transferred from the gut contents to another *G. pulex*, where they develop normally, but die in penetrating the gut wall of *G. duebeni*, subsequently becoming melanized. Similarly, encapsulated parasites can be transferred from the haemocoel of one *G. pulex* to that of another and develop normally, but die and become encapsulated as a foreign object in *G. duebeni* (see Crompton, 1964a). Incidentally, *G. pulex* tissue is treated in the same way as a foreign object by *G. duebeni*.

4. *Miscellanea*

King (1965) found that there was a significant difference in the rate of development of *M. dubius* in cockroaches which had been collected in the field and kept under uniform conditions in the laboratory during summer and winter, developing more rapidly in summer. Presumably the parasite is affected by a physiological rhythm in the host that persists under these conditions.

B. THE VERTEBRATE HOST

1. *Specificity and Pathogenicity*

Acanthocephala show a remarkable lack of specificity with regard to the definitive host. *P. minutus* has been recorded from eighty-four species of birds (Crompton and Harrison, 1965), *M. hirudinaceus* from eleven species of

mammals (Golvan, 1962), *Echinorhynchus salmonis* from fifty-seven different species and subspecies of fishes (Petrushevski and Shulman, 1958). Indeed, Shulman (1958) concluded that the host range of Acanthocephala of fish was determined ecologically rather than genetically.

An ingested cystacanth which fails to become established in the alimentary canal when ingested may migrate into the tissue and remain there viable and infective so that the host becomes an actual or potential transport host. The role and evolution of paratenic hosts was discussed by Petrochenko (1956) and Golvan (1958).

In the definitive host the proboscis penetrates deeply into the gut wall producing traumatic injury. An inflammatory response with cellular infiltration and the eventual formation of a dense fibrous nodule around the proboscis follows. In some genera the proboscis penetrates so deeply that it comes to lie beneath the serosa, e.g. *Bolbosoma*. The damage done by acanthocephalan parasites, sometimes lethal, has been described in fishes by Scheer (1935), Wurmbach (1937) and Bauer (1958), and in birds by Pflugfelder (1956) and Crompton (1963).

2. *Resistance to Infection and Competition between Parasites*

The investigation by Burlingame and Chandler (1941) of the development of *M. dubius* in the laboratory rat has provided the basis for future work in this area. They found no evidence of natural or acquired immunity, in the usual sense, but discovered some of the factors which affect the survival of the parasite in its host. Only a limited region of the intestine is suitable for the survival of the parasite, and competition, probably for nutrients, may limit the number of parasites which can become established in this zone. Those forced out are lost. Starvation of the host leads to the loss of all the parasites, but the rat remains just as susceptible to infection when returned to a normal diet.

Burlingame and Chandler found no significant difference in survival when the number of parasites administered at one time differed widely (though growth might be stunted with greater numbers), but, when repeated infections were administered, worms from later infections were less likely to become established in the favourable zone of the intestine and many were in consequence prematurely lost.

Possibly similar factors determine the outcome of superimposed infections of domestic ducks with *P. minutus* (see Nicholas and Hynes, 1958; Hynes and Nicholas, 1963) and limit the parasite burden among birds in the field.

Bulingame and Chandler (1941) also found differences in susceptibility to *M. dubius* between different litters of laboratory rats, but no evidence of age resistance. Crompton and Harrison (1965) found that a proportion of those *P. minutus* which became attached to the intestines of the duck were lost soon afterwards and this seemed to indicate some form of host resistance. They speculated that differences between strains adapted to different bird hosts, for which they had some evidence, might be manifested in this way.

Holmes (1961, 1962a) studied concurrent infections with *M. dubius* and *Hymenolepis diminuta* in rats. Both worms grew more slowly and occupied a

more restricted region of the intestine than in single species infections. These effects were more pronounced but similar to those produced by crowding in single species infections, and Holmes suggested that competition for carbohydrate was a major factor. Concurrent infections in the hamster (Holmes, 1962b), however, showed no such stunting or restriction in position, both worms occupying the same zone despite the smaller size of the intestine.

VII. Practical Importance for Man

A. Human Medicine

The Acanthocephala are rare parasites of man. *Moniliformis dubius*, a cosmopolitan parasite of rodents, has been recorded as a human parasite in Italy, British Honduras, the Sudan, Israel, Indonesia and the U.S.A. (Watson, 1960; Witenberg, 1964). Several different species of cockroaches and beetles that contaminate human foodstuffs can act as intermediate hosts and these might easily be ingested accidentally (Watson, 1960). An experimental human infection caused abdominal pain, diarrhoea, ringing in the ears and weakness (Grassi and Calandrucci, 1888).

Macracanthorhynchus hirudinaceus, a common parasite of pigs, may once have been a common human parasite in parts of Russia where the larvae of the beetle *Melolontha* used to be eaten raw (Faust and Russell, 1964). A number of human cases have been recorded (Witenberg, 1964), one recently from Thailand (Pradatsundarasar and Pechranond, 1965).

B. Veterinary Medicine

M. hirudinaceus is a cosmopolitan and common parasite of the domestic pig which also occurs in many other animals, e.g. wild pigs, peccary, cattle (rarely), monkeys, dogs, hyenas, chipmunks, muskrats, squirrels, moles and man (Petrochenko, 1956; Faust *et al.*, 1962; Golvan, 1962; Rausch, 1946). Many different insects can act as intermediate hosts and these have been listed by Golvan (1962) and Soulsby (1965). The most important are the soil-inhabiting larvae of the Scarabeidae (Coleoptera). The proboscis of the parasite causes local inflammation and ulceration of the gut of the vertebrate host which may rarely lead to peritonitis. In the pig heavy infections cause diarrhoea, emaciation and sometimes death, though sub-clinical infections are the rule and serious disease is rare (Soulsby, 1965). Heavy mortality in local areas has been recorded in the U.S.S.R. (Antipin, 1956). Because of the slow growth of the parasite clinical symptoms are not usually apparent in pigs under 10 months old. The veterinary aspects of this parasite are more fully dealt with by Soulsby (1965) and Antipin (1956).

In Texas coyotes and dogs become infected by *Oncicola canis* with harmful results (Van Cleave, 1920), probably by eating armadillos, a paratenic host, though immature worms have also been found in the oesophageal tissue of turkeys (Price, 1929). The arthropod intermediate host is unknown. Acanthocephala, because they show little host specificity, may occur occasionally in a wide range of aberrant hosts. Thus *Corynosoma semerme*, a parasite of seals in the Arctic, and *C. strumosum* in South America, have both been found in

dogs, which had presumably been fed on raw fishes (Golvan, 1959b, 1961c). Other aberrant infections from dogs and cats are recorded by Golvan (1960c, 1961c, 1962).

Domestic ducks may suffer from *Polymorphus* and *Filicollis*. *P. minutus* is common in North America, Europe and northern Asia and a second species, *P. magnus*, probably identical with *P. minutus* (see Bezubik, 1957), has been reported from Europe and northern Asia. Light symptomless infections are common in birds which have access to ponds and streams, but heavier infections leading to loss of condition or death may occur (Antipin, 1956; Petrochenko, 1958; Hynes and Nicholas, 1963). Freshwater crustacea are intermediate hosts, particularly *Gammarus* (see Hynes and Nicholas, 1957), and many wild waterfowl serve to disseminate the parasite (Crompton and Harrison, 1965). *Filicollis anatis* infects domestic ducks, geese and many wild birds in Europe, sometimes causing damage or death of the bird (Soulsby, 1965). Freshwater isopods, *Asellus*, act as intermediate hosts.

C. Parasites of Wild Birds and Fishes

Although Acanthocephala are of minor importance as parasites of domestic animals, they may be of greater importance to man as parasites of wild animals, whether these are important to him commercially for food or sport or aesthetically. However, our knowledge of their effects on such hosts is meagre. Whether, for example, *P. minutus* or *F. anatis* cause serious diseases in wild birds is unknown. Rayski and Garden (1961), however, did find evidence of high mortality among eider duck in Britain and Holland from *Profilicollis* (= *Polymorphus*) *botulus*. The crab, *Carcinas moenas*, was identified as an intermediate host.

Acanthocephala are common parasites of both marine and freshwater fishes, including many species that are exploited commercially, and fish are frequently seriously damaged or killed by them. Russian workers have made a very extensive study of the helminth parasites of fish (Dogiel *et al.*, 1958), but, although they recorded many examples of acanthocephalans infecting and damaging fish, they do not appear to consider any of them a major economic problem.

VIII. Conclusion

Acanthocephalan development differs strikingly from that of other animals, and, though there have been many descriptive studies of larval development, the significance of its many unique features remains obscure. Some of the more obvious aspects which require clarification have been touched on in Section III. Important studies have been made on the composition, notably in respect of lipids, and histochemistry of Acanthocephala. A start has been made with studies of their ultrastructure on the tegument. In view of the current interest among parasitologists in these fields they are likely to be followed up in the near future.

Acanthocephala resemble cestodes in their sensitivity to the level of carbohydrates in the gut of the vertebrate host, and in the kinetics of amino acid assimilation, which it is tempting to ascribe to their common lack of an

alimentary canal, but our knowledge is too limited to make such generalizations with confidence. In their respiratory metabolism they show interesting similarities with other parasitic worms and significant differences from higher animals. These differences may be adaptations to anaerobic or semi-anaerobic conditions in the host's gut, but, in the absence of comparable work on free-living helminths, it would be wise to keep an open mind concerning their significance.

Interesting observations on the reactions of the arthropod host to acanthocephalan parasites have thrown light on the nature of "self-recognition" in arthropods. There have been studies on the establishment of these parasites in the vertebrate host which have shown something of the sensitivity of the parasite to changes in the conditions within the gut. Finally, it is apparent that we know little about the importance of Acanthocephala in the lives of wild animals.

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Addendum

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The Post-embryonic Developmental Stages of Cestodes

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Science is always wrong. It never solves a problem without creating ten more.
George Bernard Shaw

I. Introduction 247
II. Life Histories and Development 248
A. Trypanorhyncha and Tetraphyllidea 248
B. Pseudophyllidea 249
C. Cyclophyllidea 252
D. Asexual Multiplication 262
E. Summary 264
III. Structure 267
A. Procercoids and Plerocercoids 268
B. Cysticercoids 270
C. Cysticerci 272
D. Studies in Histochemistry 276
E. Summary 277
IV. Certain Aspects of Host–Parasite Relations 278
V. Temperature and Development 282
VI. Post-embryonic Stages *in vitro* 285
VII. Excystment *in vitro* 288
VIII. Conclusions 289
References 290

I. INTRODUCTION

The purpose of this review is to discuss the advances in knowledge made in some aspects of the biology of post-embryonic stages of cestodes in the last 15 years. More importantly, perhaps, the gaps in our knowledge and some of the conflicting opinions expressed by different workers will become apparent.

In the choice and limitation of subject matter as well as in emphasis, a reviewer is frequently governed by his own special interests and research efforts in the field to be reviewed. This is one of the reasons for the selection

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of topics to be discussed here. An effort was made to include pertinent work from as many different sources as possible, but limitations of space did not permit mention of every publication relating to the general subject. The recent review by Smyth (1964) on the biology of the hydatid organisms, and the work of von Brand (1966) on the biochemistry of parasites made further discussion of these subjects unnecessary at the present time.

In the following pages an attempt will be made to summarize the information on development and structure with the purpose of emphasizing overall similarities (or differences) among the major groups of cestodes, and to indicate some of the areas in which further research is deemed essential or profitable.

Post-embryonic stages, as defined here, include all those forms habitually referred to as "larval" stages which develop in intermediate hosts.

II. Life Histories and Development

Stunkard (1953) stated of cestodes that: "Development and life histories appear to provide more substantial information for systematics than structure of the scolex."

A review of our knowledge of cestode life histories, particularly the development of post-embryonic stages in the intermediate host, shows how little we know about some of the major groups of cestodes. It also shows that the recent information on life histories and development has not substantially affected systematics. The greater present-day diversity of taxonomic schemes proposed at the ordinal level has resulted from subdivision of major groups, rather than from rearrangement substantiated by pertinent information on development.

A. Trypanorhyncha and Tetraphyllidea

One of the few recent studies on tetrarhynchid life histories is that of Riser (1956). Gravid proglottids of *Lacistorhynchus tenuis*, voided by a shark, were made to discharge eggs. Coracidia were liberated after a few days and fed to the splash-pool copepod *Tigriopus fulvus*. Almost all the copepods became infected. Developmental stages were observed at varying intervals after infection. Excretory vessels appeared after the 16th day and withdrawal of the anterior end was noted by the 18th day. A true tail was not observed. Feeding of infected copepods did not result in infection because the copepods passed through the intestines of fishes with the exoskeleton intact. Unfortunately, a detailed description of the procercoid and its developmental stages was not included in this work. Without doubt, the figures of withdrawn and presumably fully developed procercoids represent experimentally obtained tetrarhynchids, but they are very diagrammatic and show little morphological detail. However, the work does provide further evidence that the first intermediate host for tetrarhynchids should be sought among the crustaceans and, in particular, the copepods.

The life cycle of the tetraphyllid *Acanthobothrium coronatum* was discussed on the basis of observations as well as some experimental work by Reichenbach-Klinke (1956). Plerocercoids in sardines were fed to sharks in which the adult cestodes were subsequently found. Developing procercoids, presumably

belonging to the same species, were found in small crustaceans. It was concluded that tetraphyllids have a life cycle involving two intermediate hosts, the first a crustacean and the second a small bony fish.

B. PSEUDOPHYLLIDEA

Kuhlow (1953) experimentally demonstrated the life cycle of *Diphyllobothrium dendriticum*. The procercoid developed in certain copepods, the plerocercoid in fishes, and the adult in sea-gulls. Adults also developed in dogs, cats, rats and mice experimentally infected with plerocercoids. The infective plerocercoid is 4·3 cm long and never segmented. It is to be noted that, according to Kuhlow, the posterior portion of the body of the plerocercoid was *not* cast off in the definitive bird host and that patent adults 41–61 cm long developed 6 days after infection. Is there a relation between rapid growth of the adult and the retaining of all "larval" tissue? In the mammalian experimental hosts, the larval tissue was cast off 14–18 days after infection, meaning after the worms had become patent. Successive stages of plerocercoid development were not described.

Experimental demonstration of the life cycle of *D. sebago* by Meyer and Vik (1963) showed that the procercoids develop in copepods, the plerocercoids in trout, and the adult worms in hamsters, kittens and gulls. One of the observations in this careful and detailed study concerns the relation of water temperature to the growth of plerocercoids in trout. A sharp increase in the length of plerocercoids was noted when the water temperature rose above 2°C. A study of procercoid developmental stages was not included.

Guttova (1963) found that only two of eleven species of copepods examined were naturally infected with *D. latum*. One of the copepod species not naturally infected was, however, proven to be susceptible to infection under experimental conditions. Guttova stated that the littoral environment of the lake provided suitable conditions for infection of copepods, while the benthic parts of the lake which contained an efficient potential vector were not as accessible to the parasite. This is an example of ecological specificity or ecological limitation in that a physiologically susceptible host remains uninfected because contact with the parasite does not occur.

In his studies on the life cycle of *D. mansoni*, Takahashi (1959a) described an interesting technique which perhaps might be used to good advantage with other species of tapeworms. Plerocercoids from mice which had been fed to dogs were removed at different intervals from the dog's intestines and implanted subcutaneously into mice. After several days the parasites were removed from the mice to determine whether they had reverted to plerocercoids or remained young adults. Takahashi's conclusion was that the young adults could revert to plerocercoids if they had been in the dog intestine no longer than 48 h. After 48 h reversion to the plerocercoid stage apparently was not possible. It would be of interest to implant scoleces of adult worms into mice after removing all the segments. Although scolex morphology changes in the intestinal environment, it is conceivable that a sufficient number of relatively undifferentiated cells might remain in the neck region to give rise to a plerocercoid body.

The question to be answered is whether, after a period in the intestines, the determination of the adult stage is complete and irreversible for all cells, including those in the neck region of the organism.

In her study of *Bothriocephalus claviceps*, Jarecka (1959) found that the procercoid developed in copepods within 10–12 days. Further development was possible in cyprinids in which the procercoids *did not* penetrate into the body cavity but remained in the intestines. Jarecka (1963) established that procercoids which developed in copepods infected with coracidia from eels were infective to young eels in the laboratory. Adult, gravid cestodes were found in the intestines of eels 2 months after infection. Thus it may be concluded that a second intermediate host is not obligatory in the life cycle of this cestode.

Extensive life cycle studies on *Schistocephalus solidus*, including growth rates of procercoids, the copepod hosts, and of plerocercoids, were performed by Clarke (1954). Growth of individual procercoids in their transparent hosts was followed throughout development—a very fine and useful approach. Data obtained from plerocercoids showed no correlation between number of proglottids and total body weight. It appeared, however, that a certain size (bulk) had to be attained before segmentation occurred. Possibly the same holds true for tetrathyridia of *Mesocestoides* which may have to reach a certain size before transformation to the adult is possible. In cats, tetrathyridia may remain attached to the intestinal mucosa for several weeks without developing further. Also, passage of some tetrathyridia to the body cavity of the definitive host (e.g. dog) might indicate the necessity for further growth before adults can develop.

Studies on the life cycle of *Archigetes iowensis* by Calentine (1964) showed that procercoids developed in the seminal vesicles of oligochaetes. The procercoids, when fully developed, had a well-formed scolex, reproductive organs and a tail. Progenesis was not common but did occur. Development of oncospheres in eggs shed by the definitive host and procercoid developmental stages were described and illustrated. Natural as well as experimental infections were studied.

Of great interest and needing further study is the histological and cytological differentiation of the procercoid from the oncosphere. *Archigetes* and other caryophyllaeids lack the ciliary coat so common in the pseudophyllid cestodes. Thus the whole egg must be ingested by the intermediate host. A careful comparison of development of procercoids from caryophyllaeids and diphyllobothriids might reveal further differences between these two groups of cestodes.

The life history of *Archigetes limnodrili* in tubificid annelids was described by Kennedy (1965). The oncosphere lacked a ciliated envelope. Development to sexual maturity and egg production occurred in the oligochaete host.

Kulakovskaya (1962) experimentally infected oligochaetes with *Caryophyllaeus fimbriceps* and described some of the developmental stages in the intermediate host. Eggs were ingested and oncospheres hatched in the intestine of oligochaetes. Development proceeded to the formation of the male gonads but complete sexual differentiation occurred only in the definitive

fish host. When the procercoid attained a length of 2 cm, it disrupted the body of the oligochaete and died upon reaching the external aqueous environment.

Chubb (1964) reports the finding of developing plerocercoids of *Triaenophorus nodulosus* in perch during the months of March to June only. Thereafter all larvae were encapsulated (fully developed). From May to December crustaceans formed a significant part of the perch's diet but infection with procercoids was estimated to have occurred in January. Thus, the time of most intense feeding on the copepod intermediate host cannot be correlated with the time of infection of the perch. The intensity of infection of perch was generally low. The data presented showed that direct correlation between amount of a given food and infection does not necessarily exist. However, the population dynamics of the copepod intermediate host species and seasonal infection rate of the copepod host were not studied. Thus, no definite conclusions can be reached about the overall ecological situation.

Rees (1963) described the morphology of plerocercoids of *Proteocephalus niloticus* from frogs, in particular the musculature, nervous and excretory systems, and presented information on the life cycle which apparently involves three hosts, a crustacean for development of the procercoid (not observed), an amphibian for the plerocercoid, and monitor lizards for the adult stage. The latter host was established by feeding plerocercoids from frogs to *Varanus niloticus*. In view of the studies cited below, the necessity of two intermediate hosts for completion of this life cycle would have to be proved experimentally.

Evidence that proteocephalids may require only one intermediate host in their life cycle is contained in the study by Wagner (1954) on *Proteocephalus tumidicollus*. Procercoids from copepods, when ingested by rainbow trout, develop to adult worms in the fish intestines. There is no tissue phase. When eaten by larger trout, small infected trout transfer the infection to the large fish where the worms continue in growth in the intestines.

Regarding proteocephalid life cycles, the work of Hopkins (1959) on *Proteocephalus filicollis* presents additional evidence for the existence of only one intermediate host. In this species, the procercoids enter the fish final host and attach to the rectal mucosa but migrate anteriorly after a period of growth. Almost all worms found in the rectum were immature while those found in the small intestine were mature or gravid. No tissue invasion could be demonstrated.

Freeman (1964a) found that oncospheres of *Proteocephalus parallacticus* develop in different species of copepods into small plerocercoids which continue their growth as plerocercoids in the intestines of fish and subsequently reach adulthood in the same host individual. In this two-host cycle, considerable growth of the plerocercoid occurs in the intestines rather than in the tissues of fish. Freeman refers to the stage in the copepod as a plerocercoid because it does not resemble the procercoid stage of some other Pseudophyllidea but rather the plerocercoid in the fish host, although it is smaller in size. During development in the copepod, a cercomer is formed which eventually separates from the body. Of interest is the finding of numerous fine hairs on the body surface. Some of the effects of different ambient temperatures upon development of the cestode in copepods and in fishes are discussed. The

descriptions of development do not include histogenesis or cellular differentiation. A comparative study of post-oncosphere histogenesis and cytogenesis in proteocephalids and diphyllobothriids would be of great help in evaluating the relationships and present nomenclature of post-embryonic stages belonging to different cestode groups.

The question of size which must be attained by a plerocercoid before it can begin development toward the adult stage is an interesting one. Continued growth in the fish intestine may be accompanied by cellular differentiation or multiplication of certain cell types to the point where segmentation can be initiated in the intestinal environment. It is also possible that continued growth and storage of reserve foods, without differentiation, is essential for subsequent differentiation. Alternatively one might assume, for some species at least, the necessity for a period of adaptation to the intestinal environment before transformation to the adult stage can be initiated.

C. CYCLOPHYLLIDEA

1. *Linstowiidae*

Development of the cysticercoid of *Oochoristica deserti* was demonstrated experimentally by Millemann (1955) in several species of beetles, and described in some detail in *Tribolium confusum*. Developmental stages included the early spherical stage with subsequent cavity formation, elongation, scolex differentiation and scolex withdrawal. Full development, at 30°C, was reached in 16 days. Experiments to determine the effect of environmental temperature on the rate of development showed that at 26°C the required time was 20 days while at 20°C growth proceeded slowly for 60–70 days.

Gallati (1959) experimentally demonstrated complete development of infective cysticercoids of *Atriotaenia procyonis* in *Tribolium castaneum* in 10 days at 22–29°C. The developmental sequence included eccentric cavity formation, elongation of the body, posterior infolding at the site of the excretory bladder, and withdrawal of the scolex. Even after completion of development, the scolex frequently protruded from the cavity (observed also by Voge in *Oochoristica* from lizards), and the cysticercoids continued to increase in size. This sequence differs from *Hymenolepis* and other cyclophyllids in the lack of body partitions and in the development of an excretory bladder. The latter is similar to the bladder of tetrathyridia, as is the frequent "excystment" and withdrawal of the scolex.

In his study on the life history of *Oochoristica vacuolata* from lizards, Hickman (1963) described the developmental stages of the cysticercoid in carabid and tenebrionid beetles. He mentioned the evagination of the scolex in saline solution. Widmer and Olsen (1967) established that two species of grasshoppers as well as *Tribolium confusum* could serve as experimental intermediate hosts for *Oochoristica osheroffi* from rattlesnakes. In both studies, the development of the cysticercoid was very similar to that described for other species of *Oochoristica*.

2. *Anoplocephalidae*

In a detailed study, Freeman (1952a) experimentally demonstrated the life cycle of two species of *Monoecocestus* in oribatid mites. Cysticercoids reared in mites were infective to young porcupines. The sequence of the developmental stages was typical for cysticercoids and included the following: spherical organisms, cavity formation, elongation, tripartite body division, scolex formation and subsequent withdrawal. The fully developed cysticercoid had a slender tail and the scolex appeared to be surrounded by a considerable amount of fiber and other tissue. Flame cells and excretory tubules were noted at the time of sucker formation. Freeman stated that the excretory system was fully developed before the scolex withdrew into the cavity. In some hymenolepidid cysticercoids (Voge, unpublished) flame cells could not be demonstrated before scolex withdrawal even with the help of phase microscopy.

The life cycle of *M. sigmodontis* was demonstrated experimentally in oribatid mites by Melvin (1952). Developmental stages and fully grown cysticercoids were similar to those described by Freeman (1952a). At 24–26°C, development of infective cysticercoids was completed in 8 weeks.

An interesting observation (Freeman, 1952a) shows the need for "cracking" infected mites before they are fed to the definitive host. Unless the mite is damaged, it apparently passes through the digestive tract intact, failing to release the cysticercoids. Normally the chewing of food may be presumed to break up some of the ingested mites but, because of their very small size, it is likely that many mites could pass through the host intact unless affected by the host's digestive enzymes.

The study by Allen (1959) represents the first report on psocids as intermediate hosts of tapeworms or any parasitic helminths. Insects were reared experimentally in the laboratory and became infected after feeding on gravid proglottids of *Thysanosoma actinioides*. When fully developed, the cysticercoid is spherical or slightly ovoid and lacks a tail. The scolex is withdrawn but "excysts" easily in saline. At 30°C development is completed after 4 weeks. Heretofore, the life history of this species was unknown.

3. *Dilepididae and Davaineidae*

Kisielewska (1958) found natural cysticercoid infections of *Choanotaenia crassiscolex* in several species of gastropods. Development followed the cysticercoid pattern. Fully developed cysticercoids were tailless and encased in a hyaline capsule. The cysticercoid of *C. crassiscolex* from the snail host was also described in detail by Rawson and Rigby (1960). The interpretation of the sequence of development is not clear and from the description it seems doubtful that early stages of development were seen.

Enigk and Sticinsky (1959) experimentally established several new intermediate hosts for *Choanotaenia infundibulum*, *Raillietina cesticillus* and *Hymenolepis carioca*. Of interest were experiments with larvae of *Musca domestica* in which oncospheres of all three species hatched successfully but never became established. A search for developmental stages was unsuccessful. Oncospheres of all three species also hatched in the gut of the adult fly but

only *Choantoaenia* continued to develop into the cysticercoid. Perhaps the oncospheres of the other species were unable to penetrate the wall of the intestine and were eventually eliminated.

Anomotaenia ciliata, occurring as adult in wild ducks, was shown to utilize the Cladocera as intermediate hosts (Jarecka, 1958a). This is the first report of Cladocera serving as intermediate hosts for tapeworms. Attempts to infect various species of copepods and ostracods in the laboratory were unsuccessful and no natural infections were found in them. The cysticercoid has a short tail and the whole body, including the tail, is surrounded by a transparent membrane.

Cysticercoid development of *Davainea proglottina* was studied by Abdou (1958) in experimentally infected slugs. Stages described were spherical organisms with the cavity formed by the degeneration of internal cells (evidence for this statement is not included), elongate organisms with a differentiated scolex before withdrawal, and withdrawn cysticercoids. A rudimentary caudal appendage was noted in some. Figures of stained sections demonstrated a well-developed fibrous layer external to the cysticercoid cavity. Experiments to establish the effect of temperature showed that infective cysticercoids developed in 26–28 days at 15°C, in 18 days at 21°C, and in 13–15 days at 25°C. Excystment was accomplished in saline at 40°C. The tissues external to the scolex disintegrated in the presence of pepsin and hydrochloric acid.

A developmental sequence typical for cysticercoids was described by Sawada and Okada (1955) for *Raillietina cesticillus* in beetle hosts.

4. *Hymenolepididae*

Voge and Heyneman (1957) described and compared the developmental pattern of *Hymenolepsis diminuta* and *H. nana* cysticercoids in *Tribolium confusum* at 30°C. Differences between the two species were the speed of development, the extent of differentiation of the scolex just before withdrawal, and the structure of the fully developed cysticercoids. The infective cysticercoid of *H. nana* closely resembles that of *H. microstoma* (see Voge, 1964). In both species the scolex withdraws before the suckers or rostellum are fully differentiated.

In his extensive and careful study, Schiller (1959a) described the sequence of cysticercoid development of *H. nana* in *Tribolium confusum*. Comparative observations on the size of cysticercoids in different species of insects, on the effects of temperature on the speed of development, and on the numbers of cysticercoids in individual insects in relation to crowding were discussed. Data on the size of cysticercoids either from different species of insects or from the same species in heavy and light infections showed that the tail and the thickness of the capsule surrounding the scolex were the only structures affected. These structures were smaller when the host was heavily infected. The only structure to vary in different hosts was the tail, which was larger in larger hosts. Development of the cysticercoid was completed in 8 days in beetles maintained at 28°C, and in 14 days at a room temperature of 22–26°C.

Some disagreement exists between Schiller and Voge and Heyneman (1957), regarding "internal" versus "external" development of the scolex. In

hymenolepidids studied in recent years, at least the initial development of the scolex was found to occur externally. Withdrawal of a more or less developed scolex takes place rapidly (within 20–30 min in *Hymenolepis microstoma*; Voge, 1964), and frequently results in a considerable shortening of the cysticercoid. A notable decrease in length was also observed by Schiller (1959a, p. 101) during development of *H. nana*. It is possible that this decrease in length is caused by the withdrawal of the anterior end of the worm.

The fully developed cysticercoid of *Hymenolepis furcata* closely resembles that of *H. microstoma* and, according to Kisielewska (1959a) who studied the life history, the anterior or scolex part of the body withdraws before the scolex is fully differentiated. Suckers were formed and only after withdrawal did the scolex complete its differentiation. This also is similar to the situation in *H. microstoma*.

Development of cysticercoids of *H. diminuta* in the larvae of *Tenebrio molitor* was studied by Voge and Graiwer (1964), who injected oncospheres, hatched *in vitro*, into the hemocoel of the larvae and subsequently recovered normal cysticercoids. If the insect larvae were fed eggs, the embryos hatched but few reached the hemocoel. Comparative studies on the intestines of the larval and adult beetles indicated that the barrier to penetration of the larval hemocoel might be the relatively thick peritrophic membrane and the dense layer of intestinal cells. In the adult insect this barrier is not as formidable. It was also established that intestinal emptying time of *Tenebrio* larvae is much more rapid than that of adults.

In carefully conducted experiments, Heyneman (1961) demonstrated the possibility of auto-infection with *H. nana* when the original infection had occurred by ingestion of cysticercoids. From the results obtained there can be little doubt, if any, that there exists an endogenous source of infection which will result in heavy worm burdens. The most logical explanation is that eggs released in the intestinal lumen from gravid proglottids of an established infection can liberate embryos that will invade the intestinal mucosa and develop into cysticercoids. These return to the lumen and will excyst and grow into adult worms. The possibility of auto-infection thus provides *H. nana* with an additional means of increasing in number. A somewhat puzzling problem, however, is presented by the hatching of eggs liberated within the intestines. The number of oncospheres which become established in the mouse after ingestion of eggs is usually less than 10%. The adult worm is situated in the hind part of the small intestine. *In vitro* experiments have shown that a pH gradient is necessary for the hatching of *H. nana* oncospheres (Berntzen and Voge, 1965). The initial (acidic) pH required for hatching would not be expected to occur at the usual site of the adult worm, but eggs could be swept forwards by reverse peristalsis to a place where hatching could occur, and the outer egg envelope could (by some unknown means) be removed more efficiently than is usual to permit hatching of the large number of oncospheres necessary for the massive auto-infection produced by a single worm (Heyneman, 1961, p. 11, Fig. 2).

In this connection it is of interest to note the occurrence of *H. nana* in extra-intestinal sites. Mahon (1954) found cysticercoids of *H. nana* in the

liver of the mouse *Cryptomys darlingi*. Sections of liver tissue containing fully developed cysticercoids were figured. The development of the cysticercoids of *H. nana* in the lymphatics of the laboratory mouse was reported by Garkavi and Glebova (1957). Stages observed included the early spherical post-oncospheres, the pre-withdrawal stages and fully developed cysticercoids. Observations were made on naturally as well as experimentally infected mice. Both groups contained cysticercoids in the lymphatics of the mesenteries. Apparently, the developmental time in the lymphatics is a few days longer than it is in the intestinal mucosa. In one group of mice, the cysticercoids in the lymphatics numbered 1–93. Whether or not these mice also contained cysts in the intestinal mucosa was not stated. It would be of interest to obtain comparative data for the occurrence of *H. nana* in the intestine and lymphatics and to examine in more detail the rate of development of the cysticercoids in both locations. If there is indeed a delay in development in the lymphatics and perhaps a further delay before the cysticercoids can reach the small intestines, the phenomenon of "autoinfection" might be explained on the basis of two different sites and speeds of development rather than as the result of two successive generations. However, whether or not cysticercoids from the lymphatics are able to reach the intestine remains to be demonstrated.

The life cycle of *Soricina diaphana* from shrews was elucidated by Kisielewska (1960a). Cysticercoids were found in natural infections of *Geotrupes* beetles. Of interest is the tissue which grows out from the posterior end (tail?) and eventually completely surrounds the withdrawn cysticercoid in the form of a thick capsule.

Pseudodiorchis prolifer from shrews develops in myriapods (*Glomeris connexa*) and shows the typical cysticercoid developmental sequence, i.e. ball stage, cavitation, body divisions, scolex development and scolex withdrawal. The unusual feature is the occurrence of asexual multiplication so that numerous cysticercoids may arise from a single oncosphere. According to Kisielewska (1960b) this occurs by multiple budding from a single "parenchymatous" mass derived from one oncosphere, and represents a biological compensation for the small number of gravid segments and eggs produced by the adult worm. This larval form has been known as *Urocystis prolifer* Villot, 1890, but heretofore had not been related to the adult worm.

Other observations on life cycles of shrew cestodes were made by Prokopič and Groschaft (1961) whose examination of 4 030 *Gammarus pulex* yielded seven types of cysticercoids belonging to seven species of hymenolepidids which parasitize shrews. Description of the fully developed cysticercoids were included but no experiments were conducted.

Kisielewska (1961) published a fine ecological study showing the interrelationships between the habitat, the population dynamics of intermediate and definitive hosts, and the seasonal occurrence of the shrew tapeworms. An analysis of the food habits of shrews (*Sorex araneus*) at different times of the year was related to the seasonal occurrence and numbers of the intermediate host species under natural conditions. From her analyses of the pattern of distribution of the intermediate hosts and of the larval and adult stages of the parasites, Kisielewska listed the following factors which influence or determine

the dynamics of the tapeworm population: (1) the presence of suitable definitive and intermediate hosts; (2) the simultaneous occurrence of these in space and time; (3) a connection between both hosts via a food chain which must be natural and not accidental, and (4) the ecology of the intermediate host which permits a repeated contact with eggs of the tapeworms. While obvious, these principles have rarely been presented as concisely or supported by so much pertinent data.

A considerable amount of information has been published on the life histories of avian species of *Hymenolepis*. Supperer (1959) studied the life cycle of *Hymenolepis compressa* by examining crustaceans and snails from the habitat of the duck hosts. Numerous tailless cysticercoids were found repeatedly in the stomach of *Limnaea palustris*. The feeding of the cysticercoids to ducks resulted in infection with adult worms. Supperer indicated that this is the first report of cysticercoid development within the stomach of an intermediate host. He denies the possibility of initial development in the stomach wall on the grounds that a firm cyst covering would prevent the passage of the cysticercoid through tissue into the lumen. This reasoning is invalidated by what is known of *H. nana*. In no instance were any cysticercoids found attached to the stomach wall. They were always found in the food mass within the lumen. Apparently no developmental stages were ever found in any snail containing cysticercoids, but there is no mention that a search was made for them in the snail tissues.

The life cycle of *Sobolevicanthus octacantha* was demonstrated experimentally by Ryšavý (1960). Cysticercoids which developed in *Cyclops* were infective to young ducks. Figures presented by Ryšavý showed typical cysticercoid development with cavitation, elongation, body divisions and withdrawal. Experiments with another potential host, *Heterocypris*, showed that a sizable mortality of the oncospheres occurred 4–5 days after entry into the host and that very few cysticercoids completed development.

Hymenolepis furcifera developed in Cladocera experimentally infected by Jarecka (1958a). Natural infection was found in a single cladoceran only after examination of over 5000 individuals. *H. furcifera* was never found in copepods or ostracods and all attempts at laboratory infections of these crustaceans were unsuccessful. The cysticercoid is very small, bears a tail, and the whole body is surrounded by a thin membrane. The hooks in fully developed cysticercoids are always smaller than they are in the adult worm. Apparently the scolex continues development in the definitive host.

The life cycle of *Hymenolepis vistulae* from ducks was experimentally demonstrated by Czaplinski (1960) who obtained complete development of the cysticercoids in laboratory cultures of *Cyclops strenuus* at a temperature of 18°C in 18 days. The cysticercoid (according to his Fig. 9, p. 309) lacks a tail.

The pelagic and littoral zones of two lakes and their crustacean populations with naturally acquired tapeworm infections were investigated by Jarecka (1960). Parallel life cycle studies were conducted with the crustaceans in the laboratory, and the cysticercoids obtained were compared with those from natural infections. Descriptions of fully developed cysticercoids of *Hymeno-*

lepis macrocephala, *H. sacciperium*, *H. aequabilis*, *Drepanidotaenia bisacculina* and *Tatria acanthorhyncha* were included. Of interest were experimental results on *H. macrocephala*. Eggs of this species, when fed to several species of copepods, were digested. The oncospheres never reached the body cavity. When fed to ostracods, the eggs hatched and many oncospheres reached the body cavity but failed to develop further. Successful development, however, was achieved in *Notodromas monacha*. One wonders about the nature of the factors limiting or preventing development in these different hosts.

Jarecka (1958b) reported her successful results on eight new life cycles at the intermediate host level. *Hymenolepis paracompressa* developed experimentally in three species of copepods. It was found to occur naturally in eight species of snails, as well as in several species of copepods. *H. spiralibursata* developed in snails and copepods, *Diploposthe laevis* and *D. skrjabini* in copepods and ostracods. The pattern of development was of the cysticercoid type. Jarecka observed that the cyst wall of *Diploposthe* and *Fimbriarioides* developed in certain host species was thicker than in other host species, so that this variation depends on the species of intermediate host in which development occurs. This statement requires amplification in respect of the tissue layers involved.

Notably in this study by Jarecka and other works from the same laboratory, many thousands of copepods of any one species were examined in order to determine rates of natural infections. This is essential in any quest for the natural intermediate hosts of a life cycle. The perfection of Jarecka's and similar works is accomplished by the comparative study of natural and experimental infections.

Rybicka (1958) in a study of *Diorchis ransomi* described cysticercoid development in ostracods. She infected ostracods experimentally and followed the development and movements of a cysticercoid within an individual host, making the first observation of motility and displacement of a cysticercoid within such a host. The position of a cysticercoid in the host changes repeatedly and this is due to muscular contractions of either the host or the parasite. The overall developmental pattern is as known for other cysticercoids; a detailed description of the sequence of hook development is included.

The rate of development of cysticercoids within the same host individual was variable, as were developmental rates in different host individuals containing the same number of larvae and infected on the same date. Delay in development usually was observed during the early growth stages. (I have made similar observations on *H. diminuta* and *H. microstoma* in *Tribolium confusum* kept at a constant environmental temperature and with low intensity of infection. The reasons for this variable speed of development within the same host individual are not known.)

Kisielewska (1955) studied the sequence of developmental stages of *Drepanidotaenia lanceolata* within the copepod host. The pattern was comparable to that in other hymenolepidids and included cavitation, body divisions and scolex withdrawal. Particularly well illustrated is the sequence of steps leading to withdrawal of the scolex into the cavity (Kisielewska, 1955, p. 413, Fig. 12). The effect of environmental temperature upon the rate

of development in the intermediate host was noted. Development of parasites was followed within single host individuals separated in test tubes so that growth of a given parasite could be observed *in vivo* from beginning to end. The hosts were sufficiently transparent to make possible such observations. The lack of uniformity in the development of parasites in a given host individual as compared to greater uniformity in other hosts was related to the species and to the sex of the host.

An interesting investigation on interspecific competition was made by Michajłow (1958). He studied development of *Triaenophorus* and *Drepanidotaenia* in the copepod host infected with both species to determine how the presence of both parasites in the same host individual affected their development. Normal procercoids and cysticercoids developed side by side but growth of *Triaenophorus* was delayed in the presence of *Drepanidotaenia* cysticercoids, and delay could not be attributed to crowding. Similar effects were noted for *Drepanidotaenia* and were more pronounced when the number

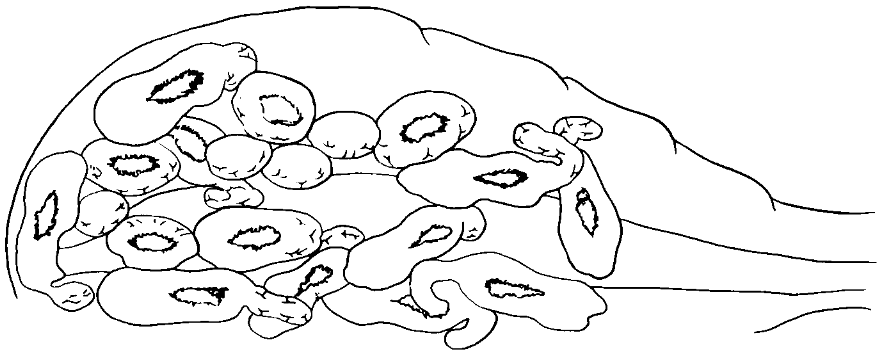


FIG. 1. Growth stages of cysticercoids of *Drepanidotaenia lanceolata* showing differences in rate of development in an experimentally infected copepod; marked delay in development is seen in organisms near the center of the host's body. (Redrawn from Kisielewska, 1957a.)

of parasites per host individual was high. It was noted further that if infection with *T. lucii* occurred when the copepod already contained *Drepanidotaenia* at a stage of maximal growth and differentiation, the development of *T. lucii* was affected by the first population. These phenomena were explained as due to interspecific competition, since they were more marked in the coexistence of two species than in the presence of large numbers of only one species.

The effect of crowding on growth of *Drepanidotaenia lanceolata* in *Cyclops strenuus* was studied by Kisielewska (1957a). When individual copepods were infected with seven to ten parasites, development of these was completed in 9–11 days which is also the developmental time for a single parasite in a host. Under conditions of overcrowding, development of the parasites was either delayed or not completed, although the parasites remained alive in the host. One of the most interesting observations is the relation of the rate of parasite development to the position within the body cavity of the copepod. Under conditions of crowding, parasites situated closer to the body surface developed much faster than those situated near the center of the

host's body (Fig. 1). Does the internal environment of the host's body cavity vary sufficiently to account for the difference in development and, if so, what are these variable factors and critical needs of the parasite? Kisielewska concluded that developmental disturbances in the parasite population may be due to the species, the sex and age of the host, the time of year at which infection occurred, the location of parasites in the body cavity, and the individual characteristics of the larvae, but we know nothing about the nature of individual characteristics which might influence the speed of growth.

The death of *D. lanceolata* at different stages of development in the copepod was also studied by Kisielewska (1957b). Death and disintegration of the parasites were observed at the solid ball stage, after cavity formation, and before and after withdrawal of the scolex. In certain host species, death occurred most frequently at the solid or hollow ball stage, in others after development had proceeded to the stage before scolex withdrawal. Analysis of the data showed that the mortality rate was not directly related to cysticercoid density but rather to the species, and the sex and age of the host, as well as the season during which infection had occurred.

From the above one could postulate the existence of stage-specific requirements during development which are only partially met by some host species. If the requirements of the parasites alter as development proceeds, some hosts may furnish an environment suitable for certain developmental processes but not for others.

Kisielewska (1959b) experimentally tested the efficiency of different species of copepods as hosts of *Drepanidotaenia lanceolata*. Different host species showed marked differences in their susceptibility to infection and in their ability to provide an environment suitable for parasite development. Differences were observed in the viability of embryos in the gut of the copepods, the speed of development at the same environmental temperature, and the intensity as well as the incidence of infection. Based on the experimental data, Kisielewska defined four types of host–parasite systems: (1) the obligatory system characterized by about 100% infection, high intensity of infection, and high viability of the developing larvae with a maximal speed of development; (2) the auxiliary system with an infection rate less than 100%, a lowered intensity of infection, most larvae viable, and the developmental speed occasionally variable; (3) the accidental system with a very low incidence and intensity infection, a certain percentage of embryos being digested in the intestine, development tending toward long delays, and with death or incomplete development of parasites, and (4) the spurious system comprising host species in which a large percentage of the embryos are digested in the intestine, and the few which do reach the body cavity are unable to complete development. While this nomenclature is somewhat awkward, the classification of hosts conveys the concept of the relative host efficiency in the laboratory. The approach is reminiscent of epidemiological studies on vector efficiency for many of the human parasitic diseases. Aside from the seasonal and age factors which were extensively studied by Kisielewska, one wonders what precisely caused the death of embryos in the intestine (mechanical destruction, pH, enzyme levels), or the arrest of development in the body

cavity (spatial relations, gas tension, food supply). Research designed to answer these questions would provide a sounder and less nebulous approach to the problems of physiological host specificity. Studies such as those by Kisielewska point the way toward better understanding of the complex interrelations between the host and parasite and help to formulate the basic questions about parasite requirements.

5. *Taeniidae*

Of the several life histories investigated in recent years, those reported below constitute the most detailed accounts noted in the literature. Freeman (1956) in his study of *Taenia mustelae* found larval stages in various rodents. The most common site was the liver where larvae occurred in host cysts containing from one to several individuals. The presence of multiscolex larvae showed that this species can multiply asexually. Numbers of scoleces per bladder varied from one to twenty-one. Development of oncospheres was observed in experimentally infected rodents. Stages noted were: (1) spherical forms with poorly defined or no cavity at 9 days; (2) forms with a pronounced cavity and thickenings in the bladder wall at 13–14 days; (3) the development of shallow scolex canals, the excretory system and calcareous granules at 17 days. Subsequent stages of scolex differentiation were described. Histology was not studied.

Freeman (1959) studied the development of post-embryonic stages of two species of *Cladotaenia*. Their gross morphology in the liver of mice was described. Freeman refers to these stages as plerocercoids, but in his descriptions and figures and in their overall structure they resemble tetrathyridia. When fully developed they had a withdrawn scolex and were surrounded by a capsule. Plerocercoids obtained from experimentally infected mice were fed to young hawks where they developed into adult worms.

In his detailed study of growth and asexual multiplication of *T. crassiceps* cysticerci in the laboratory mouse, Freeman (1962) presented data on the numbers produced from a single cysticercus in unit time. Rates of reproduction in subcutaneous foci, pleural cavity and abdominal cavity were compared. The results clearly showed the significant retardation of growth outside the peritoneal cavity, although initial rates were higher in these foci. However, for the first 4–5 months the percentages of fully developed scoleces were higher in subcutaneous sites than in forms from the peritoneal cavity. This indicates the need for defining the optimal conditions for the species as a whole, because after ingestion by the definitive host only forms with a well-developed scolex can produce adults. The seemingly unlimited capacity for asexual reproduction of the parasite and the tolerance of the host for the heavy worm burdens are astonishing features.

The developmental stages of *T. saginata* in cattle were investigated by McIntosh and Miller (1960) and the gross morphology described and illustrated. Observations indicated that the earliest time at which cysticerci could be infective was at 10–12 weeks of development. At this time the scolex and suckers were fully developed and the scolex was "withdrawn".

Study of earlier stages showed that cavity formation occurred during the 3rd week after infection. At this time there was a considerable cell density at the scolex end which terminated in a knob-like protrusion. A week later the area in which the scolex would develop had invaginated. Suckers developed at 5–6 weeks and were apparently fully formed by the end of 6 weeks. An invaginated neck portion was noted 10 weeks after infection. It is remarkable that this apparently represents the first experimental study of gross development of a species which has been known longer than most other tapeworms. An obvious reason may have been the difficulty in obtaining enough cattle for the experiments. Careful histological and cytological studies of the developmental stages with special reference to the comparative development of the scolex and bladder would be of great value.

D. ASEXUAL MULTIPLICATION

While it has been long known that certain post-embryonic forms of cestodes have the capacity for asexual multiplication, it is generally assumed that the species which are able to do so (*Echinococcus*, *Multiceps*, etc.) represent the exception rather than the rule. Although asexual multiplication in cestodes is clearly not as common as in the trematodes, it occurs more frequently than has been supposed.

In the family Taeniidae many species have the capacity for exogenous budding, an example of which is shown (Fig. 2) in a taeniid from the body cavity of a Peruvian rodent (see Voge, 1954). The manner and frequency of exogenous budding in *Taenia crassiceps* has been studied extensively by Freeman (1962). He has also described (1956) the asexual multiplication of *Taenia mustelae*, in which a single oncosphere may give rise to more than one cysticercoid by the formation of multiple scoleces, although small portions of "bladder wall", if pinched off, can also give rise to additional single or multiple scoleces.

The fact that very small pieces of the cyst tissue can initiate an infection was usefully applied by Norman and Kagan (1961) who injected homogenized *Echinococcus multilocularis* tissue into laboratory animals and produced good infections in gerbils and cotton rats. This method obviates the necessity of handling eggs in the laboratory and makes possible the maintenance of this species by intraperitoneal passage of the cyst tissue. No statement was made about the degree of disruption of the homogenized tissue. It would be interesting to determine whether an injection of completely separated cells will produce a new cyst, or if a minimal amount of intact tissue must be supplied.

Asexual multiplication in diphyllid cestodes was reported by Dollfus (1964) for *Echinobothrium affine* in *Carcinus maenas*. This represents an additional order of cestodes in which asexual multiplication can occur. The parasite appeared as a group of connected filaments bearing numerous buds, each representing a developmental stage of a young individual. Older protuberances had a cavity containing the scolex. Dollfus noted the resemblance of this type of budding to the description of *Urocystis prolifer*, which is the cysticercoid stage of the hymenolepidid *Pseudodiorchis prolifer* (see Kisielewska, 1960b). This type of budding (Fig. 3) in a species which becomes adult in

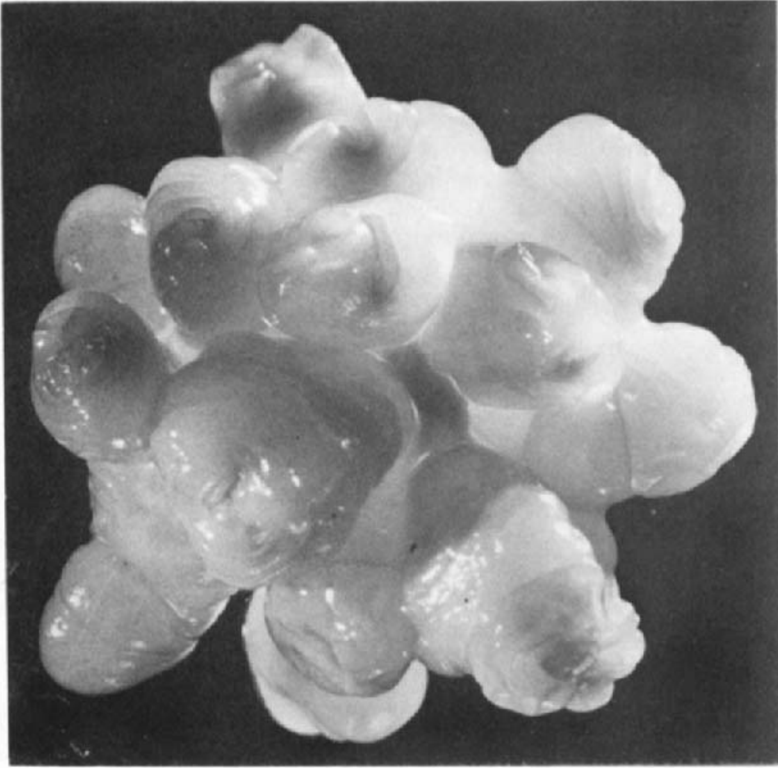


FIG. 2. Asexual multiplication of *Taenia* sp. in the body cavity of a rodent.

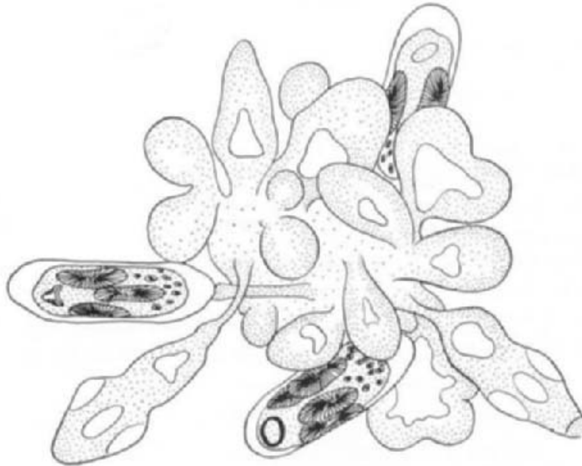


FIG. 3. Asexual multiplication in *Pseudodiorchis prolifer*. (Redrawn from Kisielewska, 1960b.)

shrews is likely to be found among other hymenolepidids of shrews, which would explain partially the heavy worm burdens frequently seen in these hosts.

A somewhat unusual type of asexual multiplication, reported by Specht and Voge (1965), occurred when mesocestoidid tetrathyridia from lizards were either fed to or injected intraperitoneally into mice. Multiplication occurred by longitudinal division, beginning at the scolex with the formation of supernumerary suckers and was followed by a gradual, longitudinal splitting (Fig. 4). In this manner two, or sometimes more, new individuals were formed.

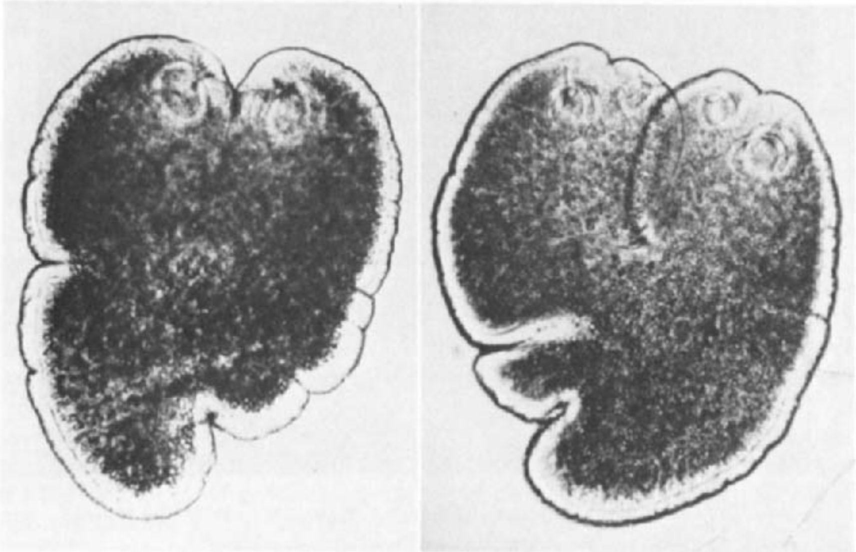


FIG. 4. Division stages of tetrathyridia of *Mesocestoides* from the peritoneal cavity of the laboratory mouse; (a) beginning of division, showing longitudinal furrow between two sets of suckers; (b) more advanced stage showing partial separation of individuals.

From an initial injection of twenty-five individuals into a laboratory mouse, more than 3000 individuals may exist 4–5 months after infection. The tetrathyridia occur throughout the liver tissue and in the peritoneal cavity. Division stages were also found in naturally infected lizards.

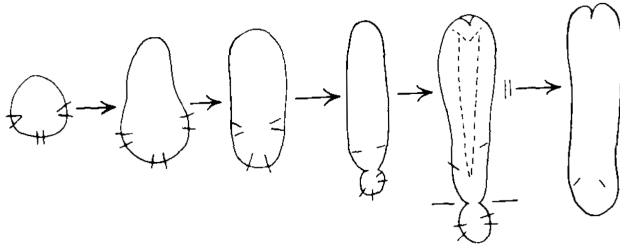
Thus, another family of tapeworms has been shown to have the ability for asexual multiplication. The manner in which this occurs is unusual and differs from what has previously been described for cestodes. The cytological events preceding the formation of new individuals need further investigation.

E. SUMMARY

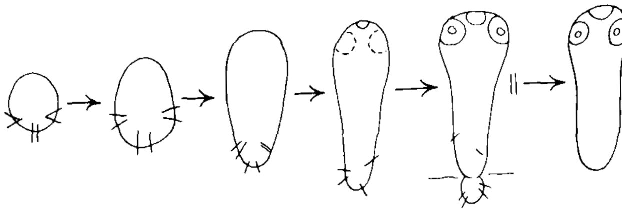
Considerable progress has been made in our knowledge of cestode life histories and development. It has been shown that certain pseudophyllids, including proteocephalids, require only one intermediate host and that

trypanorhynchs apparently utilize a crustacean as a first intermediate host. Cladocerans and psocids have been added to the groups of arthropods utilized by cestode developmental stages. Considerable information has accumulated on hymenolepidid life histories and on the development of the cysticercoid in the intermediate host. New approaches of study and careful observations under experimental conditions have yielded data on the vari-

Pseudophyllidea Trypanorhyncha



Proteocephala Catenotaenia?



Mesocestoididae

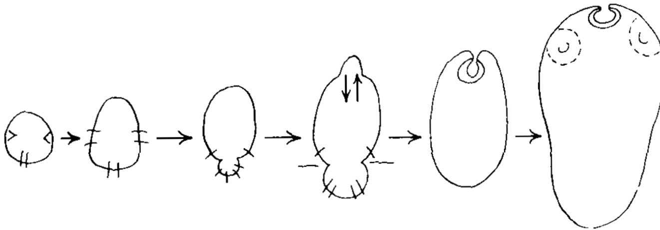


FIG. 5. Diagrammatic representation of developmental patterns in different groups of cestodes, beginning with the hatched oncosphere and ending with the plerocercoid or tetrathyridium. As far as is known, the groups listed have in common formation and loss of cercomer, elongation of body without appreciable cavitation, and development of circumscribed glandular areas. The pattern for Mesocestoididae, based on growth *in vitro*, is included for comparison.

ability of development. Correlation of this information with the host species, and with the internal as well as the external factors has been attempted. These observations have suggested many new problems to be investigated. The factors that limit or promote the development of a cestode in a host require much additional study.

From repeated observations on development, certain patterns have emerged and will be summarized briefly here, with a diagrammatic presentation of

several patterns of development, beginning with the hatched oncosphere and ending with the stage preceding the adult (Figs. 5 and 6). In pseudophyllids, possibly in trypanorhynchs, and in proteocephalids, development involves elongation of the body, cercomer formation and loss, and the differentiation of specialized glandular areas in the anterior half of the body. No central cavity has been described. The young worm is not surrounded by tissues to be discarded in the definitive host. The pattern for *Mesocestoides* was determined from *in vitro* development (see below) and is included for comparison with pseudophyllids and proteocephalids. It must be realized that almost nothing is known about the cytological and histological differentiation of procercoids

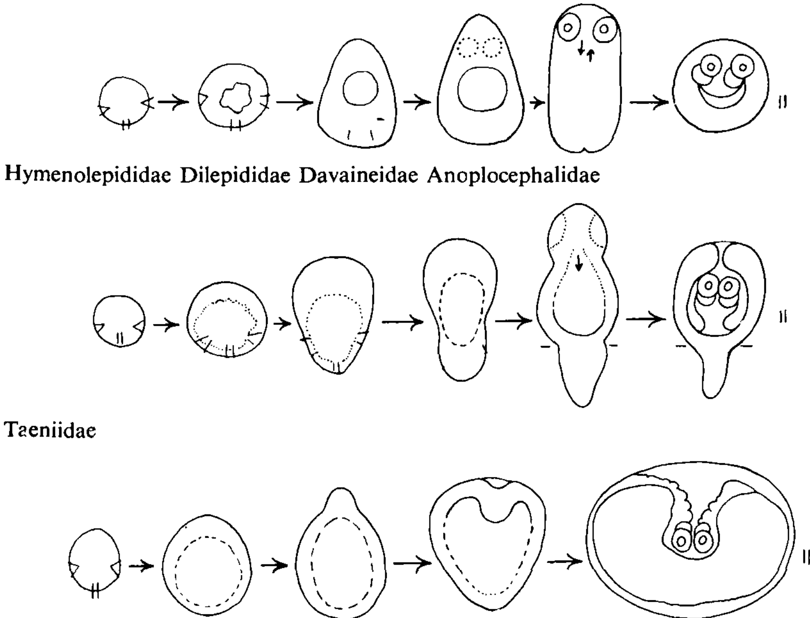


FIG. 6. Diagrammatic representation of developmental patterns in some Cyclophyllidea. Note the presence of a well-defined cavity in all groups, and the absence of body partitions and specialized cystic tissues in linstowiids. The taeniid pattern shown here is limited to cysticerci.

or of any other post-embryonic stage. Detailed studies on procercoids are badly needed for meaningful comparison of development.

The development of linstowiids entails the formation of a well-circumscribed cavity, elongation and growth without body divisions and withdrawal of the scolex. However, the scolex is not surrounded by tissues to be discarded in the definitive host and its position is not fixed after withdrawal. Essentially, the fully developed and infective linstowiid is a juvenile worm without specialized cystic tissues.

The typical cysticercoid pattern of development is characteristic for most hymenolepidids, dilepidids, davaineids and anoplocephalids. It entails the

formation of a well-defined cavity, elongation of the body with subdivision into two or three parts, withdrawal of the scolex into the cavity, and formation of specialized tissues and membranes which surround the scolex and neck. These tissues are discarded in the definitive host. After withdrawal, the scolex remains in this position until the cysticercoid has entered the definitive host.

In the taeniids (McIntosh and Miller, 1960; Šlais, 1967), development (as far as is known) includes cavity formation, differentiation of the anterior end initiated by an increase in cellular density, and growth of the scolex inward so that external development with subsequent withdrawal is much reduced or eliminated. The "bladder" tissue which surrounds the scolex is continuous with the body of the cysticercus. Very little is known about the fate of this tissue in the definitive host.

While the above summary is obviously limited by lack of knowledge and by the usual modifications or exceptions to every generalization, distinct differences in developmental patterns and in the end product of development, when established, should be sufficiently important to warrant re-evaluation of our terminology for post-embryonic stages regardless of the type of host in which development occurs. Furthermore, should differences in development be considered more important than aspects of adult morphology, or equally important, in evaluation of relationships between cestode groups? For example, development in linstowiids differs appreciably from development in *Monoceoecstus* and many other anoplocephalids and this difference could further substantiate the separation of linstowiids from anoplocephalids (Millemann, 1955). Regarding terminology, how do we define a cysticercoid or a plerocercoid? Linstowiid post-embryonic stages have been called cysticercoids and some taeniid stages plerocercoids.

Clearly, the comparative aspects of development have been neglected and further studies would be most helpful.

III. Structure

The many investigations on structure of cestode post-embryonic stages published by different authors are extremely variable in scope and purpose. Most numerous are the descriptions of gross morphology relating to life history or to taxonomic studies. Observations on the histology of post-embryonic stages are relatively few, while analyses of cellular and nuclear structure are virtually non-existent. It is reasonable to expect that cestodes have a plan of histological organization in which certain features are shared by all members of the group. Similarly, one would expect to find the recurrence of certain cell types during development and after completion of development, regardless of the species investigated.

While most of the observations to be discussed were not reported originally to demonstrate similarities in overall structure, such similarities, if obvious, will be stressed here in an attempt to determine what features are shared by most if not all cestodes.

A. PROCERCOIDS AND PLEROCERCOIDS

Very few detailed studies have been published on procercoid morphology. The structure of the procercoid of *Diphyllobothrium ditremum* was described and illustrated in Kuhlow's (1953b) study on the development of different diphyllobothriids. The procercoid had a small tail. The body, surrounded by cuticle, contained a well-developed circular musculature, excretory ducts terminating in flame cells, and a central column of glands extending along two-thirds of the body. Afferent ducts from these glands terminated at the anterior end. Detail on cell structure was not included.

Utkina (1960) briefly described the development of procercoids of *Diphyllobothrium latum* in the intermediate host and illustrated several developmental stages leading to the procercoid. No detailed description of these stages was presented.

Miscellaneous information on the size, shape and gross structure of procercoids was contained in some of the life history studies mentioned earlier. In general, the structure of the procercoid and its preceding developmental stages has had little investigation and further studies on the sequence of development and the internal cellular organization are badly needed.

Plerocercoids have been studied in much greater detail. Rees (1950) studied the anatomy of the hooks and the muscular, excretory and nervous systems of the tetrarhynch *Grillotia heptanchi*. In an attempt to differentiate the species of *Diphyllobothrium* on the basis of plerocercoid structure, Kuhlow (1953c) studied the morphology of many plerocercoids from different species of fishes. He reported the presence of cuticular hairs in three of four species studied. The only species lacking hairs was *D. latum*. In his opinion, the small cuticular processes described for some procercoids were different structures. This seems unlikely, however, but requires further study under experimental conditions. Regarding the lack of hairs in *D. latum*, perhaps young plerocercoids have hairs which later become obliterated as the organism increases in size. A fine illustration of the cuticular hairs in *D. osmeri* is given by Kuhlow (1953c, p. 191). The musculature was described in some detail. The subcuticular muscles consisted of a single layer, while the arrangement of the parenchymal muscles varied with the species. According to Kuhlow, species can be differentiated by the structure of the frontal glands, the parenchymal musculature, the length of the cuticular hairs, and other characters.

In their study of diphyllobothriid plerocercoids, Chizhova and Gofman-Kadoshnikov (1959) also described the presence of cuticular filaments over the body surface. The filaments varied in length in the two species studied. In one species the whole body surface including the scolex was covered, whereas in the other the scolex area was free of filaments.

It is possible that the presence of the cuticular hairs on the surface of the scolex indicates immaturity because, in other cestodes, the hairs may become obliterated by the fully formed cuticle (see below). Additional descriptions and illustrations of "cuticular bristles" in diphyllobothriid plerocercoids may be found in the study by Kozicka (1958).

Wikgren (1964a) studied the plerocercoids of *Diphyllobothrium dendriticum*

and *D. osmeri*. He concluded that the most valuable morphological characters for differentiation between the two species were the shape of the holdfast, the length of cuticular bristles, and the arrangement of the subcuticular muscle fibers. He stressed the importance of a histological examination in the description of plerocercoids.

Mitotic activity of plerocercoids of *Diphyllobothrium latum* was studied by Wikgren (1964b) in organisms pre-treated with colchicine. Observations designed to determine the presence or absence of a diurnal rhythm of mitotic

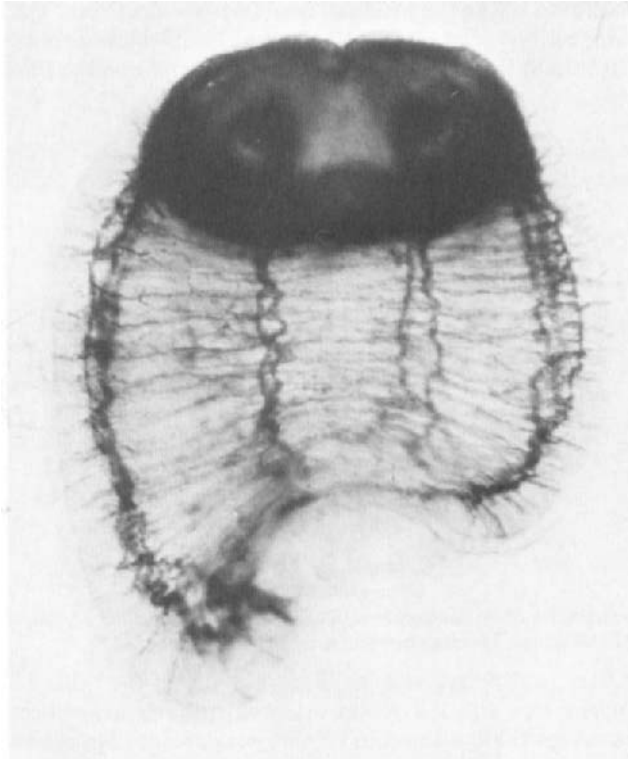


FIG. 7. Nervous system of tetrathyridium of *Mesocestoides* as demonstrated by the acetylcholinesterase technique. (Courtesy of Joseph L. Hart.)

activity showed that such a rhythm was apparently absent. Different tissues or cell types differed in their mitotic activity. No mitoses were found in the subcuticular cells or the flame cells. Metaphases were seen in the parenchyma and in particular among the cells bordering the longitudinal parenchymal muscles. Mitotic activity decreased after the plerocercoids had been kept in non-nutrient media (Hank's solution with glucose). Studies on the effect of high temperature showed that it acted as a stimulus to mitosis (see below).

The excretory systems of several proteocephalid and two bothriocephalid plerocercoids were described and illustrated by Soltynska (1964). In some of

the proteocephalids, the excretory network in the scolex was complex and different patterns were recognized in different species.

The histological organization of mesocestoidid tetrathyridia was briefly described by Voge and Berntzen (1963). The sequence of tissues, beginning with the cuticle, was similar to that observed in plerocercoids. No "cuticular bristles" were observed in fully developed tetrathyridia. Subsequent study (unpublished) showed, however, that tetrathyridia also bear cuticular hairs at certain stages of growth.

The nervous system of tetrathyridia was studied by Hart (1967) who demonstrated, in addition to the longitudinal trunks, numerous transverse rings with fine cross-connections (Fig. 7). Delicate nerve fibers were seen to extend into the cuticle at intervals, terminating close to or at the body surface.

B. CYSTICERCOIDS

Considerable variation exists in the gross morphology of different cysticercoids. Some cysticercoids lack a tail. The orientation of the scolex within the

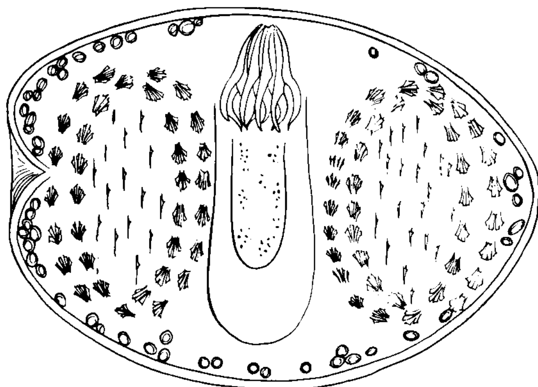


FIG. 8. Cysticercoid of *Echinocotyle clerci*, showing the absence of tail and transverse orientation of the scolex. (Redrawn from Jarecka, 1958b.)

cavity may vary in different species. Figure 8 shows the tailless cysticercoid of *Echinocotyle clerci* with the scolex oriented transversely within the cavity. Figure 9 illustrates the cysticercoid of *Drepanidotaenia bisacculina* bearing a very long and narrow tail. Shape and size of the cysticercoid body also vary with the species, as do the tissues and membranes surrounding the scolex.

Young (1952) reports tail length of *Hymenolepis californicus* as being approximately 20 mm. The function or usefulness of the cysticercoid tail is obscure. Perhaps the undifferentiated cells in the posterior part of the body just continue to divide in the absence of a stimulus for differentiation.

Histological structure of cysticercoids also varies with the species studied. For example, those species in which the scolex and neck are surrounded by a "capsule", which will be shed in the definitive host, have a pattern of organization distinct from that of species in which the scolex simply withdraws into its own body. As noted in the section on life histories, species conforming to the first type are represented by hymenolepidids, certain davaineids, and

many others. In *Hymenolepis diminuta* and *H. citelli* the structure of each body division of the cysticercoid is distinct. The scolex and neck contain the attachment organs and early developmental stages of most of the adult organ systems (nervous, muscular, excretory). External to the withdrawn scolex are several tissue layers always arranged in a comparable sequence. A delicate fibrous mesh with large nuclei immediately surrounds the scolex and neck, a thick fibrous layer next to the cavity, and an outer layer of large cells, called peripheral cells (Voge, 1960a, b), which resemble the subcuticular cells of adult cestodes. External to these is a second fibrous layer oriented differently from the inner one (Fig. 13). This organization is typical for the midbody of the cysticercoid and distinctly different from the structure of the tail. The latter usually consists of a mesh of relatively low cell density without any

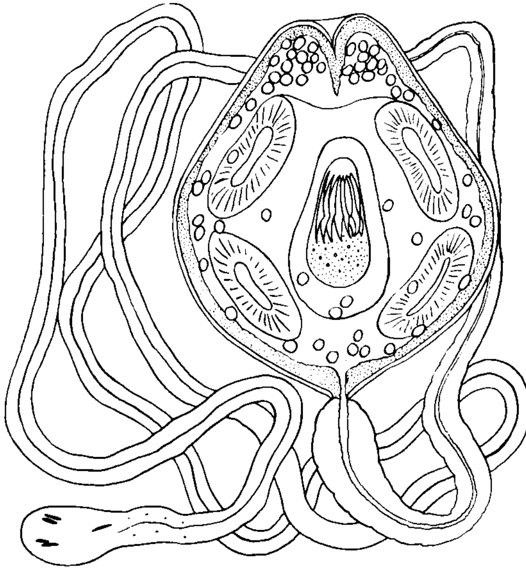


FIG. 9. Cysticercoid of *Drepanidotaenia bisacculina*, illustrating the extreme length of tail. (Redrawn from Jarecka, 1960.)

organized layers and frequently contains large fluid-filled spaces. In *H. nana* and *H. microstoma* (Voge and Heyneman, 1960; Voge, 1963a) the peripheral cell layer of the midbody is apparently greatly reduced or absent. Unfortunately, studies on the histogenesis of the two species have not been made to determine whether these cells appear but disintegrate quickly, or whether they never develop. After several months, the disappearance of peripheral cells is noted in cysticercoids of *H. diminuta*. In *Raillietina cesticillus*, the portion of the midbody of the cysticercoid external to the scolex is comparable in organization to hymenolepidid cysticercoids (Voge, 1960c). In general, the interspecific variations are primarily the thickness of the various layers, the presence or absence of external gelatinous coats, the presence or absence of a tail and, occasionally, the reduction of the peripheral cell layer. Additional studies on

different species and genera of cestodes are needed to substantiate or modify the pattern outlined here.

Perhaps the term "cysticercoid" should be restricted to those forms in which the scolex and neck are surrounded by specialized tissues (i.e. "capsule"). "Cysticercoids without a capsule" are acystic forms in which the scolex withdraws into their own bodies (*Oochoristica* and *Thysanosoma*, for example). The only tissue which might be shed in the definitive host is a portion of the posterior part of the body. Sections (Figs. 11 and 12) of these "cysticercoids" show an organization comparable to that of the withdrawn scolex and neck in other cestode groups. In different species of *Oochoristica* from mammals and reptiles, the withdrawn scolex usually rests on a bed of fibers which begin to appear at the time of cavity formation (Voge, unpublished) and in the fully developed metacestode are situated medially. Calcareous corpuscles are usually present throughout the young worm which resembles a tetrathyridium or plerocercoid in its overall structure. "Cysticercoids" of *Thysanosoma actinioides* are spherical or ovoid when withdrawn (Allen, unpublished) and sections show a scolex with a short body (Fig. 12), surrounded by only a delicate membrane.

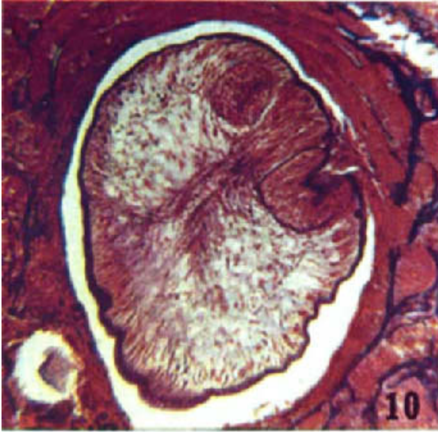
Studies on the histogenesis of cysticercoids are few and incomplete. Voge (1960d), on the basis of tissue sections, described the formation of the major tissues and the sequence of their appearance in *Hymenolepis diminuta*. Several types of nuclei were observed early in the spherical stage. The appearance of the lining tissue of the cavity was noted in the elongate stage. The alignment of the peripheral cells began with the formation of the body divisions and preceded the completion of the fibrous layers. The latter are clearly delineated only after the scolex has withdrawn. While formation and fate of some of the cell types could be determined, many others were not traced and probably not recognized.

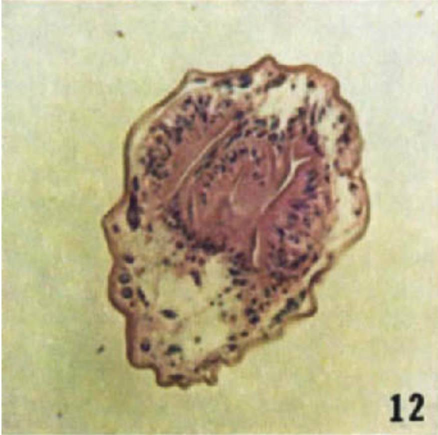
Detailed observations on cellular differentiation and organization, beginning with the early growth of the oncosphere, have not been published for any cestode species. Studies on hatched oncospheres of different cestodes and observations on their developmental stages (Voge, unpublished) clearly indicate that not just one but several of the different nuclear types of the oncosphere continue to divide in the first intermediate host and contribute to the tissues of the cysticercoid.

C. CYSTICERCI

The anatomy of the nervous, excretory and muscular systems of *Cysticercus fasciolaris* was studied by Rees (1951). Her detailed work suggests that, for the systems described, the structure of the cysticercus must be very similar if not identical to that of the adult worm. The complexity of the different organ systems of cestodes is exemplified by this careful study of *C. fasciolaris*.

Šlais (1966), in his observations on the morphogenesis of *Cysticercus cellulosae* and *C. bovis*, described the development of the bladder portion which was initially small but gradually grew anteriorly. Eventually it completely surrounded the scolex in *C. cellulosae*, but in *C. bovis* the secondary





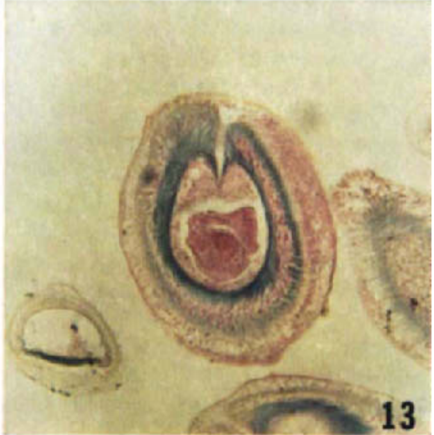


FIG. 10. Tissue section of *Mesocestoides* in liver, stained with Mallory's aniline blue.

FIG. 11. Tissue section of *Oochoristica* sp. from *Tribolium confusum*, stained with Gomori's trichrome. Scolex is withdrawn; the green area beneath the scolex is fibrous tissue.

FIG. 12. Tissue section of *Thysanosoma actinioides* from psocid, stained with hematoxylin and eosin.

FIG. 13. Tissue section of *Hymenolepis diminuta* from *Tribolium confusum*, stained with Mallory's aniline blue. Blue-staining areas are fibrous tissue. All tissues surrounding the central cavity will be discarded in the definitive host.

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enclosure of the scolex by the bladder did not occur. Presumably this difference should serve for species differentiation if appropriate sections were studied.

Review of recent work on cuticle formation and internal structure of cysticerci shows a divergence in interpretation. Conclusions differ and the terminologies used are sometimes confusing.

From studies on development of *Taenia pisiformis* cysticerci in experimentally infected rabbits, Aligon (1955) made the following observations. Forty-eight-hour stages from the liver contained two types of nuclei. At 4–6 days there was a considerable increase in size and alignment of subcuticular cells beneath the cuticle. At 8–10 days two types of nuclei were seen; large nuclei with a single nucleolus, found close to the cuticle and at opposite poles of the organism; and small nuclei with numerous chromatin granules occurring more diffusely between the poles. A distinct cavity is present. The cephalic pole was defined by a greater density of subcuticular cells and by the presence of granular zones. In somewhat more advanced stages of the same infection two layers of subcuticular muscles were noted. The author concluded that the cuticle was formed before the subcuticular muscles appeared and that it extended over the entire surface of the larva. The cuticle was composed of an external layer of varying thickness bearing cilia-like processes; a much thicker, median layer bordered by a deeply staining line (collagenous fibers?); and internal to it an area into which protrude the cystoplasmic extensions of the subcuticular cells. Aligon further concluded that the large nuclei, seen in 48-h stages, formed the subcuticular cells which were responsible for the secretion of the cuticle 1 day later.

While these conclusions appear sensible and in line with some observations by other authors on other cestode species, Aligon's definition of cuticle is somewhat misleading in that it also encompasses the extensions of the subcuticular cells. The question, where does the cuticle end and other tissues begin, could perhaps be clarified by a re-definition of the body covering of cestodes in general, taking into consideration the origin, development and final structure and composition of the cuticle.

Logachev (1959) also studied development of the cuticle in cysticerci. He concluded that the cuticle originated from connective tissue ground substance which diffused through a reticulum of muscle fibers to the surface of the body. He further stated that a "basal layer" or fibrous region was absent in the cysticercus, and that a subcuticular cell layer is usually absent. Since the preparations he used were stained with iron hematoxylin, even the distinction between muscle and fibrous layers would be almost impossible to make. Further, a subcuticular cell layer is certainly present (see below) but its thickness and orderliness in alignment vary with age and area of the cysticercus examined.

Siddiqui (1963), describing and illustrating the cuticle of the cysticerci of *Taenia saginata*, *T. hydatigena* and *T. pisiformis*, stated that the cuticle is composed of three layers: a thin external layer, the thick, homogeneous middle layer and a basal layer of thin fibers. He also described the hairs on the surface and showed their continuity with the middle layer of the cuticle. Although brief, the report does provide additional evidence for the basic

uniformity in organization of the cuticle. The major confusion concerns the nomenclature of the layers observed, particularly the fibrous layer which obviously represents the basement membrane of other authors. It goes without saying that the hairs described by Siddiqui and others are not to be confused with the microvilli demonstrated repeatedly with the electron microscope. Whether the thin external layer of the cuticle is actually a distinct entity is still questionable. In general, the problem of naming the "cuticular" layers resides in our ignorance about the development and the function of these layers. The subcuticular cells are also a constant feature of the cestode body and are always found in a comparable position.

Studies by Voge (1962, 1963b) on the histological organization of fully developed cysticerci of *Taenia hydatigena*, *T. solium* and *T. saginata* indicated that the structural plan of taeniid cysticerci is quite uniform. In the three species studied, the fully differentiated, anterior portions of the cyst were composed of the following: a cuticle with a peripheral fibrous layer (apparently collagenous, same as "basement membrane"), two layers of muscle, a layer of peripheral or subcuticular cells, and a loose fibrous network containing flame cells and ducts, calcareous corpuscles, various nuclei and thick muscle bundles. The relatively undifferentiated portions of the cysticercus (bladder region), situated posterior to the scolex, consist of hair-like processes (the "cilia" of earlier workers), the peripheral fibrous layer ("basement membrane"), spherical or ovoid cells (potential subcuticular cells) and scattered muscle fibers as yet not aligned as subcuticular muscles. Observations on *T. hydatigena* showed that the fine hair-like processes gradually become incorporated into the acellular layer of the cuticle as this layer increases in thickness. The basic, uniform organization of cysticerci makes it impossible to identify the species unless the scolex is studied.

Electron micrographs (Race *et al.*, 1965) of larval stages of *Multiceps serialis* show the microthrix and other cuticular layers as well as flame cells. The individual processes of the microthrix layer are seen to be interconnected by fine fibrils. This has not been mentioned previously in observations on cestode cuticle. These authors' statement, that the "basement membrane" or "subcuticle" was amorphous, needs further clarification. It was not clear where, precisely, this membrane was situated and whether it is identical with the "basement membrane" or "subcuticular fibrous layer" seen under the light microscope. No definitive evidence was presented to support the contention that it is amorphous.

Observations on abnormal or incomplete growth are sometimes helpful in understanding patterns of normal growth and differentiation. The "racemose" form of the cysticercus of *Taenia solium* has been the subject of much controversy. Talice and Gurri (1950) studied material obtained at surgery from the brain of a 53-year-old male in Uruguay. On microscopic examination they found irregularity in thickness of the cyst "wall", numerous infoldings, and structural irregularities such as variability in the thickness of the fibrous layers and the presence of multiple "lacunae" of variable size. The authors considered this to be a form of abnormal growth of the cyst wall without growth of the scolex. They raised the question whether the growth

potential of the cysticercus is perhaps unlimited and expressed the idea that the bladder portion develops at the expense of the atrophied scolex. Since no evidence was found that the scolex had developed at all, it seems possible that scolex differentiation had never been initiated.

Trelles *et al.* (1952) published a careful histological study of the cysticercus of *T. solium* as well as of the racemose form from the human brain. Tissues of the normal cysticercus were described as follows. The most superficial or "ciliary" (my quotes) layer is in direct contact with the host tissue, and beneath it is the cuticle. The cilia are 2–3 μ in length, protruding at regular intervals from the organism. Beneath the amorphous cuticular layer there is an external fibrous layer containing fine ducts, followed by an internal reticular fibrous layer containing large cells (subcuticular cells). The fibers are stated to be collagenous.

The racemose form is considered to be a cyst wall in which the absence of a scolex goes hand in hand with unlimited growth. The wall of the racemose form is characteristically variable in thickness and has many digitiform folds. The different layers are unevenly distributed and are disorganized. Further, the fundamental structure is often modified, although the same layers are present as in the wall of the normal cysticercus. This abnormal proliferation is compared to neoplastic growth. The question is raised whether abnormal growth is a result of the disappearance of the scolex or whether the scolex disappears because of it. Again, no evidence was found that the scolex had ever developed. Trelles's opinion is that the racemose form is an abnormal growth form of *T. solium* and not that of another taeniid species.

Biagi *et al.* (1961) stated that *Cysticercus racemosus* is characterized by the absence of a scolex and that the cyst wall is similar to *C. cellulosae*. The wall encloses a cavity. It is lobulated and varies in thickness, thus differing from *C. cellulosae*. "Ciliary" processes are seen throughout its external surface.

Biagi *et al.* argue that the relatively greater frequency of positive precipitin reactions with homologous antigen (*C. racemosus* antigen with *C. racemosus* patients) suggests differences in antigenic composition of *C. cellulosae* and *C. racemosus* and may mean that the two stages belong to two different species of *Taenia*. This argument is not necessarily valid because different antigens might appear after scolex formation of a given species. This hypothesis could be tested by correlating the appearance of antibody in experimental hosts with the degree of development of cysticerci in these hosts. Furthermore, in the many autopsies performed on cases of cerebral cysticercosis in Mexico and elsewhere, one would expect reports of at least a few cysticerci of species other than *T. solium*, if more than one species of *Taenia* occurred in the human brain in endemic areas. Conversely, one would expect the racemose type to occur occasionally in animals although, according to Biagi *et al.*, it has been sought in the brain of pigs but without success.

Voge and Berntzen (1963) reported on a case of abnormal asexual proliferation of a taeniid, probably *Multiceps*, in a dog. Neither a single scolex nor the beginnings of scolex differentiation could be seen, although many hundreds of cysts were present. The normal, orderly sequence of tissues characteristic for taeniid larvae was rarely observed. The cyst outlines were irregular.

The cyst walls had numerous infoldings and varied in thickness. There was a general disarray of the subcuticular cells and of muscular and fibrous tissues. Their proliferation was profuse and showed extreme variability in the extent of differentiation and alignment. The histological appearance was very similar to the description of the racemose form given by Trelles *et al.* (1952).

What environmental factors (if any) are responsible for this type of disorderly proliferation? One might speculate that certain substances constantly stimulate growth so that there is no time for organization or differentiation; or, that only certain cells are stimulated to divide while others cannot do so in an amount sufficient for balance. If this type of abnormal growth is environmentally induced, it should eventually be possible to replicate it *in vitro*. It is also possible that abnormal growth results from a genetic defect of the parasite.

The formation of multilocular and other types of aberrant growth in *Echinococcus granulosus* was explained by Dew (1958) as the result of a local weakening of the laminated membrane which, in unilocular cysts, is a structure of uniform thickness and extent, and thus presumably prevents extraneous proliferation of the germinative membrane. Dew also stated that the biochemical environment in certain host individuals or species does not favor normal growth of the laminated membrane and that this in turn may result in uncontrolled proliferation of the parasite.

D. STUDIES IN HISTOCHEMISTRY

Most of the histochemical studies on cestode post-embryonic stages relate to the demonstration and distribution of polysaccharides, phosphatases and fats.

Heavy glycogen deposits were demonstrated by Takahashi (1959b) in the subcuticular cells and parenchyma of plerocercoids of *Diphyllobothrium mansoni*. Other polysaccharides were present in the cuticle and muscle fibers. Alkaline and acid phosphatases were heaviest in the cuticle and subcuticular cells. Acid and alkaline phosphatases were also demonstrated in the cuticle and subcuticular cells of plerocercoids of *Ligula intestinalis* by Arme (1966). Tests for non-specific esterase were negative for the cuticle and positive for subcuticular cells, parenchyma and other body regions. Histochemical tests for lipase and leucine aminopeptidase were negative. Yamao (1952) found alkaline and acid phosphatases in the cuticle of cysticerci of *T. saginata*, *T. taeniaeformis* and *Echinococcus*, and also in the wall of the excretory ducts.

According to Erasmus (1957a), histochemical tests for alkaline and acid phosphatases in the cysticercus of *Taenia pisiformis* were negative, although biochemical tests were positive for both enzymes. He stressed the importance of biochemical tests when histochemical ones are negative, because the manner of fixation or low enzyme activity can prevent demonstration of enzymes by histochemical procedures. Alkaline and acid phosphatases were also shown to be present in *Cysticercus tenuicollis* using biochemical tests, while histochemical tests were negative (Erasmus, 1957b).

In *Hydatigera taeniaeformis*, Waitz (1963) found little or no glycogen in the cuticle, but did observe heavy concentrations in the cortical and medullary

parenchyma, and around the parenchymal muscles. The cuticle was positive for mucosubstances as were the subcuticular cells. Lipids were demonstrated in the cuticle and subcuticular cells, and within the parenchyma near the musculature.

Kilejian *et al.* (1961) found large amounts of glycogen in the scoleces and germinal membrane of hydatid cysts of *Echinococcus granulosus*. Larval scoleces contained minute amounts of acid and alkaline phosphatase. The laminated membrane was negative for glycogen and phosphatase.

Heavy glycogen deposits were demonstrated by Heyneman and Voge (1957) in the tail of fully developed cysticercoids of *Hymenolepis diminuta*, *H. nana* and *H. citelli*. Glycogen was also present in the scoleces but very little or none was seen in the tissues surrounding the scoleces. In developmental stages some glycogen was present in the early spherical forms, and the amount increased with growth of the organism. No difference in the intensity of the staining reaction was noted in organisms grown at 20° or 37°C, except that the tail is smaller at the higher temperatures and would therefore contain a lesser total amount of glycogen.

In cysticercoids, Voge (1960e) demonstrated fatty substances in the scolex and in the lining of the cysticercoid cavity of *Hymenolepis diminuta*. In some specimens a positive reaction was also obtained in the tail. Results with Sudan black indicated the presence of phospholipids, but this was not confirmed by the Smith-Dietrich test which indicated the presence of a cerebroside. The cuticle of the scolex and the wall of the cysticercoid cavity showed a positive reaction for cholesterol.

E. SUMMARY

According to the studies reviewed, cestode post-embryonic stages have in common certain structures and comparable patterns of organization: in plerocercoids, tetrathyridia and cysticerci the presence of cuticular hairs during development; in cysticercoids the presence of fibrous layers surrounding the withdrawn scolex. Cuticular hairs are also present in tetrarhynch plerocercoids (Fig. 14). The origin of the cuticular hairs has not been determined; their possible identity with fibrous or precollagenous tissue requires additional study. The cuticle and subcuticular layers in plerocercoids, tetrathyridia, and cysticerci are arranged in a comparable sequence; there is always a subcuticular fibrous layer with subcuticular muscles beneath, and subcuticular cells internal to the subcuticular muscles. Some of the existing confusion relative to the structure of the cuticle can be ascribed to differences in structure of developmental stages and those which have completed differentiation. In early developmental stages, the acellular, external layer may be very narrow or absent. Additional studies are needed to determine the relation of the cuticular hairs to the subcuticular fibrous layer and the acellular component of the cuticle. Study of the nervous system has been neglected almost completely.

The cuticle and the subcuticular cells of plerocercoids and of cysticerci contain acid and alkaline phosphatases and lipids; the subcuticular cells also contain glycogen and non-specific esterase. Because of the difficulties arising

from the use of different terms for what appears to be the same structure, a uniform terminology should be adopted. There is good evidence that the "basement membrane" is not a membrane at all but a layer of fibers, probably collagenous. The name "subcuticular fibrous or fiber layer" would be more appropriate. The term cuticle (or tegument) could be profitably restricted to the acellular layers external to the subcuticular fiber layer, with the realization that they may contain cellular components such as nerve endings.

FIG. 14. Cuticular hairs in a tetrarhynch plercocercoid. Tissue section stained with hematoxylin and eosin.

IV. Certain Aspects of Host–Parasite Relations

The overall effects of cestode parasitism on the intermediate host and those of the host upon the developing parasite have been studied but little and the mechanisms of their interaction are poorly understood. This is particularly true for the arthropod intermediate host in which development of post-embryonic stages may proceed normally in one species, while in a closely related species growth, although initiated, may never reach completion. Examples of this situation are found among the copepods and were mentioned earlier in the section on life histories. Conversely, deleterious effects upon the parasitized copepod have been reported by Mueller (1966a, b) for *Cyclops vernalis* infected with procercoids of *Spirometra mansonoides*. Infected copepods do not bear egg sacs, do not complete their last molts and are thus unable to reproduce, while uninfected individuals continue to do so.

The defense reactions of insects to metazoan parasites have been studied extensively by Salt (1963) who does not report any recent work pertinent to cestodes. One would expect that the reactions to cestodes would be similar to those elicited against trematodes or nematodes, but very little information is available on this subject. Personal observations (unpublished) have shown

that the beetle *Tribolium confusum*, pre-infected to contain fully developed cysticercoids of *Hymenolepis diminuta*, can be reinfected successfully. Development of the second infection proceeds normally and at the same speed as the first infection. Furthermore, the number of organisms developing from the superimposed infection is comparable to that of the initial infection. However, no observations were made on the cellular response of infected insects.

Studies on cold-blooded vertebrates show that the presence of cestode post-embryonic stages can have a deleterious effect on these hosts. The effect of *Ligula* and *Diagramma* infections in fish was studied by Kosheva (1956) who found considerably lower hemoglobin values, decreased fertility, and interference with the overall growth of infected host individuals. Disturbances in the carbohydrate metabolism of fish infected with the same cestode species were reported by Kosareva (1961). Pitt and Grundmann (1957) reported a marked stunting effect on the growth of yellow perch by *Ligula* plerocercoids. Most of the infected fishes contained only one plerocercoid. Marked reduction in the length and weight of parasitized fish was noted for all age groups.

Contrary to the preceding observations on cold-blooded vertebrates, a considerable weight gain of laboratory mice infected with spargana of *Spirometra mansonoides* was reported by Mueller (1963). This weight gain could not be accounted for by the weight of the parasites. Weight increase over uninfected controls (Fig. 15) occurred in mice infected with one or several spargana and also, to a lesser degree, when mice were injected with headless spargana. The longevity and activity of parasitized mice was, apparently, not affected.

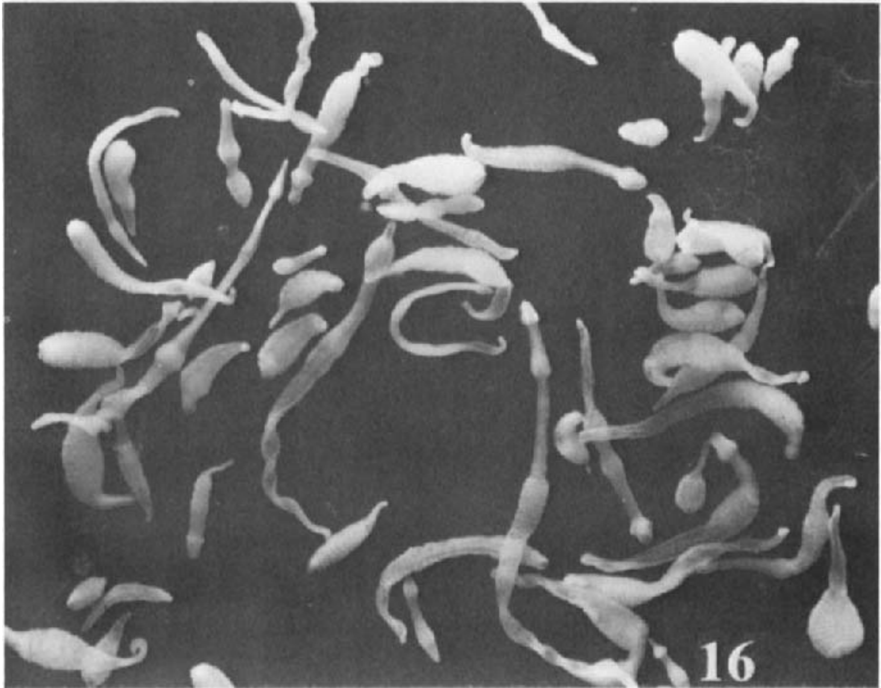
Further studies by Mueller (1965a) showed that not only infection of laboratory mice, but also of deer mice (*Peromyscus*) and hamsters with spargana of *Spirometra mansonoides* caused a striking increase in the weight of infected animals over controls. Weight increases were comparable in male and female animals. *Spirometra ranarum* infections caused even greater weight increases; the longevity and reproductive capacity of the infected animals were not affected. Precise data on the age, the initial and subsequent weights of experimental animals and controls were included in this work.

To determine whether mice infected with *Spirometra* perhaps ate more than uninfected controls, Mueller (1965b) conducted experiments to establish food intake of both groups of animals. Results showed that, while food intake of the experimental animals was somewhat greater, the difference was not sufficient to account for the increase in weight of infected mice.

In an analysis of the blood of mice infected with *S. mansonoides*, Sadun *et al.* (1965) found elevated fasting glucose levels, depressed levels of serum alkaline phosphatase and total protein, and normal levels of acid phosphatase and certain ions. How these findings relate to the presence of the spargana and the production of obesity in experimental animals is not clear.

An important aspect of host–parasite relations in experimental work is the susceptibility of different strains of the same host species to infection. Dow and Jarrett (1960) infected laboratory mice of three pure strains with *Taenia taeniaeformis* to determine the extent of resistance depending on strain, age or sex of the host. Results clearly showed that there was little, if any, difference

15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in susceptibility of male and female mice. Distinct age resistance to infection was demonstrated to occur in all. Mice of comparable age belonging to different strains showed significant differences in susceptibility to infection. The necessity for standardization of experimental animals was emphasized in this study.

Strain differences in susceptibility of rodents to infection with *T. taeniaeformis* were investigated also by Olivier (1962). Results showed the existence of marked differences in susceptibility among strains of the laboratory mouse. In some strains, oncospheres reached the liver but did not survive, while in others the proportion of surviving larvae differed considerably depending on the mouse strain used. Hamsters were refractory to infection and there was no indication that oncospheres could reach the liver. Whether or not they were able to hatch in the intestine was not stated. In guinea-pigs, oncospheres reached the liver but did not continue to develop. The nature of the different host barriers to invasion or development of the parasite presents a most intriguing set of problems which could be solved, at least in part, by observations on hatching and the penetration through the intestine and the liver, on the extent and speed of host-tissue reaction to infection, and the immune responses of the different host strains.

The relation between numbers of *Taenia taeniaeformis* ova administered and cysticerci recovered was investigated by Hinz (1962) in experiments with laboratory rats and mice which showed that mice were infected in larger numbers and more heavily than rats. The most significant results, however, related to the inverse relationship between numbers of eggs administered and cysticerci present in the liver; the larger the number of eggs fed, the smaller the number of viable cysticerci recovered. For this reason, the author recommended that only 200–500 eggs be used to infect mice, since larger numbers would decrease the chance for successful infection. While no tentative explanation of these results was given, it seems possible that the presence of a larger number of early developmental stages could induce a fairly rapid host response of sufficient magnitude to inhibit the completion of the development of most, if not all post-embryonic stages. Relatively small numbers of embryos, resulting from administration of 200–500 eggs, would induce a much milder host response, enabling a larger number of organisms to survive and complete development.

Freeman (1964b) studied the cellular response of male and female mice to infection with *T. crassiceps*. Cell counts of the peritoneal fluid and blood showed a leucocytosis in peritoneal fluid and blood, a decline of mast cells, and eosinophilia in peritoneal fluid. A high eosinophilia, when developed quickly, was correlated with the host's ability to overcome the infection. Males generally developed high eosinophilia more rapidly than did females. Eosinophilia in the peritoneal fluid was much higher than in the peripheral blood.

FIG. 15. Effect of infection with spargana of *Spirometra mansonoides* on the laboratory mouse. Left, uninfected control, weight 38·7 g; right, infected mouse, weight 68·6 g. The mice are litter-mate females. (Courtesy of Justus F. Mueller.)

FIG. 16. Spargana of *Spirometra mansonoides* grown *in vitro*, showing differences in growth rate of individuals from the same culture. (Courtesy of Justus F. Mueller.)

Observation indicated, however, that eosinophils do not act directly nor alone on the cysticerci since the latter survived for a long time in hosts having had a marked eosinophilic response.

Lubinsky (1964) described the growth of *Echinococcus multilocularis* in different strains of laboratory mice, and found striking differences in the speed and amount of parasite growth, depending on the strain of host used. Growth was especially slow in "obese", "hairless" and "dwarf" mice. Possible reasons for these differences in growth might be differences in the metabolism, in the extent of cellular reactions to infection in different strains of mice, etc. This system of host-strain susceptibility provides a most useful tool for further studies on host–parasite relationships.

V. Temperature and Development

Most of the studies on the effect of environmental temperature relate to the speed of the development of post-embryonic stages in the intermediate host and thus are a part of life history studies; some of these have been discussed earlier. Investigations on temperature tolerance ranges, on the effects of unfavorable temperatures and their influence on both the parasite and the host are relatively few. This is understandable because, in such a complex system, it is difficult to gather precise and meaningful data, or to determine which aspect of the organism's activity is primarily affected so that correct conclusions may be drawn from results observed.

In her experiments with different temperatures, Dubinina (1957) showed that development of the procercoid of *Schistocephalus solidus* in the intermediate host took 7–8 days at 22–25°C, and 13–14 days at 16–18°C. Details of procercoid structure were not included. She described the development of full segmentation of the plerocercoid in the second intermediate fish host and stressed the importance of temperature (40–41°C) as the trigger for development of the adult stage in mammals and birds.

Extensive and careful studies on the effect of temperature on cell division in plerocercoids of *Diphyllobothrium osmeri* were made by Wikgren (1966). Plerocercoids in Hank's solution with glucose were exposed to temperatures of 10–38°C and subsequently treated with colchicine for arrest of mitosis. Results showed a very rapid accumulation of metaphases at 38°C during the first 6 h, which then diminished. The latter was attributed to an exhaustion of energy supply. At the lower temperatures metaphase accumulation was less rapid but lasted for longer periods. The mean generation time of parenchymal cells in relation to temperature varied from 43 h at 38°C to 762 h at 10°C.

Freeman (1952b) studied the effect of different environmental temperatures upon development of *Monoecocestus* in oribatid mites, using the same host species (*Liacarus itascensis*) in all experiments. Infected mites were subjected to constant temperatures of 25°, 20°, 15° or 10°C. At 10°C development was arrested at the hollow ball stage; at 15° time required for development was 81 days, at 20°, 52 days, and at 25°C, 45 days. At the lower temperatures, cysticercoids were larger; this was true also for the suckers and scoleces. It is of interest that development was arrested at the hollow ball stage when the

temperature was unfavorably low. A similar observation was made by Millemann (1955) on *Oochoristica deserti* in which development did not proceed beyond the hollow ball stage if the temperature was 20°C. Perhaps beyond this point of development the organism has more stringent or different requirements which are not supplied by the host at low temperature, or certain enzymes of the parasite may be inhibited.

Studies by Voge and Turner (1956) on the relation of environmental temperature to the time required for the development of cysticercoids of *H. diminuta* in *Tribolium confusum* showed that normal development occurred between 15° and 37°C. At 15°C development was completed in 65 days and at 37°C in 5 days. Some abnormalities were noted at 37°C, so that this temperature represents the upper limit of tolerance. The optimal temperature for this species was considered to be 22–30°C.

Comparison of these results with the work by Millemann (1955) on *Oochoristica* and by Freeman (1952a) on *Monoecocestus* shows that the temperature range suitable for development varies in different cestode species, and that developmental rates of different species vary considerably within the same intermediate host species at the same environmental temperature.

The effect of environmental temperature upon development of *H. nana* in *Tribolium confusum* was studied by Heyneman (1958) who exposed infected intermediate hosts to constant temperatures, ranging from 15° to 42°C. Forty degrees centigrade was the highest temperature at which normal cysticercoid development occurred. At 42°C some of the organisms were abnormal and the tail failed to develop. Normal development occurred between 18° and 40°C, and was completed in 28 and 4 days respectively. At 15°C development usually did not progress beyond the early spherical stage, indicating perhaps that host metabolism was insufficient to supply the needs of the parasite for the subsequent major period of growth and differentiation. The author compared his results with those obtained by Voge and Turner (1956) for *H. diminuta* and concluded that *H. nana* has a wider temperature tolerance at the upper range while *H. diminuta* is better adapted for the lower temperatures.

The effect of continuous exposure to high environmental temperature on the development of *H. diminuta* and *H. nana* in *Tribolium confusum* was studied by Voge and Heyneman (1958). The abnormalities most frequently observed in *H. diminuta* at 37°C were failure of scolex withdrawal, reduction or absence of the tail, and incomplete development of the external membrane. At 38·5°C the incidence of these abnormalities was very high, and was combined with overall inhibition of growth and differentiation. In *H. nana*, a marked retardation in development was observed between 39·5° and 40·5°C. Infectivity of cysticercoids was suppressed above 39·5°C. At 42°C all organisms were abnormal. The absence of a tail in otherwise normal cysticercoids was noted at 37°C and was frequently observed at 39°C. These cysticercoids resembled the forms which develop in the villi of the mouse host.

The effect of high temperature stress at different periods of cysticercoid development in *H. diminuta* was investigated by Voge (1959a) to determine the stages of growth most sensitive to high temperature exposure. Results showed the presence of a sensitive period from days 2–6 of development, with

maximum sensitivity at days 3–5. This period coincides with the maximal growth and differentiation of the cysticercoid when allowed to develop at 30°C. Sensitivity was measured by the incidence of abnormalities and the inhibition of infectivity of normal-appearing cysts.

The possibility that the insect host of *H. diminuta* is adversely affected by exposure to high temperature, and that this effect might increase the energy requirements of the host to the detriment of the developing parasite, prompted experiments in which beetles were fed various pure sugars during the total period of cysticercoid development (Voge, 1959b). Results showed that the incidence of abnormalities due to temperature stress was markedly reduced when beetles were fed saturated aqueous solutions of various sugars instead of the standard flour diet. Some reduction in abnormalities was also noted when the host diet consisted of dry sugars. The total effect of high temperature stress, however, must be far more intricate than implied by the above results. Inhibition of enzyme activity, changes in membrane permeability, and many other impairments in the host and parasite could be responsible primarily for the end results of stress.

The effect of high temperature stress on the cells and tissues of *H. diminuta* was investigated by Voge (1961a) to determine which tissues were primarily affected, and how the abnormal histogenesis of a tissue might affect the development of the whole organism. Examination of tissue sections of stressed cysticercoids showed a delay in development, asymmetrical growth, and gross abnormalities in cell alignment. The asymmetry was clearly a result either of unequal proliferation or a complete failure of differentiation. In the large, peripheral cells of the midbody, the failure of regular alignment was particularly noticeable. Generally, it could be concluded that not all tissues were equally affected by temperature stress and that the scolex and neck appeared to be most resistant. It was also noted that temperature stress occasionally inhibited polysaccharide accumulation in the scolex and tail. An interdependence of tissue differentiation was noted between the fibrous extensions of the inner fibrous layer and the peripheral cells. Since the alignment of the latter normally precedes the outgrowth of fibers, a disturbance in cell alignment might be expected to influence fiber alignment. This view was supported by the observation that the growth of fibrous extensions was haphazard whenever peripheral cell alignment had been disturbed.

Using the failure of scolex withdrawal as an indicator of high temperature stress, Voge (1961b) studied high temperature sensitivity in *Raillietina cesticillus* and *Hymenolepis citelli* developing in *Tribolium confusum*. Since adult *R. cesticillus* in the bird host would be subjected to fairly high temperatures, it was thought that this cysticercoid might perhaps have greater tolerance than cysticercoids of *H. diminuta* of *H. citelli*. Results showed, however, that *Raillietina* was very sensitive to exposure of 38·5°C during the period of maximal growth and differentiation. In both *Raillietina*, and *H. citelli*, the temperature-sensitive period coincided with that of maximal growth and differentiation. It was shown further that this sensitive period lies approximately in the middle of the total developmental span of the cysticercoid. Similar observations were reported for *Hymenolepis microstoma* (see

Voge, 1963c). Experiments with temperature-stressed, but normally withdrawn, *H. microstoma* showed further that these organisms would not excyst *in vitro* as did controls, and that the scoleces were non-motile although structurally apparently normal.

It is of interest that some of the gross abnormalities of cysticercoids resulting from X-irradiation of *H. nana* eggs (Schiller, 1959b) resemble those obtained in experiments with high temperature (Voge and Heyneman, 1958). Schiller lists three types of alterations: inhibition of cell division, failure of differentiation and interference with structural organization. Failure of scolex development, incomplete development and asymmetry are some of the abnormalities illustrated in his report.

Very little information is available on behavior of cestodes exposed to different temperatures. Thorson *et al.* (1964) observed that spargana of *Spirometra* when placed in a thermal gradient moved toward the high temperature zone and continued to do so even when the zone of thermal damage was reached. Fragments without a scolex, although motile, did not show a thermotactic response. Since spargana in the warm-blooded host migrate toward the cooler areas of the body, these authors concluded that this migration was not stimulated by temperature.

VI. Post-Embryonic Stages *in vitro*

The comprehensive review by Silverman (1965) should obviate the necessity of a discussion on axenic cultivation, were it not for the fact that since that time several additional studies have been published. Because these experiments specifically concern "larval" cestodes, cultivation of these organisms will be briefly reviewed here, omitting those results in which maintenance only but no growth or differentiation was achieved. As pointed out by Silverman (1965) one of the major problems in assessing success of axenic cultures is the choice of criteria for success as well as their definition. For example, in their studies on plerocercoids of *Diphyllobothrium mansoni*, Takahashi *et al.* (1959) compared the glycogen content of cultured worms and controls and noted a pronounced glycogen depletion in the parenchyma of organisms maintained *in vitro*. Temperatures from 10° to 20°C were suitable for survival but best survival was obtained at 10°C, perhaps because glycogen was depleted more slowly at the lower temperature. Thus, one of the useful approaches for evaluating an *in vitro* method might be the comparison of energy reserves of controls with those of organisms from culture.

One of the most successful and complete achievements in axenic cultures of cestode post-embryonic stages is that of Mueller (1959) who was able to grow infective plerocercoids of *Spirometra mansonoides* from the procercoid stage. The increase in tissue mass was several thousand per cent and the organisms were evidently normal (Fig. 16). It is doubtful that the choice of medium (Mixture 199, calf serum and chick embryo extract) was the most important aspect for the success of this work. Perhaps different media might serve nearly as well, if other environmental factors were kept within the range tolerated by the organisms. Important features of this work are the precision and care in

handling of the cultures and the patience demonstrated in carrying on, repeatedly and uniformly, essential procedures over a period of several months. It is evident from this and other successful culture work that organisms kept *in vitro* require constant and meticulous care, daily observation, and efficiently timed procedures at transfer, cleaning, or any extraneous "disturbance" of the organisms.

Mueller (1961) described the behavior of spargana under different environmental conditions. While the major objective of his work was to relate observations on immune precipitates, the statements and illustrations concerning behavior are extremely pertinent to a discussion of culture methods and their suitability. The author stated that spargana transferred from mice to saline or basic culture medium, eventually become "hopelessly snarled", and tightly knotted. We have observed differences in behavior of *Mesocestoides in vitro*, depending on the culture medium used (see below). Not only is the initial activity of organisms greatly enhanced in the presence of "unsuitable" media, but they also adhere so tightly to each other that separation of individuals is almost impossible. This was frequently observed in liquid media with low viscosity. Slow motion and little clumping, if any, was noted in viscous media, or in those generally promoting considerable growth or multiplication of the organisms.

McCaig and Hopkins (1965) cultured young plerocercoids of *Schistocephalus solidus* in media containing Hank's salt solution, glucose, horse serum and yeast extract. Culture tubes were treated with 95% air and 5% CO_2 until the pH was stabilized at 7·1. Tubes, sealed with parafilm, were kept at 21°C. Dry and wet weight, as well as glycogen determinations, were made after 8 days of culture. Growth approaching the rate *in vivo* was obtained in a medium containing all components listed above (horse serum 25%, yeast extract 0·5%). McCaig and Hopkins observed a wide pH tolerance (6·3–8·1) and high oxygen tension tolerance.

Regarding the wide pH tolerance of plerocercoids, it would be interesting to know if this would hold also for developing procercoids in which growth and early differentiation of muscular, excretory systems, and other tissues do occur. Once the young plerocercoid stage has been reached, much of the basic cellular differentiation must already have been determined. It is possible that tolerance for certain environmental factors is considerably smaller or different in range during cellular differentiation and organogenesis. Growth *in vitro* of *Mesocestoides* procercoids (Voge, 1967) showed that growth and differentiation were abnormal above a pH of 7·4.

Oncospheres of *Mesocestoides* hatched *in vitro* grew into small procercoids (Fig. 17) in a medium (NCTC 135) containing amino acids, vitamins, glucose, and various inorganic components, as well as 20% inactivated horse serum. The culture vessels were screw cap tubes containing 3 ml of medium at a pH adjusted with sodium bicarbonate to 6·8. Tubes, sealed with parafilm, were kept at room temperature (25–30°C) and stationary in an inclined position. A rise in pH above 7·4, due to defective seals, would cause abnormal development and death of the organisms. Sensitivity to temperatures higher than 30°C was noted if organisms about 0·3 mm long were transferred from room

temperature to 37°C. At this temperature, organisms would die in a few days while controls continued to grow. As noted by Mueller (1959) for *Spirometra*, development of individuals within the same culture vessel varied considerably, some being twice the size of others. Organ systems and tissues present at 8 weeks of culture included a spinous cuticle, subcuticular muscles, excretory ducts and flame cells, subcuticular and other cells, and an apparently functional apical organ. Individuals 0·5 mm long contained many calcareous corpuscles and outlines of suckers; the latter had not yet attained full development but the organisms looked like small tetrathyridia.

Tetrathyridia of the same species of *Mesocestoides* were grown axenically by Voge and Coulombe (1966). Depending on the culture medium employed, growth could be directed either toward an increase in size of individuals, or toward asexual multiplication at a rate fairly comparable to that in the mouse host. Increase in size occurred in the presence of 10% human plasma and

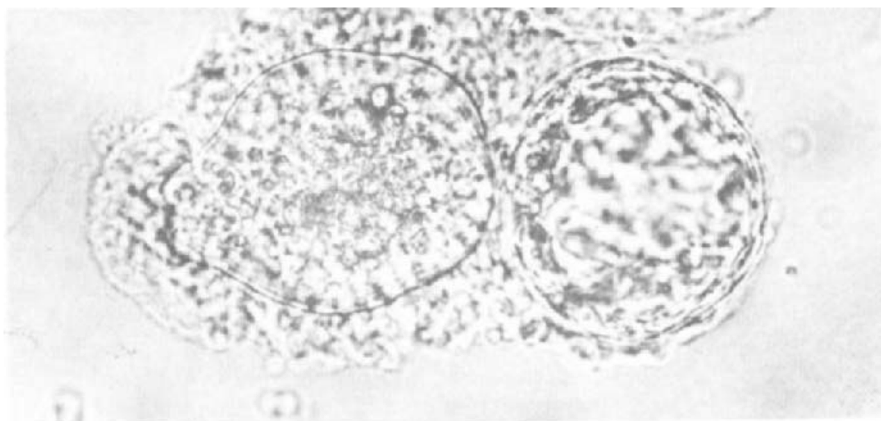

FIG. 17. Developmental stage of procercoid of *Mesocestoides* from culture, showing the spherical cercomer almost separated from the procercoid body.

asexual multiplication with 10% human whole blood, always combined with NCTC 135 (see above). Culture vessels were screw cap tubes containing 7 ml of medium. Tubes were kept sealed and stationary in an inclined position, at 37°C. Optimal pH was 7·4; a pH of 7·0 was deleterious. It should be noted that cultures of tetrathyridia were initiated with organisms removed from the body cavity of mice. To date, the transition *in vitro* between the small tetrathyridium-like organisms with an apical organ, and the stages with fully developed suckers occurring in the mouse host has not yet been accomplished. One of the difficulties is the lack of *in vivo* material for comparison, because the first portion of the mesocestoidid life cycle is unknown. Evidently a change in environment is needed after procercoids have attained a certain stage of growth, because the culture medium suitable for procercoid development does not promote appreciable growth of tetrathyridia. The timing of the necessary stimuli to accomplish the transition between the two stages of the life cycle remains to be determined.

VII. Excystment *in vitro*

Although studies on the factors facilitating excystment of post-embryonic stages are relatively few, the available data have been useful in the continuance of *in vitro* studies and have, indirectly, stimulated a greater interest in the intestinal environment of the vertebrate hosts of cestodes. Successful excystation experiments were those in which organisms were subjected to some substances or conditions also present in the host intestine.

Excystation of cysticercoids of *Raillietina kashiwarensis* was attempted by Sawada (1959) at different temperatures in the presence of different enzymes and varying hydrogen ion concentrations. Optimal results (100% excystation within 30 min) were obtained at 40–42°C in a 0·1% pancreatin solution at pH 7·0. Good results (100% in 60 min) were also obtained in a 0·1 trypsin solution. Bile salt or lipase solutions induced excystment of a few cysticercoids. Experiments clearly demonstrated the importance of temperature and pH in the production of excystment.

Rothman (1959) studied excystment *in vitro* in *Hymenolepis diminuta, H. citelli, H. nana, Oochoristica symmetrica* and *Taenia taeniaeformis*. Extensive experiments were conducted to evaluate the effect of pepsin, trypsin, bile salts and Krebs Ringer's solution, either used singly or in various combinations. Results showed clearly that the factors stimulating excystation can be related to the structural characteristics or type of cyst used. For example, cysticercoids of the three hymenolepidids all required bile salts and trypsin for optimal excystation, preferably combined with pepsin-HCl pre-treatment. Room temperature was inadequate and organisms were therefore kept at 37°C, the optimal temperature for tryptic activity. *Taenia taeniaeformis* excysted at room temperature in Ringer's solution, if cysticerci were pre-treated with pepsin-HCl. *Oochoristica* excysted at room temperature in the presence of bile salts, or in a bile salt-trypsin solution and did not require pre-treatment with gastric juice which was noxious on prolonged exposure. It was concluded that the composition of gastric and intestinal juices of the vertebrate host may well be one of the factors permitting or preventing establishment of a parasite, as prolonged exposure to noxious substances would cause destruction or elimination of the organism.

Fuentes *et al.* (1960) attempted to determine what environmental factors contributed toward excystment of cysticerci of *Taenia solium*. Temperature, pH, components of bile, and diminished O_2 concentration were tested singly over long periods of time. It was concluded that the stimulatory effects of bile salts were due to chemical properties rather than surface activity. A temperature of 37–38°C and a pH of 7·8 were most favorable. It should be noted that in these experiments cysticerci were kept in the solutions to be tested for many days; this is not realistic if the physiology of the host is considered in terms of the parasite's chance to become established. Also, the above factors were not tested in various combinations to determine optimal *in vitro* conditions for excystment.

Evagination of *Taenia pisiformis* cysticerci was achieved *in vitro* by Campbell (1963) who used surface-active agents such as Haemo-Sol and other,

chemically unrelated, compounds. A few cysticerci evaginated when placed into warm Ringer's solution. Attempts to induce excystment of *H. diminuta* cysticercoids in Haemo-Sol were unsuccessful. On the basis of sound evidence, it was concluded that a slight decrease in surface tension of the cysticercus is sufficient for its activation, and that the surface-active properties of bile salts, rather than their chemical ones, are responsible for evagination of cysticerci.

VIII. Conclusions

Progress in any field of learning is closely bound to the new or different ways of approaching problems, and to the number of appropriate questions one is able to raise concerning them. The factual information contained in the studies reviewed here has indeed raised many new questions and problems for investigation. Some problems concern the apparent similarities and differences in the developmental pattern of post-embryonic stages and in their cellular organization. Very little is known about the environmental factors which stimulate or prevent differentiation of a cestode species, and even less is known about the essential triggers that stimulate the transition from one post-embryonic stage to the next. Primarily, this is due to our ignorance of the internal environment of the hosts in which cestodes develop.

Some of the factors which form the basis of the physiological host specificity or susceptibility are suggested in this review. We may list the following: (1) enzyme concentration in the host intestine, which determines the hatching of the oncosphere; (2) intestinal emptying time in relation to time required for the hatching and establishment of the oncosphere; (3) the thickness or consistency of the host intestinal wall to be penetrated by the embryo, and (4) the environment of the host's body cavity which may permit or inhibit continued growth after the initiation of post-embryonic development. The importance of environmental temperature as a factor to limit or stimulate development has been stressed by many workers. The effects of a given temperature vary with the developmental stage and the species of cestode.

The existence of interspecific competition within the host's environment has been established but the mechanisms underlying competition are not known. Whether the developing parasites are competing for food substances, carbon dioxide, or other essentials remains to be determined. Further comparative studies are needed on intra- and inter-specific competition of developing parasites.

Areas which have been least studied are the behavior under experimental conditions, the development and morphology of the nervous system, the *in vitro* requirements of early post-embryonic stages, and the potential for regeneration of certain post-embryonic stages *in vivo* and *in vitro*.

Further application of new and established techniques or approaches of the various disciplines of biology, especially those of genetics, physiology and ecology, is essential for future additions to our knowledge of the growth and development of cestodes.

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Author Index

Numbers in italics refer to pages in the References at the end of each article

A

Aagard, K., 23, *37*
Abdou, A. H., 254, *290*
Abele, D. C., 188, *203*
Abraham, J. P., 35, *44*
Abraham, R., 146, *202*
Adam, J. P., 146, 151, *194*
Adams, A. R. D., 108, *138*
Adler, S., 117, *129*, 191, 193, *195*
Agar, H. D., 5, 9, *39*
Agosin, M., 231, *237*
Aikawa, M., 3, 6, *37*, 122, *129*, 161, 162, 163, 164, 165, 166, 169, 178, 179, 180, *195*
Akao, S., 7, 9, 11, 34, *42*, *44*
Akinshina, G. T., 3, 23, *37*
Al-Dabagh, M. A., 181, *195*
Aligon, C., 273, *290*
Alison, F., 15, 28, *38*
Allain, D., 79, *88*
Allen, K., 228, *240*
Allen, R. W., 253, *290*
Amati, L., 188, *197*
Amrein, Y. U., 103, *129*
Anfinsen, C. B., 123, 124, *132*
Angelillo, B., 24, *37*
Angus, M. G. N., 181, *195*
Anoda, T., 33, *41*
Antipin, D. N., 221, 235, 236, *237*
d'Antonio, L. E., 185, *195*
Apted, F. I. C., 67, 68, *85*
Archer, J. F., 24, *37*
Arjona, I., de 33, *44*
Arme, C., 276, *290*
Arnold, J., 164, *200*
Aronson, W. J., 34, *42*
Arya, T., 26, *44*
Ashcroft, M. T., 69, *85*, 97, *130*
Ashwood-Smith, M. J., 98, *138*
Awachie, J. B. E., 221, *237*, *245*
Ayllon-Leindl, L., 24, *41*

B

Baer, J. E., 206, 222, *237*
Bafort, J., 152, *195*
Bahler, J. W., 36, *37*
Baker, J. R., 3, 5, 6, 7, *39*, 51, 57, 65, *85*, 104, 113, 115, 121, *130*, *132*, 154, 156, 166, 167, 168, 169, 171, 172, 173, 176, 179, 180, *196*, *199*
Baker, R. H., 154, *195*
Baker, T. H., 27, *40*
Balan, J., 35, *38*
Balanovschi, G., 157, 190, *196*
Ball, E. G., 123, 124, *132*
Ball, G. H., 121, 127, 128, *130*, *131*
Ban, S., 231, *241*
Bangham, A. D., 107, *130*
Banki, G., 189, *195*
Bano, L., 166, *195*
Baqui, M., 26, *44*
Bardele, C. F., 179, *195*
Barnett, C. G., 34, *43*
Barron, E. S. G., 231, *241*
Bass, C. C., 93, 121, *130*
Bateman, H. R., 99, *131*
Baudelot, J., 15, 28, *38*
Bauer, H., 33, *41*
Bauer, O. N., 234, *237*
Bayer, M., 104, *134*
Bayles, A., 114, *130*
Beames, C. G., Jr., 229, *237*
Beattie, C. P., 1, 15, *37*
Beaver, P. C., 235, *239*
Behrens, C. A., 99, *130*
Beklemishev, V. N., 53, *85*
Benchhoff, B. M., 24, 33, *44*
Bennett, G. F., 142, 145, 147, 154, 157, *195*, *204*
Bennett, S. C. J., 80, *88*
Ben Omar, A. H., 154, 158, *196*
Ben Rachid, M. S., 20, 21, 28, *37*, *38*
Berghe, L., van den, 150, *195*

Bernstein, J., 29, *40*
Berntzen, A. K., 255, 270, 275, *290*, *296*
Beverley, J. K. A., 24, 32, *37*
Bezubik, B., 217, 236, *237*
Biagi, F. F., 275, *290*
Bickford, A. A., 23, *37*
Biering-Sorensen, U., 33, *43*
Bignami, A., 155, 181, *195*, *201*
Bird, R. G., 3, 5, 6, 7, *39*, 104, 121, *130*, *132*, 166, 167, 168, 169, 171, 172, 173, 176, 179, 180, *199*
Bishop, A., 194, *196*
Bloch, E. H., 182, *200*
Bonè, G. J., 101, 111, *130*, *137*
Bonnett, C., *40*
Bowman, I. B. R., 102, *130*
Bozdech, V., 25, *37*
Brand, T., von, 65, *85*, 96, 98, 102, 103, 108, *130*, *137*, 222, 223, 224, 228, 229, *237*, *238*, 248, *290*
Braun, J. L., 33, *42*
Bray, R. S., 5, *39*, 122, *136*, 140, 142, 146, 154, 156, 157, 160, 161, 162, 176, 179, 186, 190, *196*, *199*, *200*, *202*
Brener, Z., 78, *85*
Briceño, C. E., 275, *290*
Briggs, N. T., 159, 191, *196*, *204*
Brooks, W. H., 24, *44*
Broom, J. C., 84, *88*, 107, *130*, *131*
Brown, H. C., 107, *130*, *131*
Brown, I. N., 156, 186, *196*
Brown, K. N., 77, *85*, 156, 186, *196*
Bruce, D., 97, 99, 105, *131*
Bruce-Chwatt, L. J., 146, 155, 157, 191, *196*
Brumpt, E., 51, *85*
Brutsaert, P., 96, 100, 108, *131*
Bryant, C., 224, 227, 230, 231, *238*
Bucci, A., 189, *195*
Buchanan, J. C. R., 69, *86*
Bueding, E., 231, 232, *238*, *241*
Bukantz, S. C., 185, *201*
Bullock, T. H., 209, *238*
Bullock, W. L., 222, 223, 224, 228, *238*, *240*, *244*
Burgess, M. W., 156, *196*
Burlingame, P. L., 221, 234, *238*
Burnham, K. D., 213, *238*
Burnstein, T., 23, *37*
Burtt, E., 69, *86*
Butcher, G., 191, *197*
Buxton, P. A., 70, *86*, 101, *131*
Bykovsky, A. F., 3, *37*

C

Cadigan, F. C., 159, *203*
Calandruccio, S., 235, *240*
Celentine, R. L., 250, *290*
Camargo, E. P., 112, *131*
Camargo, M. E., 24, *37*
Campbell, J. W., 231, *243*
Campbell, W. C., 288, *290*
Capella, J. A., 35, *37*
Carrington, S. P., 190, *201*
Carver, R. K., 3, 39, 188, *200*
Cassady, J. V., 36, *37*
Castellani, O., 111, 112, *132*
Castro, M. P., da, 118, *131*
Catar, G., 35, *38*
Cavanaugh, D. C., 159, *204*
Cavanaugh, E., 27, *43*
Cavanaugh, E. N., 29, *42*
Ceccaldi, J., 96, *134*
Cedillos, R., 113, *137*
Cerna, Z., 24, *41*
Chabaud, A. G., 146, 151, 156, *194*, *200*
Chalupsky, J., 24, *41*
Chandler, A. C., 83, *86*, 221, 234, *238*
Chang, S. L., 116, *131*
Chao, J., 121, 127, 128, *130*, *131*
Chardome, M., 150, *195*
Chatton, E., 55, *86*
Chavin, S. I., 192, *196*
Cheever, A. W., 26, *37*
Cheissin, E., 166, 179, *196*
Cheissin, F. M., 176, *196*
Chelarescu, M., 157, 190, *196*
Cheong, W. H., 145, 147, 154, 158, *195*, *196*, *204*
Chin, C., 232, *238*
Chin, W., 157, 158, *196*, *197*
Chizhova, T. P., 268, *290*
Chong, T., 184, *198*
Chordi, A., 20, 21, *37*
Choudhury, D. S., 154, *196*
Chrusciel, T., 36, *37*
Chubb, J. C., 222, *238*, 251, *290*
Cit'ri, N., 111, 117, *131*
Ciuca, M., 157, 190, *196*
Clark, T. B., 48, *86*
Clarke, A. S., 250, *290*

Coatney, G. R., 142, 154, 155, 157, 158, 185, 188, *196*, *197*, *198*, *199*, *201*, *203*, *204*
Coggeshall, L., 122, *131*
Cohen, S., 190, *197*
Collins, W. E., 188, *197*
Connolly, C. S., 27, *38*
Constantinescu, P., 154, 157, 190, *196*, *200*
Contacos, P. G., 155, 157, 158, 188, *196*, *197*, *199*, *203*
Cook, M. K., 15, *40*
Coombs, G. L., 154, 158, *196*
Cooper, W. C., 185, *201*
Corradetti, A., 141, 155, 188, *197*
Corson, J. F., 70, *86*
Cortez, P., 157, 190, *196*
Cott, H. B., 68, *86*
Coulombe, L. S., 287, *296*
Couvreur, S., 15, 28, *38*
Covell, G., 191, *197*
Cowperthwaite, J., 109, *131*
Cox, F., 159, *197*
Cox, F. E. G., 161, 164, 190, *197*
Cragg, F. W., 53, 54, *86*
Cramer, D. I., 5, 9, *39*
Crompton, D. W. T., 206, 208, 214, 217, 220, 222, 223, 224, 227, 228, 232, 233, 234, 236, *238*, *245*
Crook, J. R., 217, *239*
Culwick, A. T., 97, *132*
Cunningham, M. P., 70, *86*
Czaplinski, B., 257, *290*

D

Dasgupta, B. M., 157, *197*
Davis, B. S., 76, 83, *86*
Deane, L. M., 77, *89*, *131*, 145, 154, 158, *197*
Deane, M., 115, *131*
Deane, M. P., *131*, 145, 154, 158, *197*
de Beer, G., 64, *86*
Deegan, T., 122, *131*
De Giusti, D. L., 209, 214, 215, 217, 221, *239*
Delbeke, M. J., 27, *40*
Del Campo, E., 26, *42*
Demarchi, J., 97, 99, *132*
Demina, N. A., 191, *202*
Desmonts, G., 15, 28, *38*
Desowitz, R. G., 186, *197*
Desowitz, R. S., 115, *132*, 159, 186, *202*, *204*
Despeignes, J., 25, *39*
Devakul, K., 182, *197*
Dew, H. R., 276, *290*
Dias, E., 83, *86*
Dienst, R. B., 20, *44*
Dissanaike, A. C., 142, 145, 155, *197*
Dissanaike, A. S., 154, *197*
Dixon, B. R., 224, 227, 229, *239*
Dobzhansky, T., 66, *86*
Doenhoff, A. G., von, 185, *195*
Dogiel, V. A., 236, *239*
Dollfus, R., 262, *290*
Dow, C., 279, *290*
Downes, J. A., 54, *86*
Drinnon, V. P., 126, *132*
Dubey, J. P., 16, 19, *38*
Dubin, I. N., 126, *132*
Dubinina, M. N., 282, *290*
Duggan, A. J., 69, *86*
Duke, D. S., 185, *201*
Duke, H. L., 68, *86*, 98, *132*
Dunagan, T. T., 222, 228, *239*, *245*
Duncan, D., 169, *197*
Dunn, F. L., 142, *198*
Durge, N. G., 26, *44*
Dutta, B. N., 109, *136*

E

Eaton, M. D., 185, 186, *198*
Ebringer, L., 35, *38*
Ebringerova, J., 35, *38*
Edeson, J. F. B., 152, *199*
Edmonds, S. J., 224, 227, 229, *239*, *245*
Eichenwald, H. F., 24, *38*
Elder, H. A., 157, *196*
Eliot, T. G., 182, *200*
El-Nahal, H. M. S., 186, 187, 188, 190, *196*, *198*
Emmel, L., 179, *198*
Engelbrecht, E., 29, *38*
Enigk, K., 253, *290*
Entner, N., 232, *244*
Erasmus, D. A., 276, *290*
Esch, G. W., 274, *294*
Evans, C. B., 188, *203*
Evens, F., 67, *86*
Eyles, D. E., 36, *39*, 142, 145, 146, 152, 153, 154, 157, *198*, *202*, *203*, *204*

F

Fabiani, G., 191, *198*
Fairbairn, D., 231, *239*
Fairbairn, H., 68, 69, 78, *86*, *91*, 97, 102, *132*
Fairley, N. H., 155, *198*
Falsen, E., 35, *41*
Faust, E. C., 235, *239*
Feeney, L., 5, 9, 13, *40*
Feldman, H. A., 22, 23, 32, *38*, *44*
Feletti, R., 141, *199*
Fernandes, J. F., 111, 112, *132*
Fife, E. H., 185, *195*
Fish, N. A., 29, *41*
Fisher, F. M., Jr., 229, *237*, *239*, *243*
Fleck, D. G., 22, *38*
Fletcher, K. A., 161, 165, 181, 184, *195*, *198*, *201*
Fletcher, S., 24, *38*
Folkers, C., 23, *38*
Foner, A., 191, 193, *195*
Fong, Y. L., 142, 145, 154, 157, *198*
Franceschetti, A., 29, *38*
Freeman, R. S., 251, 253, 261, 262, 281, 282, 283, *290*, *291*
French, W. L., 154, *195*
Frenkel, J. K., 21, 26, 36, *38*
Freyvogel, T. A., 172, *198*
Fromentin, H., 97, 99, *132*
Fry, B. A., 24, *37*
Fuentes, P. B., 288, *291*
Fujita, J., 24, *44*
Fukajawa, T., 33, *41*
Fulchiron, G., 191, *198*
Fulton, F., 22, *38*
Fulton, J. D., 21, 22, 24, 25, 34, *38*, *39*, 103, 104, 117, 122, *132*, 191, *197*

G

Gallati, W. W., 252, *291*
Galuszka, J., 36, *37*
Galuzo, I. G., 76, *86*
Gammage, K., 193, *199*
Garden, E. A., 236. *243*
Garin, J. P., 25, 36, *39*
Garkavi, B. L., 256, *291*
Garnham, P. C. C., 3, 5, 6, 7, *39*, 121, *132*, 140, 141, 142, 145, 151, 152, 154, 155, 156, 157, 166, 167, 168, 169, 171, 172, 173, 176, 179, 180, 181, 184, 189, 190, 191, 194, *197*, *198*, *199*, *200*, *202*, *203*
Gavin, M. A., 2, 5, 6, 7, 9, 10, 11, 14, *39*, *44*
Geigy, R., 60, *87*, 102, 103, *129*, *132*
Geiman, Q. M., 123, 124, *132*
Geiser, G., 27, *40*
Geisler, P. H., 35, *40*
Genther, L. S., 142, 157, *202*
Gerbeaux, J., 15, 28, *38*
Gettier, D. A., 222, *239*
Getz, M. E., 157, *196*, *198*
Gibson, F. D., 146, *196*
Giles, C. L., 36, *39*
Gilkeson, M. R., 27, *40*
Gilles, H. M., 182, *199*
Gilmour, J. S. L., 77, 78, *87*
Giorgi, G., 33, *45*
Glaser, P., 118, *133*
Glebova, I. Y., 256, *291*
Glenn, S., 124, *132*, *134*
Glover, J. C., 107, *130*
Glowinsky, M., 28, *39*
Goble, F. C., 77, *89*
Goddard, P. A., 193, *199*
Godfrey, D. G., 71, 78, *87*
Godoy, G. A., 113, *137*
Gofman-Kadoshnikov, P. B., 268, *290*
Goldblum, M., 140, *201*
Goldenberg, E. D., 27, *40*
Goldman, M., 3, 24, 29, *39*, *44*
Golvan, Y. J., 206, 232, 234, 235, 236, *239*, *240*
Golz, H., 179, *198*
Gordon, R. M., 99, 103, *132*
Grace, T. D. C., 129, *132*
Gracheva, L. I., 23, *37*
Graff, D. J., 220, 228, 229, 230, *240*
Graiwer, M., 255, *296*
Grant, P. T., 103, 104, 122, *132*, *133*
Grassé, P. P. 55, 57, *87*
Grassi, B., 141, *199*, 235, *240*
Gray, A. C. H., 99, *133*
Gray, A. R., 77, *87*
Greef, R., 215, *240*
Greenblatt. C., 113, *137*
Greenblatt, C. L., 118, *133*
Greenland, R., 142, 157, *196*, *202*
Grewal, M. S., 55, *87*
Griffin, R. L., *246*
Grigg, H., 214, 222, *242*

Gronroos, P., 24, *39*
Groschaft, J., 256, *294*
Grossowicz, N., 111, 117, *131*
Grundman, A. W., 217, *239*
Grundmann, A. W., 279, *294*
Guberman, V., 189, *199*
Gueft, B., 3, 6, 7, *41*
Guinn, E., 142, 145, 154, 157, *198*
Gupta, P. V., 215, 217, *240*
Gurri, J., 274, *295*
Gustafson, P. V., 5, 9, *39*
Guttman, H. N., 97, *133*
Guttova, A., 249, *291*
Gyulai, E., 29, *41*

H

Hackman, R., 34, *42*
Haley, A. J., 222, 223, 224, *240*
Hall, P. J., 189, *200*
Hallatt, J. G., 27, *40*
Hamann, O., 210, 217, 222, *240*
Hamburger, Y., 193, *204*
Hamerton, A. E., 99, *131*
Hammond, D. M., 6, *43*
Hammond, R. A., *245*
Hancki, T., 33, *41*
Hansson, H.-A., 6, 13, 35, *41*
Harada, I., 222, *240*
Hardy, C. L. S., 189, *200*
Harinasuta, T., 182, *197*
Harms, C. E., 222, *240*
Harrison, J. G., 233, 234, 236, *238*
Hart, J. L., 270, *291*
Harvey, H. P. B., 26, *39*
Hawking, F., 100, 108, 109, 112, 114, 117, 125, *133*, 193, *199*
Healey, P., 104, *130*
Heggen, D. W., 33, *42*
Heisch, R. B., 68, *87*
Held, J. R., 33, *41*, 155, *199*
Hendrickse, R. G., 182, *199*
Henneré, E., 179, *203*
Henrard, C., 96, 100, 108, *131*
Hepler, P. K., 3, 6, *39*, 122, *129*, 164, 165, 166, 180, *195*, *200*
Herbig, A., 60, *87*
Herman, R., *201*
Heslop-Harrison, J., 77, 78, *87*
Heyneman, D., 254, 255, 271, 277, 283, 285, *291*, *296*
Hickl, E. J., 29, *40*
Hickman, J. L., 252, *291*
Hickman, R. L., 183, 190, *202*
Hill, G. I., 188, *203*
Hills, G. M., 118, *133*
Hinken, M. V., 36, *37*
Hinz, E., 281, *291*
Hitchings, C. H., 36, *38*
Hoare, C. A., 48, 50, 51, 52, 53, 55, 57, 58, 59, 61, 63, 64, 65, 66, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, *87*, *88*, 95, 106, 107, *131*, *133*, 157, *200*
Hosan, M. J., 5, 9, 13, *40*
Hollingshead, S., 107, *130*, *133*
Holmes, J. C., 234, 235, *240*
Hönig, G., 224, *242*
Hoo, C. C., 152, *202*
Hoof, L., van, 68, 69, *88*
Hooft, C., 27, *40*
Hopkins, C. A., 251, 286, *291*, *293*
Hopp, W. B., 213, 214, 215, 217, *240*
Horakova, K., 35, *38*
Hornby, H. E., 78, *88*
Horridge, G. A., 209, *238*
Hotta, F., 142, *200*
Houin, R., 206, *240*
Howard, L. M., 172, *200*
Huber, M., 102, *132*
Huff, C. G., 3, 6, *37*, *39*, 122, 125, 126, 127, 129, *129*, *133*, 139, 160, 161, 162, 163, 164, 165, 166, 178, 179, 180, 184, *195*, *200*
Hughes, H. B., 35, *43*
Hutchinson, W., 15, *40*
Hutchinson, W. M., 16, 17, 19, *40*
Hutner, S. H., 109, 119, *131*, *133*
Huxley, J., 66, 78, 82, *88*
Hyman, L. H., 206, 210, *241*
Hynes, H. B. N., 209, 210, 214, 217, 219, 224, 232, 233, 234, 236, *241*, *242*, *243*

I

Ilies, M., 157, 190, *196*
Imms, A. D., 53, 54, *88*
Ingram, R. L., 188, *200*
Inoki, S., 142, *200*
In't Veld, N., 36, *42*
Ishii, T., 15, 33, *40*, *41*

J

Jackson, G. J., 194, *202*
Jacob, A., 179, *198*
Jacobs, L., 1, 2, 5, 6, 7, 9, 10, 11, 14, 15, 16, 21, 25, 26, 27, 29, 30, 33, 34, 36, *38*, *39*, *40*, *41*, *43*, *44*
Jadin, J., 117, *134*
Jaquette, D. S., 20, *43*
Jarecka, L., 250, 254, 257, 258, 270, 271, *291*, *292*
Jarrett, W. H. F., 279, *290*
Jeffery, G. M., 187, *202*
Jensen, D. V., 125, 126, 127, 133, 135
Jensen, T., 222, *241*
Jernberg, N. A., 125, *137*
Jira, J., 25, *37*
Jirovec, O., 28, *40*
Johns, F. M., 93, 121, *130*
Johnson, E. M., 103, 115, *130*, *134*
Jones, A. W., 210, *241*
Jones, J. E., 157, *197*
Jones, M. H., 27, *40*
Jones, T. C., 26, *40*
Joyner, L. P., 117, *132*
Julian, S. R., 169, *197*
Jung, R. C., 235, *239*
Justus, K. M., 27, *40*

K

Kagan, I. G., 20, 21, *37*, 79, *88*, 262, *293*
Kaiser, J. E., 210, 217, 222, *241*
Kalderon, A. E., 29, *40*
Kanai, T., 33, *41*
Kaplan, N. O., 104, *134*
Kates, K. C., 209, 214, 217, *241*, *244*
Kauffmann, M., 102, 103, *129*, *132*
Kaufman, H. E., 35, *37*, *40*
Kean, B. H., 26, *40*
Keel, H., 27, *40*
Keithly, J. S., *245*
Kelen, A. E., 24, *41*
Kelly, G. W., *245*
Kennedy, B. J., 27, *44*
Kennedy, C. R., 250, *292*
Kent, J. F., 185, *201*
Kikkawa, Y., 3, 6, 7, 29, *40*, *41*
Kikuchi, G., 231, *241*
Kilejan, A., 230, *241*
Kilejian, A., 277, *292*
Kilian, R., 210, 222, *241*
Killick-Kendrick, R., 71, *87*, 146, 147, 151, 152, 154, 166, *199*, *200*, *203*
Kilpatrick, J. W., 157, *197*
Kimball, A. C., 26, *40*
Kimball, H. C., 33, *41*
Kimball, H. R., 157, 158, *196*
King, D., 208, 214, 217, 218, 219, 220, 233, *241*
King, H. K., 157, *197*
Kirchhoff, H., 1, *41*
Kirchner, E., 115, *131*
Kisielewska, K., 253, 255, 256, 258, 259, 260, 262, 263, *292*
Kitzman, W. B., 220, *240*
Kitzmiller, J. B., 154, *195*
Kloetzel, J., 77, *89*
Kmetec, E., 231, 232, *241*
Kraubig, H., 1, *41*
Kretschmar, W., 183, *200*
Kuhlow, F., 249, 268, *292*
Kulakovskaya, O. P., 250, *293*
Kumada, M., 15, 33, *40*, *41*
Kunert, H., 33, *41*
Kusano, N., 24, *44*
Knierim, F., 20, *44*
Knisely, M. H., 182, *200*
Kobayashi, A., 15, 33, *40*, *41*
Kofoid, C. A., 112, *134*
Komiya Y., 15, 33, *40*, *41*
Kong, O. Y. C., 153, *204*
Kosareva, N. A., 279, *292*
Kosheva, A. F., 297, *292*
Koshimizu, K., 33, *41*
Koyama, T., 15, 33, *40*, *41*
Kozicka, J., 268, *292*
Kramar, J., 24, *41*
Krampitz, H. E., 83, *88*

L

Labzoffsky, N. A., 24, 29, *41*
Ladda, R., 164, *200*
Lainson, R., 9, *41*
Laird, M., 48, *89*
Laird, R. L., 126, *132*
Lamb, G. A., 23, *38*
Lamborn, W. A., 53, 54, *90*
Lambrecht, F. L., 66, 69, 70, 78, *89*
Lancisi, G. M., 181, *200*
Landau, I., 146, 151, 152, 156, *194*, *199*, *200*
Langer, H., 27, 28, *41*

Lannoo, R., 27, *40*
Laporte, M., 52, *90*
Larsh, J. E., Jr. 274, *294*
Laurie, J. S., 228, 229, 230, 231, *241*
Laveran, A., 51, 77, *89*
Lavier, G., 51, 58, 64, *89*
Lechowska, J., 28, *39*
Lee, D. L., 223, 224, 228, *238*
Léger, L., 50, 56, *89*
Lehman, D. L., 100, 102, *134*
Lelong, M., 15, 28, *38*
Lemma, A., 117, 118, *134*
Lenhoff, H. M., 104, *134*
Lester, H. M. O., 69, *89*
Leuckart, R., 217, *241*
Levy, M., 26, *42*
Lewert, R. M., 125, *134*
Lewis, A., 5, 9, 13, *40*
Lewis, D. J., 71, *89*
Lipa, J. J., 52, *89*
Lips, M., 146, *203*
Little, P. A., 111, *134*
Lock, J. A., 114, *134*
Logachev, E. D., 273, *293*
Lourie, E. M., 156, *200*
Lubinsky, G., 282, *293*
Ludlam, G. B., 32, *41*
Lund, E., 6, 13, 35, *41*
Lunde, M. N., 21, 25, 34, 36, *38*, *41*, *43*
Lunn, J. S., 157, *197*
Lupasçu, Gh., 154, *200*
Luque, O. de, *245*
Lwoff, M., 96, 109, 117, *134*
Lycke, E., 6, 13, 35, *41*

M

McCaig, M. L. O., 286, *293*
McConnachie, E., 194, *196*
McCue, J. F., 285, *295*
McCulloch, W. J., 33, *42*
Macdonald, G., 140, *202*
McGhee, R. B., 124, *134*
McGregor, I. A., 189, 190, *197*, *200*
McIntosh, A., 261, 267, *293*
Mack, A. D., 127, *135*
McKee, R. W., 123, 124, *132*
Mackie, F. P., 99, *131*
McLeod, J. G., 26, *39*
McMahon, J. P., 68, *87*
MacNeal, W. J., 51, *89*, 93, 95, 99, *134*, *135*
McNeil, E., 112, *134*
Maegraith, B. G., 122, *131*, 161, 165, 181, 182, 183, 184, *195*, *197*, *198*, *200*, *201*, *203*
Mahon, J., 255, *293*
Mahoney, D. F., 189, *200*
Makiura, Y., 142, *200*
Malone, M. F., 112, 113, *135*
Mandoul, R., 15, *42*
Mandras, A., 24, *37*
Manson-Bahr, P. E. C., 68, *87*
Manter, H. W., 215, *241*
Manton, I., 176, *200*
Manwell, R. D., 124, *132*, *134*
Marchiafava, E., 181, *201*
Marsh, C. L., *245*
Martin, J. G., 274, *294*
Martin, P., 164, *200*
Martinez, B., 275, *290*
Martius, G., 29, *40*
Martz, G., 27, *40*
Maryon, M., 154, *200*
MasBakal, P., 36, *42*
Matsubayashi, H., 7, 9, 11, 34, *42*, *44*
Mayer, H. F., 33, *42*
Mayr, E., 66, *89*
Meckel, H., 181, *201*
Mehlman, B., 96, 98, 108, *137*
Meikel, M., 34, *43*
Melton, M. L., 9, 15, 16, 29, 30, 33, 34, 36, *39*, *40*, *43*
Melvin, D. M., 253, *293*
Mer, G. G., 140, *201*
Mercer, E. H., 220, 224, 226, 228, 232, *241*, *243*
Meroney, F. C., 279, *294*
Merritt, S. V., 217, 219, *242*
Mesnil, G., 51, 77, *89*
Meyer, A., 206, 209, 210, 214, 217, 222, *242*
Meyer, H., 112, 126, *134*, *135*
Meyer, M. C., 249, *293*
Michajłow, W., 259, *293*
Micks, D. O., 169, *197*
Millemann, R. E., 252, 267, 283, *293*
Miller, D., 261, 267, *293*
Miller, J. H., 232, *241*
Miller, M. A., 223, 232, *242*
Miller, M. J., 25, 34, *42*
Minchin, E. A., 51, 52, *89*
Mitdvedt, T., 32, *42*
Miyata, I., 217, *245*

Mohan, B. N., 186, *201*
Mohiuddin, A., 155, *201*
Mohr, U., 29, *40*
Moller, T., 33, *43*
Monné, L., 224, *242*
Moon, A. P., 183, 190, *202*
Moore, D. V., 209, 214, 215, 217, *242*
Moorham, D. E., 154, *204*
Mornet, P. 71, *89*
Morska, I., 28, *39*
Morsy, T. A., 15, *43*
Most, H., 129, *138*, 150, 151, 153, 193, *201*, *204*
Moulder, J. W., 103, *134*
Moyle, G. G., 15, 33, *40*
Mueller, J. F., 278, 279, 285, 286, 287, *293*, *294*, *295*
Mühlpfordt, H., 80, 81, *89*, 104, 106, *134*
Mulligan, H., 186, *201*
Mulligan, H. W., 184, 189, *201*, *203*
Murgatroyd, F., 98, 108, *135*, *138*
Musacchio, M. O., 126, *134*
Myers, B. R., 112, 113, *135*

N

Négre, L., 20, *42*
Negrete, M. J., 288, *291*
Negulici, E., 154, *200*
Nelson, P., 142, 145, 155, *197*
Neri, I., 141, *197*
Nesbitt, P. E., 154, 176, 179, *199*
Neto, J. F., 145, 154, 158, *197*
Neva, F. A., 112, 113, *135*
Newell, J. W., 27, *43*
Newton, B. A., 109, 110, *135*
Nicholas, D. J. D., 104, *134*
Nicholas, W. L., 209, 210, 214, 217, 219, 220, 222, 224, 226, 227, 228, 230, 231, 232, 233, 234, 236, *238*, *241*, *242*, *243*
Nicolas, J., 15, *42*
Nicoli, J., 97, 99, 101, *132*, *135*
Nicoli, R. M., 51, *89*
Nicolle, C., 95, 116, 117, *135*
Niedmann, G., 20, 26, *42*, *44*
Nien, N. V., 185, *201*
Noguchi, H., 109, *135*
Norman, L., 79, *88*, 262, *293*
Novinskaja, V. F., 76, *86*
Novy, F. G., 51, *89*, 93, 95, 99, *134*, *135*
Nuorteva, P., *246*
Nussenzwieg, R., *201*
Nussenzweig, R. S., 153, 193, *201*
Nussenzweig, V., 77, *89*

O

O'Connor, R. J., 156, *200*
Ogina, N., 2, 5, 6, *42*
Okada, H., 254, *294*
Okamato, K., 285, *295*
Olbing, H., 29, *42*
Oldroyd, H., 54, 55, *89*
Oleson, J. J., 111, *134*
Olisa, E. G., 3, *42*
Olitsky, P. K., 13, *43*
Oliveira, M. X., de, 112, 126, *134*, *135*
Olivier, L., 281, *293*
Olsen, O. W., 209, 214, 215, 217, 219, *243*, 252, *296*
Orestenko, L. P., 33, *42*
Ormerod, W. E., 67, 68, 69, 70, 84, *85*, *89*, 104, 109, 110, *130*, *135*
Ormsbee, R. A., 123, 124, *132*
Osterberg, K., 27, *44*

P

Packchanian, A., 111, *135*
Packer, L., 109, *131*
Paliard, P., 36, *39*
Parent, G., 111, *130*
Pautrizel, R., 185, *201*
Payne, R. A., 22, *38*
Pechranond, 235, *243*
Peeters, E., 150, *203*
Penrose, M. E., 23, *44*
Perrin-Fayolle, M., 36, *39*
Pestre, M., 15, *42*
Peters, W., 161, *201*
Pethica, B. A., 107, *130*, *133*
Petrochenko, V. I., 206, 214, 217, 234, 235, 236, *243*
Petrushevski, G. K., 234, 236, *239*, *243*
Pflugfelder, O., 232, 234, *243*, *246*
Pickard, R., 26, *42*
Piekarski, G., 7, 9, 15, *42*, *44*
Pierce, A. E., 176, 179, 189, *199*
Pierreux, G., 117, *134*
Pinto, S. C., 118, *131*
Pipkin, A., 112, *135*
Pipkin, A. C., 125, 126, 127, *133*, *135*
Pitelka, D. R., 160, *201*

Pitt, C. E., 279, *294*
Pittam, M. D., 97, *135*
Polge, C., 98, *135*
Polyanski, Y. I., 236, *239*
Ponselle, A., 99, *136*
Porter, J. A., 159, *201*
Pradatsundarasar, A., 235, *243*
Prakash, O., 20, *42*
Pratt, I., 217, 219, *242*
Prescott, L. M., 231, *243*
Price, E. W., 235, *243*
Proieti, A. M., 188, *197*
Prokopič, J., 256, *294*
Provasoli, L., 119, *133*
Prowazek, S., von, 51, *89*

Q

Qadri, S. S., 146, *202*
Quilici, M., 51, *89*
Quinn, E. G., 188, *197*

R

Raab, J., 185, *203*
Rabson, A. S., 26, *37*
Race, G. J., 274, *294*
Raffaele, G., 166, 169, *201*
Ramachandran, C. P., 154, *198*
Ramakrishnan, S. P., 154, *196*
Ramirez, J., 231, *241*
Rao, G., 146, *202*
Rausch, R., 235, *243*
Rausch, R. L., 228, *244*
Ravens, R., 275, 276, *295*
Rawson, D., 253, *294*
Ray, H. N., 109, *136*
Rayski, C., 236, *243*
Razgha, A., von, 96, 97, *136*
Read, C. P., 228, 229, 230, *243*
Redington, B. C., 189, *200*
Rees, G., 251, 268, 272, *294*
Reichenbach-Klinke, H. H., 248, *294*
Reichenow, E., 81, *89*, 96, 97, 98, 99, 106, 110, *136*
Reid, H. A., 182, *197*
Rein, C. R., 185, *201*
Remington, J. S., 15, 25, 27, 29, 30, 33, 34, *40*, *42*, *43*
Repetto, Y., 231, *237*
Reutter, F., 27, *40*
Richards, W. H. G., 186, *201*
Rifaat, M. A., 15, *43*
Rigby, J. E., 253, *294*
Riley, M. V., 184, *201*
Ris, R. R., 15, 33, *40*
Riser, N. W., 248, *294*
Ristic, M., 115, *136*
Ritz, H., 77, *90*
Roberts, J. M., 27, *40*
Robertson, D. H. H., 68, *90*
Robertson, J. S., 27, *43*
Robertson, M., 99, *136*
Robinson, E. M., 74, *90*
Robinson, E. S., 210, *243*
Rocca, E., 275, 276, *295*
Rodhain, J., 142, 154, 157, *201*
Rogers, L., 116, *136*
Roitt, I., 176, 179, 189, *199*
Rosario, B., 224, *243*
Rose, G. G., 127, *136*
Roseau, R. M., 157, *196*
Ross, E. L., 222, *244*
Roth, W., 27, *40*
Rothman, A. H., 224, 228, 229, *243*, 288, *294*
Roubaud, E., 51, *90*
Roughley, F., 29, *41*
Rudzinska, M. A., 2, *43*, 81, *90*, 122, *136*, 160, 161, 162, 165, *202*
Russell, P. F., 140, 186, *201*, *202*, 235, *239*
Rybicka, K., 258, *294*
Ryley, J. F., 103, 104, 107, *133*, *136*
Ryšavý, B., 257, *294*

S

Sabin, A. B., 13, *43*
Sadun, E. H., 159, 183, 190, 191, *196*, *202*, *204*, 279, *294*
Saito, K., 33, *41*
Salle, A. T., 116, *136*
Salt, G., 278, *294*
Samochowiec, E., 28, *39*
Samochowiec, L., 36, *37*
Sandosham, A. A., 142, 145, 152, 154, 157, 158, *196*, *198*, *202*, *204*
Sanchez, J., 26, *42*
Sandground, J. H., 69, *90*
Sargent, J. R., 103, 104, *132*, *133*

Saurwein, J., 223, 228, 229, *238*
Sawada, I., 254, 288, *294*
Saz, H. J., 231, *243*
Scheer, D., 234, *243*
Scheibel, L. W., 231, *243*
Scheifinger, C. C., *245*
Schiller, E. L., 117, 118, *134*, 254, 255, 285, *294*
Schinazi, L. A., 277, *292*
Schmidt, C. L. A., 116, *136*
Schmidt, G. D., 209, 214, 215, 217, 219, *244*
Schmidt, I. G., 35, *43*
Schmidt, L. H., 35, *43*, 142, 157, *196*, *202*
Schmidt-Burbach, A., 22, *43*
Schneider, J., 157, *202*
Schnurrenberger, P. R., 33, *43*
Schoenbechler, M. J., 189, *200*
Scholtyseck, E., 166, 169, *202*
Scholtysek, E., 3, *43*
Schulman, S. S., 234, *243*, *244*
Schuman, L., 33, *41*
Schütz, P., 181, *202*
Schwabe, C. W., 277, *292*
Schwarz, P., 22, *43*
Scott, H. H., 75, *90*
Sebastiani, A., 188, *197*
Seidman, I., 232, *244*
Sen, A. B., 109, *133*
Sen, H. G., 109, *136*
Senekjie, H. A., 116, *136*
Sergent, G., 15, *43*
Sergiev, P. G., 191, *202*
Sever, J., 28, *43*
Sever, J. L., 27, *40*
Sezen, N., 155, *202*
Shee, J. C., 26, *43*
Sheffield, H. G., 3, 6, *43*
Sheppard, C. G., 33, *41*
Shiorii, K., 24, *44*
Shortt, H. E., 146, 155, *202*
Shute, P. G., 154, *200*
Sice, A., 76, *90*
Siddiqui, E. H., 273, *295*
Siim, J. C., 33, *43*
Sikorski, R., 28, *43*
Silva, I. I., 52, *90*
Silveira, I. P. S., 158, *197*
Silverman, P. H., 285, *295*
Simmons, J. E., Jr. 229, *243*
Singer, I., 194, *202*
Sinton, J. A., 99, *137*, 189, *201*
Skinner, J. C., 188, *197*
Slais, J., 267, 272, *295*
Smedley, R. D., 99, *136*
Smit, G. L., 20, 21, *43*
Smyly, D. P., 67, 68, *85*
Smyth, S. D., 248, *295*
Snigerevskaya, E., 166, 179, *196*
Soave, J. H., 186, *197*
Soderman, W. A., 187, *202*
Sofleta, A., 157, 190, *196*
Soliman, A. A., 15, *43*
Soltynska, M., 269, *295*
Soltys, M. A., 98, *135*
Somers, K., 32, *41*
Sonoe, M., 285, *295*
Soulsby, E. J. L., 235, 236, *244*
Sourander, P., 6, 13, 35, *41*
Southon, H. A. W., 68, *90*
Specht, D., 264, *295*
Spiecker, D., 166, *202*
Spindler, L. A., 20, *43*, 214, *244*
Spira, D., 189, 192, 193, *202*, *204*
Spooner, D. F., 21, 22, *38*, 103, 104, *132*
Sprinz, H., 3, 6, *37*, *39*, 122, *129*, 161, 162, 163, 164, 165, 166, 176, 178, 179, 180, *195*, *200*, *203*
Spurr, E. D., 118, *133*
Stahl, W., 9, 34, *44*
Stäubli, W., 161, *201*
Stein, B., 186, *197*, *202*
Steinert, M., 101, 104, *130*, *136*, *137*
Stewart, G. L., 22, *44*
Sticinsky, E., 253, *290*
Stohler, V. H., 172, *202*
Stranack, F. R., *246*
Strannegard, O., 35, *41*
Stratman-Thomas, W. K., 182, *200*
Street, J., 169, *197*
Stronach, B. W., 67, 68, *85*
Stunkard, H. W., 248, *295*
Subbarow, Y., 111, *134*
Sulli, E., 33, *45*
Sulzer, A. J., 3, *39*
Supperer, R., 257, *295*
Suto, T., 24, *44*
Sutton, R. N. P., 22, *38*
Suzuki, K., 24, *44*
Swartzwelder, J. C., 232, *241*
Sweets, H. H., 111, *135*
Szaflarski, J., 36, *37*
Szlamp, E. L., 67, 68, *85*

T

Takahashi, T., 249, 276, 285, *295*
Takemura, S., 142, *200*
Taliaferro, W. H., *184*, *203*
Talice, R. V., 274, *295*
Taraska, J. J., 29, *44*
Targett, G. A. T., 190, *203*
Tella, A., *181*, 183, *203*
Ten Pas, A., 35, *44*
Te Punga, W. A., 23, *43*
Teriteanu, E., 157, 190, *196*
Terry, B. J., 191, *203*
Terzakis, A., 176, *203*
Thalhammer, O., 28, *44*
Theodor, O., 52, 53, *90*
Theologides, A., 27, *44*
Thiermann, E., 20, 26, *42*, *44*
Thompson, P. E., 114, *130*
Thomson, J. G., 53, 54, *90*, 99, *137*
Thornham, D. N., 181, *195*
Thorson, R. E., 285, *295*
Tilden, E. B., 109, *135*
Timperman, G., 152, *195*
Tjalma, R. A., 33, *43*
Tobie, E. J., 81, *90*, 96, 98, 100, 102, 103, 108, *130*, *137*
Tobie, J. E., 188, *203*
Top, F. H., 33, *42*
Torrey, H. N., 51, *89*
Trager, W., 2, *43*, 81, *90*, 100, 101, 115, 117, 118, 122, 123, 124, 125, *134*, *136*, *137*, 160, 161, 162, 165, *202*
Trejos, A., 113, *137*
Trelles, J. O., 275, 276, *295*
Trensz, F., 185, *203*
Tsunematsu, Y., 24, *44*
Tulloch, F. M. G., 99, *133*
Turk, J. L., 22, 24, 25, *39*
Turner, J. A., 283, *296*
Turtle, S. R., 26, *39*
Tuzet, O., 52, *90*

U

Ulmer, M. J., *245*
Undeongo, I. O. K., 183, 190, *202*
Utkina, M. A., 268, *295*

V

Valsamis, M. P., 26, *37*
Van Cleave, H. J., 206, 207, 208, 215, 218, 222, 228, 235, *244*
Vanderberg, J., *201*
Vanderberg, J. P., 151, 172, 176, *203*
Van der Veen, J., 24, *44*
Van der Waaij, D., 27, *44*
Van der Zypen, E., 7, 9, *44*
Van Nunen, M. C. J., 24, *44*
Varela, G., 34, *44*
Vattier, G., 101, *135*
Velasco, R., 34, *44*
Venditti, G., 33, *45*
Verma, M. P., 20, *44*
Verolini, F., 188, *197*
Vickerman, K., 56, 70, *86*, *90*, 97, 104, 105, 106, *135*, *138*, 160, 161, 164, *197*, *202*
Vidrine, A., Jr., 231, *243*
Vik, R., 249, *293*
Villalobos, P. R., 288, *291*
Vincke, I. H., 146, 150, 152, *195*, *203*
Vivier, E., 179, *203*
Voge, M., 254, 255, 262, 264, 270, 271, 272, 274, 275, 277, 283, 284, 285, 286, 287, *290*, *291*, *295*, *296*
Voller, A., 24, *39*, 187, 188, 190, *197*, *203*
Voss, H., von, 210, *243*

W

Wagner, E. D., 251, *296*
Waitz, J. A., 114, *130*, 276, *296*
Walker, P. J., 98, *138*
Wallace, F. G., 48, 51, *90*, 97, *133*
Wallace, G. F., 48, *88*
Wallis, O. C., 110, *138*
Walls, K. W., 20, 21, 29, *37*, *44*
Walton, B. C., 24, 29, 33, *44*
Wanko, T., 2, 5, 6, 7, 9, 10, 11, 14, *39*, *44*
Ward, H. L., 210, 214, 215, 217, 219, 220, 228, *241*, *245*
Ward, R., 26, *44*
Ward, R. A., 159, 176, *203*
Warhurst, D. C., 147, *203*
Warren, M., 142, 145, 147, 152, 153, 154, 157, 158, *195*, *198*, *202*, *203*, *204*
Watson, J. M., 235, *245*
Watson, W. A., 24, *37*
Wattal, B. L., 154, *196*
Weathersby, A. B., 126, 127, 128, 129, *133*, *138*, 174, *204*
Weber, M. M., 109, *131*
Weber, R. W., 36, *38*

Weinbach, E. C., 103, *130*
Weinman, D., 96, 97, 99, 102, 117, *132*, *138*, 159, *204*
Weissbach, G., 35, *44*
Weitz, B., 68, 70, *90*
Wellde, B. T., 159, 183, 190, 191, *196*, *202*, *204*
Wentworth, B. B., 33, *43*
Wentworth, F. H., 33, *43*
Wenyon, C. M., 75, 76, *90*
Werner, H., 27, 30, 33, *41*, *45*
Wertlake, P. T., 26, *45*
Wéry, M., 24, *45*, 153, *204*
West, A. J., 213, *245*
West, L. S., 140, *202*
Wharton, R. A., 145, 154, 157, *198*
Wharton, R. G., 152, *202*
Wharton, R. H., 145, 147, 153, 154, 157, *203*, *204*
Whiteside, F. F., 94, 110, *138*
Widmer, E. A., 252, *296*
Wijers, D. J. B., 105, *138*
Wikgren, B. J. P., 268, 269, 282, *296*, *297*
Wildführ, W., 3, 5, 6, *45*
Willett, K. C., 69, *91*, 99, 103, 105, *132*
Williams, J. S., 279, *294*
Williams, K., 189, *200*
Williamson, J., 102, *132*, *138*
Winter, T. S., 26, *45*
Witenberg, G. G., 235, *245*
Wolcott, G. B., 150, 166, *204*
Wolff, E. K., 185, *204*
Wood, F. D., 112, *134*
Woodhouse, M. A., *246*
Worms, M. J., 193, *199*
Wurmbach, H., 234, *245*
Wyatt, G. R., 102, *132*

Y

Yamaguti, S., 206, 217, *245*
Yamao, Y., 276, *297*
Yoeli, M., 129, *138*, 150, 151, 153, 172, 176, 193, *201*, *203*, *204*
Yoneda, C., 2, 5, 6, 9, 13, *40*, *42*
Yorke, W., 98, 108, *135*, *138*
Young, M. D., 159, *201*
Young, R. T., 270, *297*

Z

Zardi, O., 24, 33, *45*
Zimmerman, H. E., 20, *43*
Zscheile, F. P., 29, *45*
Zuckerman, A., 126, 127, *138*, 181, 189, 192, 193, *199*, *202*, *204*
Zweigart, P., 5, 9, 13, *40*

Subject Index

A

Abortion, and *T. gondii*, 27–29
Acanthobothrium coronatum, life cycle, 248–249
Acanthocephala,
  acanthella, 216–219
  acanthor, 214–215
  biochemistry, 228–232
  carbohydrate reserves, 228
    synthesis, 229–230
  citric acid cycle, 230
  cystacanth, 219, 220
  development, 209–220
    in definitive host, 220–222
    in intermediate host, 215–220
  embryology, 210–214
  excretory organs, 209
    products, 230
  glycolysis, 230
  histochemistry, 222–224
  histology, 222
  host–parasite interaction, 232–235
  inorganic ions, 229
  lipids, 228–229
  morphology, 206–209
  nervous system, 209
  paratenic hosts, 219–220
  proteins, 229
  reproduction, 209–210
  reproductive organs, 208
  respiration, 231–232
  ultrastructure, 224–228
Acanthocephalus,
  lucii, larval development, 217
  ranae, chromosomes, 210
Anaemia, and *Toxoplasma gondii*, 29
Ancylostoma caninum, and *T. gondii* transmission, 15
Anomotaenia ciliata, life cycle, 254
Anoplocephalidae, life cycle, 253, 266
Archigetes iowensis, life cycle, 250
  limnodrili, life cycle, 250
Ascaris, respiration, 231–232
Atriotaenia procyonis, life cycle, 252

B

Besnoitia,
  micronemes, 6
  jellisoni, micropyle, 3
Blastocrithidia, classification, 50
Blood dyscrasia, and *T. gondii*, 29
Bolbosoma, histology, 222
Bothriocephalus claviceps, life cycle, 250

C

Caryophyllaeus fimbriceps, life cycle, 250–251
Catenotaenia, life cycle, 265
Centrorhynchus ptyasus,
  in intermediate host, 215
  larval development, 217
Cestodes, post-embryonic development, 247–297
Choanotaenia,
  crassiscolex, life cycle, 253
  infundibulum, life cycle, 253–254
Cladotaenia, life cycle, 261
Corynosoma,
  semerme, 235–236
  strumosum, 235–236
Crithidia, classification, 50
  oncopelti, drug action, 109–110
  see also Blastocrithidia
Cyclophyllidea, life cycle, 252–262
  Anoplocephalidae, 253
  Davaineidae, 253–254
  Dilepididae, 253–254
  Hymenolepididae, 254–261
  Linstowiidae, 252
  Taeniidae, 261–262

Cysticercus,
bovis, 272–273
cellulosae, 272, 275
fasciolaris, 272
racemosus, 275
tenuicollis, 276

D

Davainea proglottina, life cycle, 254
Davaineidae, life cycle, 253–254, 266
Diagramma interrupta, effect on host, 279
Dilepididae, life cycle, 253–254, 266
Diorchis ransomi, life cycle, 258
Diphyllobothrium,
dendriticum,
life history, 249
plerocercoid, 268–269
ditremum, procercoid, 268
latum,
life cycle, 249
plerocercoid, 268, 269
procercoid, 268
mansoni,
development, 285
histochemistry, 276
life cycle, 249–250
osmeri
effect of temperature, 282
plerocercoid, 268–269
sebago, life cycle, 249
Diploposthe,
laevis, life cycle, 258
skrjabini, life cycle, 258
Drepanidotaenia,
bisacculina, cysticercoid, 270, 271
life cycle, 258, 259
lanceolata, life cycle, 258-261
Duttonella vivax, *see Trypanosoma vivax*

E

Echinobothrium affine, asexual multiplication, 262
Echinococcus, respiration, 231
granulosus, cysticercus, 276
histochemistry, 277
multilocularis,
asexual multiplication, 262
effect on host, 282
Echinocotyle clerci, cysticercoid, 270
Echinorhynchus,
gigas, *see Macracanthorhynchus hirudinaceus*
haeruca, *see Acanthocephalus ranae*
polymorphus, *see Polymorphus minutus*
truttae, *see Metechinorhynchus truttae*
Eimeria,
intestinalis, micronemes, 6
jellisoni, micronemes, 5, 6
perforans, microgamete, 169
reproduction, 3
stiedae, reproduction, 3
Encephalitis, and *T. gondii*, 27

F

Filicollis anatis, 236
Fimbriarioides, cyst wall, 258

G

Grillotia heptanchi, plerocercoids, 268

H

Haematophagous insects, origin, 53–55
Haemoeba,
gallinaceum, *see Plasmodium gallinaceum*
relictum, *see Plasmodium relictum*
Hamanniella,
microcephala, chromosomes, 210
tortuosa, acanthor, 215
Herpetomonas, classification, 48–49
Herpetosoma lewisi, *see Trypanosoma lewisi*
Heterakis, respiration, 231
Hodgkin's disease, and *T. gondii*, 26, 27
Hydatigera taeniaeformis, histochemistry, 276–277
Hymenolepididae, life cycle, 254–261, 266
Hymenolepis,
respiration, 231
aequabilis, 258
californicus, cysticercoid, 270
carioca, life cycle, 253–254
citelli,
cysticercoid, 271
effect of temperature, 284
excystment *in vitro*, 288
glycogen, 277

Hymenolepis (*contd.*)
compressa, life cycle, 257
diminuta,
concurrent infection with *M. dubius* 234–235
cysticercoid, 271, 271
effect of temperature, 283–284
effect on host, 279
excystment *in vitro*, 288, 289
histochemistry, 277
life cycle, 254, 255
furcata, life cycle, 255
furcifera, life cycle, 257
macrocephala, life cycle, 257–258
microstoma,
cysticercoid, 271
effect of temperature, 284–285
life cycle, 254–255
nana,
cysticercoid, 271
effect of temperature, 283, 285
excystment *in vitro*, 288
glycogen, 277
life cycle, 254, 255–256
paracompressa, life cycle, 258
sacciperium, life cycle, 258
spiralibursata, life cycle, 258
vistulae, life cycle, 257

I

Isospora, and *T. gondii* transmission, 15–16

L

Lacistorhynchus tenuis, life cycle, 248
Lankesterella garnhami, micronemes, 5, 6
Leishmania,
cultivation, 116–118
effect of temperature, 117–118
agamae, cultivation, 117
brasiliensis, cultivation, 117, 118
ceramodactyli, cultivation, 117
donovani, cultivation, 95, 116–117, 118, 119
enrietti, cultivation, 118
tarentola, cultivation, 117
tropica, cultivation, 117, 118, 119
Leptomonas, classification, 48
Leptorhynchoides thecatus,
acanthor, 214, 215
in definitive host, 221
larval development, 217
reproduction, 209
Leucocytozoon marchouxi, microgamete, 167
Leukaemia, and *T. gondii*, 26, 27
Ligula intestinalis,
effect on host, 279
histochemistry, 276
Linstowiidae, life cycle, 252, 266
Lumbricus terrestris, and *T. gondii* transmission, 15

M

Macracanthorhychus hirudinaceus,
acanthor, 214, 215
assimilation, 229
carbohydrate reserves, 228
synthesis, 229
cystacanth, 219
embryology, 210–213
histochemistry, 223
in definitive host, 221
in man, 231
in veterinary medicine, 235
inorganic ions, 229
larval development, 217
lipids, 229
pathogenicity, 232
proteins, 229
reproduction, 209–210
specificity, in arthropod host, 232
in vertebrate host, 233–234
ingens,
acanthor, 217
larval development, 217
reproduction, 209
Malaria, *see Plasmodium*
Mediorhynchus grandis,
acanthor, 215
larval development, 217
Megatrypanum theileri, *see Trypanosoma theileri*
Mental retardation, and *T. gondii*, 29
Mesocestoides,
asexual multiplication, 264
development *in vitro*, 286–287
tetrathyridium, 269, 270
transformation, 250

Mesocestoididae, life cycle, 265
Metechinorhynchus truttae, in definitive host, 221
Moniliformis,
  clarki, larval development, 217
  dubius,
    acanthor, 214–216
    assimilation, 229
    capsule, 232–233
    carbohydrate reserves, 228
      synthesis, 229–230
    concurrent with *H. diminuta*, 234–235
    cultivation, 222
    cystacanth, 219, 220
    embryology, 210–214
    excretory, products, 230
    glycolysis, 230
    in definitive host, 221
    in man, 234
    larval development, 217, 219
    lipids, 229
    muscle and connective tissue, 228
    proteins, 229
    reproduction, 209
    resistance to, 234
    respiration, 231, 232
    tegument, 224–227, 228
Monoecocestus,
  effect of temperature, 282
  life cycle, 253
  sigmodontis, life cycle, 253
Multiceps serialis, cysticercus, 274
Myocarditis, and *T. gondii*, 25–26

N

Nannomonas congolense, see Trypanosoma congolense
Neoechinorhynchus,
  carbohydrate reserves, 228
  cylindratus,
    embryology, 214
    larval development, 217
    paratenic host, 220
  emydis,
    embryology, 213
    histology, 222
    in vitro cultivation, 222
    larval development, 217
  rutili,
    embryology, 210, 213, 214
    larval development, 217
Nippostrongylus muris, and *T. gondii* transmission, 15
Notodromas monacha, 258
Novyella, subgenus, 187
  juxtanucleare, see Plasmodium juxtanucleare
  rouxi, see Plasmodium rouxi

O

Onicola canis, 235
Oochoristica,
  cysticercoid, 272
  deserti,
    effect of temperature, 283
    life cycle, 252
  osheroffi, life cycle, 252
  symmetrica, excystment *in vitro*, 288
  vacuolata, life cycle, 252

P

Plasmodium,
  anomaluri, 150
  atheruri, 147–150
  berghei,
    antigens, 192
    cultivation, 129
    immunity, active, 193
      measurement,
        agglutination, 186
        complement fixation, 185
        fluorescent antibody, 188
        gel diffusion, 189
      passive, 191
    life cycle, 150–151
    pathogenesis, 183, 184
    response variation, 159
    ultrastructure,
      erythrocytic stage, 160–162, 164–165
      exflagellation, 166
      oocyst, 174–176
      ookinete, 172
    berghei, 146, 148, 149
      cross immunity, 190
      fluorescent antibody, 187

Plasmodium—berghei (*contd.*)
yoelii, 146–147, 148, 149
cross immunity, 190
fluorescent antibody test, 187
life cycle, 151
ultrastructure,
erythrocytic stage, 166
exflagellation, 167
booliati, 150
brasilianum, 154
fluorescent antibody test, 188
in man, 157
sporozoite, 176, 178
brucei, 147
bubalis, 147
cathemerium,
cultivation, 124
ultrastructure,
erythrocytic stage, 166
micropyle, 180
cephalophi, 147
chabaudi, 146, 148, 149
antigens, 192
cross immunity, 190
fluorescent antibody test, 187, 188
life cycle, 151–152
sporozoite, 179
coatneyi, 144, 145, 154
cross immunity, 190
immunity measurement,
agglutination, 186
fluorescent antibody, 187, 188
pathogenesis, 181
ultrastructure, erythrocytic stage, 161
cynomolgi, 142–145
cross immunity, 190
immunity measurement,
agglutination, 186
fluorescent antibody, 187, 188
in man, 157, 158
relapses, 156
ultrastructure, erythrocytic stage, 161, 163
bastianellii, 142, 143, 147, 153–154, 155
antigens, 192
fluorescent antibody test, 187, 188
in man, 157
response variation, 159
ultrastructure,
exflagellation, 166
micropyle, 180
ookinete, 172, 173
sporozoite, 176, 177
ceylonensis, 142, 143, 155
antigens, 192
cyclopis, 142
cynomolgi, 142, 143, 155
in man, 157, 158
elongatum,
pathogenesis, 181
ultrastructure,
erythrocytic stage, 166
micropyle, 180
sporozoite, 178
eylesi, 142, 154
in man, 157
falciparum, 147
cultivation, 93, 121, 124
immunity measurement,
agglutination, 186
complement fixation, 185
gel diffusion, 189
passive, 190–191
ultrastructure,
erythrocytic stage, 164
micropyle, 180
sporozoite, 179
fallax,
cultivation, 126–127
micronemes, 6
reproduction, 3
ultrastructure, erythrocytic stage, 166
fieldi, 145, 154, 155
fluorescent antibody test, 187, 188
foleyi, 147
fragile, 144, 145
cross immunity, 190
fluorescent antibody test, 188
pathogenesis, 181
gallinaceum,
antigens, 192
cultivation, 124, 125–127, 129
immunity measurement,
agglutination, 186
fluorescent antibody, 187, 188
pathogenesis, 181
ultrastructure,
erythrocytic stage, 163, 164
micropyle, 180

Plasmodium—gallinaceum (contd.)
ultrastructure (contd.)
oocyst, 176
ookinete, 172, 173
sporozoite, 177
girardi, 147
gonderi,
immunity measurement,
agglutination, 186
fluorescent antibody, 187, 188
pathogenesis, 181
ultrastructure, erythrocytic stage, 161
hexamerium, cultivation, 124
hylobati, 142
inui, 146, 154, 155
immunity measurement,
agglutination, 186
fluorescent antibody, 187, 188
in man, 157
jefferyi, 142
fluorescent antibody test, 188
relapses, 156
juxtanucleare,
fluorescent antibody test, 187
pathogenesis, 181
knowlesi, 144, 153
cultivation, 122, 123, 128–129
immunity measurement,
agglutination, 185
complement fixation, 185
fluorescent antibody, 187, 188
gel diffusion, 189
in man, 157, 158
pathogenesis, 181–184
response variation, 159
ultrastructure,
erythrocytic stage, 161, 162, 165–166
micropyle, 180
edesoni, 145
lemuris, 147
lophurae,
cultivation, 122–124, 125, 129
reproduction, 2–3
ultrastructure, erythrocytic stage, 166
malariae,
cultivation, 93, 121
fluorescent antibody test, 188
pathogenesis, 182
osmaniae, 146
ovale, 145
micropyle, 180
relapses, 156
relictum,
cultivation, 124, 127–128, 129
fluorescent antibody test, 187
roussetti, 150
rouxi, fluorescent antibody test, 187
sandoshami, 147
schwetzi, ultrastructure, erythrocytic stage, 161
shortti, 146
fluorescent antibody test, 187, 188
in man, 157
simiovale, 145
simium, 145
in man, 158
traguli, 147
life cycle, 152
vinckei, 146, 148, 149
antigens, 192
cross immunity, 190
fluorescent antibody test, 187
immunity, passive, 191–192, 193
life cycle, 152
ultrastructure, erythrocytic stage, 161–164
vivax, 158
cultivation, 93, 121, 126
immunity measurement,
agglutination, 186
complement fixation, 185
relapses, 156
ultrastructure,
micropyle, 180
sporozoite, 177, 178, 179
voltaicum, 150
youngi, 142
Polymorphus,
botulus, see Profilicollis botulus
magnus,
acanthor, 214
in birds, 236
in definitive host, 221
larval development, 217
minutus,
acanthor, 214
capsule, 232
chromosomes, 210
copulation, 210
cystacanth, 219
embryology, 210, 212, 213

Polymorphus—minutus (*contd.*)
enzymes, 223–224
glycogen, 223
hooks, 227
in birds, 236
in definitive host, 221
in intermediate host, 215
larval development, 217
Pomphorhynchus,
bulbicolli, cultivation, 222
laevis, chromosomes, 210
Profilicollis botulus, 236
Prosthorhynchus formosus,
acanthor, 217
larval development, 217
reproduction, 209
Proteocephala, life cycle, 265
Proteocephalus,
filicollis, life cycle, 251
niloticus, life cycle, 251
parallacticus, life cycle, 251–252
tumidicollus, life cycle, 251
Pseudodiorchis prolifer,
asexual multiplication, 262–264
life cycle, 256
Pseudomonas fluorescens, cytochrome, 104
Pseudophyllidea, life cycle, 249–252, 265
Pycnomonas suis, *see Trypanosoma suis*

R

Raillietina,
cesticillus, cysticercoid, 271
effect of temperature, 284
life cycle, 253–254
kashiwarensis, excystment *in vitro*, 288

S

Salivaria, cultivation, 95–100
Sarcocystis muris, and *T. gondii* transmission, 20
Schistocephalus solidus,
development *in vitro*, 286
effect of temperature, 282
life cycle, 250
Schizotrypanum cruzi, *see Trypanosoma cruzi*
Sobolevicanthus octacantha, life cycle, 257
Soricina diaphana, life cycle, 256
Spirometra, effect of temperature, 285
mansonoides,
development *in vitro*, 285–286
effect on host, 278–280
Stercoraria, cultivation, 110–116
Strigomonas, *see Crithidia*

T

Taenia,
crassiceps,
asexual multiplication, 262
effect on host, 281–282
life cycle, 261
hydatigena, cysticercus, 273–274
mustelae,
asexual multiplication, 262
life cycle, 261
pisiformis,
cysticercus, 273–274
phosphatase, 276
excystment *in vitro*, 288
saginata,
cysticercus, 273–274
phosphatase, 276
life cycle, 216–262
solium,
cysticercus, 274–275
excystment *in vitro*, 288
taeniaeformis,
effect on host, 279–281
excystment *in vitro*, 288
phosphatase, 276
Taeniidae, life cycle, 261–262, 266, 267
Tatria acanthorhyncha, life cycle, 258
Tetraphyllidea, life cycle, 248–249
Thysanosoma actinioides, cysticercoid, 272
Toxocara,
canis, and *T. gondii* transmission, 19
cati, and *T. gondii* transmission, 15–19
Toxoplasma gondii, 1–37
and anaemia, 29
and blood dyscrasia, 29
and encephalitis, 27
and habitual abortion, 27–29
and Hodgkin's disease, 26, 27
and leukaemia, 26, 27
and malignant disease, 26–27
and mental retardation, 29
and myocarditis, 25–26
antibody response, 29–32

Toxoplasma gondii (*contd.*)
complement fixation, 22
cyst, 6–15
cytostome, 3–5
diagnosis, 20–25
digestion resistance, 9–11, 12
dye tests, 20–22, 23
effect of pepsin on, 9–11, 12
electron microscopy, 2–15
epidemiology, 32–33
haemagglutination, 20–22
immunity, 35
immunofluorescence, 24
indirect fluorescence, 24, 25
micronemes, 4, 5–6
micropyle, 3–5
pathogenesis, 34
physiology, 35
posterior polar granule, 3
reproduction, 2–3
serology, 15–25
therapy, 35–36
toxonemes, 5–6
trophozoite, 2–6, 8, 10
transmission, 15–20
Triaenophorus,
lucii, development, 259
nodulosus, life cycle, 251, 259
Trichinella spiralis, and *T. gondii* transmission, 15
Trichuris, respiration, 231
Trypanorhyncha, life cycle, 248–249, 265
Trypanosoma,
avium, 57
cultivation, 115–116, 119, 120
blanchardi, 76
boueti, 58
brucei, 58, 59, 60, 62, 63
classification, 95
cultivation, 95, 98
infectivity, 99, 101–103
electric charge, 107
mitochondrial activity, 105
plastodemes, 77
variation, 67, 69, 70
vector infection rate, 63
subgroup,
akinetoplastic, 106
classification, 95
cultivation, 95, 96, 119, 120
effect of duration of passage, 97–98
infectivity, 99, 101–103, 119, 120
relationship to blood-stream forms, 103–105
electric charge, 106–107, 119
variation, 67
congolense, 58, 59, 60, 62, 63
akinetoplastic, 106
classification, 95
cultivation, 96, 100
infectivity, 101
drug action, 108–109
mitochondrial activity, 105
nosodemes, 78–79
quinopyramine resistance, 110
serodemes, 77
vector infection rate, 63
conorhini,
classification, 95
cultivation, 115, 116, 119, 120
cruzi, 55, 57–60
classification, 95
cultivation, 95, 110–116, 120
effect of temperature, 112–114
testing of drugs, 114
development, 52
infection rate, 62
multiplication, 64
nosodemes, 79
serodemes, 77
variation, 75
xenodemes, 78
duttoni, 76
equinum,
akinetoplastic, 81, 106
classification, 95
transmission, 94
equiperdum,
akinetoplastic, 79–81
classification, 95
quinapyramine resistance, 110
transmission, 94
variation, 72–74
evansi,
akinetoplastic, 79–82, 106
classification, 95
clinodemes, 78
serodemes, 77
transmission, 94
variation, 71–74
xenodemes, 77–78

Trypanosoma (*contd.*)
gambiense, 59, 62, 63
akinetoplastic, 106
classification, 95
cultivation, 96, 97–98, 99
infectivity, 99–100, 102
electric charge, 107
variation, 67–70
granulosum, cultivation, 99
grayi, 57, 63
vector infection rate, 52
halogaleae, 55
hippicum, *see T. evansi*
ichneumonis, 55
inopinatum, cultivation, 99
lewisi, 58, 59, 62, 64
classification, 95
cultivation, 93, 95, 99
infectivity, 99
infectivity rate, 62
multiplication, 64
variation, 75–76
mega,
cultivation, 101
mitochondrial activity, 104–105
melophagium, 57, 59, 62
vector infection rate, 52, 62
musculi, 76
nabiasi, 76
rabinowitschae, 76
rajae, 58
rangeli, 57, 60, 78
development in vector, 61–62
rhodesiense, 59, 63
classification, 95
cultivation, 96–98
infectivity, 99–100, 101
drug action, 108–109
electric charge, 107
variation, 67–70
rotatorium, 58
simiae, 63
suis, 58, 63
classification, 95
theileri, 58, 59, 64
classification, 95
cultivation, 114–115, 119, 120
theodori, 52, 62
uniforme, 63
venezuelense, 81–82
viennei, 64
vivax, 58–60, 62, 63
akinetoplastic, 106
classification, 95
clinodemes, 78
cultivation, 100, 120
mitochondrial activity, 105
quinapyramine resistance, 110
variation, 74–75
vector infection rate, 63
zapi, 76
Trypanosomes,
classification, 94–95
cultivation, 94–120
evolution, 57–66
microevolutionary divergence, 66–82
origin, 50–57
taxonomy of biological variants, 82–85
Trypanozoon, variation, 67–74

U

Urocystis prolifer, 256
asexual multiplication, 262–264