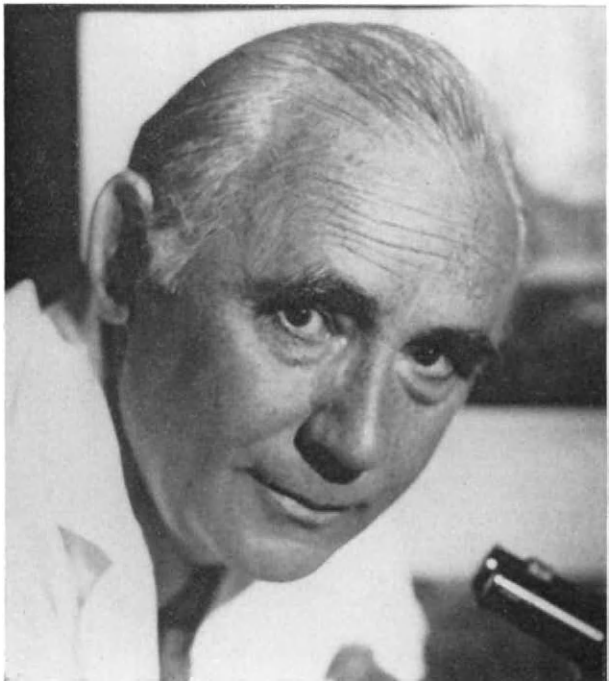

Frontispiece: (*top*) The late Dr Ignace H. Vincke (by courtesy of Prof. L. J. Bruce-Chwatt), (*bottom*) the late Prof. Meir Yoeli (by courtesy of Mrs Kitty Yoeli)

Rodent Malaria

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Preface

The publication of this book marks the thirtieth anniversary of the discovery of the first malaria parasite of a rodent by the late Prof. Ignace Vincke (*frontispiece*). Over these thirty years, malaria of rodents has been intensively studied and, as a result, many scientific papers on this subject have been published. The principal reason why the malaria parasites of rodents have attracted so much interest is because they at once provided a range of laboratory models of a disease which is still a major scourge of mankind, using parasites which were more easily handled and cheaper to study than simian malaria parasites, and which had more relevance to the disease of man than the avian parasites so widely studied in the 1930s and 1940s.

The first fifteen years' work on the malaria parasites of rodents was authoritatively reviewed in Garnham's (1966) book; the work of the second period of fifteen years, which forms the greater part of the total, is scattered throughout innumerable journals. Just before Garnham's monumental work went to press, two new discoveries were reported which were to result in an astonishing surge in activity. First, the late Prof. Meir Yoeli (*frontispiece*) and his colleagues demonstrated the complete life cycle of *Plasmodium berghei* for the first time, and thus paved the way for regular cyclical transmission in the laboratory, an achievement which had eluded many other workers. Secondly, Dr Irène Landau discovered several new malaria parasites of rodents which, until that time, were thought of as rare exotic plasmodia with a severely limited distribution. Dr Landau's findings led to renewed field work in Africa resulting in a battery of new species and subspecies being established in the laboratory. The discoveries of Prof. Yoeli and Dr Landau triggered off a second wave of research even greater than that which followed Vincke's description of the first malaria parasite of a rodent.

Prof. Garnham was just able to cover the new findings by additions to the page proofs of his book, but no comprehensive review of the fallout which followed has been published. This book fills that gap.

As in other fields of parasitic protozoology, the study of malaria has become multidisciplinary, and is no longer solely the territory of the protozoologist or the physician. Great contributions are being made by workers trained in other disciplines notably biochemistry, immunology, pharmacology, cell biology and genetics. In planning this book, therefore, it became immediately apparent that no one person could do justice to this multi-faceted subject. Accordingly, we invited leading specialists, all experts in their fields, to contribute. We have, however, tried to do more than simply gather together a series of authoritative reviews with only tenuous connections between them. The contributors were aware from the beginning of the aspects to be covered by their colleagues, and the chapters were planned to complement one another. Where there was overlap, one contributor dealt with the subject in depth, and cross references have been inserted in other chapters to guide the reader. In this way, an attempt has been made to present, against the background of the early work, reviews of all the significant new findings in a series of interrelated chapters by experts who are actively involved in the study of the murine malaria parasites.

The malaria parasites of rodents other than murids—*P.atheruri* of the African brush-tailed porcupine, three species of malaria parasites of African anomalures and three of Asian petauristine flying squirrels—have been excluded from this work. These parasites have been much neglected, and very little new information has been published since Garnham's book; what little there is can be traced from the lists of references in Killick-Kendrick (1973) (*Acta tropica* **31,** 28–69) and Chapters 1, 2 and 7 of this book.

We wish to thank the contributors for the care with which they have prepared their chapters and for the tolerance they have shown towards editorial interference. We are delighted to have this opportunity to thank Roger Farrand of Academic Press, who has been a constant source of encouragement.

April 1978

R. Killick-Kendrick
W. Peters

This book is dedicated with warm affection and deep respect to Prof. P. C. C. Garnham, F.R.S.; no other worker has taught, helped or encouraged more malariologists, and none has contributed more to the study of malaria parasites.

Introduction

L. J. BRUCE-CHWATT

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In the retrospective outline of the progress of parasitology of malaria some dates can be truly regarded as historical milestones. It was in 1880 that Laveran discovered malaria parasites in human blood, and in 1886 Marchiafava, Golgi and Celli described their developmental forms; in 1889 Danilevsky published his masterly study of avian malaria; in 1897 Ross saw the sporogonic forms of the parasite in an *Anopheles* mosquito and a year later he and the Italians observed the whole cycle of development of avian and human plasmodia in the invertebrate host.

Another major discovery, by Raffaele, of tissue forms of avian plasmodia was not made until 1934 but 14 years later two events took place that opened new vistas for malaria research. Both of them were announced in 1948, the year that can be counted as "annus mirabilis" of parasitology of malaria. In that year Shortt, Garnham and their colleagues described in England the pre-erythrocytic forms of *Plasmodium cynomolgi* in the liver of a rhesus monkey and the same forms of *P.vivax* were soon found in the liver of a human volunteer. In the far away Congo (now Zaïre) a malaria parasite of some obscure "thicket rats" was found by Vincke and Lips; they also reported that this parasite was infective to common laboratory rodents.

The names of Ignace Vincke and subsequently that of Meir Yoeli are closely connected with the fascinating story of this discovery and with the subsequent development of a remarkable scientific tool which has revolutionized research on the parasitology, immunology and chemotherapy of human malaria.

The recognition of the importance of the discovery of rodent malaria

was somewhat overshadowed in the 1950s by the great surge of our knowledge of the tissue forms of human and simian malaria, by the great progress of the treatment of the disease and by the enthusiastic acceptance of the bold promise of the global eradication of this "million murdering death". It was only during the following decade that the full significance of rodent malaria as a research tool became evident.

The International Colloquium on *Plasmodium berghei* organized in 1964 by Prof. P. G. Janssens, the then Director of the Prince Leopold Institute of Tropical Medicine in Antwerp, focused attention on the importance of the discovery made by the Belgian scientists and stressed its immense potential value. In his masterly summary of that meeting Jadin (1965a) pointed out that the early history of rodent malaria provides a perfect example of an intuitive recognition of a link between two observations, a perfect example of what Charles Nicolle called "un instant de lumière", when describing a cognitive flash that illuminates the tortuous path of a biological discovery.

Jadin was careful to emphasize that Vincke's achievement was not due to chance, but was the result of a step by step collection and interpretation of carefully observed facts. This was fully confirmed by Bafort (1971) one of Vincke's co-workers and close friends.

The origin of the story can be traced to the evening of 18 April 1937, when Dr Albert Duren was quietly fishing on the bank of the Kivubu river in the Kwango province of the then Belgian Congo. He noticed an *Anopheles* mosquito on a nearby tree trunk, caught it, and sent it to the British Museum in London where Edwards described it in 1938 as *Anopheles dureni*. In 1941 Dr Ignace Vincke, who was then in charge of malaria control at Stanleyville (now Lubumbashi), was sent on a short mission to the Katanga province where he surveyed the area between Elisabethville (now Kisangani) and Jadotville. He found *A.dureni* among the vegetation of that area, and especially in the fringing forest (also known as gallery forest) of rivers and streams. Two years later, when Vincke was appointed as malariologist in Katanga, one of the first places that he re-visited was the fringing forest of the Kisanga river where he then caught large numbers of *A.dureni*. These were slightly different from the mosquitoes collected previously in the Kwango and, in his field notes, Vincke called them *A.dureni* var. *millecampsi*; Lips described them and Gillies and de Meillon (1968) then named them as a full subspecies retaining Vincke's name. In December

1943 Vincke and the Comte d'Ursel dissected a number of females of this *Anopheles* and found a fairly high proportion with sporozoites in their salivary glands.

In 1944 several entomological surveys of the area revealed that this subspecies of *A.dureni* was exophilic, and that it fed on animals rather than man. During the following two years, much of the biology of this mosquito was elucidated by Vincke and his colleagues, but it was not until 1946 that the blood feeding habits of this *Anopheles* were properly studied by means of precipitin tests. The tests were negative for the blood of primates, cattle, horse, sheep, dog and antelopes. Rodents and insectivores remained as the only possible source of bloodmeals of *A.dureni millecampsi* collected in Kisanga.

At the end of the second world war Vincke returned to Belgium, after having spent 10 years in Africa. Lips, an entomologist, continued the field observations and noticed the presence of some apparently parasitized cells in blood smears prepared from midguts of freshly fed wild *A.dureni*. In September 1947 Vincke returned to Katanga to start a malaria control campaign by DDT spraying, but continued the trapping of small rodents in the fringing forest with the hope of finding the source of the infections in the local *A.dureni*. The fact that, at that time, sporozoite infections could not be found in the mosquitoes was probably due to a fire which had destroyed part of the fringing forest. Over two years some 360 specimens of three species of wild rats were collected and examined. Finally in February, 1948, rodent No. 173, a specimen of *Grammomys surdaster* caught at Keyberg in the Kisanga district, showed the presence of numerous blood parasites. Vincke inoculated blood from this animal into white mice and the type strain K173 of the new parasite was isolated. It was given the name of one of Vincke's friends, Dr Louis van den Berghe, the then Director of the Institute for Scientific Research in Central Africa based at Lwiro. A formal announcement of the new discovery was made by Dr van den Berghe at the Fourth International Congress of Malaria and Tropical Medicine in Washington in September 1948 (van den Berghe, 1948).*

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* It may be of bibliographical interest to quote the footnote of Dr van den Berghe's paper announcing this discovery:

"It is regretted that, because of lack of time certain restrictions made it impossible to present I. H. Vincke and M. Lips' paper at this Congress. The following summary should serve as a formal recognition of their discovery: *Anopheles dureni* infected with malaria

(continued overleaf)

Jerome Rodhain, the grand old man of Belgian protozoology, confirmed the original description of the parasite but pointed out that some proof was needed of the postulated relationship between the natural infection of thicket rats and the sporozoites seen in the salivary glands of *A.dureni millecampsi.*

Not until 1950 did the infection in mosquitoes reappear in Kisanga and, in the meantime, after two years of constant search, a suitable fringing forest was discovered astride the Kasapa river near Elisabethville. Out of 2800 dissections of *Anopheles*, one was found with infected salivary glands which, when injected into a white mouse, produced the same type of parasitaemia as the one obtained after inoculation of blood from naturally infected rodents. This confirmed the postulated natural cycle of transmission between the wild rodent and the subspecies of *A.dureni.*

Other studies by Vincke and his team showed that the natural infection with *P.berghei* was found not only in *Thamnomys* but also in two other rodents, *Praomys jacksoni* and *Leggada bella.* A large number of strains of the new parasite were recovered from the rodent hosts and anopheline vectors during that period, and the interest in the possibilities offered by *P.berghei* increased from year to year. For the first time in the history of protozoology a malaria parasite with many characteristics of a *Plasmodium* of man and infecting one of the commonest laboratory animals had become available.

Perhaps the most striking aspect of *P.berghei* was the extreme susceptibility of the white mouse in which the infection assumes a regular, fatal course. No less surprising was the wide range of animals in which the parasite survives; they varied from mice or rats to hamsters, voles, gerbils, squirrels and bats with different degrees of host restriction.

From the early days of Vincke's discovery several strains of *P.berghei*

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parasites were first found by Dr. I. Vincke in a small forest gallery near Elisabethville in the Congo. After systematic research a typical *Plasmodium* was discovered in the blood of a tree dwelling rat *Thamnomys surdaster.* White mice succumb to the parasite in 11–15 days, after very acute infection with as many as 50% of erythrocytes harbouring one and generally more (up to seven) schizonts. Numerous schizonts are also seen outside the erythrocytes in the peripheral blood. Schizonts contain from 8 to 14, sometimes more merozoites. The gametocytes are oval and slightly elongated with a typical sexual differentiation and well marked pigment granules. The erythrocytes containing the parasites (frequently of different stages, schizonts young or mature, as well as schizogony forms) are large and polychromatophilic. Different species of wild rats, including *Rattus rattus,* are also susceptible, but their resistance is greater." (van den Berghe, 1948).

were established in Antwerp and Dr Albert Dubois, the then Director of the Prince Leopold Institute of Tropical Medicine, distributed strains to all interested scientists. Within ten years the number of publications describing the morphology of *P.berghei* and its host cells, the course of infection in various animals, its response to different stimuli or antimalarial drugs rose to some 500.

There was, however, something new in Vincke's scientific store. In 1952 Vincke found at Kamena, some 600 miles north of Elisabethville (now Kisangani), a new gallery forest site with *A.dureni millecampsi*, many of which had sporozoites in their salivary glands. Vincke's assistant, Mukata, succeeded in transmitting the infection to a mouse and to some *Thamnomys* and *Aethomys* rats. But the morphology of the parasite and the course of infection in experimental animals were different from those of *P.berghei*. The new strain was sent to Jerome Rodhain in Antwerp who realized that this was a new plasmodial species and named it *P.vinckei*. As pointed out by Garnham, the presence of *P.vinckei* in the Katanga was an exceptional occurrence since all the other rodent parasites in that region proved to be *P.berghei*.

Although the importance of the two plasmodia of rodents as a laboratory tool for immunological and chemotherapeutic research was obvious from the start, one part of their cycle in the vertebrate host remained unknown. The existence of a pre-erythrocytic stage of these parasites was still one of the most elusive aspects of parasitological research. The answer could be found only if a reliable mammal–arthropod–mammal transmission model of the parasite became available.

The first attempts at inoculation into white rats of sporozoites from naturally infected *A.dureni* were made in 1950 by Vincke and Lips who observed an incubation period of three days. The report by van den Berghe *et al.* (1950) of the discovery of pre-erythrocytic forms of the parasite in the bone marrow and liver of mice inoculated with infective blood proved to be a wrong lead. Other attempts by Vincke and by Yoeli and Most (1960) were also not successful, though it soon became obvious that the prepatent period of *P.berghei* infection was shorter than the three days observed initially by Vincke.

From the very beginning of these studies there was need for an experimental vector other than the fragile *A.dureni*. Numerous attempts at substitution of this mosquito by the easily colonized *A.atroparvus*, *A.stephensi* and *A.gambiae* were disappointing. Occasionally a few

oocysts and rare sporozoites were observed, but regular mosquito transmission of the infection in the laboratory was unattainable.

One of the reasons for this failure was the disappearance of gametocytes in strains of *P.berghei* transmitted serially by blood passage. However, Jadin succeeded in reviving the gametogenesis in a strain of the parasite (Kasapa 323) after a passage through hamsters and young *Thamnomys*. This stratagem led to an infection of *A.quadrimaculatus*, *A.aztecus* and *A.stephensi*. However, the maturation of oocysts in these mosquitoes was incomplete and there was no invasion of salivary glands by sporozoites (Rodhain *et al.*, 1955).

Once again it appeared that only the use of a natural vector of rodent malaria could solve the difficulty. When, towards the end of the 1940s, the prevalence of natural infection of both *Thamnomys* and *A.dureni* declined to a vanishing point, Vincke repopulated the fringing forest in the Katanga by releasing large numbers of *P.berghei*-infected rodents and thus restored the enzootic. Nevertheless, by 1960 it appeared that the solution of the problem of cyclical transmission of *P.berghei* and of the tissue phase of this parasite were at an impasse. The inability to breed and to maintain *A.dureni* in the laboratory, and the difficulty of raising a colony of *Thamnomys* in captivity, were the main obstacles.

It was at this stage that the inspired work of Meir Yoeli opened new vistas for research on rodent malaria. In December 1963 Yoeli collected 1300 engorged female *A.dureni* from the fringing forest of Kisanga and took them to New York. They were kept in small cardboard containers at temperatures comparable to those in their natural habitat. A second generation of mosquitoes was raised without difficulty and, in addition, a thriving colony of *T.surdaster* was established. While working in the Congo, Yoeli and his colleagues were struck by the climatic conditions of the gallery forest as a major ecological factor in the life of the mosquito vector and in the transmission cycle of *P.berghei*. The environmental temperature in the forest was about 22°C, while outside the temperature was 30–31°C. In this much cooler natural environment *A.dureni* showed an oocyst rate of 23% and a sporozoite rate of 13%. It became obvious that *P.berghei* in the biotope of the fringing forest undergoes its sporogonic development in the natural vector at a temperature much lower than the one previously judged necessary for cyclical transmission. This observation of Yoeli's, striking in its simplicity, provided the clue for most of the future achievements. *Anopheles quadrimaculatus*, a North American mosquito, could now be used for

cyclical transmission and produced regularly heavy oocyst development and salivary gland infections.

Yoeli's brilliant intuition and perseverance were rewarded by his success in transmitting cyclically several strains of *P.berghei* to various animals including *Thamnomys*, white mouse, baby rat and hamster.

The secret of this good fortune lay not only in the maintenance of infected *Anopheles* at a temperature of 19° to 21°C but also in the use of newly isolated strains of *P.berghei* (such as the NK 65) with a high number of gametocytes.

Vanderberg and Yoeli (1965) described the physiological and other characteristics related to the maintenance of the cyclical transmission. From then on the way to experimental work on the problematic tissue stages of *P.berghei* was open.

It was left to Yoeli and Most (1965) to demonstrate the presence of pre-erythrocytic schizonts of *P.berghei*, first in the liver of a baby hamster and then in young albino rats and in *Thamnomys*. A remarkable aspect of this finding was the speed of development of these tissue forms. The liver schizont becomes mature in about 50 hours after the inoculation of sporozoites.

This discovery closed one of the most exciting chapters in the history of mammalian malaria, but opened countless new opportunities for research which was gathering momentum in many laboratories throughout the world.

It is impossible to mention, however briefly, all the aspects of parasitology, immunology and chemotherapy that were investigated during the two decades following Vincke's major discovery and Yoeli's splendid success in establishing the method of cyclical transmission of the parasite.

Some 20 key papers presented at the 1964 Colloquium in Antwerp are only a small proportion of a bibliography of 530 scientific articles compiled by Jadin (1965b). During that period the range of species of rodent malaria kept increasing in numbers and in complexity. Already in 1955 a *vinckei*-like malaria parasite was found in a mouse inoculated with pooled blood from several specimens of *Praomys tullbergi* caught in the Ilobi forest in Western Nigeria. Through an unfortunate accident this strain was lost but in 1967 Killick-Kendrick rediscovered this parasite during a difficult and hazardous field study at the time of the civil war in Nigeria, isolated it, and a new *berghei*-like parasite from *T.rutilans*, though the invertebrate host of these *Plasmodium* spp. still

remains unknown. Two years earlier, Landau and Chabaud (1965) had found in Maboké, near Bangui in Central African Republic, that the same species of rodent was frequently infected with a *berghei*-like *Plasmodium* and a new species which received the name *P.chabaudi*. A new strain of *P.vinckei* was then isolated by Bafort from *A.dureni millecampsi* at Kamena in Zaïre; he adapted the parasite to transmission through *A.stephensi* and other vectors and described its pre-erythrocytic schizogony in *Thamnomys* and in mice. At about the same time, Adam and his colleagues (1966) reported the finding of rodent malaria in N'ganga Lingolo in Congo (Brazzaville); once again the main mammalian host was *T.rutilans*. These discoveries, and a recent finding by Bafort of murine malaria parasites in the Cameroun Republic, confirm that rodent malaria is widespread in tropical Africa. At the same time the taxonomic position of murine plasmodia was becoming increasingly complex. Two views were expressed: one was that these parasites represent a continuous series of one polymorphic species-complex ranging from a typical *P.berghei* to a typical *P.vinckei*: the other view, supported by Vincke, is that different evolutionary pressures related to environmental conditions have led to a high degree of speciation within the species complex of murine malaria parasites. A brave attempt at putting some order into the taxonomic conundrum was made by Killick-Kendrick (1974). In tidying up the situation, the groups *berghei* and *vinckei* were retained, and three subspecies of *berghei*-like parasites from the Lower Guinea Forest were brought together as a polytypic species of *P.yoelii*. It is certainly right and proper that the two scientists should be honoured in such a way for their immense contribution to our present knowledge.

Many recent advances are due to the studies of Irène Landau and her French colleagues. Their discoveries of a number of murine malaria parasites, first in the Central African Republic and then in the Congo, pointed to the possibility of a relationship between speciation and zoogeography of the host. The further discovery by Landau (1973) that naturally infected *T.rutilans* maintain their parasitaemia throughout life and that the development of exoerythrocytic schizonts and the pattern of parasitic relapses in some rodents can be influenced by certain external stimuli affecting the metabolism of the host–parasite system (Landau *et al.*, 1975), was of special significance.

To what extent the long term infections represent an adaptive mechanism of major importance for the evolution of the parasite

remains to be established (Landau, 1973). However, new hypotheses on the phylogeny of Coccidiomorpha of the vertebrates may be formulated. A bold scheme (Landau, 1974) suggests that this group may have evolved from parasites of the coelomic cavity or from tissues of mesoblastic origin in the invertebrates. In the course of their adaptation to the vertebrate hosts the parasites have become localized first in the tissues of the same origin but have later become adapted to more specialized tissues such as the endoderm of the digestive tract and of the hepatic parenchyma.

The presence of a large number of species of plasmodia in rodents in Africa and their transmissibility by *Anopheles* opens other fields for speculation on the evolution of plasmodia of mammals. Corradetti (1963) pointed out that parasites with a relatively low degree of specificity may develop into a new race which constitutes a potentially pre-specific phase and needs only a degree of isolation to become a species. The conditions for such an isolation within a defined parasite–vector relationship appear to be largely ecological and the restrictive mechanism in rodent plasmodia is probably dependent on temperature. The recent observation by Yoeli *et al.* (1975) and Walliker (1975) of a sudden mutation of a mild strain of *P.yoelii yoelii* into a highly virulent one after a 100 days' stay in a deep-freeze compartment is one indication of the extraordinary genetic plasticity of some malaria parasites of rodents.

Moreover the alternation of host phases in the life cycle must have a selective advantage, discarding some mutants that are detrimental to the maintenance of a new species. Garnham (1964) stressed the multitude of variables that influence the development of protozoa in their alternating hosts and the lack of any precise information on this subject.

Perhaps the new possibilities of distinguishing some infraspecific groups of plasmodia by genetic methods assisted by recognition of electrophoretic patterns of their enzymes will provide a guide to the study of speciation within this important group of Haemosporidia. We can only hope that these new observations on rodent malaria, pioneered by the Edinburgh group led by Beale, may open new vistas for the study of the evolution of malaria parasites of man (Bruce-Chwatt, 1965).

Having sketched out the fascinating history of the discovery of rodent malaria one should stress the enormous contribution that it made to our knowledge. Two areas are of particular interest in this respect:

immunology and chemotherapy. In the field of chemotherapy of malaria the discovery of rodent plasmodia fulfilled the early expectations clearly stated by Schneider *et al.* (1965), even though some scientists were initially cautious in accepting such optimistic predictions.

Over many years of chemotherapeutic studies pioneered by Kopanaris in 1911 and developed by Roehl in 1926, bird malaria was the model most commonly used. *Plasmodium relictum* and *P.cathemerium* of canaries were eventually replaced by a *P.gallinaceum* model in chicks which was easier to apply. Critics of the use of rodent malaria for experimental chemotherapy stressed that the response of rodent plasmodia to the known synthetic compounds is rather different from that of primate malarias. However, parasites of birds such as *P.gallinaceum* have similar and even greater disadvantages; in spite of that they were fully and most successfully employed for large scale drug testing programmes in the UK and, especially, in the USA during the 1940s. The full vindication of the value of rodent malaria parasites for experimental chemotherapy became obvious more recently in the USA where Rane was using *P.berghei* in a superbly organized drug testing programme that assessed some 150 000 compounds during a few years, and is now ready to handle about 50 000 compounds annually (Peters, 1970). Rane's idea of using the survival time of treated and untreated mice infected by blood inoculation as an indicator of the activity of candidate antimalarial compounds, appeared to be crude in comparison with some more sophisticated laboratory techniques, but it was uniquely suitable for a mass screening programme. At present, the possibility of standardized sporozoite infections permits the screening of causal prophylactic drugs.

Today the immense advantages of screening procedures using rodent plasmodia are fully recognized, even by those whose patience and skill overcame, some 30 years ago, all the vagaries of experimental avian malaria in chicks, canaries or other birds. No less important is the role played by rodent malaria in our pursuit of basic knowledge of the action of specific drugs and in the attempts at unravelling the mystery of drug resistance. It was the availability of rodent malaria that indicated to Peters and his group in Liverpool the mode of action of 4-aminoquinolines and the changing response of parasites to these and other drugs. It is now obvious that chloroquine induces a breakdown of RNA of the plasmodia by its action on lysosomes: the observation of haemozoin clumping is good evidence of such action on the parasite,

and the converse is an indication of incipient decrease in sensitivity. The potentiation of the sulphonamide and dihydrofolate reductase inhibitors was also confirmed in the rodent model. Moreover the model showed the change of the biochemical response of blood forms of the resistant parasite from the anaerobic to the aerobic Krebs cycle pathway in the red blood cell containing chloroquine-resistant *P.berghei.*

During the past decade the progress of immunology of malaria has been enormous. Although more and more of the new knowledge is becoming incomprehensible to the non-initiated, there is no denying that much of it is of immense promise. This applies particularly to our understanding of the mechanism of acquired immunity to malaria in man.

The principles of acquired immunity of mammals to malaria were defined by Edmond Sergent and by W. H. Taliaferro in the 1930s, though it seems that the principles are more complex than at first thought. The genetic constitution of variants within one species of a murine host, let alone differences of susceptibility between various murine species, play an important role in the course of infection.

Functional aspects of immunity in rodent malaria were explored by Zuckerman who was puzzled by the greater than expected destruction of erythrocytes in malaria infections and found an explanation of it in the autosensitization occurring as a result of red blood cells having been altered by the parasite. She also found evidence of a cellular mechanism of protection, in addition to the well known humoral mechanisms so well studied by Macgregor and his colleagues in human malaria. The specificity of an immune response in relation to the stage of the cycle of development is as striking in rodent malaria as it is in plasmodial infections of other animals. Antigenic variation of blood stages confirms Charles Nicolle's inspired prophecy of a "mosaic of antigens" and represents one of nature's intricate ways in the subtle interplay between the parasite and its host. The fruitful hypothesis of antibody-producing lymphoblastic B cells stimulated by the T lymphocytes attuned, as it were, to all the determinants of the antigens has received much support from the studies of rodent malaria.

The mystery of congenital transfer of antibodies from the immune mother to her progeny has been explored in a murine model. However, the early results that appeared to be clear are now explained in a different way and this poses more questions than it gives answers. Another important field where rodent malaria model is of value

concerns the immunosuppressive role of the plasmodial infection with the consequent stimulation of the effect of oncogenic viruses leading to the appearance of tumours, such as Burkitt's lymphoma. Considering the relationship between the mechanism of protective immunity and some pathological effects of malaria, perhaps the most significant concept that emerged from studies of rodent malaria was that the function of antibody may have a Jekyll and Hyde effect, which becomes apparent when it combines *in vivo* with a soluble antigen which results in an immune-complex disease.

Much of the present immunological research in the UK and USA is directed towards the possibility of producing a protective malaria vaccine. While this achievement is still remote, the conditions needed for cultivation of plasmodia *in vitro* are being fully explored and much of the slow and painful progress in this field is due to the availability of *P.berghei.*

There can be no doubt that malaria parasites of rodents now form one of the most important sections of parasitology and that they will open many new aspects of this scientific discipline which owes so much to Ignace Vincke and Meir Yoeli.

A book encompassing a vast amount of new research on rodent malaria is bound to raise some questions concerning its relevance to one of the main diseases which inflict untold misery on the millions of people inhabiting the tropical world. Such questions were stimulated by the recent discussions at the World Health Assembly concerning the apparent failure of the global eradication of malaria.

It is perhaps more appropriate to stress the fact that out of some 2000 million people inhabiting the originally malarious areas of the world, some 1200 million are now living in areas where malaria has disappeared or where the risk of infection is very low. Another 460 million people live in areas under a reasonably satisfactory degree of malaria control especially in urban areas. But there is no denying the grim fact that nearly 400 million people of the developing world in the tropics inhabit regions where malaria is still endemic.

The present worldwide malaria morbidity is not fully known but it amounts to at least 100 million cases per annum: the mortality, though considerably reduced even in countries where the control programmes were imperfect, is still of the order of one million, a high proportion of this figure falling on the young age groups.

The initial strategy of antimalarial campaigns was to carry out indoor

spraying of residual insecticides in a given area so widely and thoroughly that the transmission of the infection would cease. The depletion of the anopheline population together with the drastic lowering of its mean longevity would prevent the resumption of the transmission: the remaining foci and cases could be dealt with during the "consolidation phase" by case detection and treatment for which the general health services would be mainly responsible. Such a simplified outline of principles of malaria eradication gives no idea of the operational complexity of the mass campaign which depends on careful planning, excellent logistic support and adequate financing.

It is also true that the ultimate success of any mass campaign such as malaria eradication depends on its results being sustained by the basic health services. Such services are defective or wanting in most of the rural areas of the developing tropics.

It has often been said that the main obstacles to success of malaria eradication in those countries are of an administrative and managerial nature such as lukewarm government support, delays in procurement of supplies, errors of planning, inadequate basic health services, shortage of trained manpower and, last but not least, the limitation and uncertainty of financial resources.

Nevertheless, it would be wrong to ignore that although the present technical means of malaria control may be adequate at the periphery of the global geographical distribution of malaria, they are certainly insufficient to deal with the extent, volume and severity of malaria in the centre of its prevalence namely in the tropical latitudes of Africa, Asia and the Americas.

Among the major technical problems, the resistance of anopheline mosquitoes to DDT and other conventional, residual insecticides must be mentioned. Alternative compounds to which *Anopheles* show some susceptibility are considerably more expensive than chlorinated hydrocarbons and involve higher operational costs as they have to be used more frequently. Exophilic patterns of behaviour of certain vector species add to the difficulties. Resistance of plasmodia to 4-aminoquinolines, confirmed in some parts of Asia, America and the West Pacific, is of growing importance for malaria eradication and remains a grave menace for tropical medicine. Human habits that interfere with spraying or surveillance, uncontrollable population movements, and the insecurity of some areas contribute to the reappearance of new foci of transmission. None will deny that our technical means of controlling,

let alone eradicating malaria from its endemic seed-bed are inadequate. Better and more acceptable insecticides and new antimalarial drugs are needed. Whether the prospective malaria vaccine will soon materialize is a moot and distant point although the recent experimental results are encouraging. Today, when smallpox is about to disappear from the world, the fight against what Ronald Ross called "the million murdering death" has not been a success, largely because of human failures of foresight and concerted action.

Malaria is the "Dog Star fever" of Homer's Iliad. Only after the 17th century A.D. did the world discover the first remedy for "ague". It took 300 years to find new ways of attack on the mosquito vector and on the parasite. And yet after 50 years of waxing and waning promise, this disease is still prevalent in its main areas of distribution and even shows a serious degree of resurgence in many parts of the world. One of the answers to the present situation, which indicates how far we are from the intended "conquest of malaria", is intensified research to improve the technical tools at our disposal and the ways of using them to the best advantage. The past few years have shown the great promise of chemotherapeutic and immunological research in many fields. It is precisely in these fields that rodent malaria constitutes a most valuable tool for applied research, though its importance for basic knowledge of parasitology may claim an equally high priority.

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1. Taxonomy, Zoogeography and Evolution

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I. INTRODUCTION

In the ten years after the publication of Garnham's classical account of malaria parasites, the number of species of *Plasmodium* known from rodents rose from six to twelve. During this period remarkable progress was made in the study of the life cycles of malaria parasites of thicket rats and this, coupled with a new genetical approach to the study of species and subspecies of the same group based on the characterization of isoenzymes, has greatly clarified the taxonomy of the species parasitizing African murine rodents. The new work led to taxonomic revisions of several of the better known parasites which may have been

confusing to workers in other disciplines. An attempt is therefore made here to summarize the new findings and trends which are leading to changes, principally in the hope of clarifying the relationships of the species and subspecies named since Garnham's book.

Except for animals at the end of a long evolution, when modern species are usually few in number, the groupings of species into hierarchies which not only reflect current views of evolution, but are also of practical use, are not immune to change. There is a common misunderstanding that taxonomic decisions are unalterable: many are not. New species are commonly discovered or novel approaches to their study evolve which force a reappraisal. This may irritate the non systematist who, having become accustomed to the names of the animals with which he works, suddenly finds he is expected to learn new names or, an even harder task, unfamiliar combinations of old names. Systematics is, however, a dynamic discipline; nowhere is this more evident than in the study of taxa such as some of the malaria parasites of rodents which appear to be in active evolution at the present time. Animals such as protozoa, with short generation times, may well evolve so fast that changes apparent in a worker's lifetime could lead to the rare opportunity of witnessing the evolution of a new species.

Taxonomy and systematics are inextricably bound to zoogeography and evolution. For this reason these subjects are grouped together in this single chapter. It will become apparent, however, that in spite of many new findings much of that which follows is highly speculative.

II. TAXONOMY AND SYSTEMATICS

A. The Subgenus *Vinckeia* Garnham, 1964, Emend. Garnham, 1971

The family Plasmodiidae Mesnil, 1903 contains the single genus *Plasmodium* Marchiafava & Celli, 1885, in which are grouped nearly 100 species of true malaria parasites of mammals, birds and reptiles. This comparatively large genus has been subdivided into 11 subgenera (Garnham, 1966; Sarkar and Ray, 1972), three of which contain the mammalian parasites. *Plasmodium* species of primates above the rank of lemurs are grouped in two subgenera, *Plasmodium* and *Laverania*, and all

other mammalian malaria parasites, including those of rodents, form the subgenus *Vinckeia*.

At the time of writing, there are 23 species in this last subgenus of which two have been only briefly described and have yet to be named (Kuttler *et al.*, 1967; Yap *et al.*, 1970). Descriptions of new, or re-descriptions of old species and subspecies published since Garnham's (1966) account are given by Lien and Cross (1968), Killick-Kendrick (1973a, b, 1974b, 1975), El-Aziz *et al.* (1975), Carter and Walliker (1975, 1977) and Garnham and Uilenberg (1975). A recent brief report of a malaria parasite of *Peromyscus maniculatus* in the USA (Wood, 1976) may be a record of a piroplasm mistaken for *Plasmodium*. Although there appear to be no published reports of piroplasms in *P.maniculatus*, *Babesia microti* has been recorded in the blood of *P.californicus* in California, USA (van Peenen and Duncan, 1968). In the absence of a full description, Wood's report is here ignored.

The subgenus *Vinckeia* as defined and later revised by Garnham (1964, 1966, 1973a) is now far from satisfactory and with the demonstration of the life cycles of four species of *Plasmodium* of murine rodents and new descriptions, it is becoming clear that *Vinckeia* is not a coherent phylogenetic taxon. Based primarily on a morphological study of gametocytes, supported by the phylogeny of mammalian hosts and the fragmentary knowledge of exoerythrocytic schizonts, Landau *et al.* (1976) have divided 40 species of Haemosporina into 3 groups each of which contains representatives of *Vinckeia*. One difficulty in accepting such a revolutionary approach is that the morphology of gametocytes of many species of Haemosporina changes so rapidly after blood is shed that comparisons are best made on material prepared by a single worker in standard conditions. Of the 40 species, gametocytes of 12 were in material prepared by the authors, 22 were in diverse slides in the collections of P. C. C. Garnham and W. Peters and 6 were characterized from published descriptions. Nevertheless, although conclusions based on such varied material require confirmation, Landau *et al.* (1976) propose a phylogenetic tree of the evolution of Haemosporina which is a considerable contribution to the continuing debate on the origins of malaria parasites. Since in the new groupings they ignore subgenera, their novel proposals do nothing to resolve dissatisfaction with *Vinckeia*.

One at present insuperable obstacle to a revision of the subgenus is that the type species is a poorly known parasite of the water buffalo,

P.(V.)bubalis Sheather, 1919. It was chosen because it was one of the first malaria parasites of mammals other than primates above the lemurs to be described and named.* Subgenera, like genera, are defined by the characters of the type species but a number of important criteria of *Vinckeia* apply only to species better studied than *P.bubalis*. Thus, in the diagnosis the prepatent period following the inoculation of sporozoites is given as 3 days or less (Garnham, 1964), about 48 h (Garnham, 1967) or just over or just under 2 days (Garnham, 1973a). But this is known to be true for only four species, *P.berghei*, *P.yoelii*, *P.vinckei* and *P.chabaudi* (Yoeli, 1965; Landau and Killick-Kendrick, 1966; Bafort, 1967); the prepatent period of the type species of *Vinckeia* is not known.

Similarly, in the definition of *Vinckeia* the presence of "secondary" exoerythrocytic schizonts was considered typical of the subgenus. Although there is reason to suppose that such forms may be part of the life cycle of *P.yoelii* (see p. 80), there is no evidence for their presence in *P.bubalis* nor, indeed, in any other species of the subgenus with the possible exception of *P.traguli* of the mouse deer (see Garnham, 1966).

With the publication of new or revised descriptions of the erythrocytic stages of parasites of thicket rats or anomalures, another character of the subgenus is no longer tenable. Erythrocytic schizonts were described, with few exceptions, as small with 8 or less merozoites. However, ten of the 21 named species, and one of the two as yet unnamed, are exceptions (Table I), and the morphology of these stages is no longer a valid character separating *Vinckeia* from the other two subgenera of mammalian malaria parasites.

Vinckeia has now become a taxonomic quandary with no immediate prospect of solution. One answer would be the demonstration of the life cycle of *P.bubalis* followed by a new definition of the subgenus based more clearly on the characters of the type species. Ill-defined species could be classified in *Vinckeia incertae sedis* pending the study of their life cycles and isoenzymes. The better known parasites, *P.berghei*, *P.yoelii*, *P.vinckei* and *P.chabaudi*, might well be found to differ sufficiently for this apparently closely related group to be moved to a new subgenus.

Poorly known malaria parasites of rodents other than murines are generally accepted as readily recognizable species. This is partly because so little is known of them that possible overlap with the

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* *Plasmodium (V.) cephalophi* Bruce, Harvey, Hamerton & Bruce, 1913, of the duiker (*Sylvicapra grimmia*) was the first.

Table I

Species of *Vinckeia* grouped according to the size and number of merozoites of the erythrocytic schizonts

Small schizonts or schizonts with < 8 merozoites	Large schizonts or schizonts with > 8 merozoites
bubalis (water buffalo)	*cephalophi* (duiker)
girardi (lemurs)	*brucei* (duiker)
foleyi (lemurs)[a]	*voltaicum* (bat)
sandoshami (colugo)	*anomaluri* (*Anomalurus*)
traguli (chevrotain)	*pulmophilum* (*Anomalurus*)
rousseti (bat)	*landauae* (*Anomalurus*)
atheruri (porcupine)	*watteni* (*Petaurista*)
booliati (*Petaurista*)	*Plasmodium* sp.[b] (*Petaurista*)
chabaudi (thicket rats)	*berghei* (murine rodents)
aegyptensis (*Arvicanthis*)	*yoelii* (thicket rats)
	vinckei (murine rodents?)

[a] *P.(V.)lemuris* Huff & Hoogstraal, 1963 is a synonym of *P.(V.)foleyi* Bück, Coudurier & Quesnel, 1952 (Garnham and Uilenberg, 1975).
[b] Yap *et al.* (1970).

characters of related parasites is concealed, and partly because they are exotic oddities of interest to few. The situation with the intensively studied murine malaria parasites is markedly different. Isolates have been made available to any interested worker, and strains are now found in scores of laboratories in dozens of countries. As is obvious from the contents of this book, intensive study is illuminating many of the corners of the parasites' biology and enough has now been revealed for differences of opinion to have arisen over the taxonomic status of several different populations of malaria parasites of murine rodents. Before summarizing what now appears to be a consensus of opinion, it is helpful to consider the concepts of species and subspecies of animals as a whole and to judge to what degree they apply to malaria parasites, in particular to those of rodents.

B. The Species Problem

The conventional view of an animal species has arisen from studies on metazoa, and doubt is sometimes expressed as to whether or not such a view is applicable to protozoa (e.g. Carter, 1951). But the general

conclusions reached in metazoan taxonomy inevitably affect the thinking of protozoologists (Corliss, 1967), and notions of species of protozoa have always been related to those of the metazoa; indeed no other concept has gained universal acceptance. When difficulties have been met by protozoologists, new terms applicable to populations of protozoa, such as Sonneborn's syngens or Hoare's various demes, have been coined.

Efforts to find an absolute definition of the species taxon, whether of metazoa or protozoa, are comparable to the search for the Philosopher's Stone and as likely to succeed. There is no hard and fast definition; there are bound to be border-line cases because evolution is often gradual, and because species appear to arise in more than one way (Huxley, 1942). Darwin (1872) commented that "no one definition [of species] has satisfied all naturalists; yet every naturalist knows vaguely what he means when he speaks of a species". Tate-Regan (1926) and Cain (1963) pragmatically suggested that a species was simply a taxon defined by a competent systematist; this is, however, an over simplification.

The first species of animals to be named by the Linnean system were, like palaeontological species, wholly phenotypic in that they were based on morphology alone; if an animal looked different from another it was given a different name. From the time of Wallace and Darwin, the idea slowly grew of the "biological species" which, in addition to morphological and general biological characters, greatly depended upon the ability or lack of it for populations to interbreed freely in natural conditions; the important point became whether a population shared, or could potentially share, the gene pool of another. Mayr (1963) defined species as "groups of actually or potentially interbreeding populations which are reproductively isolated from other such populations". This broadly summarizes the views of most leading systematists (e.g. Dobzhansky, 1937; Huxley, 1942; Emerson, in Riley, 1948; Sheppard, 1967). Some, however, have said that definitions based on abilities to interbreed in nature are applicable only to metazoa (Carter, 1951).

It is seldom possible to find out if an animal will interbreed with related groups and, in practice, a species is generally recognized by morphological and other biological characters which are sufficiently marked to make it reasonable to assume that the animal does not share the gene pools of related populations. In difficult groups precise

methods of investigating genotypes, such as observing the electrophoretic mobility of enzymes or the buoyant densities of DNA, or mapping chromosomes, are valuable tools.

Several difficulties arise when attempting to apply modern definitions of a species to the protozoa. The greatest of these is the position of agamospecies, an aspect outside the scope of the present account. Definitions of species based on the inability to share other gene pools obviously do not apply to such organisms (see Poljansky, 1977).

A second difficulty, even with protozoa such as malaria parasites in which genetic recombination occurs, is the question of distribution. Huxley (1942) thought that a species should have a "geographical area consonant with a single origin". With parasites, this must be interpreted in the light of the distribution, habits and phylogenetic relationships of the hosts. The zoogeography of hosts may be the key to the origins and relationships of a parasite rather than simply the geographical area in which the parasite is now encountered. Examples are *Trypanosoma evansi* of the dromedary and *Polychromophilus deanei*, a haemoproteid of Neotropical bats, the identities and taxonomic positions of which were not understood until the zoogeography, and in particular the migrations, of their hosts were considered (Hoare, 1957; Garnham *et al.*, 1971).

To sum up, there seems to be little difficulty in applying the concept of the genospecies to the parasitic protozoa which reproduce sexually. As Manwell (1937) said of malaria parasites, "we should conceive of a species of malaria in genetic terms as we do most other types of living things. It should be a strain of malaria plasmodium which will not hybridize with others in the invertebrate host, and it should, of course, have certain more-or-less unique morphological characters". At present it is seldom practicable to test the ability to interbreed, and reliance is usually still placed upon the phenotype and biological characters other than interbreeding. New methods of recognition of enzymes of protozoa pioneered by Tait (1970) and Carter (1970) are, however, more precisely measurable expressions of genes than are many characters of the phenotype, and are becoming more widely used.

C. Trinomial Nomenclature

Trinomials were first used by the ornithologist Hermann Schlegel in 1844 to designate geographical subdivisions of species of birds (Sibley,

1954). Since that time subspecies have been a constant cause of polemic (see Wilson and Brown, 1953 and later papers on subspecies by other authors in *Systematic Zoology*, 1953–1958), and there is perhaps no aspect of the taxonomy of malaria parasites which causes more argument and strong feeling than the use of trinomials. Much of this feeling is tilting at windmills: there are circumstances when subspecific designations appear to be not only helpful in providing a universally understood name for an infraspecific population, but also to reflect current thinking on evolutionary positions and relationships.

The subspecies problem is partly one of temperament: "splitters" like them, "lumpers" do not. Prolonged study of a group of animals may lead a specialist to the point when he confidently gives subspecific status to certain populations, but workers in other specialities may find it difficult to accept his conclusions. The validity of a trinomial depends to some degree upon the reputation of the taxonomist who gives the name (Tate-Regan, 1926) and, since the recognition of an infraspecific population is often a nice question of judgement, perhaps that is as it should be.

The subspecies is the lowest taxon given formal recognition in the International Rules of Zoological Nomenclature. It has been defined by many workers, and, although it is sometimes thought to be an arbitrary subdivision of a continuum (Simpson, 1945; see also Huxley, 1942), the present generally accepted view is that a subspecies is a population or group of populations inhabiting a geographical subdivision of the range of a species and differing from other populations by diagnostic morphological characters (Mayr, 1963). It follows that a subspecies of a free-living animal cannot be sympatric with other subspecies of the same species, since their identities would be lost by interbreeding.

As with the distribution of vertebrates (Huxley, 1942), the distribution of parasitic protozoa may be either geographical or ecological. There are instances in which the first step in speciation may be considered to have taken place when a parasite has acquired a strong host restriction (e.g. *T.lewisi* of rats and *T.musculi* of mice). Such a parasite may be found in a vertebrate host in the same place as another which harbours a related parasite. One factor giving rise to this ecological separation of sympatric populations could be marked feeding preferences of two haematophagous vectors resulting in parallel evolution in the same locality. Subspecies of parasites could possibly be sympatric, but only if they were parts of different biocenoses, and did not have

hosts in which genetic exchange took place in common. In this respect the concept of subspecies of parasites differs from that of Mayr's (see Garnham, 1973b). The mobility of hosts led Manwell (1957) to reject Mayr's largely geographical definition of the subspecies when applied to parasites, but if the host is considered to be part of the "geography" of a parasite, Mayr's definition would seem to be acceptable to the parasitologist.

There are a few observations which give an idea of the time taken for a subspecies to appear in populations isolated from others of the same species. A population of the normally marine seal *Phoca vitulina* isolated in a fresh-water lake in Canada 3000–8000 years ago is now recognized by its morphology as a subspecies. This period represents 300–1600 generations. Scattered data for rodents isolated on islands suggest that subspecific differentiation may occur in even less than 300 generations—the lower figure for the seal (Simpson, 1944). If it took as few as 300 generations for a morphologically recognizable subspecies of a malaria parasite to arise, and if there were 5 generations from zygote to zygote each year, the time taken would be only 60 years; with continual cyclical passage in the laboratory it could be as short as 17 years. Final separation into species with a discrete gene pool, if indeed this point in evolution is reached, may be assumed to take longer. Speculations on the evolution of the malaria parasites of Neotropical monkeys (Coatney *et al.*, 1971) suggest that changed selective pressures on an isolated population of a mammalian malaria parasite may lead to the evolution of a new species in only a few hundred years. This may not, however, be invariable since genetic polymorphism is important in the ability to exploit new habitats and in the response to new selective pressures; lack of genetic plasticity would presumably hinder or prevent the evolution of new species.

The vagueness of the subspecies is a reflection of evolution. Darwin (1872) thought in terms of a hierarchy of infraspecific populations leading towards, but not necessarily attaining, the rank of species. A subspecies will always be a vague taxon but while it is a useful means of naming a population it will, no doubt, continue to be used.

D. Species and Subspecies of Murine Malaria Parasites

For reasons given above (p. 4) the species of malaria parasites of rodents other than murines are here ignored; they have been little

studied and, since none of their complete life cycles has been seen, there is insufficient material available for critical comparisons to be made.

In considering the murine parasites, the enigmatic species *P.aegyptensis* Abd-el-Aziz, Landau & Miltgen, 1975 of *Arvicanthis* in Upper Egypt is also excluded. It is undoubtedly a true species of *Plasmodium*, but its provenance is uncertain and it is impossible to speculate on its relationships to others. Levine (1971) thought that another mysterious parasite of a murine rodent, *Anthemosoma garnhami* Landau, Boulard & Houin, 1969 of *Acomys*, should be considered as a malaria parasite but, for reasons given elsewhere (Killick-Kendrick, 1974a), it is preferred that this parasite is left for the moment in the comparative obscurity of the family Dactylosomidae. This conservatism is supported by recent observations by H. Momen (personal communication) who found that the buoyant density of the DNA of *Anthemosoma* differed greatly from that of malaria parasites (and piroplams) of murine rodents. The unequivocal malaria parasites of murine rodents fall into three groups, *berghei*, *vinckei* and *chabaudi*, the complete taxonomic citations of which are given in Table II together with their known distribution.

The blood stages of the *berghei* group are morphologically indistinguishable and are notable for their marked predilection for immature erythrocytes in the blood of experimentally infected laboratory rodents (Garnham, 1966). Parasites of this group (*P.berghei* and *P.yoelii* sspp.) are readily distinguished by this character from *P.vinckei* sspp. and *P.chabaudi* sspp., neither of which has a predilection for immature erythrocytes (Carter and Walliker, 1975, 1977).

Until recently, the separate identities of *P.vinckei* and *P.chabaudi* were far from clear. In the original description (Landau, 1965), *P.chabaudi* was named as a species, but was later reduced to a subspecies of *P.vinckei* by Bafort (1968); this revision at first received general support (Landau *et al.*, 1970; Garnham, 1973b; Carter, 1973; Killick-Kendrick, 1974a). However, in recent studies of the isoenzymes of many new isolates from the Central African Republic (CAR), Carter and Walliker (1974) found it possible to separate the parasites of mature erythrocytes into two readily recognizable groups by the electrophoretic mobilities of the enzymes GPI, 6PGD and LDH. A close examination of the morphology of the blood stages revealed that the two parasites were conventionally distinguishable by, principally, the appearance of the trophozoites and schizonts and, having confirmed their observations by studying clones, Carter and Walliker concluded that the material on

Table II

Geographical distribution of species and subspecies of African murine malaria parasites

Places	Groups of parasites			
	P.berghei group	*P.vinckei* spp.	*P.chabaudi* spp.	Other
Katanga	*P.berghei* Vincke & Lips, 1948			
	P.yoelii sp.(?)[a]	*P.v.vinckei* Rodhain, 1952		
CAR	*P.y.yoelii* Landau & Killick-Kendrick, 1966	*P.v.petteri* Carter & Walliker, 1975	*P.c.chabaudi* Landau, 1965 *partim* Carter & Walliker, 1975	
Brazzaville	*P.y.killicki* Landau, Michel & Adam, 1968	*P.v.lentum* Landau, Michel, Adam & Boulard, 1970	*P.c.adami* Carter & Walliker, 1977	
Nigeria	*P.y.nigeriensis* Killick-Kendrick, 1973	*P.v.brucechwatti* Killick-Kendrick, 1975		
Cameroons	*P.yoelii* ssp.[b]	*P.vinckei* ssp.[b]		
Kenya	*P.yoelii* ssp.[c]			
Egypt				*P.aegyptensis* Abd-el-Aziz, Landau & Miltgen, 1975

[a] Peters *et al.* (1978); Chance *et al.* (1978).
[b] Bafort (1977); M. Wéry (personal communication).
[c] B. Gardener (personal communication).

which Landau (1965) based the original description of *P.chabaudi* must have been a mixed infection of *P.chabaudi* and *P.vinckei*. They re-described *P.chabaudi* and described and named the *vinckei*-like parasite from CAR *P.vinckei petteri*. It is clear that *P.vinckei* and *P.chabaudi* must now be accepted as distinct species which, since they retain their identities in the same localities and vertebrate host, are presumed not to interbreed (Carter and Walliker, 1975).

The new observations on the murine malaria parasites of CAR were

confirmed by the results of a similar study on the isoenzymes and morphology of the malaria parasites of thicket rats from Brazzaville. Once again, in addition to a subspecies of *P.yoelii*, Carter and Walliker (1977) found two parasites with no predilection for immature erythrocytes. One was named as a new subspecies of *P.chabaudi* (*P.c.adami*), while the other was recognized as an already named subspecies of *P.vinckei* (*P.v.lentum*).

In a third enzootic lowland locality, Nigeria, only two isolates of a parasite of mature erythrocytes have been studied. Both are clearly *P.vinckei* for which the name *P.v.brucechwatti* has been proposed (Killick-Kendrick, 1975). It is tempting to speculate that if more isolates are studied from this locality, another subspecies of *P.chabaudi* may well be found.

1. *The* berghei *group*

Seventeen years after *P.berghei* was named, an obviously related parasite of thicket rats of the Central African Republic was discovered and, a year later, described and named as a subspecies namely: *P.berghei yoelii* Landau & Killick-Kendrick, 1966. There followed the discovery of a third parasite, this time from Brazzaville, which was again named as a subspecies of *P.berghei* namely: *P.b.killicki* Landau, Michel & Adam, 1968. Yet a fourth was found in a thicket rat in Nigeria and named *P.b.nigeriensis* Killick-Kendrick, 1973.

These four populations of *berghei*-like parasites have one important character in common; the blood forms are morphologically indistinguishable. Their separation into subspecies was at first based on morphological differences in the sporogonic stages, differences in rates of growth of exoerythrocytic schizonts in standard conditions, geographical distribution and differences in hosts (Landau and Killick-Kendrick, 1966; Garnham *et al.*, 1967; Landau *et al.*, 1970).

One authority was at first unwilling to recognize the subspecies and postulated that the strains studied represented a continuous spectrum extending from *P.berghei* on one side to *P.vinckei* and related parasites on the other (Bafort, 1971). The principal problem at the time was the difficulty of reconciling some of the observations made in different laboratories due, in some degree, to differences in methods of demonstrating and examining stages of the life cycle; conservatism and a dislike of trinomials may also have been an influence.

A major taxonomic advance was the characterization of isoenzymes of murine parasites used as markers in the study of the genetics of *Plasmodium* (Chapter 5). Laboriously obtained conclusions on the differences of phenotypes were rapidly confirmed, thus leading to a taxonomic revision of the *berghei* group. This was heralded at a round table discussion on the taxonomic problems relating to malaria parasites held in Bethesda, Ma. USA on 22–24 November, 1971, where participants in general felt that the biochemical analyses of malaria parasites of murine rodents had confirmed the presence of species and subspecies (Garnham, 1973b). The highland parasite, *P.berghei* from Katanga, was seen to be separate from the lowland subspecies (*yoelii*, *killicki* and *nigeriensis* from the Central African Republic, Brazzaville and Nigeria respectively) and, three years later, the three lowland populations were moved from species *berghei* and placed as subspecies of the elevated species *yoelii* (Killick-Kendrick, 1974b). The present position, therefore, is that *P.berghei* Vincke & Lips, 1948 is thought to be a monotypic species restricted, as far as is known, to the highlands of Katanga: and the allopatric lowland subspecies, *P.y.yoelii* Landau & Killick-Kendrick, 1966, *P.y.killicki* Landau, Michel & Adam, 1968 and *P.y.nigeriensis* Killick-Kendrick, 1973 are grouped together in another species. The differences in the species *berghei* and *yoelii* are summarized in Table III. The subspecies of *P.yoelii* are distinguishable by morphological characters other than those of the blood stages, their geographical distribution and differences in isoenzymes, notably GDH (see Table IV). Interbreeding between subspecies of *P.yoelii* (Chapter 5) confirms the infraspecific position of these populations. There is no report of successful hybridization of *P.berghei* and *P.yoelii*.

Another locality in which a *berghei* group parasite is enzootic was discovered in the eastern part of the Republic of Cameroun by Bafort (1977). An isolate from a wild rat* was established in laboratory mice and cryopreserved, and from a study of its life cycle M. Wéry (personal communication) concludes it is *P.yoelii*; its subspecific designation awaits a study of its isoenzymes.

The apparent restriction of *P.yoelii* to lowland localities of the Lower Guinea Forest may not be as absolute as it appears. By chloroquine

* Bafort (1977) isolated two strains of malaria parasites—one of the *berghei* group and another of the *vinckei* group—from two rodents in the Cameroun. One rodent was *Thamnomys* sp. and the other was *Hylomyscus* sp., but in his report there is no indication which parasite came from which rodent.

selection, Peters *et al.* (1978) repeatedly selected lines with isoenzymes like those of *P.y.nigeriensis* from stock derived from the type strain of *P.berghei* (K173) and strain NK65, both of which were originally isolated in Katanga. Hybridization of DNA (Chance *et al.*, 1978b) and cross-immunity studies, however, revealed that the drug-selected lines (NS) were not identical to the only available strain (N67) of the Nigerian parasite. They postulated that the original isolate must have been a mixture which has survived for more than thirty years in the laboratory. Accidental contamination in the laboratory, of which Peters *et al.* (1978) were well aware, is not a plausible explanation of their observations. No lines with enzymic similarities to *P.yoelii* were

Table III

Principal differences between *P.berghei* and *P.yoelii*

	P.berghei	*P.yoelii*
Distribution	Highlands of Katanga Province, Republic of Zaire	Lowlands of parts of West and Central Africa, including CAR, Brazzaville and Western Nigeria
Vertebrate hosts	*Grammomys surdaster*; also *Praomys jacksoni* and *Leggada bella*	*Thamnomys rutilans*
Invertebrate hosts	*Anopheles dureni millecampsi*	*?A.cinctus*
Optimum temperature of sporogony	<21°C	24°C
Mean diameter of mature oocysts	<45 μm	60 μm or more
Mean length of sporozoites	<13 μm	>14 μm
Normal minimum maturation time of tissue schizonts in white rats	>50 h	<50 h
Mean diameter of 50-h-old tissue schizonts in white rats	<30 μm	>30 μm
Electrophoretic forms of enzymes of blood stages[a]		
GPI	3	1 or 2
6PGD	1	4
MDH	2	1
AK	2	1
HK	2	1
GDH	3	1, 2 or 4

[a] In the *berghei* group, no differences have yet been found in the enzyme LDH, several forms of which have been demonstrated in subspecies of *P.vinckei* and *P.chabaudi* (Chapter 5).

From Killick-Kendrick (1974).

Table IV

Principal differences between named subspecies of *P.yoelii* in standard conditions

	P.y.yoelii	*P.y.killicki*	*P.y.nigeriensis*
Distribution	CAR	Brazzaville	Western Nigeria
Mean diameter of mature oocysts	75 μm	60 μm	60 μm
Mean length of sporozoites	15 μm	14–15 μm	17 μm
Mean diameter of tissue schizonts in liver of white rat			
at 36 h	21 μm	18 μm	20–25 μm
at 50 h	37 μm	35 μm	42–50 μm
Effect on nucleus of infected parenchymal cell	Not enlarged	Usually enlarged	Usually enlarged
Electrophoretic form of enzyme GDH	4	1	2

From Killick-Kendrick (1974).

selected from two other strains of *P.berghei* (ANKA and RLL). They conclude that there may be a relict population of *P.yoelii* ssp. which is sympatric with *P.berghei* in parts of Katanga, but that this hypothesis requires confirmation by a study of new isolates.

The present classification of the *berghei* group is neat and plausible, but it is based on too few isolates. Although the isoenzymes of more than 16 isolates of *P.y.yoelii* have been examined, only one strain of *P.y.nigeriensis* and two of *P.y.killicki* are available. In spite of this, as a result of the detailed studies of the murine malaria parasites there is now more information on their phenotype and genotype than any other group of malaria parasites, mammalian, avian or saurian. They are thus in the forefront of taxonomy and systematics of *Plasmodium*, and the genetical studies of the workers in Edinburgh are clarifying the concepts of species and infraspecific populations of malaria parasites; their work has also stimulated the characterization of populations of several other parasitic protozoa of man and domestic animals by examining their isoenzymes (e.g. Godfrey, 1976; Miles *et al.*, 1977; Chance *et al.*, 1978a).

2. P.vinckei *and subspecies*

Four years after *P.berghei* was described and named, a second malaria parasite of rodents was found in Katanga. It was named *P.vinckei* and, with the later discovery of parasites placed as subspecies of this species,

the Katangan parasite became the nominate subspecies, *P.vinckei vinckei*. Many years after the first isolation Bafort (1967) rediscovered the parasite in its type locality and established a second strain in the laboratory. Both strains originated from isolations made from wild-caught specimens of *Anopheles dureni millecampsi* and it is curious that, in spite of intensive studies by Belgian workers, the nominate subspecies, unlike other subspecies of *P.vinckei*, has never been found in a naturally infected rodent. However, since *A.d.millecampsi* is thought to feed solely or principally on rodents and, furthermore, *P.v.vinckei* is highly infective to laboratory mice, it is confidently assumed that the natural vertebrate host of this parasite is a sylvatic murine rodent from the gallery forests of the highlands of Katanga. Rodhain (1952) suggested that the probable host was *Grammomys surdaster*, the principal, though not sole, rodent host of the sympatric parasite, *P.berghei*.

The second *vinckei*-like parasite to be discovered was found in Nigeria in 1954 by Bruce-Chwatt and Gibson (1955). The rodent from which it was isolated was reported to be *Praomys tullbergi*, but Bruce-Chwatt and Gibson's inability to find the parasite again by the routine inoculation of blood from over 1000 specimens of *P.tullbergi* from the same locality into laboratory mice (Killick-Kendrick, 1973a), and the later finding of an apparently identical parasite in 6 out of 18 thicket rats (Killick-Kendrick *et al.*, 1968) led to the suggestion that Bruce-Chwatt and Gibson's rodent was a misidentified specimen of *Thamnomys rutilans* (Killick-Kendrick, 1975). Two strains from thicket rats were studied in naturally infected rats, laboratory mice and experimentally infected *A.stephensi*, and the writer (1975) then named the Nigerian *vinckei*-like parasite *P.v.brucechwatti* in honour of one of its discoverers. *P.v.chwatti* Garnham, 1973, an earlier name for the Nigerian parasite, was published without a description or bibliographical reference to a description and, on these grounds, was declared a *nomen nudum* in accordance with the International Code of Zoological Nomenclature (Killick-Kendrick, 1975).

Before the identity of the Nigerian parasite was determined, Adam *et al.* (1966) found a *vinckei*-like parasite in *T.rutilans* captured near Brazzaville, a discovery which stimulated the later work in Nigeria. The complete life cycle of the parasite from Brazzaville was described, and it was aptly named *P.vinckei lentum* because of the comparatively slow development of its exoerythrocytic schizonts (Landau *et al.*, 1970). Carter and Walliker (1976) redescribed the sporogonic and erythrocytic

stages of *P.v.lentum* and examined the isoenzymes of four isolates. Their findings confirmed the parasite's closeness to *P.v.vinckei* and enabled them to differentiate the strains from Brazzaville from a superficially similar sympatric subspecies of *P.chabaudi* (see p. 12).

The most recent subspecies of *P.vinckei* to be described and named was first found, but not recognized, in the CAR in 1964 by Landau (1965). As recounted above (p. 10), in studies on the isoenzymes of new isolates of murine malaria parasites from the CAR, Carter and Walliker (1975) detected two series of distinct electrophoretic mobilities in parasites which, since they had no predilection for immature erythrocytes, were at first thought to be *P.chabaudi*. A close examination of the erythrocytic stages revealed the presence of a parasite distinguishable from *P.chabaudi* by, principally, the morphology of the trophozoites and schizonts. Carter and Walliker (1975) noted that the illustrations accompanying the first description of *P.chabaudi* showed a *vinckei*-like parasite mixed with undoubted *P.chabaudi* and they then described and named the newly recognized parasite of thicket rats of the CAR *P.v.petteri*. Mixed infections of *P.v.petteri* and *P.c.chabaudi* were common, and Carter and Walliker found it necessary to confirm their observations by studying clones.

Blood stages of the four subspecies of *P.vinckei* are indistinguishable. Minor morphological differences in the sporogonic and exoerythrocytic stages which permit identification of the subspecies are discussed by Killick-Kendrick (1975). From the extensive studies of Carter and Walliker (1975, 1977) a more certain way of distinguishing the subspecies would appear to be by a study of the isoenzymes (Table V). All

Table V

Forms of isoenzymes of blood stages of subspecies of *P.vinckei* and *P.chabaudi*

Species	Subspecies	Origins	No. of isolates examined	Enzymes			
				GPI	6PGD	LDH	GDH
vinckei	*vinckei*	Katanga	2	7	6	6	6
	lentum	Brazzaville	4	6,11	5	7,9	6
	petteri	CAR	4	5,9	5	7	6
	brucechwatti	Nigeria	2	6	6	9	6
chabaudi	*chabaudi*	CAR	22	4	2,3,7	2,3,4,5	5
	adami	Brazzaville	2	8	2	8,10	5

subspecies of *P.vinckei* share the common form GDH-6, a feature which clearly separates *P.vinckei* spp. from *P.chabaudi* spp. *P.v.brucechwatti* shares 6PGD-6 with *P.v.vinckei* from which it may be distinguished by differing forms of GPI and LDH. *P.v.lentum* and *P.v.petteri* are separable by differences in the forms of GPI, but share the same form of 6PGD and have one form of LDH in common. Both differ from *P.v.vinckei* by the forms of GPI, 6PGD and LDH.

P.vinckei has also been found in the eastern part of the Republic of Cameroun where Bafort (1977) isolated a single strain from a wild rat (see footnote to p. 13). M. Wéry (personal communication) has studied the sporogonic and exoerythrocytic stages of the life cycle of this parasite and has confirmed its identity. As the isoenzymes have not yet been examined, this parasite has not been assigned to a subspecies.

3. P.chabaudi *and subspecies*

The reasons for restoring *P.chabaudi* to full specific status, rather than placing this parasite as a subspecies of *P.vinckei*, are given on p. 10. In her original description, Landau (1965) had no hesitation in naming *P.chabaudi* as a species, but the then current, somewhat rigid, separation of murine malaria parasites into two categories (*berghei* and *vinckei*) based on a predilection or otherwise of erythrocytic stages for immature red cells, coupled with a common mixture of *P.chabaudi* with unrecognized infections of *P.vinckei* misled workers into rejecting full specific status for *P.chabaudi*. The discovery of the differences in isoenzymes of *P.chabaudi* and *P.vinckei* was followed by the realization that, although neither parasite exhibits a predilection for immature erythrocytes, they may be readily distinguished by the morphology of their blood stages (Carter and Walliker, 1975, 1977).

At the time of writing, *P.chabaudi* has been found in only two localities in Africa. The nominate subspecies, *P.c.chabaudi*, is a parasite of thicket rats (*T.rutilans*) in the CAR (Landau, 1965; Carter and Walliker, 1975), and *P.c.adami* is found in the same host near Brazzaville (Carter and Walliker, 1977).

A full comparison of all stages of the life cycles of the two subspecies has not yet been made, and it is not therefore known if there are morphological differences between *P.c.chabaudi* and *P.c.adami*. No important differences in their erythrocytic and sporogonic stages were found in the detailed comparisons made by Carter and Walliker (1977).

The recognition of the parasite from Brazzaville as a new subspecies is based on differences in the patterns of isoenzymes of *P.c.chabaudi* and *P.c.adami* (Table V) which, from extensive studies on the other murine malaria parasites and on zoogeographical grounds, are sufficiently marked to warrant taxonomic recognition if all members of the three groups of African murine malaria parasites are to be treated similarly. Both subspecies have a common form of GDH (GDH-5), but differ in the forms of GPI, 6PGD and, most notably, LDH. This last enzyme displays a wide range of differences in subspecies of *P.chabaudi* and *P.vinckei*, but is apparently constant in the *berghei* group (Carter, 1973).

III. ZOOGEOGRAPHY AND EVOLUTION

The diversity of the murine malaria parasites suggests that they are in a state of active evolution. On zoogeographical grounds and on the assumption that their present geographical distribution is seldom or never contiguous with that of another subspecies of the same species, it appears as if subspeciation is taking place in discrete populations which, it may be assumed, are subjected to different selective pressures in spite of, in some instances, somewhat similar habitats. It cannot, however, be inferred that new species will necessarily evolve from allopatric populations at present judged to be sound subspecies. There may be occasions, no matter how rare, when the geographical range of a population of these malaria parasites temporarily overlaps with that of a related parasite with which interbreeding is possible. Such changes in distribution could be caused by, for example, variations in climate or by increases in the populations of either vertebrate or invertebrate hosts leading to migrations and changes in distribution.

If, on the other hand, ecological barriers such as those it has been suggested exist between the murine malaria parasites of Nigeria and those of other parts of the Lower Guinea Forest (Killick-Kendrick, 1973a) are never now breached, it can be anticipated that speciation may well continue to the point where interbreeding between clearly related populations may no longer be possible. By definitions of species which, although principally devised for metazoa, seem equally to apply to gamospecies of protozoa (p. 7), new species will then be seen to have arisen. The phenotypic and genotypic plasticity of the murine malaria parasites, revealed in the detailed morphological and genetical

studies reviewed in Chapters 2 and 5, suggests that this diverse group probably has sufficient genetic polymorphism to respond to varying selective pressures to a point in evolution at which isolated populations could be assumed to have attained specific status. The chance of this point being reached is doubtless not the same for each presently recognized subspecies. It does, however, already appear to have been attained within the *berghei* group in which two obviously related species—*P.berghei* from highland forests and *P.yoelii* from lowland forests—are now thought to have diverged sufficiently far for cross-breeding now to be unlikely or impossible. The same drift may now be taking place between two or more of the subspecies of *P.vinckei* or *P.chabaudi*.

To create plausible hypotheses of the recent evolution of the murine malaria parasites, the present distribution of the parasites and that of their vertebrate and invertebrate hosts must be considered. When contemplating the early evolution of this group of parasites, it is necessary to search among the malaria parasites of other African small mammals for possible relationships, and to assess the possible effects of changes in the climate and geology of the continent on the delicately balanced biocenoses of rodent, parasite and vector. Among the more important lacunae which inhibit confident speculation are the following:

1. the vectors of all lowland subspecies of murine malaria parasites are unknown;
2. knowledge of the true distribution, both geographical and hostal, of malaria parasites of murine rodents and other African small mammals is incomplete (there are still parts of the continent unsurveyed);
3. virtually nothing is known of the migrations of the mammalian hosts;
4. much of the information on the geological history and climatic changes of Africa is highly speculative.

In spite of these formidable gaps, an attempt is made here to seek patterns in the morphology and distribution of the parasites of African mammals and in the evolution and habits of their hosts, and to suggest ways in which the murine malaria parasites might have evolved.

A. Distribution of Murine Malaria Parasites

1. Geographical distribution

The known distribution of murine malaria parasites is limited to the continent of Africa and, with one exception, to that part of the continent south of the Sahara desert. Although a number of surveys of the blood parasites of murine rodents have been undertaken in other parts of the World, which have resulted in many reports of haemogregarines and piroplasms in the blood of these and other rodents (Killick-Kendrick, 1974a), there are still no unequivocal records of murine malaria parasites from anywhere except Africa. It is certain that they will not be found in the New World because, except for introduced species of *Rattus* and *Mus*, murid rodents are absent from North and South America. It is, moreover, unlikely that other rodents will be found to be hosts of malaria parasites in the New World since there is a remarkable paucity of plasmodiids of mammals other than man and monkeys in that continent. The species parasitizing man in North and South America are thought to have been introduced, and the two species in Neotropical monkeys probably arose from *P.vivax* and *P.malariae* of man (Coatney *et al.*, 1971). For reasons given above (p. 3), Wood's (1976) report of a malaria parasite of *Peromyscus* in the USA is considered to be so doubtful that, without confirmation, it must be ignored.

It is less easy to understand the apparent absence of murine malaria parasites from Asia. In this continent plasmodiids are known in apes, monkeys, the water buffalo, the chevrotain, the colugo and, among rodents, in sciuromorphs—petauristine flying squirrels of the genera *Petaurista*, *Petinomys* and *Hylopetes* in Peninsular Malaysia and *Petaurista* in Taiwan (see Killick-Kendrick, 1974a). Nevertheless, no malaria parasites of murids or other myomorphs have yet been discovered in Asia, in spite of surveys which have revealed many other blood parasites.

In the reasonably well surveyed parts of Africa—Katanga, Western Nigeria, southern Central African Republic and Brazzaville (Figure 1) —there are two or three species of murine malaria parasites in each place. In the CAR and Brazzaville, *P.yoelii*, *P.vinckei* and *P.chabaudi* are parasites of the thicket rat, *T.rutilans*, in which animals mixed infections are common. In Nigeria, *P.yoelii* and *P.vinckei* co-exist in the same

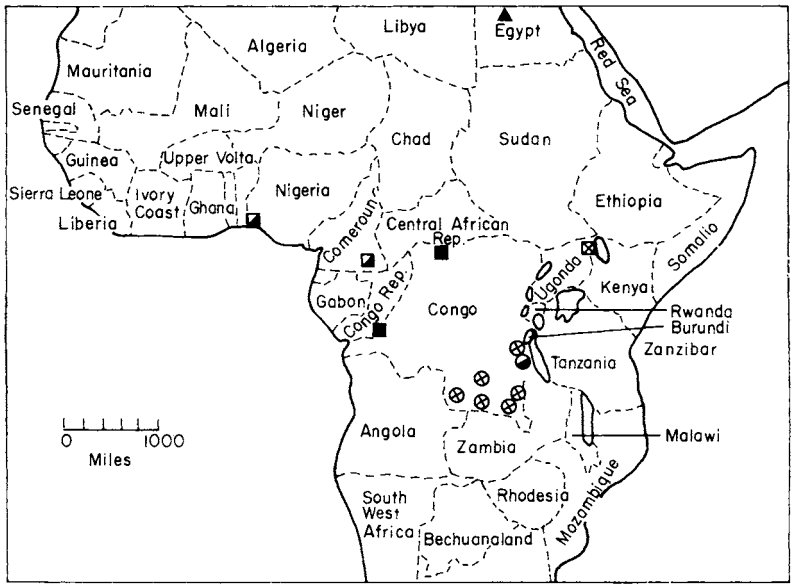

Figure 1. Map of Africa showing localities in which murine malaria parasites have been found. ⊗, *P.berghei*; ◒, *P.berghei* and *P.vinckei*; ⊠, *P.yoelii* (?); ◪, *P.yoelii* and *P.vinckei*; ■, *P.yoelii*, *P.vinckei* and *P.chabaudi*; ▲, *P.aegyptensis* (Killick-Kendrick, 1971, 1974a, b, 1975; Bafort, 1977; Abd-el-Aziz *et al.*, 1975; Carter and Walliker, 1975, 1977; P. J. Gardener, personal communication).

species of rat, but *P.chabaudi* has not yet been found. In Katanga, where Belgian and American workers have carried out extensive surveys, *P.berghei* is the common species infecting another thicket rat (*Grammomys surdaster*), *P.vinckei* is apparently rare and *P.chabaudi* is unknown. There is some evidence that the distribution of *P.yoelii* may overlap with that of *P.berghei* in the highlands of Katanga (Peters *et al.*, 1978; Chance *et al.*, 1978), but this requires confirmation. The subspecific identities of *berghei*-like parasites found in two other localities, the Cameroons and Kenya, have not yet been determined although from the morphology of the blood stages in laboratory mice both are thought to be in the species *P.yoelii*. The subspecific names of parasites, the distribution of which are shown in Figure 1, are listed in Table I. The record of *P.aegyptensis* from Assuit, Egypt, is based on a single blood film and it is not possible to speculate on the relationship of this species to the better known murine malaria parasites.

With the exception of *P.aegyptensis*, the African murine malaria parasites have been found only around the periphery of the Lower

Guinea (or Congo) Forest which covers the Congo Basin; it extends from Katanga in the south to the CAR in the north, and from Nigeria in the west to Kenya in the east. Zoogeographically, the recent finding by P. J. Gardener (personal communication) of a malaria parasite of the *P.berghei* group in a rodent from the Kakamega Forest in Kenya is of special interest. Some of the flora and fauna of this forest are isolated West African forms (Garnham *et al.*, 1945) and, in spite of its high altitude (1500–1700 m), this forest is considered as the eastern boundary of the "Congo Forest" (Milne-Redhead, 1954). The explanation of this modern East African island of West African animals and plants (now mixed with more dominant East African forms) is that, about 25 000 to 18 000 years ago, there was a link of montane forest between the Cameroons and the Kenya Highlands (Moreau, 1966). The connection was a result of the climate of Africa at that period which permitted montane forest to flourish at an altitude as low as 500 m rather than, as now, of 1500 m (Figure 2). Perhaps Gardener's parasite is a relict population of a West African species of *Plasmodium* which became isolated when the link between the West and East was broken by a change in climate and a reduction in montane forest with a consequent intrusion of lowland flora and fauna.

Bafort's (1977) *P.berghei*-like parasite of the Cameroons was found at a low altitude and, from the limited information at present available, an especially close relationship between this parasite and the one from Kenya cannot be assumed on the grounds of the continuous stretch of montane forest which once existed between the two sides of the continent.

From the present knowledge of the distribution of the murine malaria parasites other than *P.aegyptensis*, several patterns emerge. Of the four species, parasites of the *P.berghei* group, represented by *P.yoelii* in the well prospected lowland localities of the CAR, Brazzaville and Nigeria and *P.berghei* in the highlands of Katanga, have a wide but focal distribution with, in some places, a high prevalence. Subspecies of *P.vinckei* have the widest distribution and are the most prevalent in the lowland localities. By contrast, in the highlands of Katanga *P.vinckei* appears to be rare. *P.chabaudi* has the most limited distribution with subspecies at present known only from the CAR and Brazzaville.

In studies on the distribution of animals in Africa (and their more recent evolution), account must be taken of the profound changes in terrain, climate and vegetation which took place in the Early and

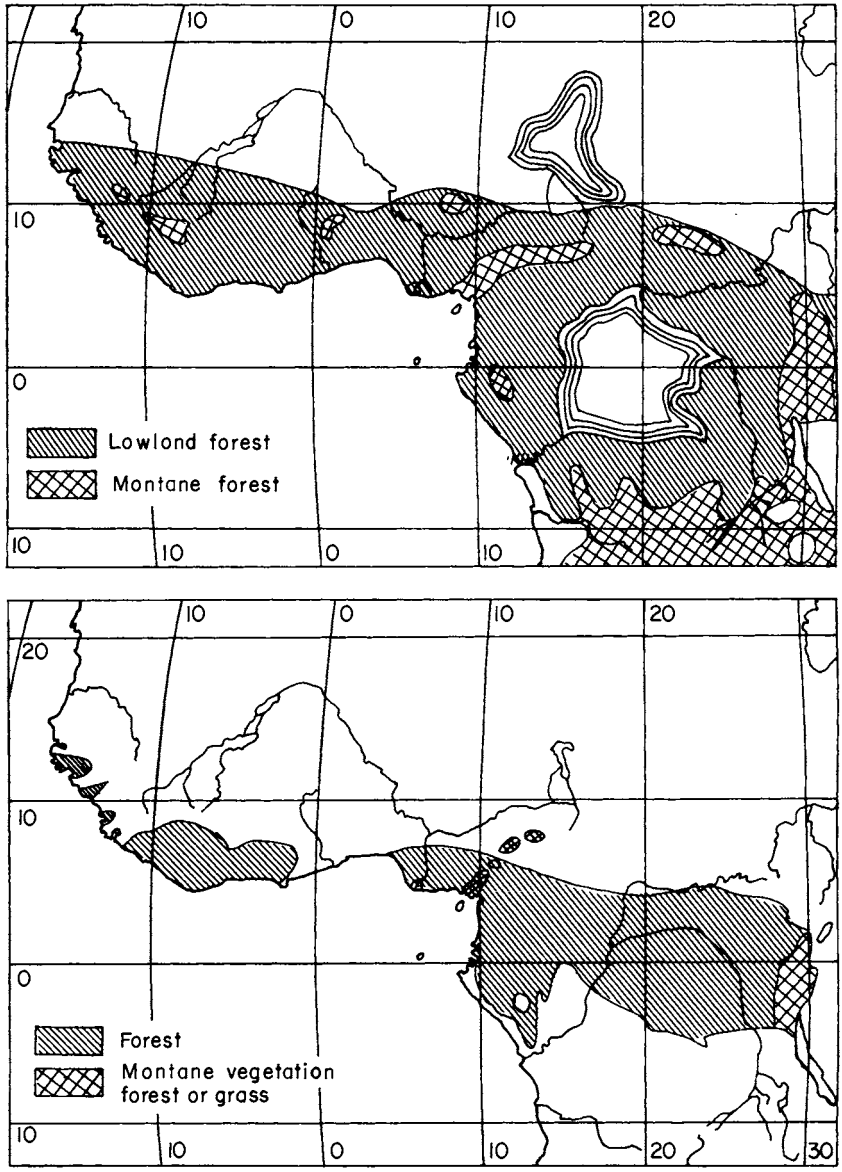

Figure 2. (*upper*) Map showing the presumed montane vegetation in West Africa in the Pleistocene; about 22 000 years ago, the montane vegetation is thought to have been continuous from West to East Africa, and the lowland forests of the Upper and Lower Guinea were divided; note the presumed absence of forest in the Congo Basin. (*lower*) Map showing present rain forests of West Africa; the Dahomey Gap between the Upper and Lower Guinea Forests is believed to be less than 22 000 years old, and may be as recent as only 12 000 years (Rand, 1951, after Chapin). (Courtesy Field Museum of Natural History, Chicago.)

Middle Pleistocene (Moreau, 1963). An explanation then emerges for the present distribution of the malaria parasites to the periphery of the Congo Basin and a time can be fixed by which murine plasmodiids may be assumed to have evolved. From soil surveys it is known that near the end of the Middle Pleistocene, about 75 000 to 25 000 years ago, almost all the vast Congo Forest of today was covered by Kalahari sand redistributed by wind. It is unimaginable that this could have happened under a forest climate and at this time the Basin must have been a semi-arid area utterly different from today (Moreau, 1966). This dryness would have profoundly affected the fauna of the previously forested area. Moreau (1966) points out that the sylvatic animals and plants survived in only a few refuges, namely:

1. in relict forest along rivers;
2. on the eastern edge of the Basin where the dryness would have been ameliorated by rainfall at high altitude;
3. towards the still wet Atlantic Coast of the Gabon and the Gulf of Guinea;
4. towards the northern edge of the Basin.

It is remarkable that these are the very areas in which murine malaria parasites are found today. Moreover, in two localities, Katanga and Brazzaville, the landscape epizootiology of murine malaria is characterized by forest galleries. In the former locality, a later period of dryness in this montane area in the second part of the Upper Pleistocene, 35 000 to 10 000 years ago (Moreau, 1963), would have been a selective advantage to rodents and mosquitoes already well adapted to a riverine habitat, and the framework within which murine malaria parasites could survive and evolve in this locality may well have been reinforced.

A possible series of events explaining the present distribution of the murine malaria parasites is, therefore: the parasites or the stock from which the modern species have arisen evolved in the Congo Basin some time before 75 000 years ago; changes in climate led to the desiccation of the Basin; the sylvatic fauna, including the rodent and mosquito hosts of the parasites, survived only in refuges to the north, east and west of the Basin and in the highlands of Katanga; the Basin became re-afforested and was re-invaded by forest animals from the periphery, but the delicately balanced biocenose of rodent–parasite–mosquito did not become re-established in the middle of the Congo Basin. The

pattern of the modern distribution of murine malaria parasites was then set.

There is, however, an alternative explanation of the present distribution which dates the cause very much earlier than the Middle Pleistocene. At the end of the Miocene, about 12 million years ago, the Congo Basin was filled by a vast lake (Chapin, 1932; Moreau, 1966). If the murine malaria parasites had occupied the Basin before this time, a peripheral distribution could have been a consequence of the formation of the lake. Murid rodents branched off from a cricetid stock in the Miocene (Wood, 1959), and it is probable that anopheline mosquitoes had evolved by this time. Nevertheless, the apparent absence of malaria parasites of myomorphs in the Neotropics suggests that the malaria parasites of these animals probably did not arise long before the final separation of the Old and New Worlds brought about by continental drift. Since the drift was progressive and took place over a long period, it is difficult to decide when faunal barriers arose which would have affected the evolution of terrestrial animals; however, the final separation perhaps took place only about one million years ago (Tarling and Tarling, 1971). The formation of a lake in the Congo Basin in the Miocene seems, therefore, far too early to have been the cause of the modern distribution of the murine malaria parasites, which had probably not evolved by that time. Desiccation of the Congo Basin in the Middle Pleistocene is a more plausible explanation.

The notion that populations of the three of the four species of murine malaria parasites, those of the Lower Guinea Forest, are correctly classified as subspecies is based partly on the assumption that their ranges seldom or never overlap, and that there are, or have been, faunal barriers of greater or lesser importance between the known foci. The degrees of isolation, however, seem unequal. *P.berghei* and *P.v.vinckei* of Katanga are found only at altitudes of 1000–1700 m and the flora, fauna and climate of this montane locality are so different from those of the lowland localities that ecological isolation of the Katanga parasites is easily accepted. The possibility of the range of the essentially lowland parasite, *P.yoelii*, extending into the highlands of Katanga seems unlikely. However, by drug pressure Peters *et al.* (1978) and Chance *et al.* (1978) selected lines (NS) from two strains of *P.berghei* which they concluded had many of the characters of *P.yoelii*. Their tentative identification of the new line was made before the discovery by Gardener of a parasite which is probably *P.yoelii* from another

highland locality. If these unexpected observations are confirmed, it appears as if the distribution of *P.yoelii* is even wider than previously supposed. On present evidence *P.yoelii* can be regarded primarily as a lowland parasite with, possibly, rare relict populations in a few highland localities.

Western Nigeria is the most isolated of the lowland localities. Present faunal barriers between the forest there and other parts of the Lower Guinea Forest include the Cameroon Highlands, the River Sanaga, the River Cross and the Niger delta, all of which have profoundly influenced the distribution of mammals, birds and tree-frogs of the coastal forest of West Africa (see Killick-Kendrick, 1973a).

Zoogeographically, Nigeria may be considered either as the western limit of the Lower Guinea Forest (Chapin, 1932) or as the eastern limit of the Upper Guinea Forest from which it is presently separated by the recently formed Dahomey Gap (Rand, 1951) (Figure 2). If it is accepted that the murine parasites evolved in the Congo Basin, an early spread to Nigeria may have at first been prevented by the link of montane forest at the northern rim of the Congo Forest which joined the eastern and western highlands of Africa. A spread would also have been hampered by a barrier formed by the proximity of Lake Mega-Chad to the Bamenda Highlands and Mandara Mountains (Figure 2). It is difficult to date the Mega-Chad, but it is believed to have been at its maximum size about 22 000 years ago (Moreau, 1966). The disappearance of the link of montane forest was accompanied by a dimunition in the size of the lake. These two changes would then have extended the potential range of the Congolese fauna westwards into Nigeria, but would not have allowed unimpeded passage. Even today the gap between Mount Cameroon, near the sea, and the mountains to the north-east is only about 16 miles wide (Rand, 1951), and other faunal barriers further west of the Cameroons (Killick-Kendrick, 1973a) appear to have impeded the westward spread of a number of different West African vertebrates. The later formation of a break in the coastal forest—the Dahomey Gap—left the forest of Southern Nigeria linked to the Lower Guinea Forest, but separated from the Upper Guinea Forest. The barriers between the Nigerian habitat of murine malaria parasites on the one hand, and the enzootic localities in the Cameroons, the CAR and Brazzaville on the other, though not individually insurmountable, are collectively formidable. It follows, therefore, that the range of the Nigerian parasites now seldom or never overlaps with

the others, and the relative isolation has led to the evolution of subspecies which well fit Mayr's (1963) geographical definition of the taxon.

In the Congo Basin, there are no notable faunal barriers between the localities in the CAR and near Brazzaville. Both localities lie in the watershed of the River Congo, and no cogent argument for a separation on geographical grounds can be made. Perhaps the apparent subspeciation of murine malaria parasites in these two localities will be better understood when the vectors are known. At the moment, however, it is notable that these are the only two localities in which *P.chabaudi* has been found; moreover, the morphological differences between *P.y.yoelii* of the CAR and *P.y.killicki* of Brazzaville are the least marked of any pair of subspecies of *P.yoelii* (Killick-Kendrick, 1974b). On these grounds, it can be assumed that the degree of isolation between the CAR and Brazzaville is, and has for some time, been much less than between any other two of the localities. It would, however, be absurd to expect identical degrees of separateness between all of the places, and subspeciation could be expected to proceed in populations of genetically polymorphic malaria parasites with ranges which seldom overlap. Selective pressures in the CAR and Brazzaville differ; in the latter locality, the habitat is in gallery forest, but in the former it is secondary forest. On balance, therefore, it may be concluded that, although there are differences in the measure of geographical isolation of the murine malaria parasites all, with the exception of *P.berghei* (which may well have evolved from *P.yoelii* stock), are sufficiently separate for subspeciation to be expected and, indeed, to have taken place.

2. *Hostal distribution*

Restriction of a parasite in nature to a narrow range of hosts can be a strong selective pressure. The distribution of the murine malaria parasites in both their vertebrate and invertebrate hosts is, therefore, a factor which must be examined to understand the present point of evolution of the parasites.

Although some clear differences in hostal distribution are apparent, confident speculation on their influence on speciation is undermined by the belief that the list of known vertebrate hosts is almost certainly incomplete and, moreover, by the fact that the identity of the vector or

vectors of the parasites in all localities other than Katanga are unknown. It is nevertheless, possible to draw some conclusions which support the general notion that varying degrees of isolation, geographical and ecological, are leading to speciation of the parasites.

a. Vertebrate hosts. Murine plasmodiids are principally parasites of thicket rats. Among many thousands of murids examined in Africa, only few others have been found infected. In the CAR the only known host is the thicket rat, *T.rutilans*. Other rodents have been examined in the CAR, but no parasites have been found; in contrast, almost every adult thicket rat captured near the experimental station of La Maboké, near Bangui, was infected with one or more species of *Plasmodium* (Landau and Chabaud, 1965). A lower but nevertheless high prevalence was recorded by Carter and Walliker (1975) who examined 50 specimens of *T.rutilans* from the same locality. They found *P.chabaudi* in well over one-half of the animals, *P.y.yoelii* in about one-half and *P.v.petteri* in about one-quarter; infected animals commonly harboured more than one species of parasite. Although it cannot be said that other species of rodents are never hosts of malaria parasites in the CAR, the surveys undertaken would certainly have revealed any with a prevalence of infection approaching that of thicket rats.

Less is known of the rodent hosts of the parasites from Brazzaville and, since the only forest gallery in which infected rodents have been trapped is being cleared for farming, studies on the epizootiology of murine malaria in that locality are no longer possible. As in the CAR, *T.rutilans* is the only rodent known to harbour malaria parasites in this locality. Adam *et al.* (1966) examined single blood films from 24 thicket rats and diagnosed infections in ten; they found no other infected rodents.

The most detailed results of surveys are from Western Nigeria where, by the inoculation of blood into laboratory mice, Bruce-Chwatt and Gibson (quoted by Killick-Kendrick, 1973a) examined more than 1500 rodents from the type locality at Ilobi and over 1000 from another nearby secondary forest at Agege. A further 40 rodents from Ilobi were similarly examined by Killick-Kendrick *et al.* (1968). Six out of 18 *Thamnomys* were infected with *P.v.brucechwatti* and one was additionally infected with *P.y.nigeriensis*. Although the single *Praomys* Bruce-Chwatt & Gibson, 1955 found infected with *P.v.brucechwatti* is now thought to have been a misidentified thicket rat (Killick-Kendrick, 1975), the possibility

that it was a rare infection in an unusual host cannot be totally dismissed. However, since over 1000 *Praomys* from Ilobi and more than 500 from Agege were found to be uninfected, it can be safely assumed that *Praomys* is not a common host of malaria parasites in Western Nigeria. Although the numbers of other rodents examined were smaller, the same conclusion can be reached for five other species, namely, *Lemniscomys striatus*, *Hybomys trivirgatus*, *Stochomys longicaudatus*, *Malacomys edwardsi* and *Lophuromys sikapusi*. African forests are remarkably rich in murine rodents, and even in Nigeria new hosts may yet be found; a number of species, notably *Leggada bella*, have never been examined. On present evidence, however, it seems as if *T.rutilans* is the principal and probably the sole vertebrate host of *P.y.nigeriensis* and *P.v.brucechwatti* at Ilobi.

In the recently discovered lowland locality in the Cameroun, the identity of the single rodent infected with a parasite of the *P.berghei* group has not yet been determined (see footnote to p. 13).

T.rutilans is absent in highland localities (Figure 5) and in Katanga the commonest host of *P.berghei* is another thicket rat, *G.surdaster*. Unlike the lowland localities, there are two other rodents which are natural hosts in Katanga. By the inoculation of blood into laboratory mice, Vincke (1954) diagnosed infections of *P.berghei* in 7 out of 61 *Grammomys* (11·5%) and 5 out of 99 specimens of *Praomys jacksoni* (5·1%). He also reported that *Leggada bella* was a natural host of *P.berghei*, but gave no details of the prevalence of infection in this tiny forest mouse. The vertebrate host of the second murine malaria parasite of Katanga, *P.v.vinckei*, is unknown; the only two isolations of this parasite were from wild-caught mosquitoes (Rodhain, 1952; Bafort, 1967).

In the second highland locality, Kenya, the single rodent P. J. Gardener (personal communication) found infected with a *P.berghei* group parasite was identified as *Praomys jacksoni*—one of the hosts of *P.berghei* in Katanga.

The restriction of murine malaria parasites to only a few species of rodents in nature is not because of a lack of infectivity to all but a few murids. On the contrary, the parasites have been shown to have a surprisingly wide range of infectivity which is not, however, the same for each of the four species. The range of hosts experimentally infected by the inoculation of sporozoites of *P.berghei* or *P.yoelii* includes, among others, white mice, laboratory rats, hamsters, multimammate rats and laboratory-bred thicket rats (*G.surdaster*) (see Chapter 2). Sporozoites of

P.y.yoelii are also infective to rabbits and guinea pigs, but the tissue schizonts in the liver fail to mature (Wéry *et al.*, 1968).

The range of infectivity of sporozoites of *P.vinckei* spp. and *P.chabaudi* spp. is less wide. Laboratory rats and hamsters are insusceptible, but mice, multimammate rats and *G.surdaster* are all readily infected; *P.v.lentum* (and probably other subspecies), is infective to *Hybomys* (Landau *et al.*, 1970). Thus, although the infectivity of sporozoites of *P.vinckei* and *P.chabaudi* appears to be limited to fewer species of rodents than that of *P.berghei* or *P.yoelii*, it is by no means confined to the natural host.

The ability of murine malaria parasites to infect a wide range of hosts has been taken as evidence that the restriction in nature to only a few species of rodents has little relevance to the evolution of subspecies (Bafort, 1970). This view was based on the results of inoculating infective blood, which has revealed an astonishingly long list of hosts susceptible to *P.berghei* (see Cox, 1967; Killick-Kendrick, 1975). These results, however, have less relevance to the natural distribution than the observations on the infectivity of sporozoites which, nevertheless, reveal that there must be many murine and cricetine rodents in the enzootic localities which, although susceptible, are never found infected. Presumably the explanation lies in marked preferences of the vectors in the selection of the animals on which they will feed. This must surely be considered as a selective pressure since, from the differences in hosts in highland and lowland localities, it is clear that the rodents preferred by the vectors in these two areas are not the same. The wide range of infectivity of the parasites can be viewed as evidence of adaptability and genetic plasticity of the parasite, similar to the morphological and enzymic polymorphism so characteristic of murine malaria parasites.

b. Invertebrate hosts. The only known vector of murine malaria parasites is *A.d.millecampsi*, the invertebrate host of *P.berghei* and *P.v.vinckei* in Katanga. This rodentophilic mosquito has a distribution limited to the high altitudes of that part of Africa (Figure 3), and is not, therefore, the vector of *P.yoelii*, *P.chabaudi* or the lowland subspecies of *P.vinckei*.

In the locality near Brazzaville, Adam (in Killick-Kendrick, 1971) found small numbers of unusually long sporozoites in the salivary glands of a single specimen of *A.cinctus*. J. P. Adam kindly showed me

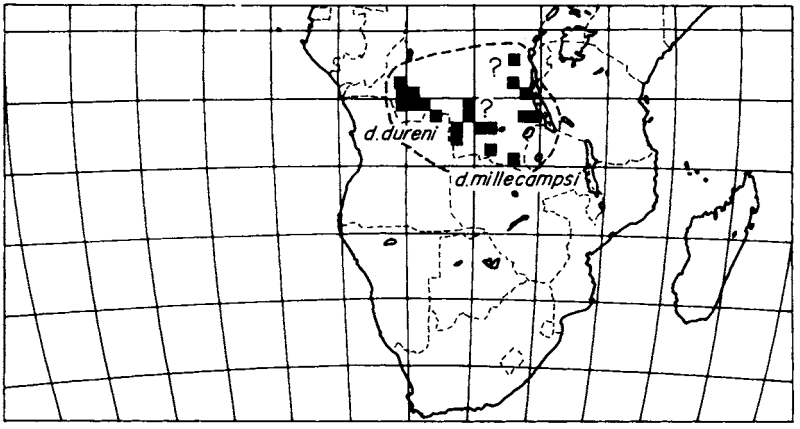

Figure 3. The geographical distribution of *A.d.millecampsi* (Gillies and de Meillon, 1968).

photographs of four of these sporozoites, and pointed out that they bore a striking resemblance to the long sporozoites of *P.v.lentum* of that locality. Evidence incriminating *A.cinctus* is slightly weakened because this species is thought to feed on galagos and to transmit a trypanosome of these primitive primates (Frezil and Adam, 1971). The vector or vectors in the lowland localities would be expected to feed solely or predominantly on thicket rats (see p. 39). Galagos do, however, share the arboreal habitat of *T.rutilans* (and are often taken in traps set for thicket rats), and a mosquito preferentially feeding above ground could be in close enough contact with both animals to be a vector of parasites of both.

Three species of zoophilic anophelines other than *A.cinctus* have been recorded in one or other of the three well known lowland localities, namely *A.coustani*, *A.paludis* and *A.obscurus* (Landau and Killick-Kendrick, 1966; Adam *et al.*, 1966; Killick-Kendrick *et al.*, 1968). Mattingly (1973) has noted that the few known vectors of malaria parasites of mammals other than man are grouped in the series Neomyzomyia of the subgenus *Cellia*. Of the mosquitoes listed above, only *A.cinctus*, like *A.d.millecampsi*, is in this series (both in the *ardensis* section). Moreover, *A.cinctus* occurs in all the lowland places where murine malaria parasites have been found (Figure 4). In spite of the assumption that *A.cinctus* feeds sufficiently often on galagos to act as a vector of a trypanosome, Adam's observation and the closeness taxonomically of *A.cinctus* to *A.d.millecampsi* suggest that this mosquito

could be a vector of *P.yoelii*, *P.chabaudi* or *P.vinckei* in one or more of the lowland localities. There are, however, reasons for believing that the vector is more likely to be a mosquito which feeds solely on thicket rats (see p. 39).

As with vertebrate hosts, it may be argued that a constant difference in invertebrate hosts is a powerful selective pressure. Bafort (1970) belittles the possible effect of a natural restriction of vectors on subspeciation, and compares the transmission of murine malaria parasites with that of the parasites of man which have many anopheline hosts. It is difficult to see the relevance of this comparison. Malaria parasites of mammals other than man are characteristically one member of a delicately balanced biocenose of mammal–parasite–mosquito in sylvatic or cavernicolous habitats markedly different from the typically peri-domestic habitat of the parasites of man.

The range of infectivity of murine malaria parasites to anopheline mosquitoes is much less than it is to vertebrates. Many of the species of *Anopheles* established in the laboratory are partially or totally refractory to infection (Killick-Kendrick, 1971) and, even in the mosquito which is the best experimental vector (*A.stephensi*), the

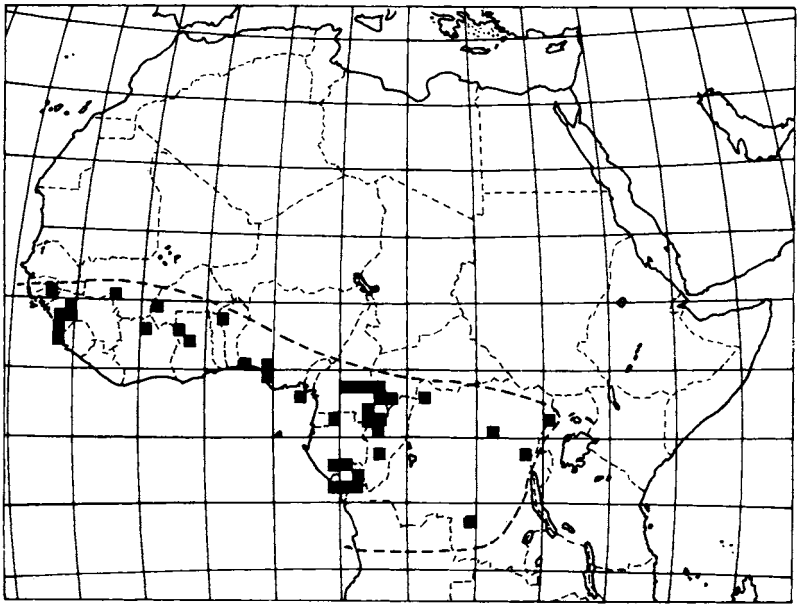

Figure 4. The geographical distribution of *A.cinctus* (Gillies and de Meillon, 1968).

comparatively low number of sporozoites which invade the salivary glands of the mosquito (Wéry, 1968) suggests that the conditions for sporogony in this surrogate host are not ideal. The massive infections of sporozoites of *P.berghei* in the salivary glands of *A.d.millecampsi*, the parasite's natural vector (see Yoeli *et al.*, 1965), are not seen in other species of mosquitoes. This inability to flourish in mosquitoes which are not the natural vectors suggests that a restriction in nature to perhaps no more than one vector in any given place has left a mark on the parasites. It is, therefore, reasonable to suppose that the difference in vectors in the highland and lowland localities has been, and still is, one of several selective forces in the speciation and subspeciation of the murine malaria parasites. Until the vectors of the lowland subspecies are discovered, this argument is too speculative to apply to subspeciation of *P.yoelii* or *P.chabaudi*.

B. Habits of Hosts

Bray (1974) developed the concept that the relationships between invertebrate and vertebrate hosts, and the biotype of which they are part, form a web of causation (MacMahon and Pugh, 1970) within which vector-borne parasites evolve and flourish. To speculate on the origins and evolution of the murine malaria parasites it is illuminating to look at the biocenoses in this way and, in particular, to consider what is known of the habits of the hosts with special reference to possible points of physical contact between the vertebrate and invertebrate.

With the exception of one of the parasites of the Cameroun and *P.berghei* of Katanga, the principal and probably sole host of murine malaria parasites in Africa south of the Sahara is the thicket rat, *T.rutilans*, the distribution of which is shown in Figure 5. Genest-Villard (1972) described this rodent as the model of a murid highly specialized for tropical forest conditions. Superficially it is a typical rat in appearance, with a body length of about 140 mm; its tail is naked, tipped with hair and longer than the body. As with all thicket rats, its hind feet are longer (about 24 mm) than most terrestrial murids of the same size (Kingdon, 1974; see also Rosevear, 1969); this is an adaptation to its arboreal habitat.

The typical habitat of *T.rutilans* is thick secondary forest, or forest galleries, with abundant lianas, small shrubs and trees. Genest-Villard's (1972) study of the behaviour of this thicket rat in its natural

habitat in the CAR includes many observations pertinent to the role of *T.rutilans* as a host of malaria parasites. Fortunately, its ecological niche is much more clearly defined than that of most other sylvatic African rodents. It is a highly arboreal animal and probably spends not more than one-tenth of its active time at ground level where it is very seldom taken in traps. Its nest, made of shredded leaves, is built in bushes or trees 2 to 3 m from the ground (Rosevear, 1969; Killick-Kendrick, 1971; Genest-Villard, 1972). *T.rutilans* is strictly nocturnal, and passes the day in a lethargic state within the nest. Except when mothers have young, it is a solitary animal with a defended territory; such behaviour encourages dispersal (Genest-Villard, 1972).

Figure 5. The geographical distribution of *T.rutilans* (Kingdon, 1974).

The nocturnal activity of *T.rutilans* is nicely governed by humidity and ambient temperature, two factors likely to influence the biting activity of the unknown vector. As the ambient temperature falls after sunset, the relative humidity rises and reaches near saturation at about 2100 h. This is the trigger for *T.rutilans* to leave the nest; occasionally an animal may leave as early as 1800 h, but it then either quickly re-enters the nest, or remains immobile on a branch among leaves until the humidity rises. Throughout the night, until about 0500 h, the rat's activity is broken by periods of rest in the nest of 15–30 min duration. At the low nocturnal temperature of the habitat (21°C), *T.rutilans* is unable to maintain its body temperature and is forced to warm itself in the nest at regular intervals. While out of the nest, it has periods of inactivity lasting as long as 30 min when it remains immobile in branches of the trees and shrubs (Genest-Villard, 1972).

The activity cycle of *T.rutilans* compared to that of other rodents studied in similar conditions is remarkable in several ways which perhaps explain why, although there are a number of susceptible species of rodents in the same macrohabitat, the thicket rat is the only one which is commonly infected with malaria parasites. Notable features include:

1. the total period of activity is unusually long;
2. activity is triggered by a rise in humidity;
3. the animal's body temperature must be maintained by regular periods of withdrawal into the nest;
4. when outside the nest, there are periods of immobility which may last as long as 30 min.

Since the closely woven nest would not easily be invaded by mosquitoes,* contact between the vector and vertebrate presumably takes place in conditions of high humidity some time between 2100 h, when the rat's nocturnal movements begin, and 0500 h, when it finally returns to the nest until the next evening. The vector, although unknown, can be assumed to feed on the thicket rat during its periods of inactivity outside the nest.

Further speculation on the habits of the vector can be made from the vertical distribution of *T.rutilans* compared with that of other murids in the same macrohabitat, examples of which are shown in Figure 6. Two main factors, height and rain, seem to govern selection of the niches. If the vector fed mainly at one height and at a particular humidity, rodents other than *T.rutilans* in the same macrohabitat might never be in sufficiently close regular contact with the vector—a vital thread in Bray's web of causation—for them commonly to acquire infections. Their susceptibility to infection would, therefore, be irrelevant. If the habits and habitats of the vector and the thicket rats match as closely as suggested, it is possible that the mosquito would develop a degree of dependence on this particular rat as a source of blood, which would reinforce the association and further reduce the chances of other species of rodents becoming infected.

In Katanga, where *T.rutilans* is absent, another thicket rat, *Gram-*

* Killick-Kendrick, Otitoju and Lambo (unpublished observations) collected twelve nests of *T.rutilans* in plastic bags at Ilobi, Nigeria, and searched for mosquitoes resting during the day; none was found.

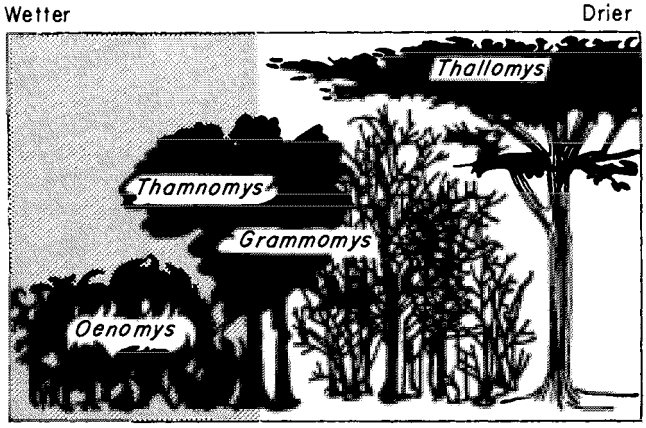

Figure 6. The vertical distribution of *Thamnomys*, *Grammomys*, *Thallomys* and *Oenomys* in their forest habitats in relation to rainfall (Kingdon, 1974).

momys surdaster,* is the principal rodent host of *P.berghei*. The distribution of this rat and its close relatives (Figure 7) is wider than that of *T.rutilans*, with which there is overlap, but nowhere except in Katanga has it been found infected with malaria parasites. This suggests that the distribution of *P.berghei* (and *P.v.vinckei*) is restricted by the limited range of the vector, *A.d.millecampsi* (Figure 3).

Although less is known of the behaviour of *G.surdaster* than of *T.rutilans*, the wider distribution of the former species and its relatives of the *dolichurus* group, and their ability to thrive in many varied habitats except high forest indicate that thicket rats of the *dolichurus* group (including *G.surdaster*) are less specialized than *T.rutilans*. This is supported by the few observations on its behaviour discussed by Genest-Villard (1972) and Kingdon (1974). Like *T.rutilans*, it is mainly arboreal, but since it is commonly trapped on the ground, its range of movement within the habitat is presumably wider than that of *T.rutilans*. It has a greater tolerance to dry conditions, and tends to select a stratum of forest below that of *T.rutilans* (Figure 6). Nests of *G.surdaster* are similar to those of *T.rutilans*, but it also builds in tree holes or the holes of other rodents, or it renovates birds' nests. Unlike *T.rutilans*, the variable temperatures in the habitat do not make it

* *G.surdaster* is called *Thamnomys dolicurus* or *G.dolicurus* by some workers (e.g. Genest-Villard, 1972; Kingdon, 1974). The taxonomy of thicket rats is in a state of flux (Rosevear, 1969) and revision is overdue. I use here the name to which murine malariologists are accustomed.

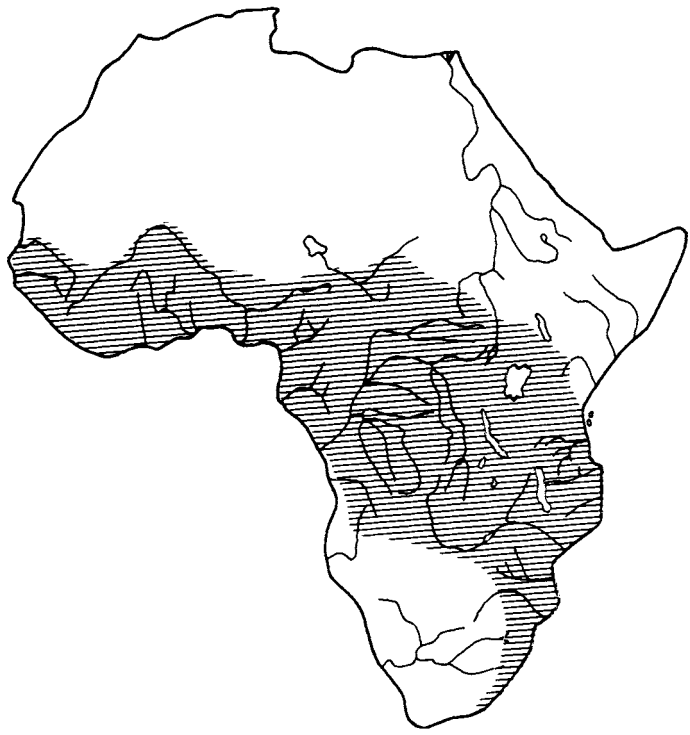

Figure 7. The geographical distribution of the *G.dolicurus* group of thicket rats (Kingdon, 1974), of which *G.surdaster* is a part.

necessary for *G.surdaster* regularly to warm itself in the nest, and its ability to control its body temperature is, therefore, more efficient than that of the other thicket rat.

In Katanga, the less specialized behaviour of *G.surdaster*, in comparison with that of *T.rutilans* elsewhere, would presumably provide a wider range of opportunities for contact between forest rats and the vector of murine malaria parasites. This looser fit between the habits of the rat and the mosquito suggests that in Katanga the whole biocenose is less specialized than in the lowland localities. The idea is supported by the wider range of vertebrate hosts of *P.berghei* in the highland locality. One rat other than *G.surdaster* which is not uncommonly infected, *P.jacksoni*, is an unspecialized highly successful rodent which inhabits both the floor and higher levels of the forest. If *A.d.millecampsi* is the only anopheline in Katanga which transmits *P.berghei*, it is clearly not restricted to feeding upon only one species of

rodent, and its biting activity would not necessarily be limited to one level above the ground.

Although too little is known of the biology of thicket rats for their roles as hosts of malaria parasites to be fully understood, enough has been revealed to illustrate that there are fundamental differences in the physiology, behaviour and habitats of the two principal hosts in lowland and highland localities; the apparent similarities of the ecology of the hosts of murine malaria parasites in the two kinds of habitat are clearly no more than superficial. The most notable differences are:

1. the humidity of the habitat;
2. the selected stratum of forest;
3. the responses to nocturnal falls in temperature;
4. the selection of nesting sites;
5. degrees of specialization to a woodland habitat.

All these factors greatly affect the activities and behaviour of the two rats and their points of contact with the sylvatic mosquitoes which are the vectors. From the differences in the behaviour of the two main vertebrate hosts in the two groups of localities, it seems probable that the vector in the lowland places has a restricted preference for feeding on thicket rats alone, but in the highland locality the vector additionally feeds on other rodents.

C. Evolution of Murine Malaria Parasites

1. Origins

Modern species of parasites can be thought of as having arisen in one of two principal ways. Some seem to be "heirlooms" which have evolved from parasites of the ancestors of the modern hosts, the evolution of which has been accompanied by speciation of the parasites. An example is the *vivax* group of malaria parasites of man, apes and macaques; the stock from which the present species arose is thought to have been a parasite of a common primate ancestor (Peters *et al.*, 1976).

Other species appear to have been acquired as "gifts" from another animal sharing the same habitat. Once received, the "gift" may, for ecological or other reasons, become separated from the stock from which it came, and then evolve into a form recognizably different from the parasite from which it originated. *P.brasilianum*, the quartan malaria parasite of Neotropical monkeys, is an example; it is thought to have

arisen from *P.malariae* of man taken to the New World during the European conquest of Latin America (Coatney *et al.*, 1971).

It seems more likely that the murine malaria parasites have evolved from gifts rather than from heirlooms. The principal reason for supposing this is that malaria parasites are unknown in the Cricetidae from which the Muridae are thought to have evolved as recently as the Miocene (Figure 8). Cricetids themselves probably branched off from

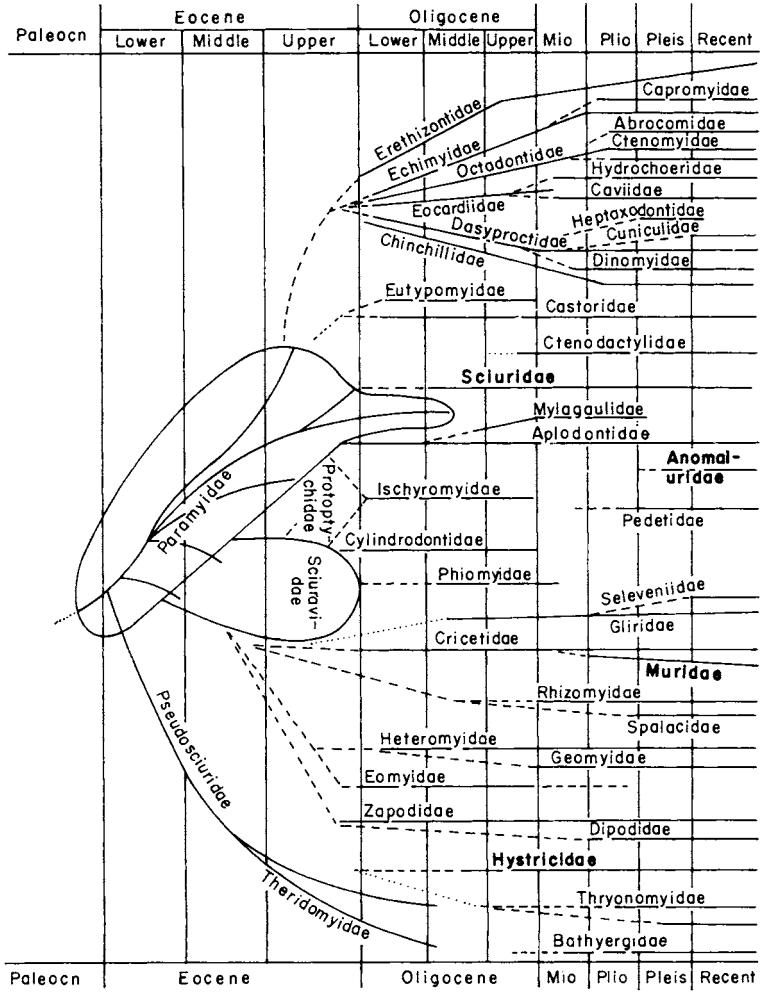

Figure 8. Wood's (1959) suggested phylogeny of the families of rodents; families in which one or more genera of rodents are known to be hosts of malaria parasites are shown in bold type.

neighbouring lines some time in the Eocene, the time when it is believed that mammalian malaria parasites first arose (reviewed by Garnham, 1966). Since the only families of rodents other than Muridae to harbour malaria parasites (Sciuridae in parts of Asia, and Anomaluridae and Hystricidae in Africa) are phylogenetically distant from Muridae (Figure 8), there is no evidence to suggest that the murine malaria parasites evolved from "heirlooms" inherited from the ancestors of murids.

If the murine parasites were indeed acquired as one or more "gifts", it is possible to suggest several ways by which this might have happened by looking at the plasmodiids of other mammals. A speculative tree showing possible evolutionary pathways of malaria parasites of mammals in relation to their modern distribution and the known or presumed habits of the vectors is shown in Figure 9. The subgenus *Laverania* is excluded from the tree because of its striking differences in several ways from the other two subgenera of mammalian malaria parasites, *Plasmodium* and *Vinckeia*; *Laverania*, with only two species, *P.falciparum* of man and *P.reichenowi* of African apes, presumably arose

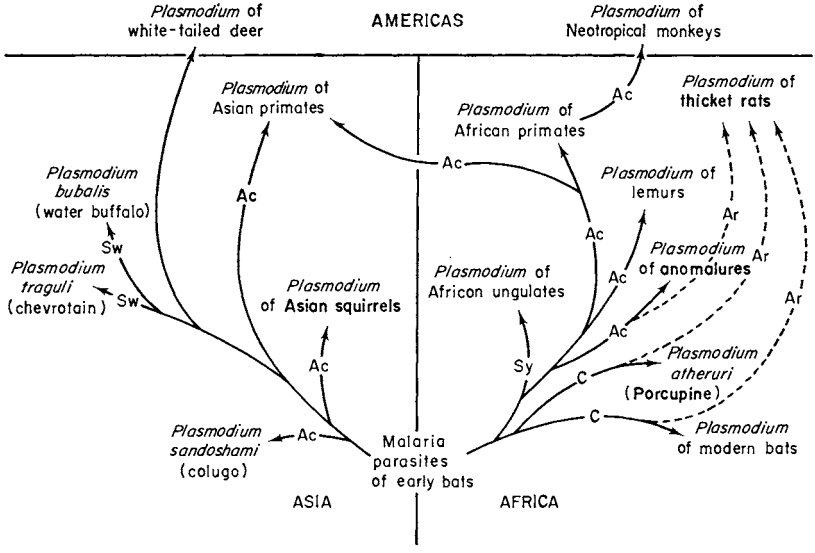

Figure 9. Speculative tree of the evolution of malaria parasites of mammals (subgenera *Plasmodium* and *Vinckeia* only) in relation to (i) the present continental distribution of the parasites and (ii) the habitats of the anopheline vectors. C, cavernicolous; Sy, sylvatic; Ac, acrodendrophilic; Ar, arboreal; Sw, swamps. Rodent hosts are shown in bold type. Dotted lines indicate three possible evolutionary origins of murine malaria parasites.

in Africa relatively recently and, since the ultrastructure of its gametocytes appears to be closer to that of avian rather than other mammalian plasmodiids (Sinden *et al.*, 1978), it perhaps evolved separately from the subgenera *Plasmodium* and *Vinckeia*.

In the evolutionary tree, bats are assumed to be the mammals in which the genus *Plasmodium* first became established. Reasons for this are:

1. the comparative antiquity of bats;
2. the modern diversity of malaria parasites *sensu lato* in bats (*Plasmodium*, *Hepatocystis*, *Nycteria* and *Polychromophilus*; see Garnham, 1966);
3. the migratory habits of bats which could have led to a dispersal of ancestral forms of *Plasmodium*;
4. the common cavernicolous habits of bats which could bring them into close contact with specialized mosquitoes (*A.caroni* and *A.hamoni* are modern examples) in a closed habitat.

There is no evidence to suggest in which continent an ancestral malaria parasite could have arisen in bats. However, the rarity of haemoproteids in North and South America suggests that the event took place no earlier than the separation of North and South America during the continental drift; the presence of many species of mammalian malaria parasites in both Africa and Asia points to the period when these continents formed a more compact land mass than at present.

In the evolutionary tree (Figure 9) the key factors determining the relative positions of the parasites are the geographical distribution and relative ages of the mammalian hosts, and the habits of the vectors. Each set of mammal–parasite–mosquito constitutes a discrete biocenose which is presumed to form an evolutionary unit. When the habitat of two mammals is partially shared (e.g. bats and porcupines or the colugo and Asian squirrels), it is easy to imagine the parasites of one gaining a foothold in the other. Another means of spread could be by mosquitoes with unspecialized habitats which could, for example, carry a parasite of an acrodendrophilic* mammal to another with a less specialized arboreal habitat. Once established in a new vertebrate host, transmission might then be by a second mosquito in close contact with the

* Acrodendrophily is a term first used by Garnham *et al.* (1945) to describe the "tendency that is shown by certain sylvan mosquitos for haunting tree-tops".

newly infected mammal, and a new evolutionary unit would thus be formed.

With these principles in mind, three possible routes of evolution of murine malaria parasites present themselves. These possibilities are not mutually exclusive and, indeed, if more than one had occurred it could explain the existence of the three groups of modern malaria parasites of thicket rats. Of the three routes, that from malaria parasites of bats transmitted by cavernicolous mosquitoes seems the least likely. There are no striking morphological similarities between modern plasmodiids of bats and thicket rats, and a cavernicolous habitat does not closely overlap an arboreal one. The latter point is also an argument against the evolution of parasites of thicket rats from the stock which gave rise to *P.atheruri* of the African brush-tailed porcupine. In this instance, however, there are some notable morphological similarities between *P.atheruri* when established in mice and *P.berghei* group parasites in the same animals. The erythrocytic stages of both have a predilection for immature red cells, and the only striking differences are in the size of mature schizonts and the number of merozoites to which they give rise (personal observation). *P.atheruri* in mice retains the small size of schizonts and low number of merozoites so typically seen in the blood of porcupines. Cross-immunity tests, however, point to a closer relationship between *P.atheruri* and *P.chabaudi* than between *P.atheruri* and the other murine malaria parasites. Mice which had recovered from infections of *P.atheruri* were shown to have no protection against *P.berghei*. The only murine malaria parasite conferring immunity against *P.atheruri* in mice was *P.chabaudi* (Cox, 1970). On morphological evidence, therefore, it could be claimed that the *P.berghei* group evolved from the stock which gave rise to *P.atheruri*, but on immunological grounds it could be suggested that *P.chabaudi* arose in this way. Such cross-immunity studies, however, are probably of little help in determining evolutionary relationships since the immune response to *P.chabaudi* protects against a wide range of intraerythrocytic parasites, some of which are clearly not phylogenetically close to *P.chabaudi* (see Chapter 7).

Three species of malaria parasites are known in African anomalures (Killick-Kendrick, 1973b), a comparatively old group of animals which, although superficially resembling flying squirrels, are in reality so different from them that Wood (1955) removed the anomalures from the suborder Sciuromorpha and grouped them in a

suborder of their own (Theridomyomorpha). Anomalures are specialized arboreal rodents which never normally come to ground. The vector or vectors of their malaria parasites are unknown but, because the vertebrate hosts feed in the forest canopy at night, the invertebrate hosts are presumably anopheline mosquitoes with nocturnal, acrodendrophilic habits. Such mosquitoes might well have carried malaria parasites from anomalures to other arboreal rodents such as thicket rats. One anomalurine malaria parasite, *P.landauae*, gives rise to a pink flush, but no stippling, of infected erythrocytes (Killick-Kendrick, 1973b), a rare effect otherwise seen only in infections of *P.chabaudi*. This similarity is, however, too slender evidence to postulate that *P.chabaudi* branched off the evolutionary line of *P.landauae*.

The three possible routes by which the murine malaria parasites might have evolved, as shown in Figure 9, are no more than suggestions based on slim, sometimes contradictory, evidence. It may become possible to make more confident speculations when the complete life cycles and vectors of the malaria parasites of bats, the brush-tailed porcupine and anomalures are known. Since exoerythrocytic schizogony is assumed to be a primitive part of the life cycle of plasmodiids (Garnham, 1966), comparisons of these tissue stages may reveal unsuspected affinities between the murine malaria parasites and other species from the ancestors of which they may have branched.

For completeness, possible evolutionary pathways of *Plasmodium* species of all groups of mammals are shown in Figure 9. There has been much speculation on the evolution of the malaria parasites of primates including man, some believing they arose in Asia, while others—no doubt influenced by the hominoid fossils recently discovered in Africa—think the parasites more probably arose in Africa as man himself evolved. The two hypotheses are discussed by Peters *et al.* (1976), and this is not the place to consider them in great detail. In the tree, it is assumed that plasmodiids of Asian primates (notably the uniquely Asian *P.pitheci* group parasites of apes which are unknown in Africa) were augmented as a result of migrations of infected primates from Africa and, in new habitats, the introduced parasites underwent an evolutionary burst resulting in the richness of species seen in Asia today. In addition, it is here somewhat arbitrarily assumed that the malaria parasites of man evolved in Africa.

From the grouping of the species of malaria parasites of mammals in Figure 9, those at present classified in the subgenus *Vinckeia* (all except

the parasites of primates) do not fall naturally into places suggesting subgeneric affinities. This reinforces the writer's view that *Vinckeia* is not a coherent phylogenetic taxon (see p. 3).

2. *Speciation and subspeciation*

There can be no doubt that there are different selective pressures in different localities of sufficient force to influence speciation and subspeciation of the murine malaria parasites.

Morphological and other similarities between *P.berghei* and *P.yoelii* support the notion that one evolved from the other comparatively recently. *P.yoelii* has by far the greater geographical range, which suggests that *P.berghei* arose from *P.yoelii* in response to the selective pressures in the Katangan habitat which are clearly different from those in the lowland localities. In the latter places, varying degrees of geographical isolation appear to have resulted in subspeciation of *P.yoelii*.

From the little that is known of *P.v.vinckei* of Katanga, only two isolations of which have ever been made, there is no evidence to suggest that this parasite should, like its sympatric partner *P.berghei*, be considered as a species separate from related populations of *P.vinckei* in the lowland localities. Presumably speciation of *P.vinckei* of Katanga has not reached the same point as *P.berghei*, in spite of the clear differences in selective pressures between the highland and lowland localities.

The superficial similarities in the morphology of the blood stages of *P.vinckei* and *P.chabaudi* could be taken as evidence of a close relationship between the two, and it is possible that one branched off from an ancestral form of the other. The apparent similarity, however, is far from as obvious as that which is seen between *P.berghei* and *P.yoelii*, and the separate specific status of *P.chabaudi* and *P.vinckei* is not in doubt. As with *P.yoelii* and *P.vinckei*, the geographically defined populations of *P.chabaudi*, of which only two are presently known, are considered to be correctly classified as subspecies.

IV. SUMMARY AND CONCLUSIONS

Cricetid rodents, from the stock of which the Muridae are thought to have evolved, appear not to be naturally infected with malaria para-

sites. In addition, the murine rodents which are hosts of malaria parasites are phylogenetically distant from all other rodents in which malaria parasites have been recorded. From this it is assumed that the four species of malaria parasites of murine rodents south of the Sahara desert evolved from the ancestors of the modern malaria parasites of African bats, the African brush-tailed porcupine or anomalures. Early murine rodents presumably became infected when they came into contact with the vectors of the other parasites and new evolutionary units of vertebrate–parasite–invertebrate have evolved.

From the geological history of Africa and the present distribution of the murine malaria parasites, the Congo Basin seems the most likely place for the precursors of the four modern species (*P.berghei*, *P.yoelii*, *P.vinckei* and *P.chabaudi*) to have arisen. The present distribution of these parasites at the periphery of the Basin may have been a consequence of climatic events 75 000 to 25 000 years ago, when the centre of the Basin is believed to have become so arid that the forest disappeared. Present localities are all in refuges in which the sylvatic fauna and flora survived, and from which they spread back to the centre of the Basin when changes in climate led to reforestation; the biocenoses of rodent–malaria parasite–mosquito—as evolutionary units—appear, however, not to have become re-established in the place of their evolution.

There are ecological barriers of varying importance between the localities in which murine malaria parasites have been discovered. Such partial or total isolation is thought to have led to speciation and subspeciation of the *P.berghei* group, and subspeciation of *P.vinckei* and *P.chabaudi*. There are reasons for supposing that such developments are in response to several known or suspected differences in selective pressures in the varied localities. Of these, the most clearly understood are between the highland localities (in the highlands of Katanga) and the lowland localities (in Western Nigeria, the Central African Republic, Brazzaville and the Republic of Cameroun). In the lowland localities, where the principal vertebrate host is the shining thicket rat, *T.rutilans*, there is evidence that the "fit" between the rat and the unknown vector or vectors is remarkably close. In Katanga, however, where the principal vertebrate host is another thicket rat (*G.surdaster*), two other rodents are also known to be infected and the relationship between rodents and the vector (*A.d.millecampsi*) is looser.

There are obvious differences in climate between the high and low

localities. Rainfall and ambient temperature are among the most notable differences and, since these factors greatly influence the habitats of thicket rats and mosquitoes, and therefore their distribution, they are considered as important selective pressures of great influence in the two kinds of biotope. In addition, temperature has a profound influence on the sporogonic development of malaria parasites which has resulted in a selection in the highlands of Katanga of parasites able to undergo sporogony at unusually low temperatures (Vanderberg and Yoeli, 1964; Vanderberg *et al.*, 1968).

In considering the zoogeography and evolution of the murine malaria parasites, it is concluded that the recent revisions of the taxonomy of the murine malaria parasites, in which *P.yoelii* was elevated from a subspecies of *P.berghei* and subspecific recognition was given to geographically defined populations of *P.vinckei* and *P.chabaudi* (Landau *et al.*, 1970; Killick-Kendrick, 1974b, 1975; Carter and Walliker, 1975, 1977), have resulted in a taxonomy which is not only practical, but which also reflects the presumed relationships between the species and subspecies of the parasites.

Finally, it must be noted that there is much work to be done before it is possible satisfactorily to classify the murine malaria parasites in such a way that both clear and subtle differences can be fully shown. In particular, it is hoped that widespread surveys will be undertaken in Africa in a search for new enzootics, and steps will be taken to discover the vectors in the lowland localities. A full appreciation of the epizootiology, zoogeography and evolution of the murine malaria parasites could well lead to a greater understanding of other malaria parasites, in particular those of man.

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2. Life Cycles and Morphology

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INTRODUCTION

A. Historical Notes

Discoveries concerning the nature of the life cycles of the murine *Plasmodium* parasites in the laboratory have been intimately interwoven with the epidemiological investigations that were being carried out in the field. The first step was Vincke's finding of malaria sporozoites in the salivary glands of *Anopheles dureni millecampsi* in Katanga in 1943. Examination of the bloodmeals of these anophelines showed that most

of them had fed on rodents. In 1948 Vincke and Lips captured two infected *Grammomys surdaster* near Elizabethville, and succeeded in transmitting *P.berghei* Vincke & Lips, 1948 from them to white rats and white mice by the inoculation of blood. Subsequently Vincke made numerous isolations of the same parasite from the salivary glands of *A.d.millecampsi* and, in 1952, a second species, *P.vinckei vinckei* Rodhain, 1952, was found in a white mouse that had been inoculated with the salivary glands of one of these insects.

In 1964 Yoeli *et al.* obtained the laboratory transmission of *P.berghei* through the bite of *Anopheles quadrimaculatus*. This success was achieved in several steps.

1. Some 2300 *A.d.millecampsi* were captured in Katanga and transferred in containers which were maintained at the same conditions of temperature (22°C) and relative humidity (90%) as those in which the insects were found in Nature. The sporozoites from these anophelines were inoculated into a variety of rodents. The strains after isolation were conserved both by syringe passage and by freezing in liquid nitrogen. With the isolates thus obtained Yoeli and his colleagues were able to ascertain that the incubation period of *P.berghei* in the rodent lay between 72 h and 8 days.
2. As it was found impossible to raise a colony of *A.d.millecampsi* in the laboratory the more readily handled species *A.quadrimaculatus* was used for further transmission experiments. *A.quadrimaculatus* were fed on mice that had been infected 3 days previously by sporozoite inoculation, and were then held at 22°C. In these mosquitoes ripe oocysts were found on the eighth day after the feed, and sporozoites were seen in their salivary glands on the thirteenth day. Yoeli and his collaborators then succeeded in transmitting the infection from *A.quadrimaculatus* to *Grammomys* in which patent parasitaemia was detected between 4 and 7 days after infection. However, the nature of the tissue schizonts of *P.berghei* could not be determined on the basis of these first experiments since, while oocysts were readily obtained on the midguts of *A.quadrimaculatus*, few sporozoites invaded the salivary glands of the mosquitoes which were held at a temperature normally found to be suitable for the development of other mammalian malaria parasites. Thus too few pre-erythrocytic

schizonts were produced to be demonstrable on examination of the rodent livers.

3. The next step was only achieved after Yoeli, in the course of a visit to Katanga, was struck by the coolness of the gallery forest in which *A.d.millecampsi* lives. At that altitude, i.e. between 1000 and 1700 m, the temperature never exceeds 21 to 22°C in the daytime and falls to 18°C at night, even in the summer, whereas the mean diurnal temperature outside the gallery forest is in the region of 29°C. Yoeli therefore decreased the temperature at which he held his *A.quadrimaculatus* to between 18 and 21°C throughout the entire duration of sporogony, and thus for the first time succeeded in obtaining massive infections in the salivary glands. When these were dissected and inoculated into laboratory rodents microscopically detectable numbers of tissue schizonts developed in their livers. Thus it was found that *P.berghei* possesses a mammalian type of pre-erythrocytic schizont which develops within the liver parenchymal cells, but matures within a remarkably short time (average, 50 h).

These unusual biological characteristics led to the belief, which held sway for a long time, that the Katangan rodent malaria focus was unique, and that these parasites could only survive in the very restricted biotope of the gallery forest, because of the limitations set by the association between the rodent host and a vector species which feeds almost exclusively on it.

In 1964 and 1965, in a different biotope, namely the fringes of primary rainforest in the Central African Republic at an altitude of 600 m and a mean temperature of 25°C, *P.chabaudi chabaudi* and *P.yoelii yoelii* were discovered (Landau and Chabaud, 1965). This changed the situation in that it could now be anticipated that transmission of rodent malaria could take place in conditions that would be considered as normal for other species of mammalian *Plasmodium*. It was then shown by Landau and Killick-Kendrick (1966) that these two parasites were readily transmitted through mosquitoes at 25°C, although the cycles were otherwise in most ways comparable to that of the Katangan *P.berghei*. While in the Katanga the majority of isolates were made from wild-caught *Anopheles*, in the Central African Republic they were obtained from the rodent host (*Thamnomys rutilans*) in which the infection rate was found to approach 100%.

Since that time infected *Thamnomys* have been captured in other parts of tropical Africa, i.e. Congo Brazzaville at 300 m altitude (Adam *et al.*, 1966), and in Nigeria at 100 m altitude (Killick-Kendrick, 1973). In these cases too it has proved possible to complete the life cycle in the laboratory, sporogony taking place normally at 25°C.

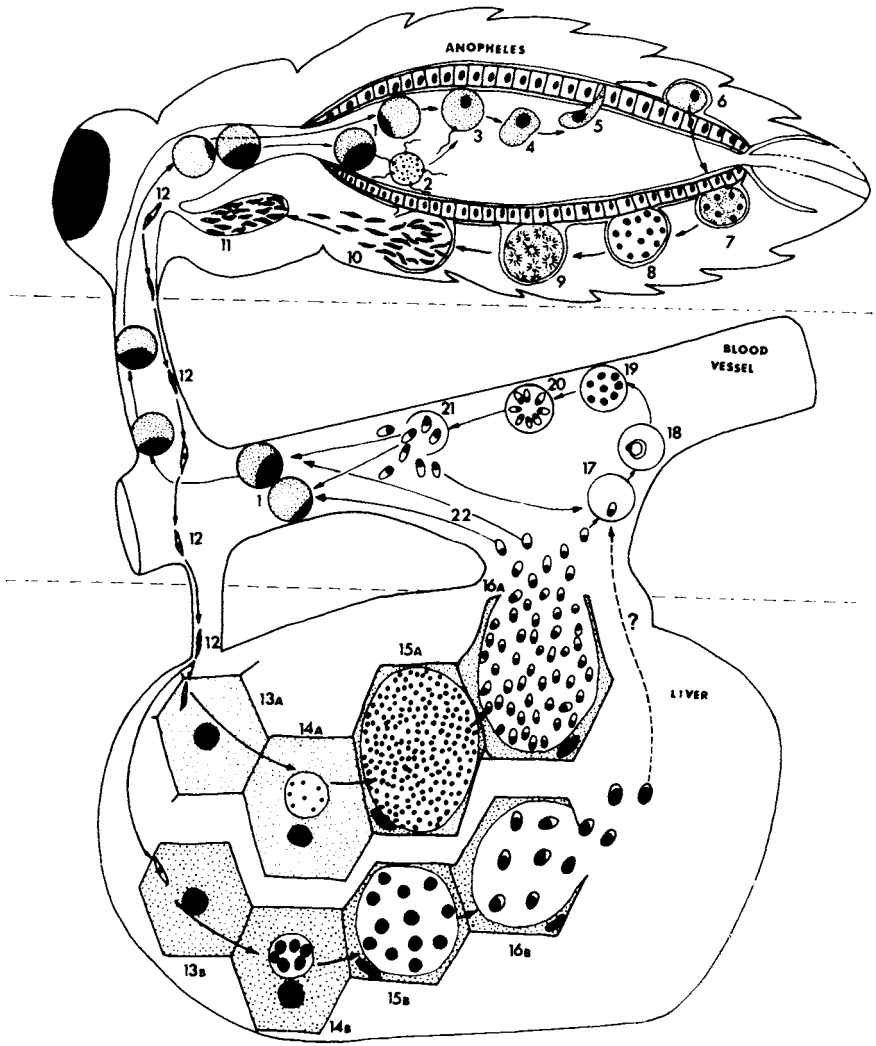

Figure 1. The life cycle of murine malaria (see text for explanation of numbers).

B. General Remarks on the Life Cycle

The life cycle of the murine *Plasmodium* species is summarized in Figure 1. Gametocytes (1) are ingested by the *Anopheles* mosquito with a bloodmeal. In the midgut of the mosquito the macrogametocyte transforms into the female (macro-) gamete while the microgametocyte undergoes exflagellation (2), liberating male (micro-) gametes which fertilize the female gametes (3). Fertilization is followed by the formation of the motile ookinete (4, 5) which passes through the midgut wall. On the outer side of the midgut the ookinete develops into an oocyst which matures over a period of 8 to 15 days depending upon the environmental temperature (6–9). When mature, the oocyst ruptures (10) liberating sporozoites which move towards the salivary glands (11)

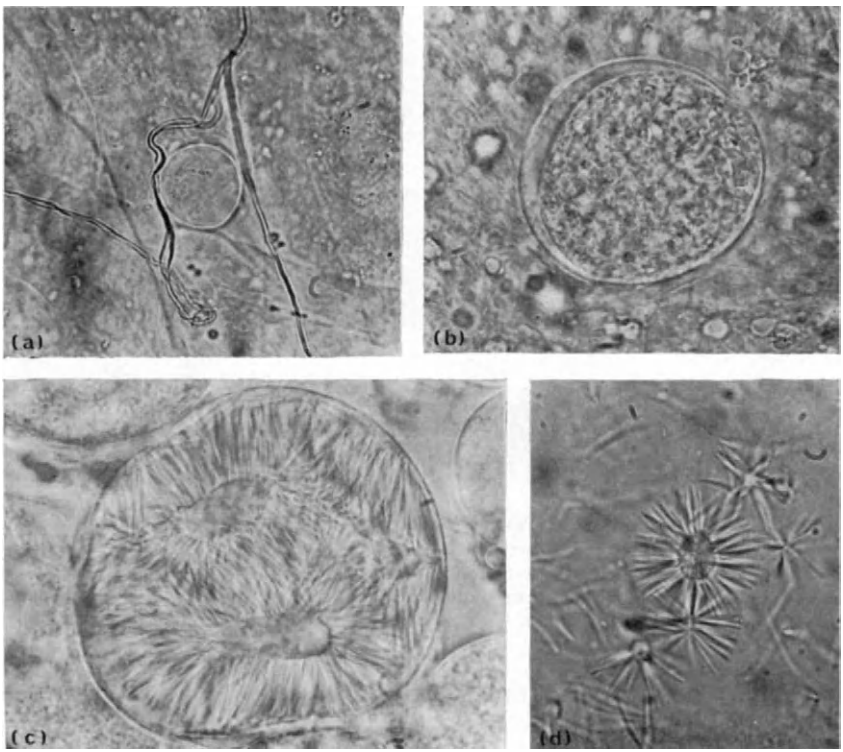

Figure 2. Sporogony of *P.y.yoelii* in *A.stephensi*. (Fresh preparations in physiological saline.) (*a*) Immature four day oocyst. (*b*) Older developing oocyst. (*c*) Mature oocyst. (*d*) Sporozoites still attached to the blastophores following mechanical rupture of the oocyst.

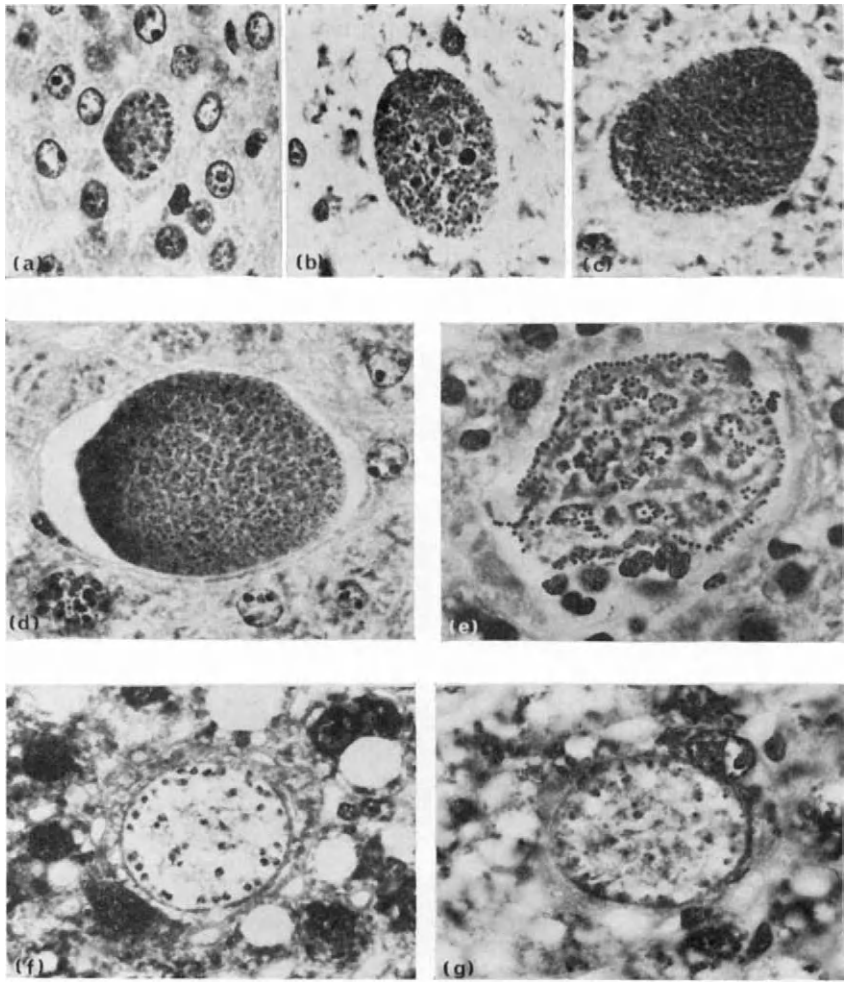

Figure 3. Sections of tissue schizonts in liver (Carnoy fixation, Giemsa-colophonium staining). (*a–c*) *P.y.yoelii* rat at 36 h (*a*), 45 h (*b*) and mature schizont (*c*). (*d–e*) *P.c.chabaudi*. (*d*) Almost mature schizont in mouse. (*e*) Schizont in the liver of an albino rat (a host refractory to the blood stages but receptive to the tissue stages). (*f–g*) *Plasmodium* sp. Delayed schizonts found in hepatocytes of *Thamnomys* 8 months after capture.

where they accumulate prior to being inoculated into another rodent when the mosquito again feeds.

The sporozoite (12) arrives via the bloodstream to the liver where, in some so far unknown manner, it reaches the interior of a hepatocyte in which it gives rise to the pre-erythrocytic schizont. This schizont

matures some 42 to 72 h after the infective bite (13A–15A), releasing between 2000 and 20 000 merozoites into the circulation (16A).

The merozoites enter red blood cells (17) in which they undergo several cell divisions (18–21) to form schizozoites. These in turn invade further red cells and again undergo schizogony. A certain number of schizozoites develop into gametocytes (1). Killick-Kendrick and Warren (1968) have shown that merozoites formed in hepatic schizonts can develop directly into gametocytes (22).

There is, finally, a second type of hepatic schizont (13B–16B) that, according to the hypothesis of Landau *et al.* (1975), may be the underlying cause of relapses of parasitaemia of *P.yoelii* (see p. 79).

II. METHODS OF STUDY

In 1971 Killick-Kendrick gave a detailed account of the majority of the techniques that are required for the isolation of rodent malaria parasites, their adaptation to laboratory hosts, their transmission and their study. We will therefore restrict ourselves to a number of points that merit stressing here.

It is often very difficult to define precisely the optimum conditions that will enable one to achieve the normal biological cycle with any degree of regularity. Many factors are concerned and even a single strain handled in apparently identical conditions in two laboratories may behave quite differently.

A. Isolation and Maintenance of Strains in the Laboratory

Petter *et al.* (1964) described simple techniques for breeding African rodents (in warm, humid conditions), which open up new opportunities for the isolation, maintenance and study of murine malaria parasites; a translation of their account is given below.

The rodents are placed in couples in cages made from a cubic metallic box, volume 11 litres, which acts as their "dayroom", to which is attached a cylindrical container of 800 cc capacity, placed horizontally and communicating with the main cage via a hole which is just large enough for the rodents to pass. The cylinder acts as a refuge. One side of the cage is fitted with a sheet of glass which slides from bottom to top, and gives access to the interior. The bottom is formed

by a removable sheet on which food can be placed on a bed of sawdust. The back of the cylinder which is 10 to 11 cm in diameter is formed of a "Chauvancy" trap which serves as a readily detachable cover. By this means the condition of the rodents can be checked at any time without disturbing them, or they may be removed in order to measure them. This part of the cage contains wood shavings.

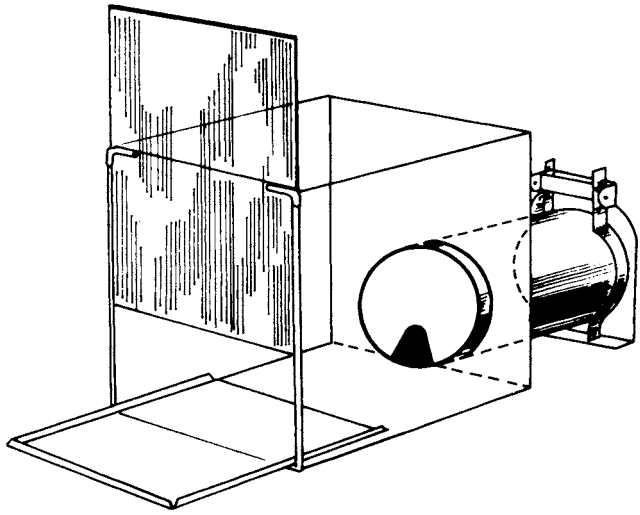

Figure 4. A breeding cage for tropical rodents (after Petter *et al.*, 1964).

The minimum exchange of air that is produced by the tight fit of the whole assembly results in ideal conditions of isolation and of relative humidity for the breeding of tropical rodents. The temperature of the animal room should be maintained between 20 and 25°C; serious fluctuations due to defects in the heating in winter or ventilation in summer have not produced any apparent consequences. A particularly warm, humid microclimate is maintained by the rodents in their nest. The shavings in the nest should normally be renewed weekly except when the animals have produced a litter. The sawdust on the floor of the dayroom should be replaced once or twice a week. It serves both to absorb excess condensed humidity and urine and acts therefore as a humidity balance.

An almost identical diet is provided daily throughout the year consisting of a small sliced apple (certain rodents, in particular

Lemniscomys, may completely ignore whole apples), one or two mouse biscuits and a helping of germinated barley grains. It is sometimes useful to add a little condensed milk to the diet of lactating females. It is unnecessary to provide drinking water.

The isolation of strains from wild rodents is made relatively easy since the albino mouse has so far proved receptive to all murine plasmodia. Patent infections usually appear in the blood in less than a week, but the prepatent period in the first passage of *P.v.lentum* from wild *Thamnomys* to white mice may be as long as two weeks. Although the white rat and hamster are naturally receptive only to *P.berghei* and *P.yoelii*, by splenectomizing the animals Adler and Foner (1963) adapted *P.v.vinckei* to both these rodents.

While splenectomy is commonly employed for adapting *Plasmodium* to hosts which are not naturally receptive, there is no way of foretelling what success may follow. An apparently close biological relationship between the various rodent species that are being used is not essential for success. Thus, for example, Adam and Landau (1969) adapted *P.atheruri* of the porcupine to *Calomys* (a South American cricetid) and to the white mouse. *P.berghei* can be adapted to numerous mammalian species (Rodhain, 1953; Vincke *et al.*, 1953; Cox, 1967).

B. Cyclical Transmission

It is difficult to prescribe a specific procedure since parasite strains vary considerably not only from one laboratory to another, but even within a single laboratory. Moreover, selection or mutations influencing, for example, the degree of virulence, or the tendency to produce greater or smaller numbers of gametocytes may appear, necessitating at times modifications to the experimental conditions. Thus a strain that is cyclically transmitted through a particular vector and vertebrate host tends to become adapted to this system and may thus become an excellent experimental model. On the other hand, strains transmitted solely by continual syringe passage eventually fail to produce gametocytes, and virulence often becomes enhanced. Other procedures may also affect virulence and result in a strain becoming difficult to transmit cyclically. Yoeli *et al.* (1975) give the example of a line of a normally avirulent strain of *P.yoelii* that became hypervirulent after cryopreservation and thawing to produce fulminating infections and death of white mice after an infection of only 3 or 4 days.

Table I

Optimal conditions to ensure cyclical transmission of rodent *Plasmodium*

Parasite	Suitable gametocyte carriers other than natural hosts	Best days of blood-induced infections for mosquitoes to be fed	Suitable laboratory mosquitoes	Unsuitable laboratory mosquitoes	Best temperatures for sporogony (°C)	Days glands first invaded
P.berghei (Katanga)	Some strains of white mice, white rats, hamsters, muskrats	4–5	*A.stephensi* *A.l.atroparvus* *A.annulipes* *A.gambiae* A?B *A.aztecus* *A.quadrimaculatus*	*A.albimanus* *Aedes aegypti* *Culex salinarius*	19–21	14
P.y.yoelii (CAR)	Mice, white rats, hamsters	4–5	*A.stephensi* *A.sundaicus*	*A.l.atroparvus* *A.b.balabacensis* *A.quadrimaculatus*	24	9
P.y.killicki (Brazzaville)	Mice, white rats	3–6	*A.stephensi*		22–24	10
P.y.nigeriensis (Nigeria)	Mice, white rats	3–10	*A.stephensi*		24	9

P.v.vinckei (Katanga)	Splenectomized or hairless mice	4–8	*A.stephensi*	*A.gambiae* *A.albimanus* *A.quadrimaculatus* *A.l.atroparvus*	20–21	13
P.v.lentum (Brazzaville)	*Hybomys univittatus*	5	*A.stephensi*		24–25	10
P.v.brucechwatti (Nigeria)	Mice, multimammate rats	10	*A.stephensi*	*A.stephensi*[a] *A.l.atroparvus*[a] *A.quadrimaculatus*[a] *A.gambiae*[b]	24–26	13
P.v.petteri (CAR)	Mice		*A.stephensi*		24–26	11
P.c.chabaudi (CAR)	Mice, multimammate rats	10–18	*A.stephensi*	*A.aztecus* *A.freeborni* *A.sundaicus*	26	10
P.c.adami (Brazzaville)	Mice		*A.stephensi*		24–26	11

[a] At 23–25°C sporozoites which were never infective first invaded the salivary glands of these mosquitoes on the twelfth day of infection (Killick-Kendrick, 1970).

[b] Insusceptible.

Modified from Killick-Kendrick (1971).

Among the factors influencing cyclical transmission the following are the most important:

1. vector species: *A.stephensi* appears to be the best experimental vector for murine malaria parasites;
2. optimal temperature for sporogony: the primary importance of this factor was demonstrated by Vanderberg and Yoeli (1965, 1966) and Yoeli (1965);
3. contaminating infections: *Eperythrozoon* and *Haemobartonella* in the vertebrate (Peters, 1963), and viruses or microsporidia in the invertebrate host tend, in general, to inhibit development of the malaria parasites (see Chapter 7);
4. choice of vertebrate host: this will depend on the type of study required, e.g.

   G.surdaster—parasitaemia is rapidly lethal;

   G.surdaster (Ippy strain)—the blood infection is much less intense and may last a long time (up to 6 months);

   Hybomys univittatus (the one-striped rat)—this species is very easy to breed and handle. The level of blood infection is high with the early production of gametocytes that are readily infective for mosquitoes;

   Mastomys natalensis—produces a low grade parasitaemia but with a high proportion of gametocytes;

   Ondatra zibethica (the musk rat)—a large animal that is highly receptive to *P.berghei* (Wellde *et al.*, 1966).

Table I summarizes the principal factors influencing cyclical transmission, including the vertebrate and invertebrate host pairs that best favour transmission.

C. Differential Criteria for Species and Subspecies

The morphological description of the blood stages of rodent *Plasmodium* is often an inadequate means of distinguishing them. Other stages or factors related to the complete life cycle may have to be taken into consideration such as:

1. the rate of maturation of exoerythrocytic schizonts: Landau and Killick-Kendrick (1966) and Landau *et al.* (1970) used this criterion to distinguish between *P.y.yoelii* and *P.berghei* and between *P.v.lentum* and *P.c.chabaudi*, respectively (see Figure 5);

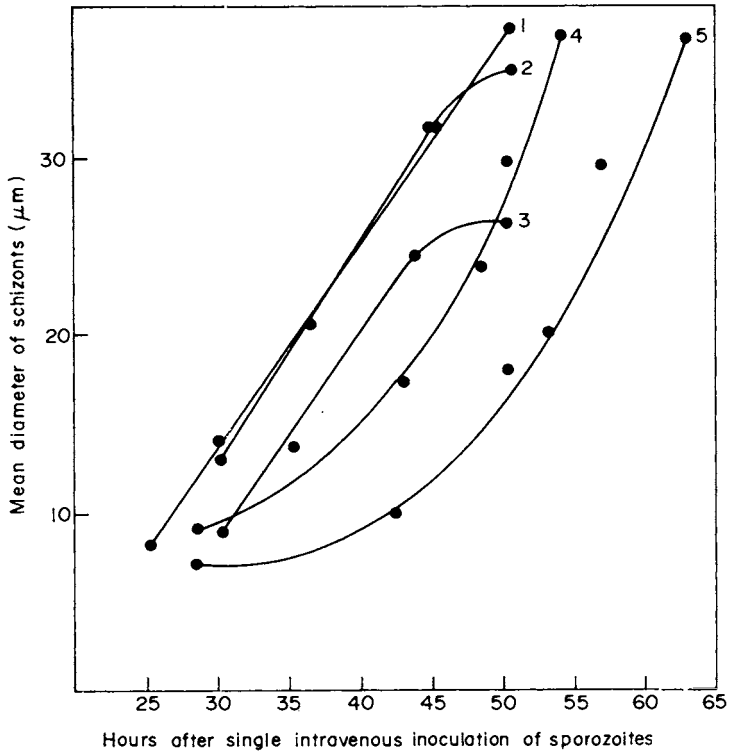

Figure 5. The rate of growth of the pre-erythrocytic schizonts of five subspecies of rodent plasmodia. 1, *P.y.yoelii*; 2, *P.y.killicki*; 3, *P.berghei*; 4, *P.c.chabaudi*; 5, *P.v.lentum* (Landau *et al.*, 1970, reproduced by permission of the editors, *Annals of Tropical Medicine and Parasitology*).

2. optimal temperature at which infective sporozoites are obtained;
3. mean diameter of mature oocysts (see Table II);
4. mean length of salivary gland sporozoites (see Table II);
5. biochemical criteria.

The addition of isoenzyme characterization to the classical criteria has proved extremely valuable in separating and identifying various strains of *Plasmodium* (see Chapter 5).

III. DIAGNOSES AND LIFE CYCLES OF MURINE MALARIA PARASITES

A. *P.berghei–P.yoelii* Complex

The species of the *berghei* complex have the following characters:

1. trophozoites show a predilection for reticulocytes in the circulation;
2. the exoerythrocytic cycle is of short duration (under 50 h);
3. sporogony is relatively rapid (on average 9 days);

Table II

Comparison between the sizes of mature oocysts and the sporozoites of 10 subspecies of rodent plasmodia

	Mean diameter of fresh mature oocysts (μm)	Mean length of sporozoites in dried preparations (μm)
P.berghei	37[a]	11[b]
P.y.yoelii	75[a]	14[c]
P.y.killicki	60[a]	14[d]
P.y.nigeriensis	60[d]	16·7[d]
P.v.vinckei	40[d]	15[d]
P.v.lentum	47[f]	21[f]
P.v.petteri	50[e]	16·2[e]
P.v.brucechwatti	54[g]	14·7[g]
P.c.chabaudi	75[a]	13[c]
P.c.adami	51[h]	11·6[h]

[a] See Killick-Kendrick (1974).
[b] See Garnham (1966).
[c] See Landau and Killick-Kendrick (1966).
[d] See Killick-Kendrick (1973).
[e] See Carter and Walliker (1975).
[f] See Landau *et al.* (1970).
[g] See Killick-Kendrick (1975).
[h] See Carter and Walliker (in press).
Modified from Landau *et al.* (1970).

4. the rat and hamster are naturally receptive to the blood stages;
5. the isoenzymes have characteristic electrophoretic mobilities.

The two species of the complex, *P.berghei* and *P.yoelii*, are indistinguishable on the basis of the morphology of the blood stages but are readily separated by a number of other characters such as those of the sporogonic and exoerythrocytic stages, and their isoenzymes (Table III, p. 14).

The following descriptions of the members of the *P.berghei–P.yoelii* complex are reproduced with minor modifications from those of Killick-Kendrick (1974).

1. P.berghei *Vincke & Lips, 1948*

a. Sporogonic stages. Exflagellation of microgametes takes 10–15 min at 27°C. The microgametes measure about 15 μm in length (Garnham, 1966). The optimum temperature of sporogony is typically 18–21°C; at less than 16°C or more than 24°C sporozoites are either not formed or have a low infectivity (Vanderberg and Yoeli, 1965, 1966). The ookinetes are shaped like a tapering banana with one blunt end and measure 10–12 μm in length (Garnham, 1966). The pigment in 3-day-old oocysts is in 1 or 2 curved lines (Yoeli, 1965). The mean diameter of unsquashed mature oocysts is typically less than 45 μm (Rodhain *et al.*, 1955; Yoeli and Most, 1960; Garnham, 1966; Landau and Killick-Kendrick, 1966) and sporozoites in stained preparations of salivary glands of *A.d.millecampsi* measure 11–12 μm (Garnham, 1966). In similar preparations from experimentally infected *A.stephensi* the mean length of sporozoites of line RLL of strain SP11 is 10·90 ±s.e. 0·22 μm, and of ANKA strain, 12·04 ±s.e. 0·16 μm (Killick-Kendrick, 1973).

b. Exoerythrocytic stages. In the liver of the white rat the minimum maturation time is typically not less than 50 h (Yoeli, 1965; Wéry, 1968). The mean diameter of tissue forms of strain NK65 (from the type locality) is 9 μm at 30 h, 14 μm at 36 h, 24 μm at 45½ h and 26 μm at 50½ h (Landau and Killick-Kendrick, 1966). Estimates of the numbers of merozoites produced in the white rat are from 4000 (Landau and Killick-Kendrick, 1966) to 5000–8000 (Yoeli *et al.*, 1966). In *G.surdaster* the mean diameter of mature forms is larger (38–57 μm) and

the estimated number of merozoites is 10 000–18 000; in white mice and hamsters the size is 24–27 μm and the merozoite number has been estimated at 1500–2000 (Yoeli *et al.*, 1966). The nuclei of infected parenchymal cells are not enlarged (Yoeli, 1965).

c. Blood stages (Garnham, 1966). The early infections in white rats and mice occur in normocytes, but later there is a predilection for immature erythrocytes. Polyparasitism, often causing hypertrophy of the host cell, is common. Ring forms occasionally have twin nuclei. The trophozoites and young schizonts are compact, not amoeboid; schizonts produce varying numbers of merozoites according to the host, namely in mice 6–10, in rats and hamsters 16 (Garnham, 1966) and in *G.surdaster* 16–18 (Yoeli and Most, 1960). The asexual cycle takes 22–25 h. Macrogametocytes are 8–9 μm in diameter and fill the host cell; their nuclei are eccentrically placed and consist of a darkly staining homogeneous centre surrounded by a pink areola. Microgametocytes are slightly smaller and have a larger similar nucleus but with the deeply staining material in the form of threads. The pigment is described as black (Vincke and Lips, 1948), dark (Garnham, 1966) or golden-yellow (Yoeli and Most, 1960). Electrophoretic forms of enzymes of five strains (K173, SP11—and line RLL, see Bafort, 1970—NK65, LUKA and ANKA) are: GPI-1, 6PGD-1, LDH-1, MDH_a-2, AK-2, HK-2 and GDH-3 (Carter, 1973, 1974).

2. P.y.yoelii *Landau & Killick-Kendrick, 1966*

a. Sporogonic stages. Microgametes and ookinetes are undescribed. The pigment in 4-day-old oocysts lies in four or more straight or curved lines grouped in one part of the oocyst (Landau and Killick-Kendrick, 1966). At 24°C mature oocysts have a mean diameter of 75 μm (60–82 μm) and sporozoites first enter the salivary glands of *A.stephensi* on the tenth day (Landau and Killick-Kendrick, 1966; Wéry, 1966); the mean length of sporozoites of the strain 17 X in stained smears of crushed salivary glands is 14·72 ±s.e. 0·12 μm (Killick-Kendrick, 1973). The optimum temperature of sporogony is 24°C; sporozoites produced at 28°C are not infective (Wéry, 1966).

b. Exoerythrocytic stages. In the liver of white rats the minimum maturation time is 43 h and the mean diameter of schizonts of the type strain is 8 μm at 25 h, 14 μm at 30 h, 21 μm at 36 h, 32 μm at 45 h and

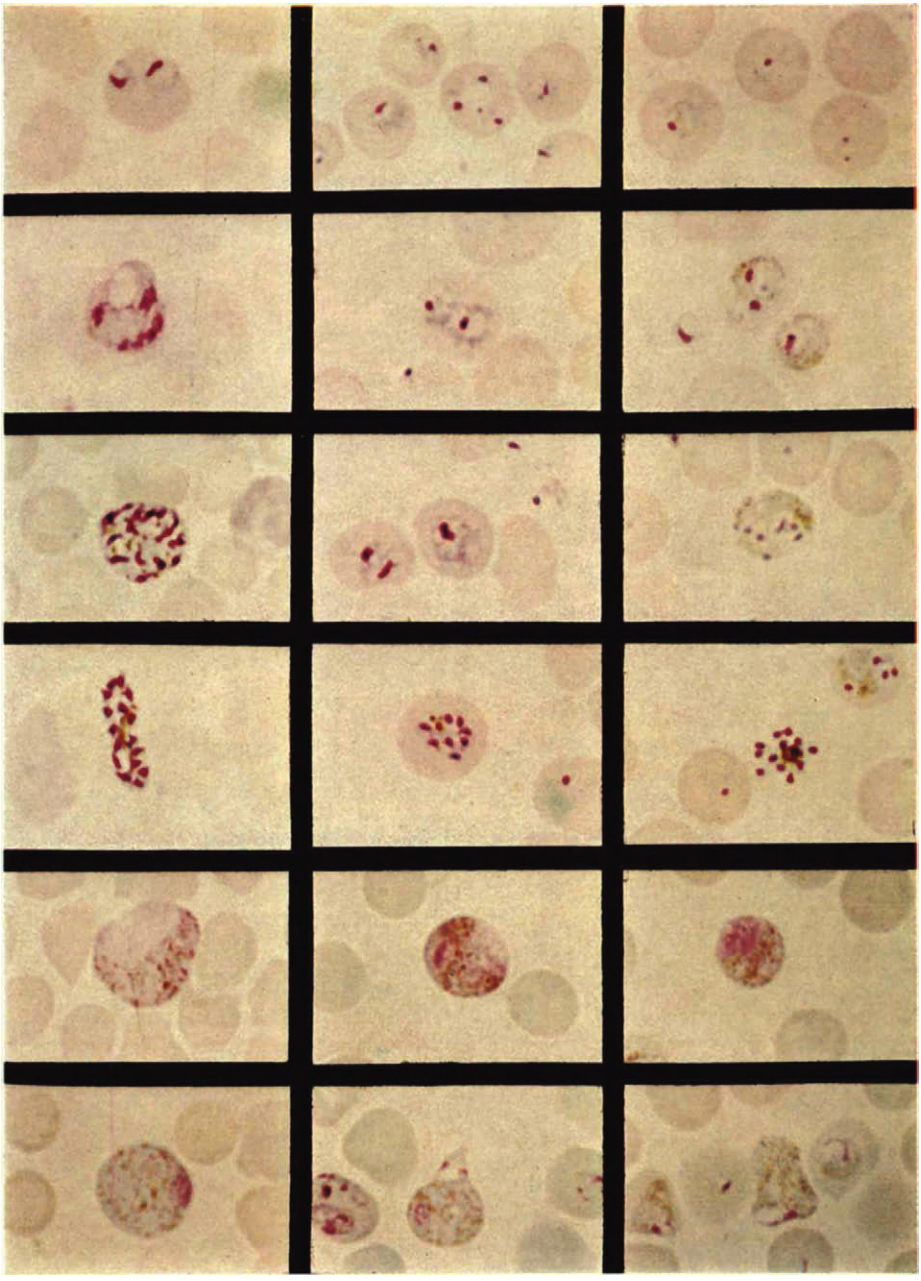

Plate 1. Comparative morphology of blood stages of rodent plasmodia in the mouse (Giemsa's stain). (*a–f*) *P.y.yoelii*, (*g–l*) *P.c.chabaudi*, (*m–r*) *P.v.petteri*. (*a, g, m*) early ring stages, (*b, h, n*) young trophozoites, (*c, i, o*) segmenting late trophozoites, (*d, j, p*) mature schizonts, (*e, k, q*) microgametocytes, (*f, l, r*) macrogametocytes. (*P.c.chabaudi* and *P.v.petteri* infections originated from cloned material kindly supplied by D. Walliker and R. Carter.)

37 μm at 50 h. In the same host, the approximate number of merozoites at maturity is 7500–8000. Nuclei of infected parenchymal cells are not enlarged (Landau and Killick-Kendrick, 1966). A few of the merozoites from primary exoerythrocytic schizonts grow directly into gametocytes with no intervening schizogony in the blood (Killick-Kendrick and Warren, 1968).

c. Blood stages. (Plate 1, a–f) Morphologically indistinguishable from those of *P.berghei*. The growth of sexual and asexual stages takes 22–24 h (Killick-Kendrick and Warren, 1968). The electrophoretic forms of enzymes of the type strain (17 X) are: GPI-1, 6PGD-4, LDH-1, MDH_a-1, AK-1, HK-1 and GDH-4; in 21 other strains the only variant found was in GPI which is occasionally GPI-2 (Carter, 1973, 1974).

3. P.y.killicki *Landau, Michel & Adam, 1968*

a. Sporogonic stages. Microgametes and ookinetes are undescribed. The pigment patterns in young oocysts are the same as those of *P.y.yoelii*; at 22–24°C mature oocysts have a mean diameter of about 60 μm and sporozoites first enter the salivary glands of *A.stephensi* on the tenth day. The mean length of sporozoites of the type strain (193L) in stained smears of crushed salivary glands is 14·32 ±s.e. 0·22 μm and of strain 194ZZ, 14·9 ±s.e. 0·25 μm (Killick-Kendrick, 1973). The optimum temperature of sporogony is in the range 22–26°C.

b. Exoerythrocytic schizogony. In the liver of white rats the minimum maturation time is 46 h and the mean diameter of schizonts is 13 μm at 30 h, 18 μm at 36 h, 32 μm at 45 h and 35 μm at 50 h. At maturity, the approximate number of merozoites is 8000, and nuclei of infected parenchymal cells become enlarged.

c. Blood stages. Morphologically indistinguishable from those of *P.berghei*; electrophoretic forms of enzymes of two strains (193L and 194ZZ) are: GPI-2, 6PGD-4, LDH-1, MDH_a-1, AK-1, HK-1 and GDH-2 (Carter, 1973, 1974).

4. P.y.nigeriensis *Killick-Kendrick, 1973*

a. Sporogonic stages. Exflagellation of microgametes takes place in 6–14 min at 16°C. The mean length of microgametes is 16·8 ±s.e.

0·29 μm. The ookinetes are leaf like, have pigment anterior to the nucleus and measure 11·4 ±s.e. 0·27 × 3–4 μm. In 4-day-old oocysts the pigment lies in one place in short beaded lines. Infective sporozoites are produced at temperatures of 21–28°C, but the optimum is 24°C; at this temperature the mean diameter of mature oocysts is 60 μm and sporozoites first invade the salivary glands of experimentally infected *A.stephensi* after 9–11 days of infection. The mean length of sporozoites in stained smears of crushed salivary glands is 16·72 ±s.e. 0·22 μm.

b. Exoerythrocytic stages. The minimum maturation time in the liver of the white mouse is 47–48 h; at 36 h the parasites in the liver of white rats fill the host cell and have a mean diameter of 20–25 μm; the same measurement of mature forms at 49–50 h in the same animal is 42–50 μm. The nuclei of infected parenchymal cells are commonly enlarged.

c. Blood stages (Killick-Kendrick, 1973). Morphologically indistinguishable from those of *P.berghei*. In white mice there is usually, but not always, a predilection for immature erythrocytes; polyparasitism is common. Ring forms only occasionally have twin nuclear masses. Trophozoites are not amoeboid. The mature schizonts almost completely fill the host cell, and produce 8–16 merozoites; pigment is golden or light brown. The infected cells are neither enlarged nor stippled. The electrophoretic forms of enzymes of the type strain are: GPI-2, 6PGD-4, LDH-1, MDH_a-1, AK-1, HK-1 and GDH-2 (Carter, 1973, 1974).

B. *P.vinckei* and Subspecies

The subspecies of the *vinckei* group possess the following distinguishing characters:

1. the merozoites have a predilection for mature erythrocytes;
2. the trophozoites are not amoeboid;
3. the erythrocytic schizonts typically produce an average of 8–10 (range 6–16) merozoites in white mice;
4. the blood stages have abundant golden malaria pigment;
5. exoerythrocytic schizogony lasts longer than 50 h (usually more than 60 h);
6. the duration of the sporogonic stages (averaging 11 days);

7. insusceptibility of the rat and hamster to the blood stages;
8. characteristic electrophoretic mobilities of the isoenzymes.

1. P.v.vinckei *Rodhain, 1952*

The following description is based on that of Bafort (1969) who, having isolated a new strain, was able for the first time to obtain the complete life cycle of this species in the laboratory.

a. Sporogonic stages. The ookinetes which have a sharp anterior and a rounded posterior pole are about 8–10 μm long. The pigment is usually concentrated in a large mass, and the nucleus occupies the posterior half of the body. At 20°C the diameter of the oocysts is 15 μm at 7 days, 40 μm at 9 days and 50–56 μm at 12 days. The optimum temperature for sporogony in *A.stephensi* is 21°C (20–24°C) in which oocysts, mature in 14 days, may measure between 40 and 73 μm (Bafort gives no mean diameter for them). At 21°C sporozoites varying from 11–21 μm first appear in the salivary glands on the thirteenth day of infection. Although Bafort gave no mean value for the length of the sporozoites, Killick-Kendrick (1973) found that this was 15 μm in dried preparations.

b. Exoerythrocytic stages. Schizonts matured in a minimum of 53 h in the thicket rat (*G.surdaster*) and 61 h in the white mouse. In the liver of the thicket rat the mean diameter of the tissue forms was 11 μm at 30 h, 19 μm at 42 h, 24 μm at 48 h, 33 μm at 52 h and 37 μm at 54 h. By 58 h most of the schizonts had disappeared but the mean size of those that remained was 45 μm and it was estimated that the schizonts contained approximately 9000 merozoites. A number of schizonts persist as long as 74 h; these are polymorphic, some appearing normal and others atypical with smaller numbers of large nuclei. In the white mouse the schizonts are a little smaller than in *Grammomys* (average 24 μm at 57 h against 27 μm at 54 h, respectively) and the prepatent period is longer (61 instead of 53 h). Polymorphism is less apparent in mice than in thicket rats.

c. Blood stages. The parasites develop in mature erythrocytes which, in stained blood films, are paler than uninfected ones. A few reticulocytes are invaded in very heavy infections. Young ring forms often have two unequally sized nuclei. The trophozoites remain compact through-

out their development. The schizonts, which are small, occupy only two-thirds of the host cell, and produce on average 8 merozoites in the white mouse (from 6 to 10) and 12 to 14 in *Grammomys*. Abundant pigment develops early. Macrogametocytes have a compact, peripherally situated nucleus which is smaller than that of the microgametocytes. Pigment is regularly distributed throughout the cytoplasm which stains light blue with Romanowsky stains. Microgametocytes fill the host cells which are sometimes enlarged; they have a diffuse nucleus and rose-staining cytoplasm with abundant and regularly dispersed pigment. The electrophoretic types of the enzymes of two strains (V52 and V67 from Kamena) are GPI-7, 6PGD-3, LDH-6 and (in V67 only) GDH-6 (Carter, 1973, 1974).

2. P.v.petteri *Carter & Walliker, 1975*

a. Sporogonic stages. Carter and Walliker found that the oocysts in *A.stephensi* maintained at 24–26°C measure up to 50 μm in diameter on the ninth day of infection, and that sporozoites measuring 16·2 μm (s.d. ±2·2 μm in stained smears) appear in the salivary glands on the eleventh day.

b. Exoerythrocytic stages. Not yet described.

c. Blood stages. (Plate 3, m–r) Except in late infections the young ring forms invade mature erythrocytes in which they grow to occupy up to one-third of the diameter, polyparasitism being rare. The ring forms usually have a single nucleus, although two chromatin dots are commonly observed. The pale blue cytoplasm forms a thin circle surrounding a central vacuole. The cytoplasm remains pale in the trophozoite and is speckled throughout with golden-brown, dense pigment. Up to 3 small vacuoles may be present. Trophozoites and young schizonts become somewhat condensed and deeply stained, the pigment forming a single central mass. Mature schizonts possess on the average 10 merozoites (8 to 16), the maximum numbers being seen around midnight and the asexual cycle occupying 24 h. Gametocytes appear in small numbers occupying the whole erythrocyte but causing no host cell enlargement. The macrogametocytes which stain pale blue have a small nucleus which is often elongated and frequently contains a clear vacuole. Microgametocytes have a deep pink cytoplasm and nucleus,

the large area occupied by the latter being distinguished mainly by the absence of pigment over it. The electrophoretic enzyme types of 4 lines are as follows: GPI-5, 6PGD-5, LDH-7.

3. P.v.lentum *Landau, Michel, Adam & Boulard, 1970*

The description of the sporogonic and exoerythrocytic stages is based on the work of Landau *et al.* (1970), and that of the blood stages and enzyme characters on that of Carter and Walliker (1977).

a. Sporogonic stages. The complete sporogonic cycle was achieved in *A.stephensi* gorged on the fifth day of infection on a splenectomized *Hybomys univittatus* and maintained at 23–25°C, few gametocytes being produced in the white mouse. Oocysts measured 9 μm on the fourth, 12·5 μm on the fifth, 19·5 μm on the sixth, 21·5 μm on the seventh and 44 μm on the eleventh day. Sporozoites were first visible in the oocysts on the eighth day. When mature, the oocysts measured on the average 44 μm (up to 56 μm). Sporozoites first appeared in the salivary glands on the tenth day, measuring, in stained smears, an average of 21 μm (18–25 μm range).

b. Exoerythrocytic stages. Liver schizonts seen at 28 h after infection measured 7 μm in diameter; older stages measured a mean of 11 μm at 42 h, 18 μm at 50 h and 20 μm at 53 h. They contained no vacuoles and the host cell nuclei were unmodified. By 62 h, the schizonts measured an average 39·9 × 33·5 μm (range 46·5 to 32·5 × 40·3 to 26·3 μm). The cytoplasm stained pale blue and contained numerous, small and evenly distributed nuclei. In contrast to *P.y.yoelii* and *P.c.chabaudi*, no cytomeres were seen. Merozoites appeared in the circulation after 72 h, and by 73 h almost all the schizonts had burst and were seen to have become invaded by macrophages. A few abnormal forms were present containing lightly staining cytoplasm and scanty, large pyknotic nuclei.

c. Blood stages. The blood forms show a marked preference for mature erythrocytes, and multiple infections are rare. Ring forms consist of a thin circle of blue cytoplasm around a central vacuole, and have nuclei containing a single- or a double-chromatin dot. The mature trophozoites are compact and not amoeboid. They occupy up to three-quarters of the volume of the host cell, the membrane of which

frequently appears crenated. Mature schizonts possess 6–16 merozoites (average 10), the host cell membrane usually breaking down. The asexual cycle takes 24 h with a maximum number of schizonts appearing at midnight. Infections are not usually lethal. Macrogametocytes, which may be difficult to distinguish from large trophozoites, contain pale blue cytoplasm with abundant pigment granules, and have a small slightly elongate nucleus lying in a clear region of the cytoplasm. The microgametocytes stain pink and contain conspicuous pigment in the cytoplasm (the large nuclear region being recognizable by an absence of pigment over it). Carter and Walliker (1977) examined the enzymes of lines originating from four isolates of *P.v.lentum*. Each was characterized by 6PGD-5 and GDH-6. Three lines possessed GPI-6 and LDH-7, while the fourth (derived from isolate 408 XZ) contained GPI-11 and LDH-9.

4. P.v.brucechwatti *Killick-Kendrick, 1975*

a. Sporogonic stages. The optimum temperature for sporogony is 25°C; Killick-Kendrick (1975) described the pigment in 3-day oocysts as abundant and lying in short lines that are sometimes fan shaped. The mean oocyst diameter was 9·5 μm (range 8·2–10·8 μm). By the fifth day, the diameter of the oocysts had increased to 16 μm (11–19 μm) and on the eighth day to 32 μm (24–43 μm). The mean diameter of mature oocysts was 54 μm (40–70 μm), the extremes rarely being encountered, and the normal range being 50–60 μm. Sporozoites appeared in the salivary glands of a few mosquitoes on the twelfth day, but in the majority invasion took place on the thirteenth day. The mean length of sporozoites in a stained smear of the salivary glands of *A.stephensi* was 14·7 μm (10·0–24·0 μm). Sporozoites from *A.quadrimaculatus* and *A.l.atroparvus* were not infective and from *A.stephensi* rarely so.

b. Exoerythrocytic stages. According to Bafort (1971) the exoerythrocytic stages in the liver of white mice showed mature forms at 61 h with a mean diameter of 43 μm. Merozoites invaded the blood from 61 to 65 h after the inoculation of sporozoites.

c. Blood stages. There is no predilection for immature erythrocytes nor enlargement or stippling of the host cell. Ring forms commonly have double nuclei and an accessory dot is occasionally present. The young trophozoites are vacuolated, old ones being compact and not amoeboid.

At the time of the first division of the nucleus, the diameter of the schizonts is at least one-half that of the erythrocyte. The schizont nuclei are irregular in shape with one deeply stained part; the schizonts almost fill the host cell and produce 12 (8–14) merozoites in mice or 8–12 in naturally infected thicket rats. Gametocytes are dimorphic, fill the host cell and measure 6–7 μm in diameter. Their nuclei are always peripheral. Yellow pigment appears early and is mainly at the periphery. In the schizonts, pigment clumps into a mass before the merozoites are fully formed; Carter's characterization of the enzymes of strains N48 and 1/69 is: GPI-6, 6PGD-6, LDH-9 and GDH-6.

C. *P.chabaudi* and Subspecies

We have classified the *P.chabaudi* complex as a separate group on the basis of differences in the morphology of the blood stages and the electrophoretic mobilities of their enzymes as compared with those of the *vinckei* group. On the other hand, the geographical distribution of the *chabaudi* group may be as wide as that of the other groups since, in addition to the Central African Republic, Carter and Walliker (1977) have also found it in Congo Brazzaville. In our opinion the concept of parasite pairs comprising members of the *berghei* and *vinckei* groups may have to be replaced by that of triple associations between *P.berghei* (or *P.yoelii*), *P.vinckei* and *P.chabaudi*.

The subspecies of *P.chabaudi* possess the following distinguishing characters:

1. the merozoites have a predilection for mature erythrocytes;
2. the trophozoites are amoeboid and may cause reddening of the host cell;
3. the erythrocytic schizonts are small and, in white mice, typically produce an average of 6 (range 4–10) merozoites;
4. pigment in trophozoites is notably fine;
5. rats and hamsters are insusceptible;
6. exoerythrocytic schizogony takes longer than 50 h.

1. P.c.chabaudi *Landau, 1965* partim *Carter & Walliker, 1975*

In her original description Landau failed to detect that her specimens contained a mixture of the two parasites, *P.c.chabaudi* and *P.v.petteri*, and

she described the two as a single species. In the course of their survey of the isoenzymes of numerous strains isolated from wild caught *Thamnomys* from the Central African Republic Carter and Walliker (1975) were able to obtain pure infections of each species and re-describe them separately. Although the Central African Republic strains were often mixtures of *P.vinckei* and *P.c.chabaudi*, strain 54 X utilized by Landau and Killick-Kendrick (1966) in their experimental study of *P.chabaudi* did in fact contain only a single species. We have very carefully re-examined the blood films made from the mouse that was used to feed *A.stephensi* for this study and there is nothing to suggest the presence of a *Plasmodium* of the *P.v.petteri* type. It is therefore believed that the descriptions given by these workers of the sporogonic and exoerythrocytic stages are indeed those of *P.c.chabaudi*.

a. Sporogonic stages. The 4-day-old oocysts in *A.stephensi* held at 25°C measure 8–12 μm and contain pigment in large, nearly black grains which tend to lie in loose clumps, or form short lines that cross each other. A wide range of oocyst sizes from 45 to 74 μm was recorded on the tenth day of infection. The mature oocysts measure up to 80 μm on the eleventh day. On the eleventh day, sporozoites measuring 15 μm in saline preparations or 13·2 μm in stained smears (range 10–15·5 μm) appear in the salivary glands.

b. Exoerythrocytic stages. In the liver of white mice the minimum primary schizont maturation time is 52–53 h. The mean diameter of tissue forms is 9 μm at 28 h, 23×11 μm (26–19×19–10 μm) at 42 h, 36·2×23·7 μm (43·7–26·2×31·2–18·7 μm) at 50 h and 44·2×29·7 μm (53·5–33·7×33·7–24·5 μm) at 53 h. The estimated number of merozoites is 18 000 to 20 000. Rupturing schizonts are very quickly invaded by leucocytes, and the cellular response of the host is the same as that seen in infections of *P.berghei* (Yoeli and Most, 1965) and *P.y.yoelii*. Hydrolysis revealed two types of nuclear patterns. Firstly, in large immature schizonts apparently in the final nuclear division, the nuclei are large, triangular or irregular in shape, and are arranged in rosettes and secondly, when the last nuclear division is complete the nuclei appear as small, oval or round dots arranged in circles or tubes and tend to form a palisade at the periphery of the schizont.

c. Blood stages (Plate 1, g–l). According to Carter and Walliker (1975), the ring forms are seen in mature erythrocytes, particularly

during early infections when single infections of the host cells predominate. In late infections multiple infections occur. Both single- and double-chromatin nuclear masses are seen. Trophozoites increase in number during the morning, and predominate over the ring forms by late afternoon. The pigment is inconspicuous. The trophozoites do not become very large, tend to be amoeboid, and seldom exceed one-half the host cell diameter. Reddening of the infected erythrocyte may be seen as the trophozoites mature. The schizonts, with a diameter approximately one-half that of the red cell, normally produce only 4–8 merozoites (average 6), although large numbers (up to 16) have been seen. The host cell membrane may become ragged as the schizont matures, but it usually remains intact as the merozoites separate. In mice and thicket rats infections are synchronous, with a periodicity of 24 h. Schizogony commences around midnight, and continues into the early morning hours. Infections are often fatal; gametocytes become more frequent after the peak of infection, approximately 10 days after the inoculation of blood forms. They are similar morphologically to the gametocytes of *P.v.petteri*. The macrogametocytes normally have a pale blue cytoplasm with an oval nucleus. The microgametocytes have a typically deep pink cytoplasm and a large nucleus. Fifteen strains of *P.c.chabaudi* isolated from individual wild-caught thicket rats, were examined for isoenzyme types. Only one form of GPI (GPI-4), two forms of 6PGD (6PGD-2 and -3) and four forms of LDH (LDH-2, -3, -4 and -5) were found. All but one of the eight possible combinations of forms of GPI, 6PGD and LDH were found among the fifteen lines examined, suggesting that all the lines belonged to a single interbreeding population.

2. P.c.adami *Carter & Walliker, 1977*

a. Sporogonic stages. In *A.stephensi* maintained at 24–26°C mature oocysts first appeared on the ninth day with an average diameter of 51 μm (range 45–64 μm). Sporozoites which first appeared in the salivary glands on the eleventh day, had an average length in fresh preparations (prepared from mature oocysts) of 13 μm (s.d. ±1·8 μm) and 11·6 μm (s.d. ±1·6 μm) in fixed and stained smears.

b. Exoerythrocytic stages. Not yet described.

c. Blood stages. The early infection occurs in mature erythrocytes, but

immature cells may also be invaded. Occasionally reddening of the infected cell is seen as trophozoites mature. The host cells do not enlarge. In ring forms both single- and double-chromatin dots are seen. The trophozoites are amoeboid with a ragged outline and contain inconspicuous pigment. Fine granules are laid down in older and mature forms. The schizonts produce 4–10 merozoites (average 6). Infections in mice and thicket rats are synchronous, with a periodicity of 24 h and the maximum number of schizonts appears around midnight. Of the two parasite lines studied, one (408 XZ) gives rise to virulent infections in mice which die, while the other (556 KA) produces a mild infection with no deaths. Gametocytes are present throughout the infection, but are particularly numerous after the peak of parasitaemia. They occupy the entire volume of the host cell which does not become enlarged. In the macrogametocytes, the cytoplasm is very pale blue and contains numerous golden-brown pigment granules. The nucleus is small and sometimes elongated, and it usually lies in a small region of clear cytoplasm. The microgametocytes possess pink cytoplasm with abundant pigment granules. The large nuclear region is distinguished from the cytoplasm by an absence of pigment. Both lines are characterized by the enzyme forms GPI-8; 6PGD-2 and GDH-5. The LDH for 556 KA is LDH-8 and for 408 XZ is LDH-10.

D. *P. aegyptensis* Abd-el-Aziz, Landau & Miltgen, 1975

This new species was described on the basis of a single blood film made from an *Arvicanthis niloticus* which was one of many rodents captured by Abd-el-Aziz at Assiut in Egypt between 1972 and 1973. However, the true origin of this parasite remains a mystery. Numerous *Arvicanthis* have been examined previously in Egypt by various experienced workers who have never reported the presence of a *Plasmodium*. Moreover Miltgen undertook a field study at Assiut in April 1975 where he found no parasitaemias in 25 *Arvicanthis* that were captured and subsequently splenectomized. Accidental contamination of an *Arvicanthis* held in the laboratory by *P.berghei* has also been eliminated. The morphology of *P.berghei* in this host, in fact, differs considerably from that of *P.aegyptensis*. *P.vinckei* has never been maintained in the laboratory at Assiut.

If the eventual rediscovery of *P.aegyptensis* confirms the data on its origin, this parasite will assume great interest in that it will extend the

host spectrum and geographical distribution of the *Plasmodium* of murine rodents far beyond those that we currently accept.

a. Sporogonic stages. Not yet described.

b. Exoerythrocytic stages. Not yet described.

c. Blood stages. The parasite develops in erythrocytes that themselves are unmodified unless they contain several parasites. A few reticulocytes have been observed that are enlarged and contain a number of young parasites mostly devoid of pigment. The young ring form is compact with a single, round nucleus, bright blue cytoplasm and a tiny vacuole. Yellow-brown pigment appears very early; it is at first uniformly distributed but then tends to occupy limited areas and to conglomerate in two or three compact masses of rather coarse granules. The trophozoite is compact and rounded with only a slight tendency to become amoeboid. The schizont usually contains 6 merozoites (from 4 to 8) which are rounded or slightly elongated, and the pigment forms a single well defined black mass. The immature gametocytes occupy one pole of the red cell, the outer membrane of which disappears as the gametocyte matures to a size greater than that of unparasitized erythrocytes. The macrogametocyte has a rounded nucleus with fine granules situated in the centre of a rather large and clearly defined clear zone. The cytoplasm stains bluish-grey and the fine, yellowish pigment leaves several more or less extensive areas uncovered at the edges of the clear zone. No mature microgametocytes have been seen. The isoenzyme characteristics are unknown.

IV. THE PROBLEM OF RELAPSES AND CHRONIC INFECTIONS

Studies both in Nature and in the laboratory have shown that malaria in wild *Thamnomys* produces a chronic infection of long duration. All the adult *Thamnomys* captured in the Central African Republic have been found to have malaria, and the infection has persisted throughout their lives. In *P.yoelii* the parasitaemia fluctuates, subinoculation of blood from wild *Thamnomys* into white mice showing that the blood is alternately positive and negative. On the other hand, strains of *P.chabaudi* or *P.vinckei* can be isolated at each subinoculation even up to 2 or 3 years following the capture of the original hosts. This chronic condition of natural infections contrasts with the lability of experimental infections

which are always limited to under 6 months (Landau *et al.*, 1975) irrespective of their mode of origin (e.g. sporozoite inoculation or blood passage).

As in the case of the primate *Plasmodium*, it is necessary to consider that the relapses may be due to the presence of latent tissue forms that are capable of reseeding the peripheral circulation. In wild *Thamnomys* of the Central African Republic schizonts with a very distinctive appearance and slow evolution are found in the liver (Figure 3, f–g). In serial biopsies and autopsies of *Thamnomys* Landau *et al.* (1968) found that the morphology of these schizonts was as follows.

Smaller than normal pre-erythrocytic schizonts, the schizonts stain lightly and have only a small number of nuclei (30–300). Many of the nuclei have a very special appearance with beading of the chromatin around the nucleolus. This forms a sort of crown of granules surrounding a bluish-violet staining central mass which is as well defined as the nucleolus. The small amount of cytoplasm stains lightly. The schizonts are surrounded by a limiting membrane. As followed in serial biopsies, they appear to develop slowly. Although the majority disappear at the end of 2 or 3 months, in one *Thamnomys* they were numerous at autopsy $3\frac{1}{2}$ months and in another 8 months after capture.

In the laboratory, however, under the usual conditions used for transmission only rapid schizogony is obtained in the liver and the schizonts disappear in less than 3 days. No "chronic schizonts" have been found. Parasitaemia is continuous until self-cure by the sixth month.

A number of attempts have been made in the laboratory in order to establish whether there is a relationship between chronic liver schizonts and parasitaemic relapses by trying to reproduce chronic liver and blood infections. Landau *et al.* (1975) proposed that, in the course of the natural life cycle, certain ecological conditions exist that favour the development of chronic parasitism, or that lead to a modification of the parasite metabolism (e.g. cooling, dietary intake). Landau and Michel (1970) and Yoeli *et al.* (1975) demonstrated that cold can induce a slow growth of exoerythrocytic schizonts which morphologically resemble the forms seen in chronic infections in Nature. However, in spite of being associated with a slight delay in the appearance of parasitaemia, these retarded schizonts disappear relatively rapidly (in less than a week) and the overall parasitaemia seems little modified. Dunn *et al.* (1972) were able to inhibit the hepatic development of *P.berghei* in white rats by dosing them during the pre-erythrocytic phase with ethionin.

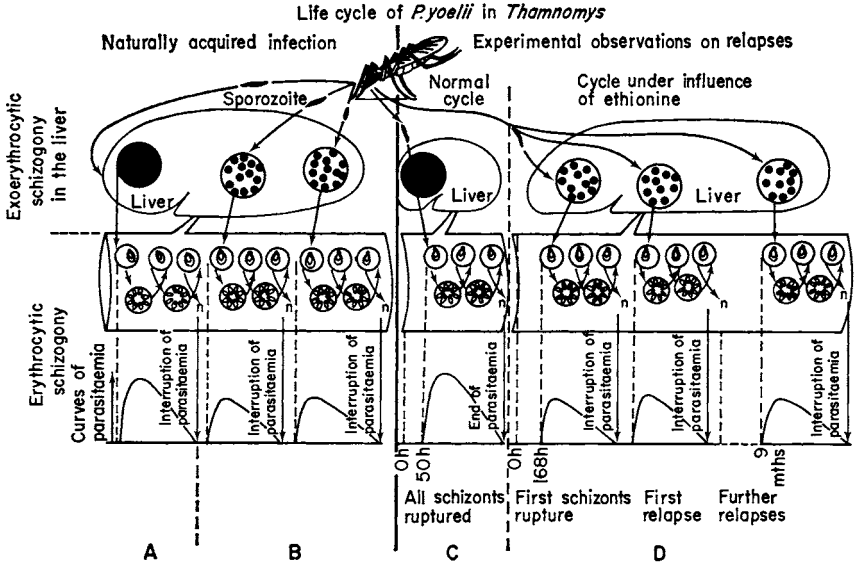

Figure 6. An hypothetical explanation for relapses in murine malaria. A. The majority of liver schizonts mature and rupture in 50 h. B. Delayed liver schizonts may remain latent for several months. When they rupture merozoites are liberated to invade erythrocytes and initiate relapses of parasitaemia. C. Infection of *Thamnomys* by sporozoites in the laboratory invariably leads only to the first phase of the cycle, and all the schizonts undergo a rapid development. The rodent host does not undergo relapses and tends towards spontaneous cure. D. The maturation of a proportion of the liver schizonts is delayed. Their morphology is identical to that of those delayed liver schizonts that have been found in naturally acquired infections. The *Thamnomys* treated with ethionine show relapses of parasitaemia up to nine months after sporozoite inoculation.

Landau *et al.* (1975), by using lower doses given over a longer period, obtained schizonts in *Thamnomys* that were identical to those observed in wild-caught *Thamnomys*, and a parasitaemia that fluctuated with alternating periods of positivity and negativity for 9 months following sporozoite inoculation.

It has not been shown that the mechanism of relapse is the same in other mammalian species of *Plasmodium*. For example Shute *et al.* (1976) believe that there are two types of sporozoites of *P.vivax*, one developing quickly and the other slowly; the latter may exist as latent intracellular parasites within the host.

Whatever the mechanism of relapse is in *P.vivax*, *P.cynomolgi*, or the *Plasmodium* species of the Muridae, the fact is that exoerythrocytic schizonts of rodent plasmodia have been found in the liver several months after every possibility of contamination has been ruled out.

In conclusion, the study of experimental models such as the murine *Plasmodium* has led to considerable advances in our knowledge of malaria parasites of mammals from many points of view, including their ultrastructure, immunology, enzymic characterization and chemotherapy. Some of the studies have begun to shed light on two of the major problems posed by the true biological cycle of *Plasmodium*, namely the basis for relapses and the origin of gametocytes; these questions, however, remain far from being finally answered.

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3. Cell Biology

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I. INTRODUCTION

In 1942 Emmel *et al.* published the first description of the ultrastructure of malaria parasites, a study of sporozoites of *Plasmodium falciparum* and *P.vivax*; blood forms were similarly examined in the same year (Wolpers, 1942). Since then advances in microscope technology and preparative techniques have led to new insights into the organization of all stages of development of the malaria parasites. Several excellent reviews of the

progress made have been produced by Garnham (1967), Rudzinska and Vickerman (1968) and Rudzinska (1969) and Aikawa (1971). In the last decade the rodent malaria parasites, particularly *P.berghei*, *P.vinckei* and *P.yoelii*, have contributed most to our understanding of this diverse group of organisms.

Although most of the more fundamental aspects of the fine structure of malaria parasites have been known for a number of years, their functional organization is less well understood. Garnham (1966b) commented "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". Little advance in this field had been made by 1971 when Aikawa stated "Understanding of the function will make it possible to achieve a meaningful and dynamic analysis of the parasite morphology". Happily considerable advances have been made in the application of histochemical techniques to studies on the fine structure of murine malaria parasites. This has led to a new understanding of the macromolecular organization of *Plasmodium*.

In spite of the unique nature of each of the developmental stages in the life cycle of malaria parasites, the remarkably conservative organization of *Plasmodium* is impressive. This conservatism is particularly striking when the fine structure of the vegetative stages (the pre-erythrocytic schizont, erythrocytic schizont and oocyst) is compared with that of the invasive stages (the merozoite, ookinete and sporozoite), "les germes infectieux" of Porchet-Heneré and Vivier (1971). Departures from the basic or common organization can be viewed as special adaptations to the unique microenvironments of the differing host tissues. Sexual development alone interrupts the repetitive cycle of vegetative growth and tissue invasion. It is perhaps within this focal point of the life cycle that the rodent malaria parasites continue to provide an unique insight into the subcellular organization of *Plasmodium* species.

II. METHODS

In electron microscopy the method of preparation selects the information gathered. Currently four combined aldehyde–osmium fixatives are routinely applied in transmission electron microscope (TEM) studies of malaria parasites. These are the glutaraldehyde–osmium techniques of

Aikawa (1971) for erythrocytic stages, and of Sterling *et al.* (1973) for the sporogonic stages; the glutaraldehyde–osmium–uranium fixative of Terzakis (1968) for the sporogonic stage and a modified Karnovsky fixative (formaldehyde–glutaraldehyde–osmium–uranium) of Sinden *et al.* (1976) for all stages (with the exception of unclotted blood suspensions).

For scanning electron microscopy (SEM) a wide variety of fixatives has again been used. Arnold *et al.* (1971) evaluated numerous techniques and concluded that a five-step fixative was desirable for preparations of blood cells. Most workers, however, use a single glutaraldehyde fixation (Strome and Beaudoin, 1974; Sinden, 1975a, b; Bodammer and Bahr, 1973). In SEM studies the use of critical point drying techniques to avoid shrinkage artefacts is as important as the choice of fixative (Sinden, 1975a). In comparison with TEM investigations, SEM studies are in their infancy and considerable caution still has to be exercised in the interpretation of SEM micrographs; the identification of technical artefacts must be vigorously pursued.

High voltage electron microscopy (HVEM) has been used to study the three-dimensional relationships of various organelles of the parasite (Aikawa, 1972; Sinden, unpublished), and is of special value when looking at long tortuous structures which may be difficult to examine in thin sections, e.g. microgametes (Aikawa and Sterling, 1974b) or sporozoites (Sinden, unpublished).

III. FINE STRUCTURE

A. The Zygote and Ookinete

The zygote is the fertilized female gamete lying in the bloodmeal in the midgut of the mosquito vector (Figures 1 and 2). At the moment of fertilization the plasmalemmas of the two gametes fuse and the condensed microgamete nucleus and axoneme enter the macrogamete cytoplasm. Within 15 min the nucleus of the microgamete becomes enlarged and the chromatin decondensed (Figures 64 and 65). Consequently nuclear fusion, so readily recognized in *Haemoproteus* by the joining of the small dark male nucleus with the large pale female nucleus (Gallucci, 1974a), has not been recorded at the ultrastructural level. On light microscope evidence Garnham (1965) suggests nuclear

fusion may be delayed in some cases for $47\frac{1}{2}$ to $48\frac{1}{2}$ h. The axoneme of the microgamete and its kinetosome are rapidly depolymerized and cannot be detected within the ookinete 12–24 h later (Canning and Sinden, 1973; Davies, 1974a).

The pellicle of the zygote is composed of a single unit membrane beneath which lies a patchy network of flattened sacs of endoplasmic reticulum. No micropore has been found in the pellicle. The endoplasmic reticulum (ER) is largely confined to a single part of the cytoplasm, and is associated with numerous ribosomes which are synthesizing proteins. The proteins are detected as amorphous dense material within the expanded cysternae of the ER. A newly developed expanse of smooth membraned vesicles is associated with the ER; it may occupy up to one-half of the cross-sectional area of the parasite (Davies, 1974a). Electron-dense particles 20–35 nm in diameter are interspersed between these vesicles (Canning and Sinden, 1973; Davies, 1974a). These particles (Figures 1 and 2) rapidly increase in number and become the characteristic crystalloid (Figures 7 and 10). A similar sequence of crystalloid formation has been described in *Haemoproteus* (Gallucci, 1974b) but Trefiak and Desser (1973) suggest that crystalloid material may already be present in the mature macrogametocyte of *Leucocytozoon*. The exact function and composition of this organelle has been the subject of much speculation; Garnham (1966b) suggested it was a precursor of the oocyst wall; Vickerman and Cox (1967b) believed it to be crystalline ribosomes, whereas Terzakis (1969), Davies *et al.* (1971), Davies and Howells (1973) and Terzakis *et al.* (1976) considered the organelle to be a viral infection. However, Trefiak and Desser (1973) demonstrated that the crystalloid of several haemosporina is composed of lipoprotein and may therefore act as a store of energy.

During development of the zygote, marked changes occur in mitochondrial organization, namely the gradual transition from a predominantly acristate state in the young zygote to a totally cristate state within the young oocyst. This transformation accounts for the varied descriptions of cristate (Garnham *et al.*, 1962), acristate and partially cristate forms in the ookinete (Figure 7) (Canning and Sinden, 1973; Davies, 1974a). The transition in mitochondrial morphology is accompanied by the reappearance of succinic dehydrogenase activity in the oocyst (Howells, 1970a).

The zygote nucleus is limited by a double-membraned nuclear envelope in which there are numerous nuclear pores (Davies, 1974a).

This large (2 μm diameter) eccentric organelle contains a diffuse nucleoplasm with numerous scattered groups of large particles (18 nm diameter) in a fibrous matrix. Occasionally the large particles, which readily incorporate ^{3}H-adenosine, are aggregated in nuclei of both zygote and ookinete and form a nucleolus-like structure (Figures 2 and 6); this is not unexpected in view of the rapid expansion in the ribosome population within the ookinete and oocyst. Division of the nucleus of the zygote has been seen both in parasites still within the bloodmeal (Davies, 1974a) and in ookinetes traversing the midgut wall (Figure 3) (Canning and Sinden, 1973). This division, which is thought to be a one step meiotic division (Bano, 1968; Canning and Anwar, 1968; Canning and Sinden, 1973; Sinden and Canning, 1973), is marked by the appearance of spindle poles 2–2·5 μm apart on the flattened or slightly invaginated nuclear envelope. In thin sections there are 2 to 5 electron-dense kinetochores (see Table I on p. 154) on the short spindle microtubules which radiate from an electron dense centriolar plaque situated in a single enlarged nuclear pore. Contrary to the suggestions of Garnham *et al.* (1969a) and Bafort (1971), there is no evidence for the presence of a centriolar pinwheel on the cytoplasmic side of the centriolar plaque.

While still in the bloodmeal, the spherical zygote transforms to the banana- or leaf-shaped mature ookinete (Figures 4 and 5). This transformation is preceded by the rapid appearance of the subpellicular organelles and the apical complex. On the basis of the light microscope studies and by analogy with *Haemoproteus* (Gallucci, 1974b) and *Leucocytozoon* (Trefiak and Desser, 1973), this pellicular synthesis might itself be expected to precede the budding of the anterior region so frequently found in the "retort form" ookinete. Gallucci (1974b) has demonstrated the origins of the apical complex of *Leucocytozoon* in an electron-dense mass found on the cytoplasmic face of the centriolar plaque of a nuclear spindle which marks the first division of the zygote genome. This conoidal anlage, which bears a striking resemblance to the microgametocyte MTOC of *Plasmodium* (see p. 145), migrates towards the plasmalemma and gives rise to the polar rings and conoid. The subpellicular microtubules are derived from fibrous material beneath the conoidal canopy. As with the formation of the merozoite and sporozoite, the position of the formation of the apical complex in the cell is determined by the intranuclear spindle. In *P.yoelii*, synthesis of the inner pellicular membranes begins with the deposition of short

segments of membrane 14–20 nm thick beneath the plasmalemma (8–10 nm thick) of the zygote (Figure 1) (Canning and Sinden, 1973; Davies, 1974a). These segments ultimately fuse to form an apparently continuous inner doublet membrane (Figure 2). These inner layers are synthesized before the subpellicular microtubules (Davies, 1974a). The

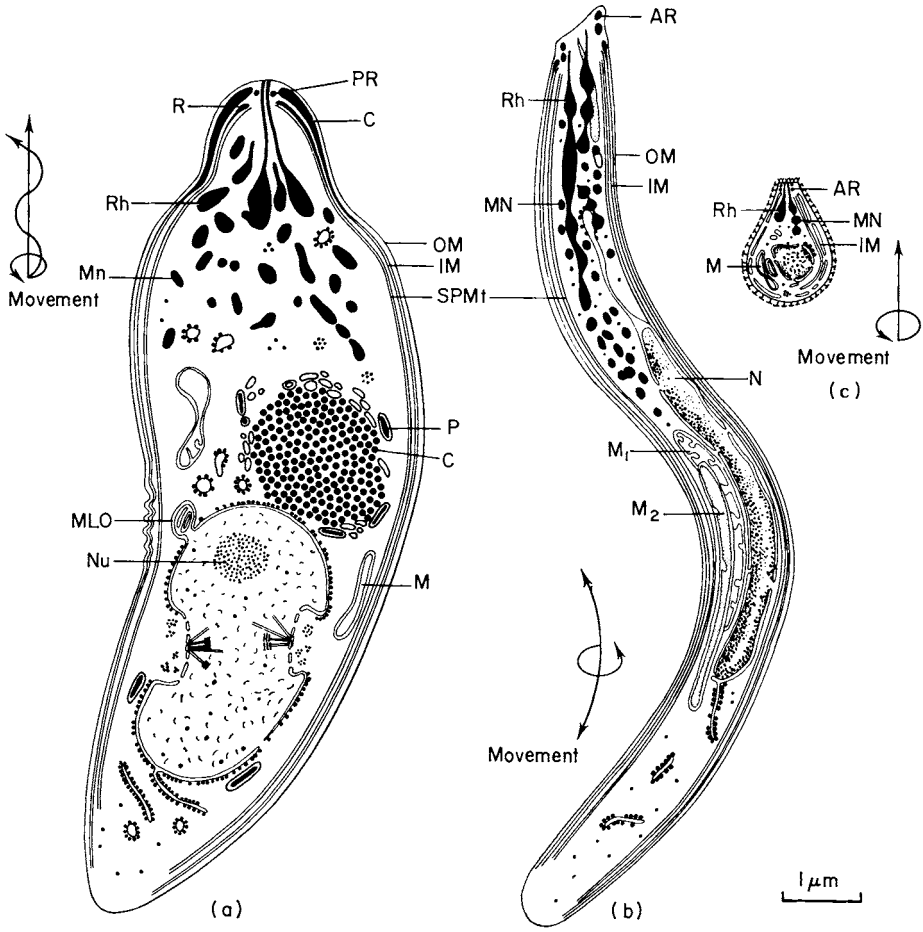


Figure 4. The invasive stages. (*a*) The ookinete. (*b*) The sporozoite. (*c*) The erythrocytic merozoite. *Abbreviations.* AR, apical ring; C, collar; Cr, crystalloid; IM, inner pellicle membrane; M, mitochondrion; MLO, multilamellate organelle; Mn, microneme; N, nucleus; Nu, nucleolus; OM, outer pellicle membrane; P, pigment; PR, polar ring; R, electron dense ring; Rh, rhoptry; SB, spherical body; SPMt, subpellicular microtubules.

flexibility of this pellicular complex (Figure 7 insert) is dramatically emphasized by the cinephotographic studies of Freyvogel (1965) and the ultrastructural studies of Garnham *et al.* (1969a). The function of the inner pellicular membranes may be to provide a structural support for the movement of the 55–65 subpellicular microtubules. Neither these membranes, nor the subpellicular microtubules appear to extend over the whole surface of the ookinete. The posterior third of the cell is possibly covered only by a single plasmalemma, which would account for the apparent "fragility" of this region seen in SEM preparations (Figure 5) (Speer *et al.*, 1974; Sinden, 1975a).

Figure 8. Reconstruction of the anterior portion of ookinete (from Canning and Sinden, 1973).

In the mature ookinete the apical complex comprises four structures (Figure 8): the polar ring; a collar continuous with the inner pellicle membranes; an electron-dense ring, 90 nm thick, to which the subpellicular microtubules are attached and the rhoptry–microneme complex (Figures 8–11). The collar (= polar cavity of Garnham *et al.*, 1969 and Davies, 1974a) covers the anterior conical projection, is 46 nm deep at its anterior border and tapers to nothing over the "shoulders" of the ookinete. The unusual appearance of this organelle in oblique section led Garnham *et al.* (1962) to describe a shark-like slit for a mouth, a misnomer corrected in their later publication (Garnham *et al.*, 1969a). The apical complex appears to be extendable and retractable on the subpellicular microtubules (Garnham *et al.*, 1969a; Speer *et al.*, 1974). A similar retractable structure has been described in the ookinete of *Parahaemoproteus* (Desser, 1972). It is possible this apical complex is used much in the same way as the conoid of sporozoites or the intestinal coccidia in boring into the host tissues. The rhoptries and micronemes lie beneath the pellicular components of the

apical complex. These electron-dense organelles are limited by a single unit membrane and extend as ductules through the polar ring to the plasmalemma. The inner pellicular membranes do not extend over the polar ring but terminate in the electron-dense collar.

The function of the rhoptry–microneme complex has always been regarded as secretory (Garnham *et al.*, 1961), presumably to facilitate penetration of the midgut wall (Canning and Sinden, 1973); however, in contrast to the observations of Jensen and Edgar (1976b) on the sporozoite of *Eimeria magna* during penetration of cultured cells, there is little loss of electron density or change in distribution of these organelles after penetration of the gut wall (Figure 9). It is not known if the rhoptries are responsible for the production of the viscous threads recorded in *in vitro* studies (Garnham, 1965).

Locomotion of the ookinete, which in its mature form varies from 7 to 18 μm in length and is 2·4 μm in diameter, has been described as a linear gliding motion (Freyvogel, 1965) or as a snakelike wriggling or gliding movement (Rosales-Ronquillo and Silverman, 1974), but is clearly rotational and is at its most efficient when the ookinete is in a cellular environment. Speer *et al.* (1975) have suggested the ookinete may move against a substrate secreted by the rhoptries, a mechanism reminiscent of that described in the gregarines. Speer *et al.* (1974), in an SEM study, illustrate that the ookinete is often helically coiled and described spiral waves in the pellicle. It is not known if the movement of the rigid subpellicular microtubules against the inner pellicular membranes or the activity of membrane-associated microfilaments is responsible for these waves.

The ookinete is capable of penetrating the newly formed, but not the thickened and hardened, peritrophic membrane (Freyvogel, 1965); failure to cross this barrier results in the enzymic destruction of the parasite (Gass, 1977). If successful, the ookinete subsequently brushes aside the microvillar border of the midgut epithelium and penetrates the epithelial cells.

Garnham *et al.* (1962) describe the ookinete as traversing the midgut epithelium intracellularly, but Canning and Sinden (1973) suggested that an intercellular route should not be discounted. However, an intracellular route (Figure 6) is most frequently seen in ultrastructural investigations. The ookinete is commonly found beneath the basement cell membrane with the apical complex pressed firmly against an extended basal lamina of the midgut wall (Figures 7 and 9). Clearly any

secretion from the rhoptry–microneme complex is incapable of disrupting this fibrous structure (Rosales-Ronquillo and Silverman, 1974, have demonstrated how a freely suspended ookinete *in vitro* is capable of invaginating an erythrocyte which strongly suggests that the function of the rhoptries and micronemes is similar in both merozoite and ookinete—see p. 141). Subsequently the ookinete usually differentiates into an oocyst extracellularly between the basement cell membrane and the basal lamina of the mosquito midgut wall (Figures 10–19). However, intracellular development of oocysts has been described in *P.vinckei* (Bafort, 1971) and *P.berghei* (Beaudoin *et al.*, 1974).

B. The Oocyst

Redifferentiation of the ookinete to the oocyst (Figures 10–14) is predominantly extracellular (Rudzinska, 1969; Howells and Davies, 1971; Sinden, 1975a), but occasionally occurs within the midgut epithelial cell (Vanderberg *et al.*, 1967; Bafort, 1971; Beaudoin *et al.*, 1974). Weathersby (1960), however, demonstrated that contact between the ookinete and the midgut epithelium is not essential for oocyst development. Bafort (1971) suggested that the intracellular forms become progressively extracellular as they enlarge and their delayed development may produce a second brood of sporozoites.

The ookinete usually comes to rest beneath the basal lamina 18–22 h after the bloodmeal was taken. Here the parasite rapidly rounds up (Figure 10) and the apical complex is resorbed into the oocyst cytoplasm (Figures 11–13). The subpellicular microtubules and electron-dense collar become progressively detached from the inner pellicle membranes, detachment beginning at the "posterior" edge of the collar. Eventually the whole complex together with a reduced crystalloid lies free in the oocyst cytoplasm (Figure 12) where it may be detected for up to three days (Garnham *et al.*, 1969a; Canning and Sinden, 1973). The inner pellicle membranes remain attached to the oocyst plasmalemma (Garnham *et al.*, 1969a) but are broken into small fragments which are slowly resorbed into the cytoplasm (Canning and Sinden, 1973). The young oocyst (1–2 days) is enveloped by a single 7·5 nm thick plasmalemma, which on the haemocoelomic surface is covered by the "zipper-like" fibrous basal lamina (Figure 14) (Vanderberg *et al.*, 1967). This covering is clearly revealed in SEM studies (Figure 20) (Strome and Beaudoin, 1974; Sinden, 1975a). The basal lamina itself

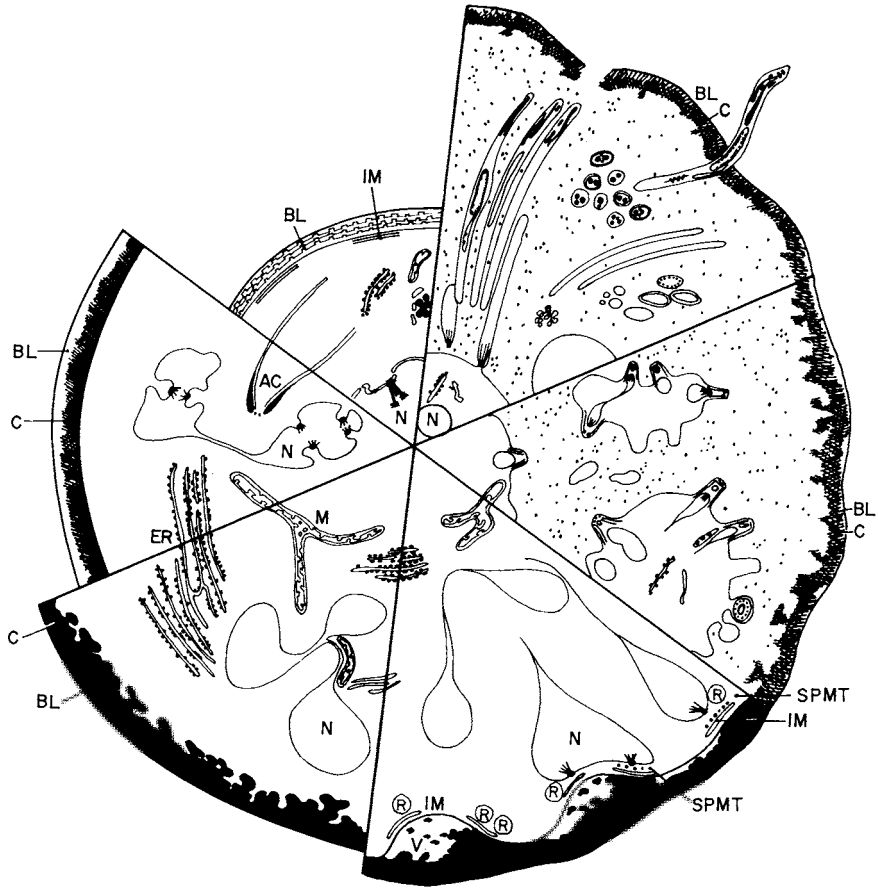


Figure 13. (*not to scale*) Oocyst development and sporozoite formation. *Abbreviations.* AC, apical complex; BL, basal lamina of midgut; C, capsule; ER, endoplasmic reticulum; IM, inner pellicular membrane; M, mitochondrion; N, nucleus; R, rhoptry; SPMt, subpellicular microtubules; V, vacuole.

has been described as being covered by an additional thin membrane (Garnham *et al.*, 1969a).

As the oocysts enlarge they lift the basal lamina away from the midgut epithelial cells and are themselves displaced by the muscle basket around the gut (Figure 15). With increasing maturity the expanding oocysts may break down the distended basal lamina (Figure 20) which would then provide little obstacle to the emerging sporozoites (Sinden, 1974, 1975a).

Oocysts from the second day onwards are covered by a capsule the thickness of which has been variously described as 0·1–0·2 μm (Vanderberg *et al.*, 1967), 0·35–0·49 μm (Sinden, 1975a) or 1 μm (Aikawa, 1971). The capsule is for the most part amorphous, but occasionally long fibrous molecules may be found. Terzakis *et al.* (1976) have described virus-like particles in the oocyst wall which they suggest are derived from the crystalloid present in the ookinete and young oocyst. In the mature oocyst the capsule, which becomes reduced to a thickness of approximately 0·1 μm, is no longer seen as a continuous smooth layer (Figure 18) but is a stretched thin fibrous network deformed by the contained sporozoites (Figure 33). There is controversy about the origin of the oocyst capsule. Rudzinska (1969) suggests the capsule is of parasite origin, whereas Bafort (1971) argues that the capsule of extracellular oocysts is derived from host connective tissue and that of intracellular oocysts from host cell cytoplasm. Bafort (1971) also discusses the possible role of a host immune response in capsule formation. In scanning electron micrographs Strome and Beaudoin (1974) have illustrated haemocytes attached to the basal lamina covering oocysts, an appearance which may, however, be coincidental. The constancy of the oocyst capsule irrespective of the site of development surely indicates a parasitic origin.

As the oocyst matures the plasmalemma is noticeably evaginated into the capsule as microvilli (Figure 16) (Vanderberg *et al.*, 1967; Rudzinska, 1969; Bafort, 1971) which may enhance uptake of nutrients into the oocyst. Bafort (1971) provides dubious evidence for surface phagotrophy in the oocyst. Even though the presence of digestive vacuoles or lysosomes in the oocyst is indicated by the demonstration of aryl sulphatase activity (Davies, 1974b), other lysosomal enzymes (e.g. acid phosphatase and β-glucouronidase) were not detected in the same study. A very rapid uptake of nutrients into the oocyst has been demonstrated (Vanderberg *et al.*, 1967). Davies and Howells (1975) studied the uptake and incorporation of ^{3}H-adenosine and concluded there was no permeability barrier; they showed that the oocyst, like the erythrocytic stages, does not incorporate ^{3}H-thymidine.

As the oocyst matures, the cytoplasm contracts away from the capsule leaving vacuolar extracellular spaces around the periphery of the cyst (Figures 13 and 18). In the SEM this change may be recognized by the appearance of wrinkled oocysts which are presumably less rigid than the more immature solid oocyst (Figure 20). Wrinkled oocysts

might, however, be artefacts of preparation or degeneration. Progressive cytoplasmic retraction results in the coalescence of the peripheral vacuoles and the formation of clefts between the developing bands of sporoblast cytoplasm. The vacuolar space becomes filled with a fibrous and vesicular material (Figure 22) which is almost certainly derived from the oocyst wall. A nutritive role proposed for the extracellular vesicular material (Aikawa, 1971), which is presumably secreted by the oocyst, is most unlikely.

Nuclear division in the oocyst has been subdivided by Howells and Davies (1971) into three distinct phases: proliferation (Figures 16, 18 and 19); nuclear fission (Figure 22) and finally division into the budding sporozoites (Figure 24). However, Schrevel *et al.* (1977) suggest that the final two phases in fact occur simultaneously.

A young (9 μm diameter) oocyst (Figure 14) contains a nucleus 5–6 μm in diameter, which is surrounded by a double nuclear envelope 80 nm wide, the outer surface of which is usually devoid of ribosomes (Bafort, 1971). Failure to detect a nuclear envelope in some young oocysts (Garnham *et al.*, 1969a) was correctly attributed to inadequate fixation. Only once has a classical interphase nucleus been described in a young oocyst (Canning and Sinden, 1971, 1973). A nucleolus has never been detected in a normal oocyst nucleus. Within two days the oocyst nucleus becomes enlarged and irregularly lobed (Figures 16 and 19); inside each nuclear lobe numerous short (0·5 μm) spindles are formed (Figure 19).

Mitosis is synchronized throughout the nuclear syncytium (Canning and Sinden, 1973a). The spindle microtubules extend between characteristic 0·5 μm diameter gastrula-like invaginations of the nuclear envelope. Within these invaginations, which are excellently preserved in permanganate fixed material, there are numerous large polyribosomes (Vanderberg *et al.*, 1967; Garnham *et al.*, 1969a; Bafort, 1971; Howells and Davies, 1971; Canning and Sinden, 1973). However, the suggestion of Garnham *et al.* (1969a) and Bafort (1971) that a centriole is present in the adjacent cytoplasm remains unsubstantiated. As mitotic spindle formation begins with the appearance of a single hemispindle, 20–25 microtubules originate in a single large centriolar plaque situated in a nuclear pore. The bipolar spindle is formed by the fission of the centriolar plaque and the apposition of the two daughter plaques by the expansion of the intervening nuclear envelope (Fouchet and Schrevel quoted by Vivier and Vickerman, 1974; Schrevel *et al.*,

1977). This nuclear division is classified as cryptomitotic (Hollande, 1972). Interzonal microtubules, which extend from pole to pole (Canning and Sinden, 1973), are presumably polymerized only after apposition is complete. The 5–10 kinetochores are 50–55 nm wide and 48–85 nm long and are composed of 3 electron-dense bars, the outermost being connected either to the spindle microtubule (inner diameter 10–13 nm, outer diameter 18–23 nm) or to the chromosome (Figure 19). The kinetochores have been confused with chromosomes (Aikawa and Beaudoin, 1968) or chromosome-like bodies (Howells and Davies, 1971). However, the chromosomes, at best visible as a vague electron-dense condensation around the kinetochore (Howells and Davies, 1971), are more usually undetectable either morphologically, by DNAase extraction or by autoradiographic localization of ^{3}H-adenosine (Canning and Sinden, unpublished). It must be assumed that the chromosome condensation cycle in mitosis of malaria parasites is abnormal, much as it is in *Aggregata* (Grell, 1973). Chromosome separation is achieved by the extension of the interzonal microtubules and a retraction or resorption of the kinetochore microtubules (Canning and Sinden, 1973). It is clear that the numerous small spindles maintain a haploid organization within the polyploid endomitotic nucleus.

At the time of sporoblastoid formation, mitotic activity is reduced and the attentuated nuclear lobes may separate into numerous small nuclei (with a diameter of 1 μm) which migrate to the surface of the sporoblastoid (Howells and Davies, 1971). However, from my observations, I think that attenuated nuclear lobes may persist throughout the development of the oocyst (Figure 22).

The final nuclear division is intimately associated with the appearance of the budding sporozoite at the plasmalemma of the sporoblast (Vanderberg and Rhodin, 1967; Vanderberg *et al.*, 1967). Flattened nuclei approach within 160 nm of the plasmalemma. The subpellicular structures of the sporozoite develop immediately above a single nuclear spindle pole of each nucleus (Figure 22) (Howells and Davies, 1971). Vanderberg and Rhodin (1967) and Schrevel *et al.* (1977) have demonstrated numerous spindle poles within such nuclei. As the sporozoite evaginates, the nucleus is drawn spindle first into the bud (Figures 22 and 24). The spindle becomes unusually long (1·5–2·5 μm) as the nucleus extends into the sporozoite. After separation of the sporozoite nucleus from the residuum, the chromatin very rapidly

becomes condensed as heterochromatin on the nuclear envelope, where it is readily detected by DNAase digestion (cf Figures 28 and 29) (Canning and Sinden, unpublished). The residual body of the oocyst invariably contains some Feulgen positive DNA which Vanderberg *et al.* (1967) interpret as an overproduction of nucleic acid by the oocyst. Aikawa (1971) has suggested that nuclear division in the oocyst and erythrocytic schizont is similar. Although the accelerated endomitotis of the nuclei of the oocyst differs from the classical mitotic division so frequently described in erythrocytic schizonts, more detailed ultrastructural studies of erythrocytic schizogony may yet substantiate Aikawa's suggestion.

The cytoplasm of the oocyst contains a high density of ribosomes, which explains both its strong basophilia and sensitivity to RNAase (Vanderberg *et al.*, 1967). In the young oocyst ribosomes are associated with a single region of endoplasmic reticulum (Figure 10), but, as the oocyst matures, numerous peripheral islands of endoplasmic reticulum develop (Figure 16). These regions have been termed Golgi bodies by Bafort (1971). The same author has demonstrated continuity between the endoplasmic reticulum and the nuclear envelope and, most surprisingly, suggests a similar continuity with the outer mitochondrial membranes. This marked expansion of endoplasmic reticulum shows there is considerable synthesis of secreted proteins which probably form the cyst wall.

After the transition of the mitochondria in the ookinete from a subcristate to a cristate state, there is a marked increase in number, size and electron density of the mitochondria in the oocyst (Figures 16 and 18), some of which become as long as 3 μm (Vanderberg *et al.*, 1967). Bafort (1971) describes aggregates of organelles limited by two unit membranes, strikingly similar to the spherical body found in the merozoite, which he suggests may be involved in mitochondriogenesis. Within the oocyst the mitochondria are frequently found lying adjacent to the nuclear envelope and, during sporozoite evagination, they closely follow the nucleus into the budding sporozoite.

Other cytoplasmic organelles described are few, but include: the multilamellate organelle (Vanderberg *et al.*, 1967) which is usually attached to other membrane systems; toxonemes (rhoptries and micronemes) (Rudzinska, 1969); pigment crystals within single membrane-bound vesicles (Bafort, 1971; Canning and Sinden, 1973a); and dense spherules (Figure 18), which when examined by energy

dispersive X-ray analysis are found to be devoid of iron and are therefore distinguished from pigment crystals (Figure 17).

Sporozoite formation occurs synchronously over the whole surface of the sporoblastoids. Initially narrow (2 μm) diameter plates composed of 2 tightly appressed unit membranes appear, closely applied to the plasmalemma above the nuclear spindles. Beneath these inner pellicular membranes the subpellicular microtubules rapidly develop. As the sporozoite begins to evaginate (Figures 23 and 24), the 2 or 3 apical rings form at the apex, and the short subpellicular microtubules attach to the posterior ring and radiate from it over the sporoblastoid surface. Extension of the inner pellicle membranes, and the subpellicular microtubules, is strictly confined to the junction between the sporozoite bud and the sporoblastoid plasmalemma; neither organelle extends into the sporoblastoid cytoplasm. In marked contrast to other species of *Plasmodium*, a micropore (cytostome) has never been detected in the sporozoites of murine malaria parasites. An immature sporozoite (Figure 24) is a rigid vase-shaped structure with a smooth plasmalemma. The distal (apical) pole is flattened and inclined at an angle to the longitudinal axis of the cell (Figures 30–32) (Sinden, 1975a). The 1–3 rhoptries are first recognized as clear membrane-bound spheres lying immediately anterior to the nucleus in the sporozoite bud (Figure 23). These spheres become progressively more electron dense and elongate as sporozoite formation proceeds (Figures 24 and 31). Ductules finally develop and extend anteriorly to the plasmalemma through the apertures of the apical rings (Bafort, 1971; Sinden and Garnham, 1973). There are 2–5 rhoptries, 0·1 μm in diameter, in fully grown sporozoites in oocysts (Figures 25 and 26). Rhoptry morphogenesis continues until the sporozoite enters the salivary glands, the organelles becoming branched (Sinden and Garnham, 1973) or subdivided into beaded strands (Figure 30); this morphogenesis transforms the rhoptries into numerous micronemes. After the incorporation of the mitotic nucleus, 1 or 2 mitochondria migrate into the sporozoite (Vanderberg *et al.*, 1967; Bafort, 1971; Howells and Davies, 1971; Sinden and Garnham, 1973). Finally endoplasmic reticulum is incorporated behind the severed nucleus. Sporozoite formation may be accompanied by a vacuolization of the residual body (Vanderberg *et al.*, 1967).

Oocyst degeneration, which is sometimes associated with heavy laboratory infections in unnatural vectors, usually takes one of four

forms: viral infections (Davies *et al.*, 1971; Davies and Howells, 1971); vacuolization of the cytoplasm and its membranous organelles; condensation of the nucleic acids within the nucleus; and the formation of Ross' black spores (Bafort, 1971; Sinden and Garnham, 1973). Ross' black spore formation is not a cellular response by the haemocytes, but is a humoral response. The oocyst capsule and membrane adjacent to the haemocoele first being "melanized", this reaction subsequently extending into the oocyst along its internal membranes (Sinden and Garnham, unpublished).

C. The Sporozoite

The overall dimensions of the mature sporozoites of rodent malaria parasites differ between species (see Chapter 2) and the type of preparation examined. Giemsa-stained salivary gland sporozoites of *P.yoelii nigeriensis* are 16·72 $\pm$1·56 nm long (Killick-Kendrick, 1973), whereas scanning microscopy studies on sporozoites released from oocysts showed smaller sporozoites 9–12 μm in length and 0·5 μm in diameter (Sinden, 1975a). Abnormal sporozoites of *P.yoelii nigeriensis* which may have vacuolated cytoplasm and condensed nuclei varied from 0·4 to 2·7 μm in diameter and may be up to 40 μm long (Hulls, 1972; Sinden and Garnham, 1973; Sinden, 1975a).

The normal sporozoite viewed in the SEM (Figure 34) is devoid of any permanent surface architecture such as the microtubular ridges of the merozoite of *Sarcocystis* (Mehlhorn and Scholtyseck, 1974), or the micropores of *Eimeria* (Vetterling *et al.*, 1971). The absence or extreme rarity of a micropore in the sporozoites of rodent plasmodia contrasts with the primate and avian malaria parasites and has been regarded as an indication of incomplete development which may be related to the relatively poor infectivity of the sporozoites of murine malaria parasites (Garnham, 1972; Sinden and Garnham, 1973). This highly mobile and flexible cell is usually curved or coiled in a single turn of a helix, and contrasts markedly with the straight immature form. Vanderberg (1975) describes the movement of sporozoites from the oocyst as flexing, alternate end thrusting, and rarely circular gliding, and makes an interesting distinction between this and the movement of the salivary gland sporozoite which he described as limited to circular gliding and attached waving. However, Yoeli's description (1964) of movement of salivary gland sporozoites is very similar to that of Vanderberg's oocyst form, suggesting that the mobility of the sporozoite may differ quanti-

tatively rather than qualitatively between the two forms. We have always thought that the sporozoite from the oocyst was more "active" and flexible than the form from salivary glands. Vanderberg (1975a) also makes the fascinating observation that the sporozoite moves with the rhoptry microneme complex trailing. The polarity of the sporozoite is revealed by a localization of the circum sporozoite precipitate to the rhoptry complex end of the cell. This "backward" movement he suggests may be related to secretions from the rhoptry–microneme complex, both he and Schaudinn (1903) having observed droplets released from the trailing pole of the sporozoite. However, in contrast to Vanderberg's observations Cochrane *et al.* (1976) have shown the circum sporozoite precipitate forms about the posterior end of the sporozoite. Vanderberg's results therefore show the sporozoite to move with the rhoptry–microneme complex foremost, as do the ookinete and the merozoite. Vanderberg (1975a) also suggests that the circular gliding of the sporozoite is in one direction only, showing that the sporozoite has a dorsal–ventral polarity. The morphological basis for this may well be the asymmetry of the apical complex which is inclined to the longitudinal axis of the sporozoite.

Despite the widely varying environments to be tolerated and the numerous cellular barriers to be crossed, the basic morphology of the sporozoite (Figure 4b) does not differ remarkably from that of the ookinete or merozoite (Figures 4a and 4c), the migrations of which are, by comparison, trivial. The sporozoite is covered by a single unit membrane plasmalemma 7·5 nm thick, the outer surface of which is covered by a disperse fibrous material which is probably derived from the cystic fluid. Occasional blebs in the membrane are visible by both TEM and SEM (Figures 25 and 34). Beneath the plasmalemma lie the two continuous unit membranes of the inner pellicle, the thickness and duplex structure of which is clearly revealed by some fixatives but not others (cf. Figures 25–27) (Terzakis, 1968). These membranes extend over the whole cell surface with the exception of an apical pore, and possibly a similar region at the posterior pole. The inner membranes often have invaginations, which have erroneously been described as micropores (Vanderberg *et al.*, 1967). When examined by negative staining techniques, the pellicle of the sporozoite appears reticulated. This suggests the wall of the sporozoite has a labyrinthine structure similar to that of the merozoite (Cochrane *et al.*, 1976). The inner pellicle membranes of the sporozoite are, however, continuous and not

labyrinthine as in the merozoite. The segmented pattern seen using the negative staining technique may therefore be produced either by the topography of the sporozoite plasmalemma, or by the negative staining of the innumerable underlying micronemes (Figure 35). The inner membrane is recurved at the anterior pole; in the recurved region lies a ring of electron-dense material (the posterior "apical ring") to which the subpellicular microtubules are attached (Figure 31). Anterior to the electron-dense ring lies another apical ring of smaller diameter which, contrary to the suggestion of Scholtyseck *et al.* (1970), is not continuous with the inner pellicle layers. The subpellicular microtubules are spaced 2–4 nm from the inner pellicular membranes by regular paired electron-dense linkages analagous to the dynein arms of flagellar microtubule doublets (Figure 25). The microtubules commonly lie in electron-lucent regions of cytoplasm, presumably the movement of the tubule having pushed material from this zone. Each tubule has an external diameter of 25 nm with a wall thickness of 8·5 nm (Vanderberg *et al.*, 1967). The distribution of these tubules, although symmetrical in the anterior third of the sporozoite, assumes a typical asymmetry in the region immediately anterior to the nucleus. Here all the tubules but one are equally spaced around two-thirds of the circumference, the remaining tubule occupying the other one-third (Figures 26 and 27). Emmel *et al.* (1942) suggest this tubule is on the "outside" of the sporozoite coil. The number of tubules may vary between species or subspecies, e.g. 16 in *P.v.vinckei* (Bafort, 1971), 16–17 in *P.berghei* (Vanderberg *et al.*, 1967; Aikawa and Sterling, 1974), 14 in *P.yoelii* and 15 in *P.chabaudi* (Sinden and Garnham, 1973). Aberrant sporozoites may contain as many as 32 microtubules. Posterior to the nucleus the microtubules decline in number suggesting they are of variable length, and that there is no common posterior anchoring organelle.

The rhoptries found in sporozoites from the oocyst transform to micronemes in the salivary gland form (Figure 35) (Sinden and Garnham, 1973). A similar transition in rhoptry organization has been recorded in sporozoites of *Leucocytozoon* (Wong and Desser, 1976) and the merozoites of *Sarcocystis* (Mehlhorn *et al.*, 1975), *Toxoplasma* (Vivier and Petitprez, 1972) and *Eimeria* (Heller, 1972). The function of these organelles, which occupy most of the cytoplasm anterior to the nucleus, is unknown but by analogy with the rhoptries of *Eimeria* (Jensen and Edgar, 1976b), they probably aid penetration of at least some of the cell barriers crossed by the sporozoite.

The sporozoite nucleus is 2–3 μm long and 0·3–0·7 μm in diameter, and is centrally situated in the cell. The nuclear envelope in which there are few compound nuclear pores may extend for a considerable distance toward the anterior pole. A concentric membraned organelle may occasionally be detected continuous with the nuclear envelope. A DNAase-sensitive layer of heterochromatin up to 0·15 μm deep is applied to the inner surface of the nuclear envelope. The remainder of the nucleus contains a fibrous matrix with interspersed particles 16–25 nm in diameter. There is no nucleolus in the sporozoite nucleus.

Other cytoplasmic structures include: numerous small membrane-bound vesicles, termed Golgi bodies by Vanderberg *et al.* (1967); dense spherules (0·16 μm diameter) similar to those described in gametocytes of avian parasites (Sterling and Aikawa, 1973); a single elongate cristate mitochondrion (a second electron-dense mitochondrion, see Figure 27, has been described in *P.chabaudi*—Sinden and Garnham, 1973—which may be analagous with the spherical body of the merozoite, see Figure 46) and one or two large cisternae of rough endoplasmic reticulum posterior to the nucleus.

The passage of the mature sporozoite from the salivary gland to its eventual site of development in the liver parenchymal cell is the most remarkable aspect of the whole of the life cycle of the malaria parasite. Unfortunately it is this phase of development which is the least understood when considering the fine structure of murine malaria parasites.

Escape of the highly motile sporozoite from the oocyst has long been considered a passive event, the thin oocyst wall presumably being ruptured by the activity of the gut musculature (Garnham, 1966a). However, Sinden (1974, 1975a) has suggested the sporozoites may actively participate in the breakdown of the fibrous oocyst wall. SEM studies revealed the oocyst wall peppered by small 0·25–0·65 μm diameter holes through which sporozoites protruded (Figure 33). In normal oocysts the numerous holes would weaken the oocyst wall so drastically that the oocyst could be torn apart by external mechanical forces. Large openings in the oocyst wall have been seen in the SEM studies of Sinden (1974, 1975a) and Strome and Beaudoin (1974). While all authors are aware of the possible artefact produced by preparative techniques, these openings may well be involved in the breakdown of the oocyst wall *in vivo*. In degenerate oocysts, a few active sporozoites could emerge singly through scattered pores without causing a total breakdown in the cyst wall. Although it was suggested that the

sporozoite activity was directed toward the haemocoelomic surface of the oocyst (Sinden, 1974), it is this surface which has the least mechanical support and is therefore the more liable to disruption. Studies on the release of sporozoites from oocysts freely suspended in culture would perhaps more clearly reveal how they escape. The basal lamina of the midgut is so shredded by the expansion of the growing oocyst that it offers no barrier to sporozoite migration. Indeed, were it not ruptured it would be as impenetrable to the sporozoite as it obviously is to the ookinete—unless the rhoptries secrete different substances at each stage of development. Sporozoites in common with the ookinete are clearly capable of migrating through the midgut epithelium. Beaudoin *et al.* (1974), in their study on the ectopic development of *P.berghei* oocysts, found infective sporozoites and oocysts in the lumen of the gut. Within the midgut epithelial cells the sporozoite, like the intraerythrocytic merozoite, was in a parasitophorous vacuole limited by host cell membrane. This is in marked contrast to the ookinete which is intracytoplasmic. We have made similar observations on the sporozoites of the primate parasite *P.vivax* (Sinden and Garnham, unpublished). The excysted sporozoite has to avoid the activity of the mosquito's phagocytes and humoral responses, "a task in which it invariably succeeds" (Weathersby, 1975). However, we have observed a most unusual concentration of sporozoites of *P.vivax* inside a cell, tentatively identified as a haemocyte, in the midgut epithelium of *A.atroparvus* which suggests that not all sporozoites successfully reach the haemocoelomic fluid. It is apparently unknown whether the sporozoites actively migrate toward the salivary glands, a surprising omission in the studies of sporozoite physiology. However, they must actively penetrate the basement membrane of the salivary gland cell, a penetration achieved with exceptional rapidity for it has never been recorded in ultrastructural studies (Garnham *et al.*, 1960, 1961; Sinden and Garnham, 1973; Sterling *et al.*, 1973). The basement membranes of some distal salivary gland cells are disrupted into small vesicles and vacuoles by the entry of numerous sporozoites (Sterling *et al.*, 1973). Other damage produced by the migration of sporozoites through the cells (Figure 35) includes an increase in the number of random microtubules and vesiculation of the secretory cavities.

Within the salivary glands the sporozoites accumulate in the distal cells of both median and, to a lesser extent, lateral lobes—possibly a behavioural adaptation to the sporozoites inability to penetrate the

thick chitinous lining of the secretory duct found in the proximal cells. Sporozoites are found packed in parallel bundles within the secretory matrix. They enter the lumen of the duct in the distal cells where the duct wall is not chitinized, and are subsequently found in proximal regions (Figure 36). The duct narrows down to 1·25 μm in places (Sterling *et al.*, 1973), barely wide enough to allow passage of the sporozoite. Remarkably few signs of degeneration have been noted in sporozoites from salivary glands beyond surface blebbing and the presence of a few giant sporozoites (Sinden and Garnham, 1973). This contrasts markedly with the sporozoites seen in oocysts and suggests that the degenerate forms fail to migrate to, or penetrate, the salivary glands. In marked contrast to the merozoite, after penetration of the host cell (salivary gland) the rhoptries, micronemes and the apical complex of the sporozoite are unchanged. It must be assumed, therefore, either that they function solely during infection of the vertebrate host or that they aid penetration of several barriers.

The sporozoites from the oocyst which contain rhoptries also have an immature (10 day) antigen which is released from the apical pole and may be involved in salivary gland penetration. In contrast, the salivary gland forms, which contain micronemes, possess a different (18 day) antigen which is similarly released and may be involved in penetration in the vertebrate host (Vanderberg *et al.*, 1972). However, there is at present no morphological explanation for the increased antigenicity of the salivary gland sporozoite over that of the oocyst form. The dramatic increase in infectivity of the sporozoite population from the salivary glands (Vanderberg, 1975b) is perhaps explained by the failure of degenerate and non-infective parasites to reach this site. Of the sporozoites in the glands, Vanderberg (1977) has shown that in the case of *A.stephensi* infected with *P.berghei* between 0·1 and 17·8% are injected during a single feed of the mosquito and subsequently develop into exoerythrocytic parasites. The exact proportion inoculated, he suggests, is dependant upon specific host skin factors.

In a study of the morphology of various antigenic preparations of salivary gland sporozoites Jakstys *et al.* (1974) found little change induced by gentle homogenization; cytoplasmic and nuclear flocculation induced by heat inactivation and severe leaching produced by freeze thawing techniques.

The major omission in ultrastructural studies of all malaria parasites is the total absence of information on the process of infection of the

vertebrate host and the subsequent development of the intracellular parasite—problems so elegantly studied in the Eimeridae by Roberts *et al.* (1971) and Jensen and Hammond (1975). Hepler *et al.* (1966) and Aikawa *et al.* (1968) have, however, studied the early development of the pre-erythrocytic schizont of avian plasmodia *in vitro*.

D. The Pre-eyrthrocytic Schizont

Since electron microscope studies on the pre-erythrocytic schizont of murine *Plasmodia* have been confined to the mature schizont, i.e. 42–52 h following infection, the interesting phases of sporozoite redifferentiation have yet to be examined.

The 48 h schizont of *P.berghei* and the 52 h schizont of *P.vinckei vinckei* (Figure 37) are contained within a membrane-limited parasitophorous vacuole within a liver parenchyma cell (Bafort and Howells, 1970; Bafort, 1971). The host cell has a reduced density (Bafort, 1971) and its nucleus is displaced (Desser *et al.*, 1972). A system of vesicles between the parasite plasmalemma and the parasitophorous vacuole membrane (Bafort, 1971) has not been confirmed by either Desser *et al.* (1972) or Garnham *et al.* (1967b, 1969b). Desser *et al.* (1972) suggest the uptake of nutrients is by surface phagocytosis and Beaudoin and Strome (1972) have clearly demonstrated surface phagotrophy through a micropore in the avian parasite *P.lophurae*. No micropore has been detected in the murine parasites, neither have food vacuoles been identified within the cytoplasm. Clearly diffusion of nutrients must play a significant role in the nutrition of the pre-erythrocytic schizont.

Within the immature schizont the endoplasmic reticulum proliferates rapidly forming large whorls in the mature schizont. The high ribosome density also reflects the active protein synthesis by this rapidly growing cell. The numerous mitochondria have been variously described as being acristate (Bafort, 1971; Bafort and Howells, 1970), cristate (Terzakis *et al.*, 1974) or as smooth membraned organelles (Garnham *et al.*, 1969b; Desser *et al.*, 1972). Terzakis *et al.* (1974) suggest that it is in the pre-erythrocytic schizont that the mitochondrion rapidly regresses from the cristate form of the sporozoite to an acristate condition. Indeed, although histochemical analysis revealed cytochrome oxidase, G6PD and 6GPD activities in this stage, succinic dehydrogenase activity was not demonstrable (Howells and Bafort, 1970).

Nuclear division in the pre-erythrocytic schizont has been poorly

studied. The nuclei in a 48 h schizont are discrete rounded bodies surrounded by a double nuclear envelope, poorly endowed with nuclear pores. Neither mitotic nor interphase nuclei with condensed heterochromatin have been described.

The only other cytoplasmic structures of note are: lipid droplets and a virus body (Bafort, 1971) identical to that described in the oocyst of *Plasmodium* by workers from the same laboratories (Davies *et al.*, 1971).

Pseudocytomere formation begins with the appearance of vacuoles between the membrane of the host cell and the cytoplasm of the parasite. Subsequent cytoplasmic contraction and cleft formation is less marked than in the oocyst (Bafort, 1971). The vacuolar space is filled with fibrous and granular materials. Desser *et al.* (1972) suggest that pseudocytomere formation increases the surface area for the absorption of nutrients, but this is surely unlikely as cytoplasmic retraction is occurring at this stage. However, pseudocytomere formation does allow the development of a vastly increased number of merozoites within the schizont (Figure 37). Merozoite formation as described by Garnham *et al.* (1967b, 1969b), Bafort (1971) and Desser *et al.* (1972) is identical to that described in the erythrocytic schizont. Points of note are that subpellicular microtubules, which are rare in the erythrocytic merozoite, have not been detected in the exoerythrocytic form. However, a micropore has once been described in the exoerythrocytic merozoite (Garnham *et al.*, 1969b), but has not been reported in the erythrocytic form. The merozoite plasmalemma is coated with a layer of microfibrillae which, in surface view, show a cross-hatched distribution. This coating, which must be homologous with that of the erythrocytic form (Bannister *et al.*, 1975), was denser than its erythrocytic counterpart, as was the content of the parasitophorous vacuole. The subpellicular labyrinthine membrane has been described as a single thick membrane (Garnham *et al.*, 1969b; Desser *et al.*, 1972). Further studies with other fixation techniques are required to clarify the exact nature of this structure, which is probably composed of two closely applied unit membranes.

The mature merozoite, which frequently has both rhoptries and micronemes, is highly motile (Huff *et al.*, 1960), and may therefore contribute to the rupture of mature schizonts. As merozoites of tissue schizonts are shed into sinusoids of the liver, they quickly invade erythrocytes. At the time of maturation of the schizonts, parasitized erythrocytes are readily detected in the blood of the liver (Garnham

et al., 1969b). Redifferentiation of the intracellular merozoite occurs as rapidly as that of its erythrocytic counterpart.

E. The Trophozoite

The trophozoite and intraerythrocytic schizont are the most extensively studied stages of development, primarily because of their pathological significance, but not least for the ease with which they are obtained (Rudzinska, 1969). However, numerous subjects of controversy remain, including the mechanism of uptake of nutrients and the functions of numerous ill-defined structures.

The trophozoite (Figure 38) differentiates from the merozoite following invasion of the erythrocyte. Initial redifferentiation results in the sequential loss of the rhoptry–microneme complex, apical rings, inner labyrinthine membrane and subpellicular microtubules. This process differs from that of the saurian parasite *P.mexicanum* where the subpellicular organelles persist into schizogony (Moore and Sinden, 1974). Blackburn and Vinijchaikul (1970) are alone in suggesting that resorption of the rhoptries is delayed in the murine parasites. The fate of these apical organelles is unknown; the microtubules are presumably depolymerized and the inner pellicular membranes may be resorbed into the cytoplasmic membrane systems to form the multilamellate organelle. Their loss, however, allows the trophozoite to become amoeboid (Ladda, 1969). The parasite lies in a vacuole the limiting membrane of which is evaginated from the erythrocyte plasmalemma during merozoite invasion (Ladda *et al.*, 1969). This unit membrane, which was not recognized in the pioneer study of Fulton and Flewitt (1951), is 5·0–7·5 nm thick and has an irregular surface. When examined by freeze-fracture techniques, it differs from the plasmalemma of both parasite and erythrocyte, in being devoid of particles on either internal face of the unit membrane (Mazen *et al.*, 1975; Sterling, unpublished), although Ladda and Steere (1969), in a similar freeze-fracture study, were unable to detect such differences. Sterling (unpublished) further suggests that the extrinsic membrane-associated protein—spectrin—may also be absent from the parasitophorous vacuole membrane. Weidekamm *et al.* (1973) found a specific degradation of 3 out of 9 erythrocyte plasmalemma proteins in infected cells, which they suggest may result in a changed flux of Na^{2+}, K^{2+} and amino acids across the membranes.

The parasite plasmalemma is 5·0–7·5 nm thick depending on the fixation technique. Its surface is roughened (Ladda and Steere, 1969; Mandahar and van Dyke, 1975) with an appearance described as "brick-like" in freeze-fractured specimens (Seed *et al.*, 1973b), and "cog-like" in TEM specimens (Blackburn and Vinijchaikul, 1970). The latter authors suggest the interdigitation of the host and parasite membranes produces "hold fast" regions which may represent specialized regions for host–parasite exchange. Seed *et al.* (1973a) show that the surface irregularity is produced by the intramembranous protein particles each 25–35 nm in diameter, and contrast the parasite membrane structure with that of the erythrocyte in which the intramembranous particles are 8·5 nm in diameter. Seed and Kreier (1976), using electrophoretic and lectin-binding techniques, were able to show the parasite plasmalemma has a net negative surface charge with an isoelectric point of approximately 3·0, and a surface characterized by a scarcity of lectin-binding receptors and sialic acid residues, with a correspondingly high lipid contribution to the surface charge properties.

The vacuolar space is from 6 to 17 nm in width (Rudzinska and Trager, 1959) dependent upon the fixative used (Blackburn and Vinijchaikul, 1970). The plasmalemma of the amoeboid trophozoite is often invaginated. Rudzinska and Trager (1959), in a study of *P.berghei*, suggested such invaginations were phagocytic vacuoles. Similarly, Killby and Silverman (1969a) describe a single large food vacuole in the ring stage and 2–7 such vacuoles in larger parasites. These and other authors (Theakston *et al.*, 1968a) suggest that secondary vacuoles, limited by only a single membrane, bud off from this invagination (which is limited by two unit membranes). Cox and Vickerman (1966) propose that the large invagination simply increases the surface area of the parasite upon which the pinocytotic vesicles may form. In marked contrast cytostomal feeding is the normal mechanism of food intake in avian malaria parasites (Aikawa *et al.*, 1966a, b; Langreth, 1976). Cytostomal feeding also occurs in murine parasites (Figure 42) (Sinden and Garnham, 1973; Howells *et al.*, 1968b). Aikawa and Thompson (1971) have readily demonstrated the lysosomal enzyme acid phosphatase in small food vacuoles of both avian and murine parasites, but were unable to detect such activity on the plasmalemma invaginations. Trager (1966) contested Aikawa's hypothesis on two points, firstly, invaginations of the plasmalemma persisted following release of the parasite from the erythrocyte and are not therefore amoeboid intuckings

(as suggested by Aikawa) and secondly, the hypothesis did not explain the observed connection between the small food vacuole and the plasmalemma. A compromise suggested by Scalzi and Bahr (1968) is that pinocytotic feeding occurs in the small trophozoite but cytostomal feeding occurs in the larger asexual parasites. Although surface phagotrophy (or pinocytosis) may be a method of nutrient uptake in murine parasites, as it is in *Babesia microti* (Langreth, 1976), its role is in all probability secondary to cytostomal feeding combined with active transport and diffusion of materials across the plasmalemma. The suggestion that the plasmalemma may secrete enzymes inducing external food vacuoles (Jerusalem and Heinen, 1965) has received little support from other studies. However, Rudzinska (1976) has suggested that the multilamellate organelle of *B.microti* may similarly secrete digestive enzymes into the host cell.

The trophozoite normally has a single cytostome, but 2 have been recorded (Theakston *et al.*, 1968a). Although usually regarded as a permanent organelle (Theakston *et al.*, 1968a), Ladda (1969) has suggested it may appear *de novo* beneath the trophozoite plasmalemma. This organelle (Figure 42) is recognized as two concentric electron-dense rings lying approximately 16 nm beneath the plasmalemma, with an internal diameter of 50–80 nm and an external diameter of 100–220 nm. Both parasite plasmalemma and parasitophorous vacuole membrane are drawn through this structure during ingestion. Ladda (1969), Sterling and Aikawa (1973) and Aikawa and Sterling (1974) suggest that a Golgi complex attached to the endoplasmic reticulum produces electron-dense spherules (primary lysosomes) which fuse with the food vacuole (phagolysosome). Acid phosphatase, a lysosomal enzyme, has been detected in the cisternae of the endoplasmic reticulum (Theakston *et al.*, 1968b; Aikawa and Thompson, 1971). Howells *et al.* (1968b) have recorded the continuity of vesicles with the cytostomal vacuole, but suggested the vesicles were secondary food vacuoles budding from the vacuole and not lysosomes fusing with the vacuole. Following digestion of the ingested haemoglobin, malaria pigment is formed (Figure 41). Although widely assumed to be a simple catabolic product, Moore and Boothroyd (1974) suggest pigment or haematin may be a synthesized excretory substance. Contrary to the observation of Blackburn and Vinijchaikul (1970), malaria pigment is readily recognized in section as single or multiple crystalline rods lying within a unit membrane-limited vacuole. The crystals which are 190–200

× 30–90 nm (Rudzinska and Trager, 1959; Aikawa and Antonovych, 1964) have a crystal lattice of 1·05–1·10 nm (Moore and Boothroyd, 1974). Pigment crystals are larger in *P.vinckei* than *P.berghei* (Cox and Vickerman, 1966), in which the crystals have been described as being below the resolution of light microscopy when peripherally distributed in the young trophozoite (Rudzinska and Trager, 1959). As the trophozoite matures the pigment becomes centralized and ultimately the pigment vesicles fuse (Cox and Vickerman, 1966; Theakston *et al.*, 1968a; Ladda, 1969). The incorporation of various radioactive substances into pigment has been studied by high resolution autoradiography; listed in order of increasing specificity these are leucine, glycine, δ-aminolaevulinic acid and ^{55}Fe (Theakston and Fletcher, 1968; Theakston *et al.*, 1968b, 1970a).

Exposure of trophozoites to chloroquine significantly alters the formation of food vacuoles and pigment. Peters *et al.* (1965) and Howells *et al.* (1968a) suggest that food vacuoles are more numerous in the P and RC strains of *P.berghei* than in drug-sensitive strains, although the latter authors question whether the trophozoite has simply become more amoeboid. Peters *et al.* (1965) and Howells *et al.* (1968b, 1969) noted that in a chloroquine-resistant line of *P.berghei* the high prevalence of vacuoles persisted even in the absence of drug pressure. Irrespective of whether or not food vacuoles increase in number, there is a marked increase in vacuole size. Aikawa (1972) found vacuoles increased from 0·4 to 1·0 μm, whereas Howells *et al.* (1968b) saw vacuoles 0·1–1·0 μm diameter in immature trophozoites. Using EM autoradiography, Aikawa (1972) demonstrated the binding of ^{3}H-chloroquine to the membrane of a food vacuole; Warhurst (1973), however, suggests that the membrane binding illustrated involved an autophagic vacuole. This membrane retained bound label for 24 h during which period it represented the major binding site of the drug. This study did not describe an initial binding to the lysosomes (dense spherules) as proposed by Warhurst and Hockley (1967), and the results were inconsistent with the hypothesis of Macomber *et al.* (1967) that haematin formation concentrates the drug within the parasite. The suggestion that there was a reduced pigment production in a chloroquine resistance RC line of *P.berghei*, despite an observed increase in the number of food vacuoles (Peters *et al.*, 1965), has been questioned by Howells *et al.* (1968a, b) who correlated the decline in pigment formation with the preference of the RC strain for immature red cells. In

Unless otherwise stated all figures are of *P. yoelii nigeriensis.*

Figure 1. Autoradiograph of zygote within 24 h bloodmeal in mosquito gut. Labelled with ^{3}H-adenosine. Crystalloid, inner pellicular membranes and subpellicular microtubules are being formed (×14 700).

Figure 2. Autoradiograph of 24 h zygote in mosquito gut bloodmeal. Inner pellicular membrane fully formed. Intense peripheral labelling of nucleus suggests transcription is in progress (Fakan, 1976). Nucleolus (Nu) (×18 000).

Figure 3. Intranuclear spindle within nucleus of ookinete fixed while traversing midgut epithelium (×61 600).

Figure 5. SEM micrograph of ookinete from bloodmeal, specimen taken from Giemsa-stained bloodfilm. Apical complex arrowed (×5120).

Figure 6. Autoradiograph of ^{3}H-adenosine labelled ookinete passing through midgut epithelium. Nucleolus (Nu) is heavily labelled (×20 100).

Figure 7. Ookinete closely applied to basal lamina following penetration of midgut. Crystalloid (C), acristate mitochondria (M) nucleus (N) (×13 100). *Inset.* Pellicular folds (×38 500).

Figure 9. Autoradiograph of ookinete following penetration of midgut wall, illustrating the persistence of the rhoptry–microneme complex (×13 600).

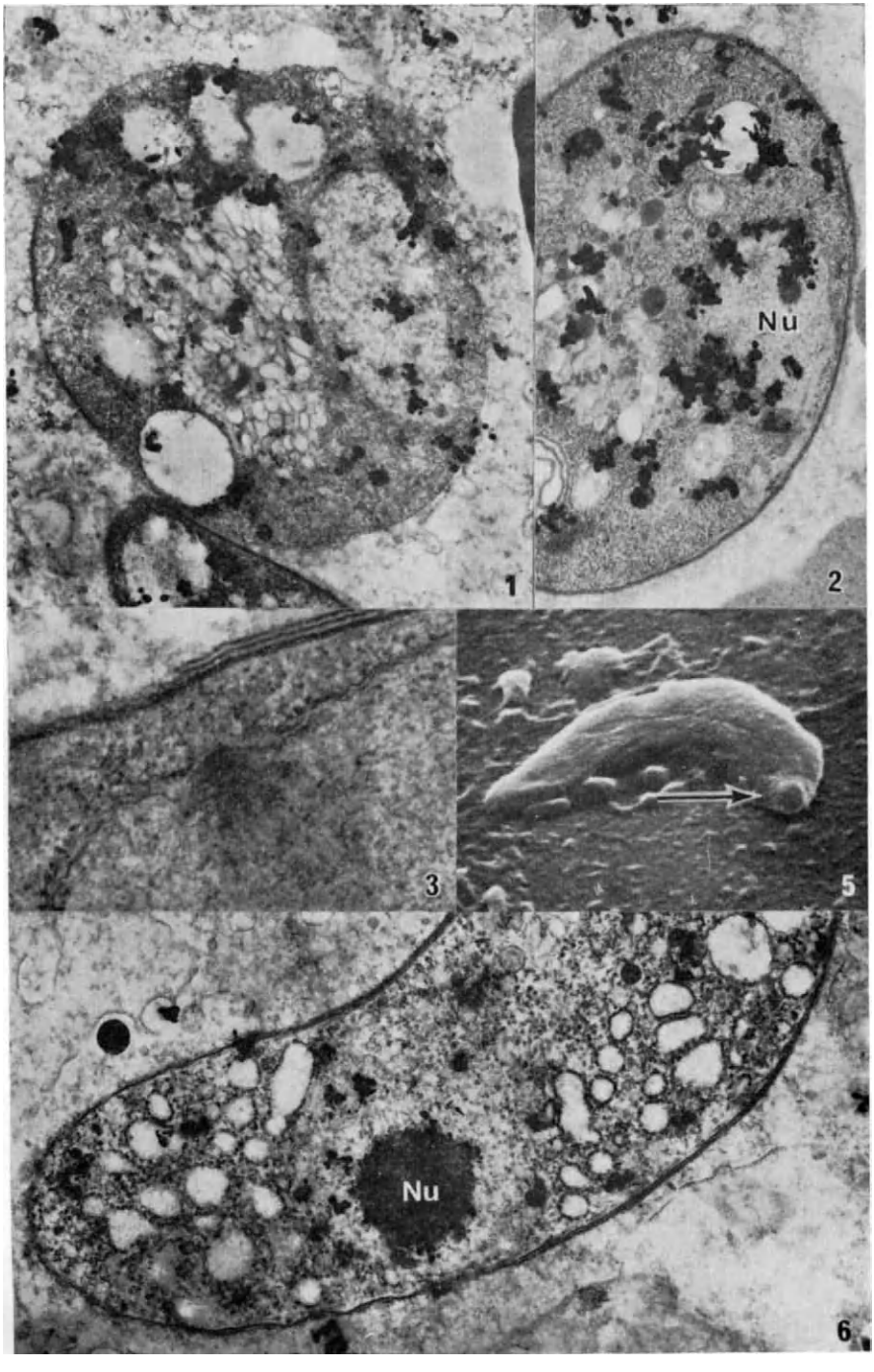
Figure 10. Ookinete undergoing transition to oocyst between basal lamina (B) and basement cell membrane (BM) of midgut epithelium (×22 700).

Figure 11. Apical complex (A) becoming detached from pellicle in young (18 h) oocyst containing partially cristate mitochondria (M) (×23 100).

Figure 12. Young oocyst showing totally resorbed but persistent apical complex (A) (×20 000).

Figure 14. Young (18 h) extracellular oocyst covered by "zipper-like" basal lamina. The inner pellicle membrane of the ookinete is broken (arrows) and the nucleus is undergoing mitosis (SP) (×14 500).

Figure 15. SEM micrograph of 4-day oocyst infection. Oocysts can be seen concentrated on posterior midgut where they develop between the muscle bands, and beneath the tracheoles (×300).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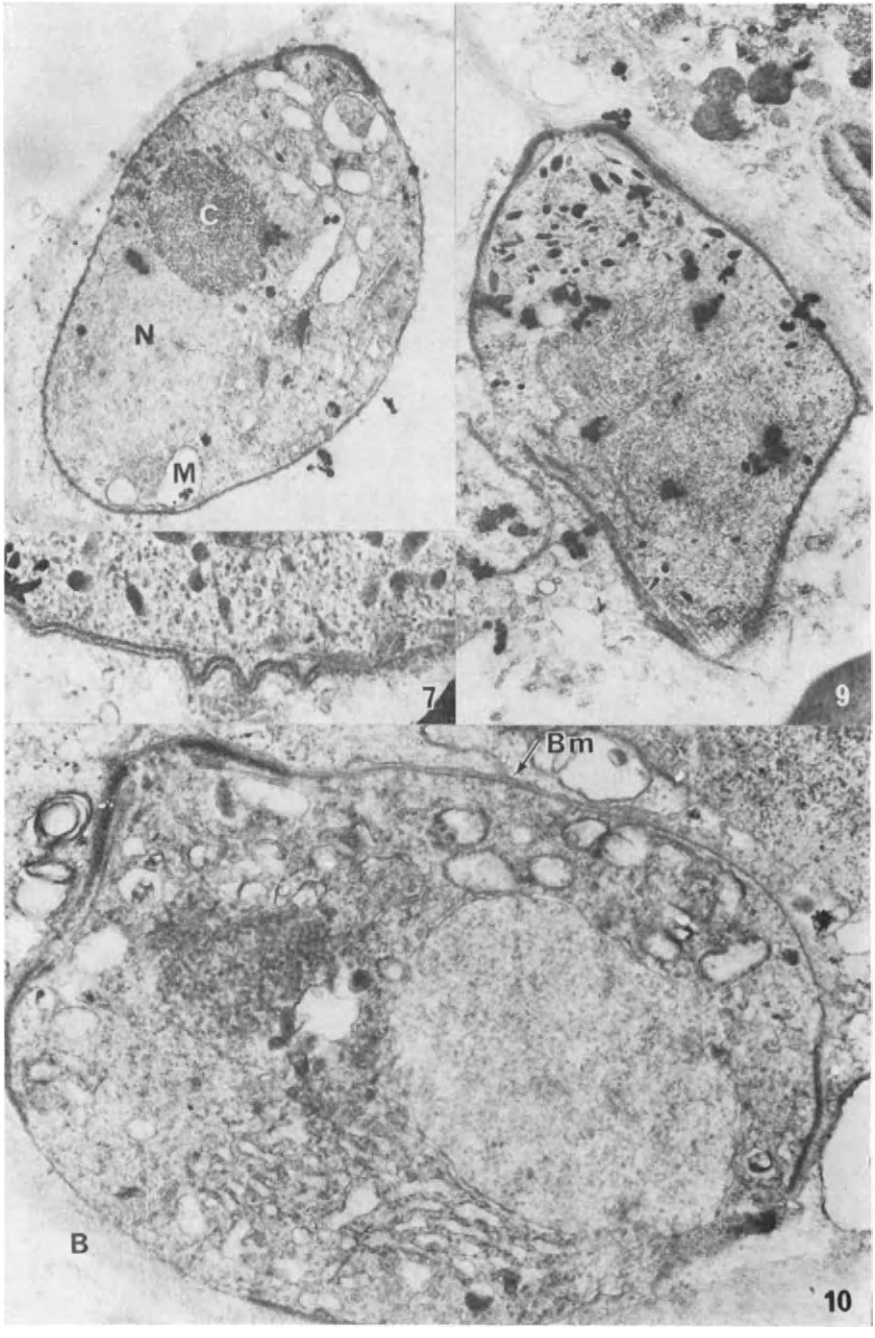
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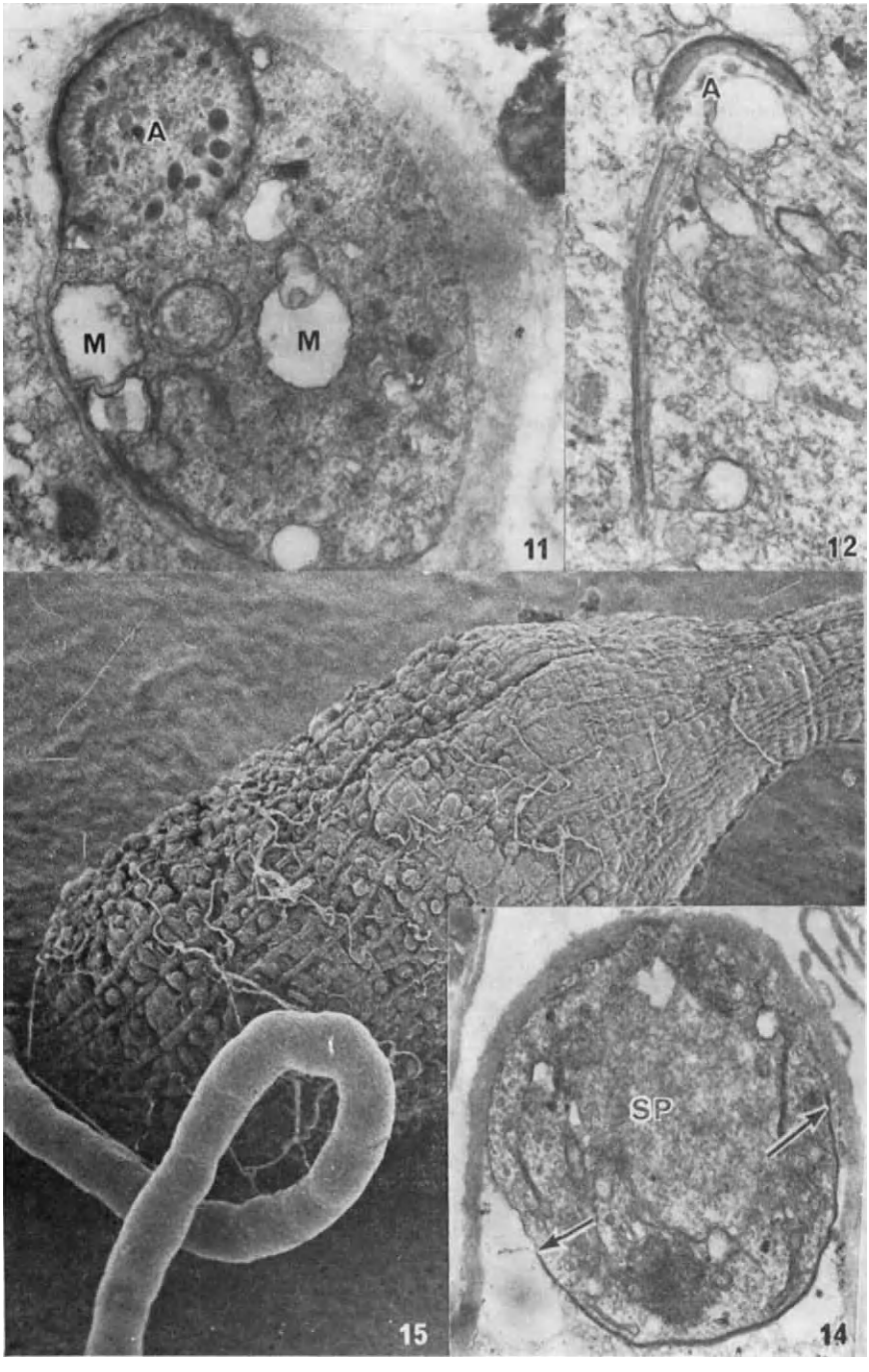
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Unless otherwise stated all figures are of *P.yoelii nigeriensis*.

Figure 16. Sterographic pair of high voltage (650 keV) micrographs of 1 μm thick section of mosquito gut bearing normal and degenerate 9-day oocysts, and spores of the microsporidian *Nosema algerae* (×1300).

Figure 17. Energy dispersive X-ray analysis of electron-dense spherules, the absence of a peak of emission at the location of iron (arrow) shows these are not haemoglobin residues. Cytoplasm dotted plot, dense spherule barred plot.

Figure 18. Nine-day oocysts at earliest stage of peripheral vacuolization. Numerous nuclear lobes present with mitotic configurations and nuclear envelope invaginations. Abundant cristate electron-dense mitochondria. Dense spherules (DS) (×11 200).

Figure 19. Mitotic spindle extending between centriolar plaques in nuclear envelope invaginations (NEI). Paired kinetochores (K) not associated with condensed chromatin (×36 600).

Figure 20. SEM micrograph of 6-day oocysts showing varied topography. Basal lamina torn by expansion of forest of oocysts (×610).

Figure 21. SEM micrograph of 11-day infection showing some liberated sporozoites (×360).

Figure 22. Sporozoites forming on surface of sporoblast. Attenuated nuclei peripherally situated with hemispindles leading into budding sporozoite. Newly formed inner pellicular membrane confined to sporozoite buds (×9300).

Figure 23. Budding sporozoites showing sequential elongation and darkening of rhoptries. (R, R′, R″) (×11 600).

Figure 24. Budding sporozoites showing rhoptry development, inclusion of mitotic nucleus and parallel incorporation of a mitochondrion (×11 000).

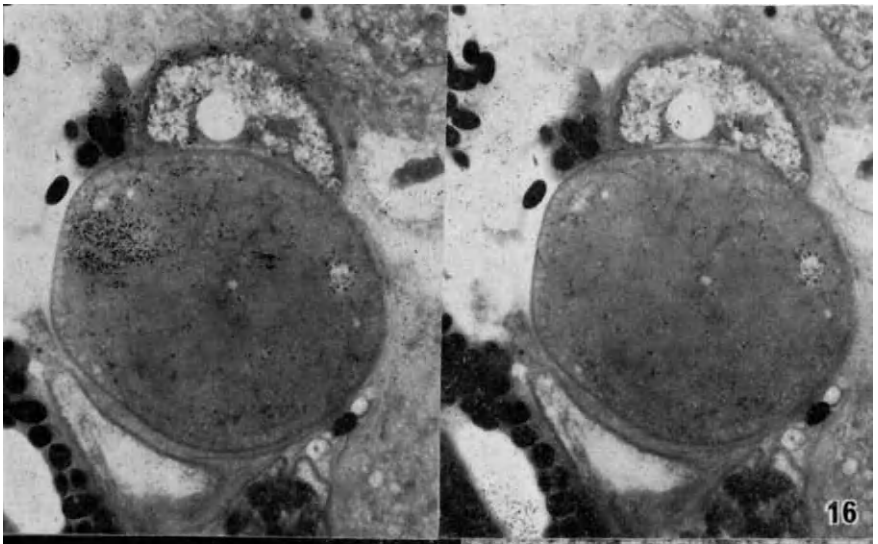
Figure 25. TS of sporozoites in 9-day oocyst, showing numerous rhoptries and asymmetrically distributed subpellicular microtubules attached to paired inner pellicular membranes by double electron-dense linkages (arrows). Fixation: Karnovsky's solution (×36 300).

Figure 26. TS of sporozoites fixed in 2·4% glutaraldehyde, followed by 1% osmium tetroxide in phosphate buffer (×27 900).

Figure 27. TS of sporozoites of *P.c.chabaudi*. Note mitochondrion and second electron-dense "mitochondrion" running parallel to nucleus. Fixation: 2·4% glutaraldehyde, followed by 1% osmium tetroxide in phosphate buffer (×30 600).

Figure 28. TS of sporozoites in 8-day oocyst. Thin section incubated in DNAase (Aikawa *et al.*, 1972) which has removed peripheral condensed heterochromatin in nucleus (×31 900).

Figure 29. Control section to Figure 28, incubated in RNAase (×26 300).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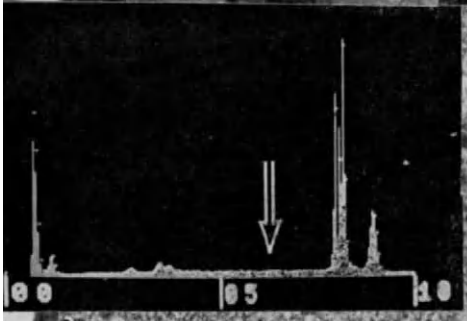
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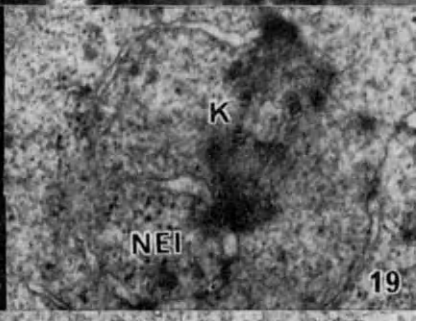
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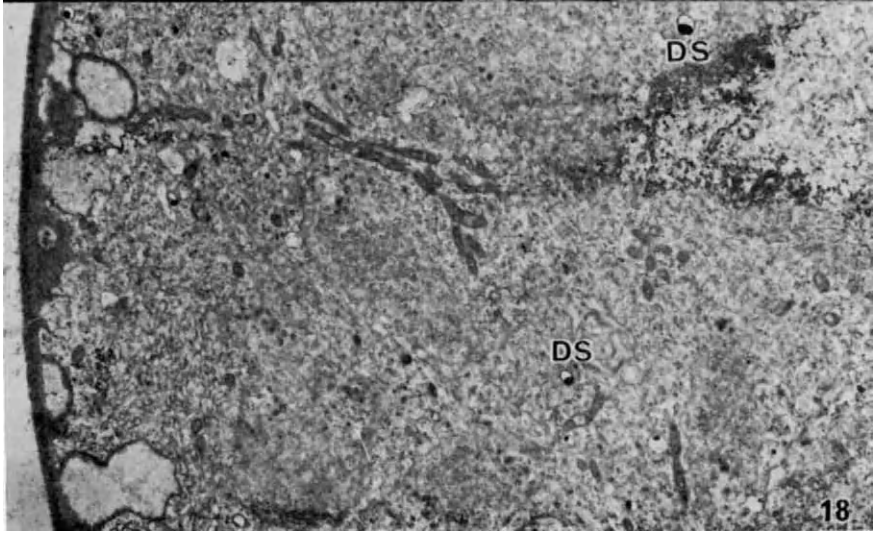
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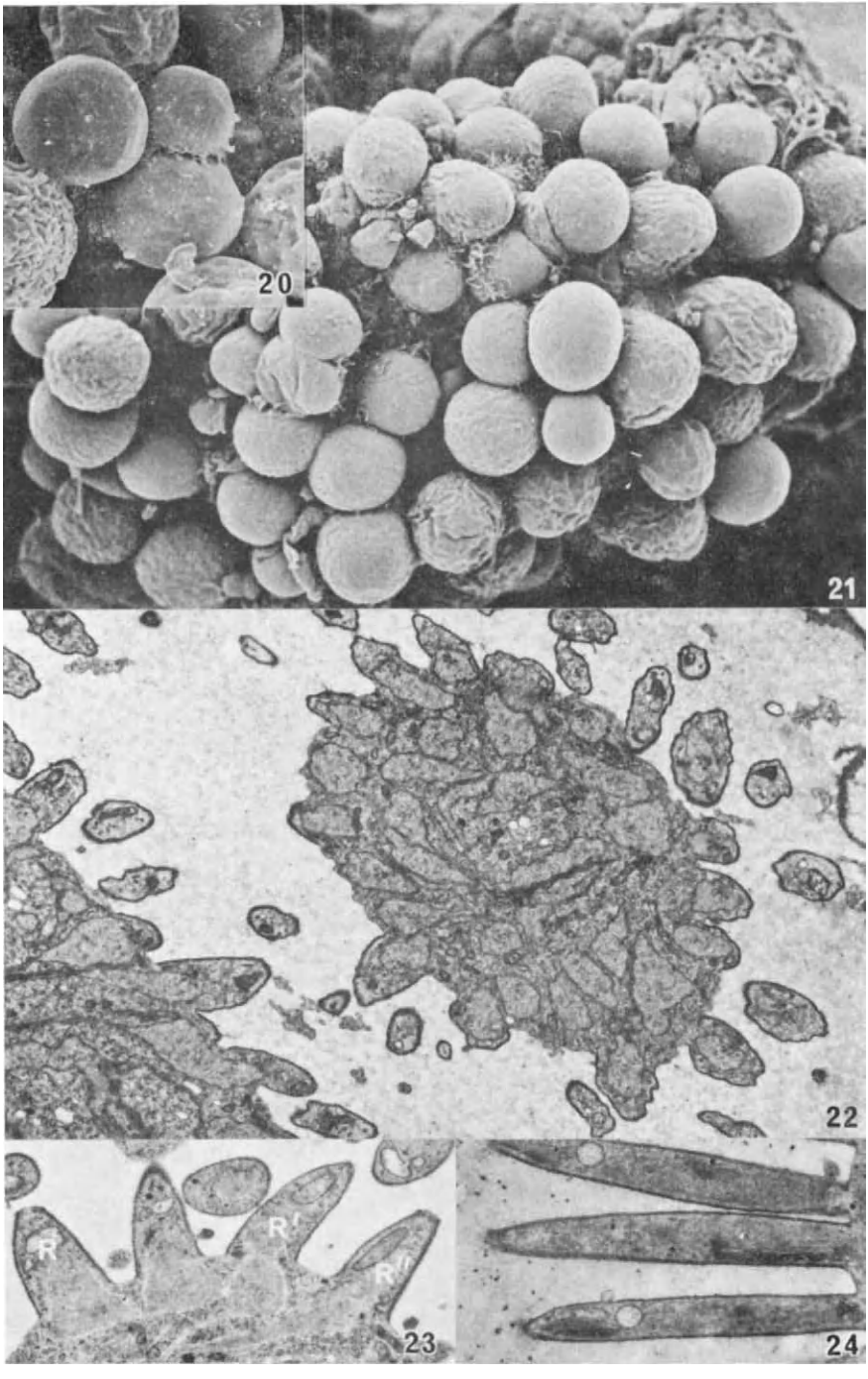
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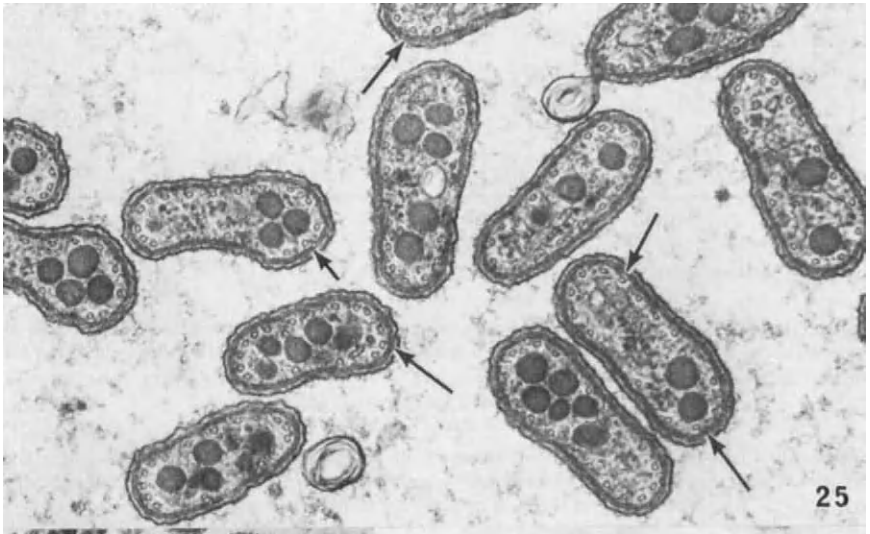
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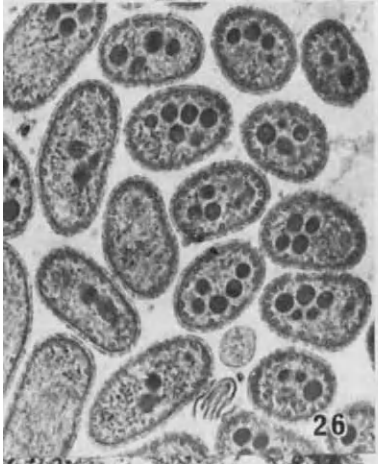
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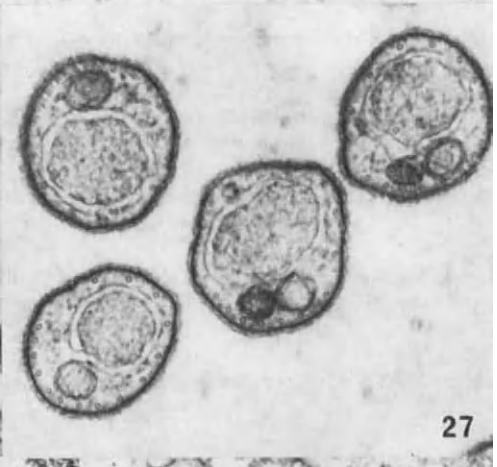
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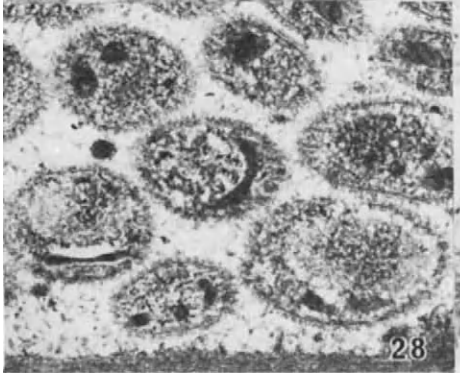
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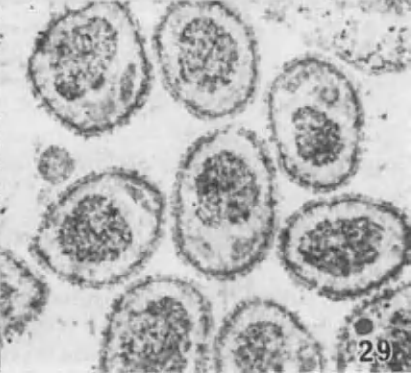
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Unless otherwise stated all figures are of *P. yoelii nigeriensis*.

Figure 30. HVEM (650 meV) stereomicrographs of 1 μm thick section of distal portion of sporozoites in 13-day oocyst. Note inclined flattened end within which lies the apical ring on which the subpellicular microtubules converge. Electron-dense rhoptries now have a markedly "beaded" structure (×31 300).

Figure 31. TEM of thin section sporozoites as for Figure 30. Anterior thickening of inner pellicle membranes forming apical ring, at which point subpellicular microtubules attach. Rhoptries run through apical ring to pellicle (×34 900).

Figure 32. SEM micrograph of sporozoites as for Figures 30 and 31 emphasizing asymmetry of anterior pole (×33 700).

Figure 33. SEM micrograph of contorted sporozoites emerging through holes produced in stretched fibrous wall of 11-day oocyst (scale interval 1 μm) (×11 000).

Figure 34. SEM micrograph of haemocoelomic form of free sporozoite. Note spiral shape and increasing diameter at position of nucleus (N) (×11 900).

Figure 35. Sporozoite in cells of distal lobe of salivary gland. Sporozoite is distending a membrane with the anterior end which contains numerous micronemes. Gland tissue is grossly distorted (×9200).

Figure 36. TS of sporozoites in proximal region of salivary gland duct which has a thin cuticular lining (×9100).

Figure 37. Mature pre-erythrocytic schizont of *P.berghei berghei* in liver of experimentally infected mouse (from Garnham *et al.*, 1969b) (×1600).

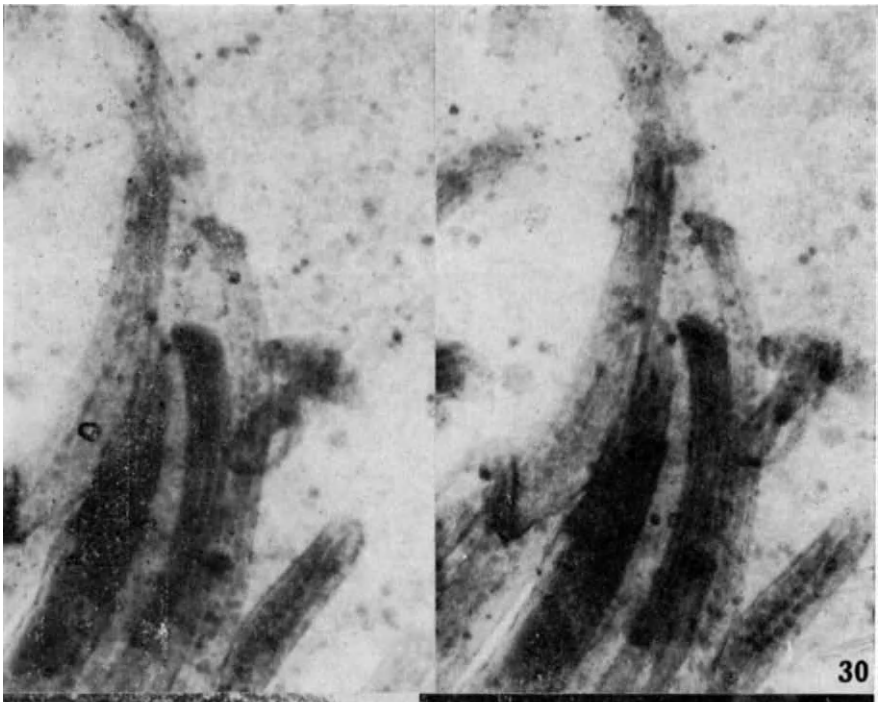
Figure 38. Thin section of intraerythrocytic trophozoite. Multilamellate organelle (MLO) associated with pellicle; nucleus (N); endoplasmic reticulum (ER) (×30 000).

Figure 39. SEM micrograph of small intraerythrocytic parasites (? trophozoites) (×6000).

Figure 40. Trophozoite with "metabolic window" (arrow) beneath which lie the multilamellate organelle (MLO) and nucleus (×12 540).

Figure 41. Crystal of malarial pigment in membrane limited vacuole (×80 000).

Figure 42. Active cytostome in young trophozoite (×71 800).



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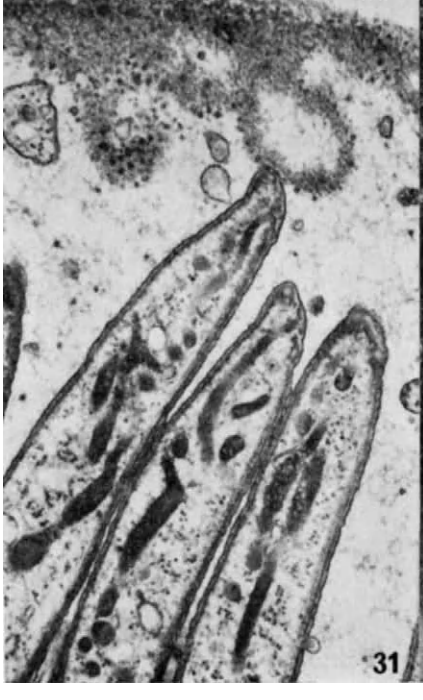

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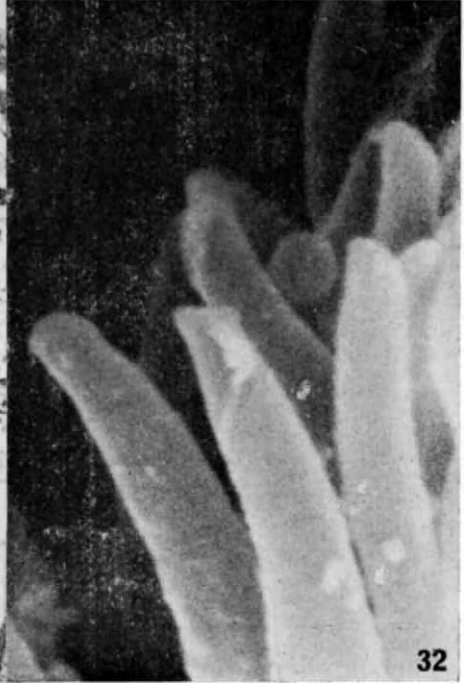

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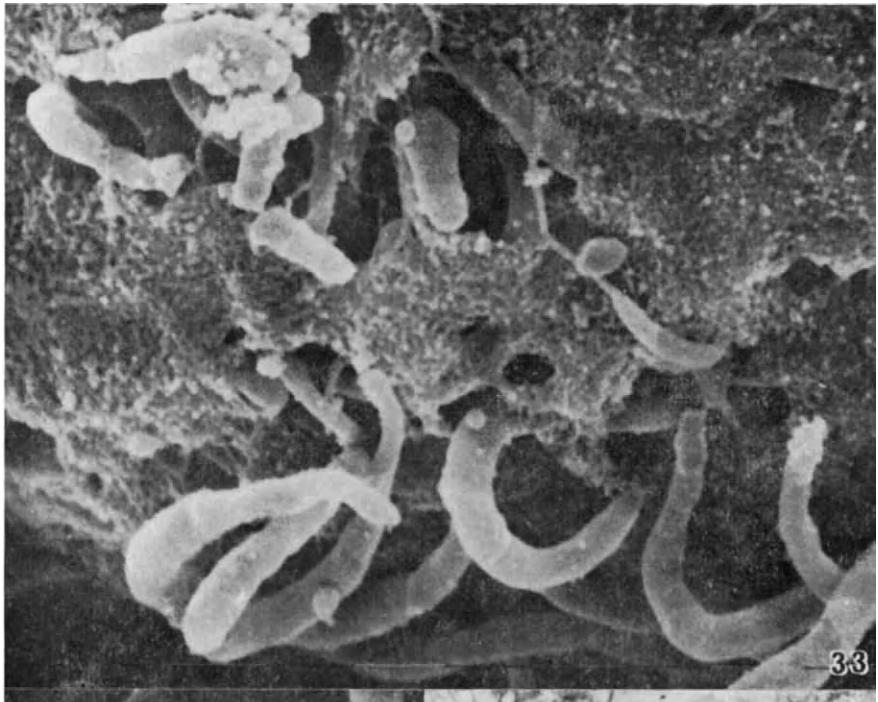
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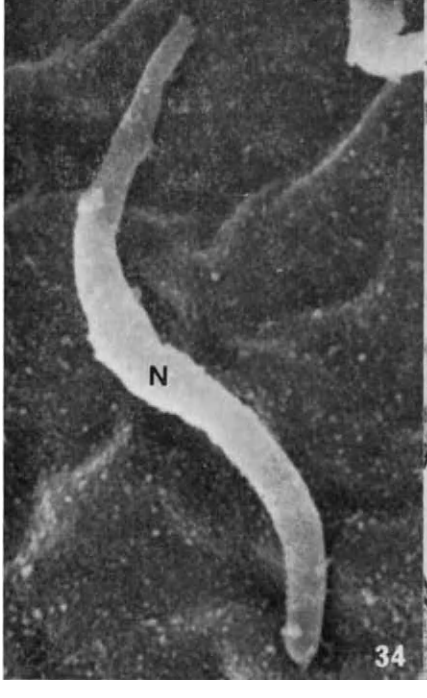
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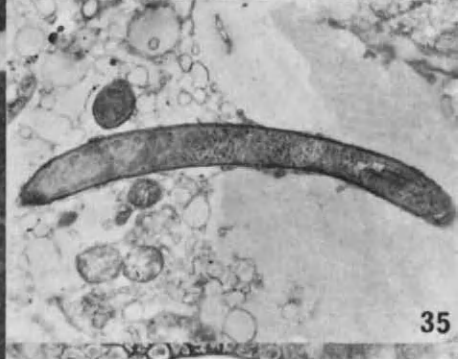
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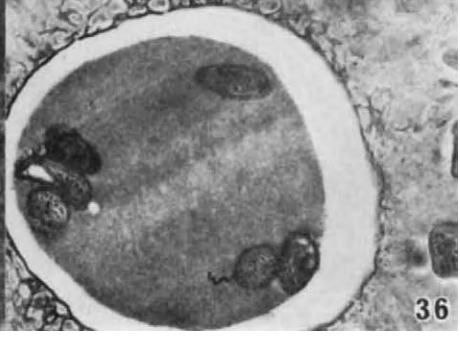
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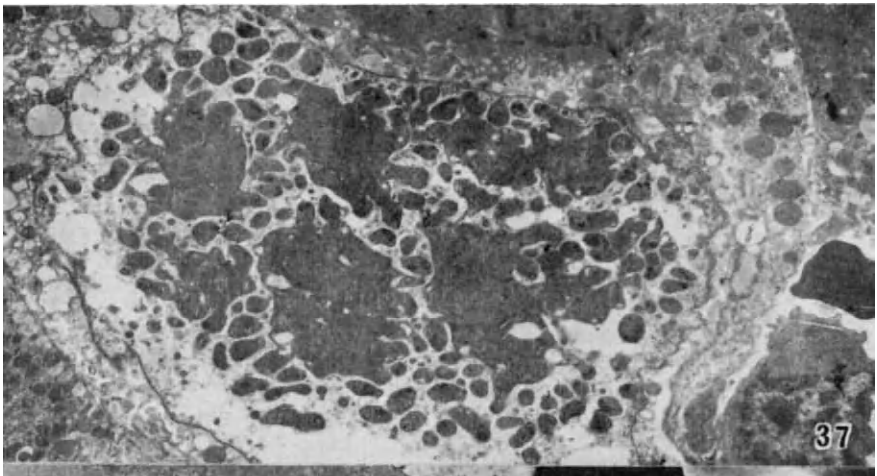
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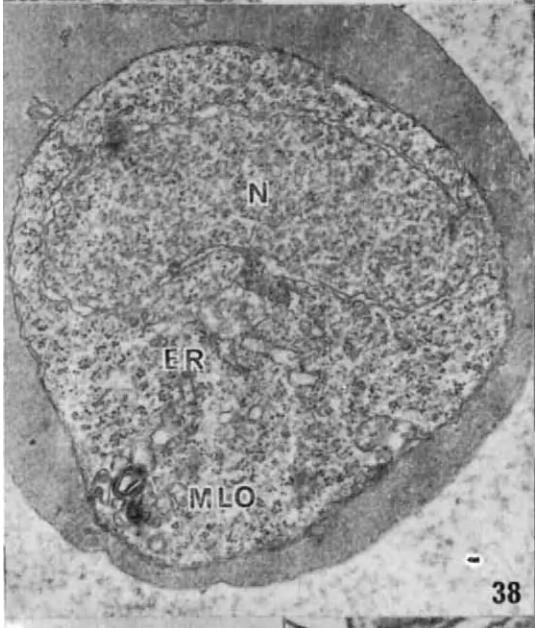
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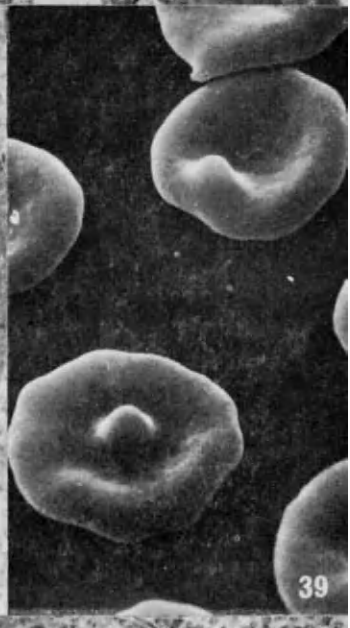
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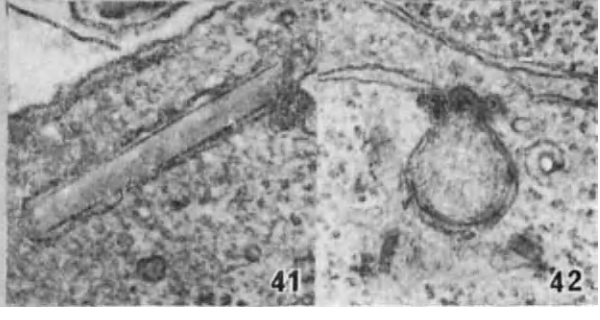
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Unless otherwise stated all figures are of *P. yoelii nigeriensis*.

Figure 43. Erythrocyte with probable multiple parasitaemia. Young schizont (S) with intranuclear spindle (arrow) and large acristate mitochondria (M) (×20 800).

Figure 44. Detail of schizont mitotic spindle. Microtubules seated in electron-dense centriolar plaque in nuclear pore, short microtubules bear electron-dense kinetochores, longer radiating or interzonal microtubules without kinetochores. Chromosomes are not condensed about kinetochores (×60 200).

Figure 45. Erythrocyte containing two schizonts, *left*—presegmenting, *right*—segmenting. Inner pellicle membrane (IM) laid down above peripheral nucleus, developing rhoptry (R) contains partially condensed matrix. Budding merozoite (*right*) contains large nucleus and electron-dense rhoptries (×18 900).

Figure 46. LS of mature intraerythrocytic merozoite tenuously attached to residual body. Rhoptries and micronemes extend through gap in labyrinthine membrane (IM) and apical rings. Pellicle covered in light amorphous coat (×38 500).

Figure 47. Near spherical merozoites released from ruptured schizont, showing paucity of subpellicular microtubules (arrows) (×26 200).

Figure 48. TS of mature intraerythrocytic merozoite showing prominent apical rings (arrow) and the close association of mitochondrion (M) and spherical body (SB) (×31 200).

Figure 49. Merozoite ingested by macrophage (×31 600).

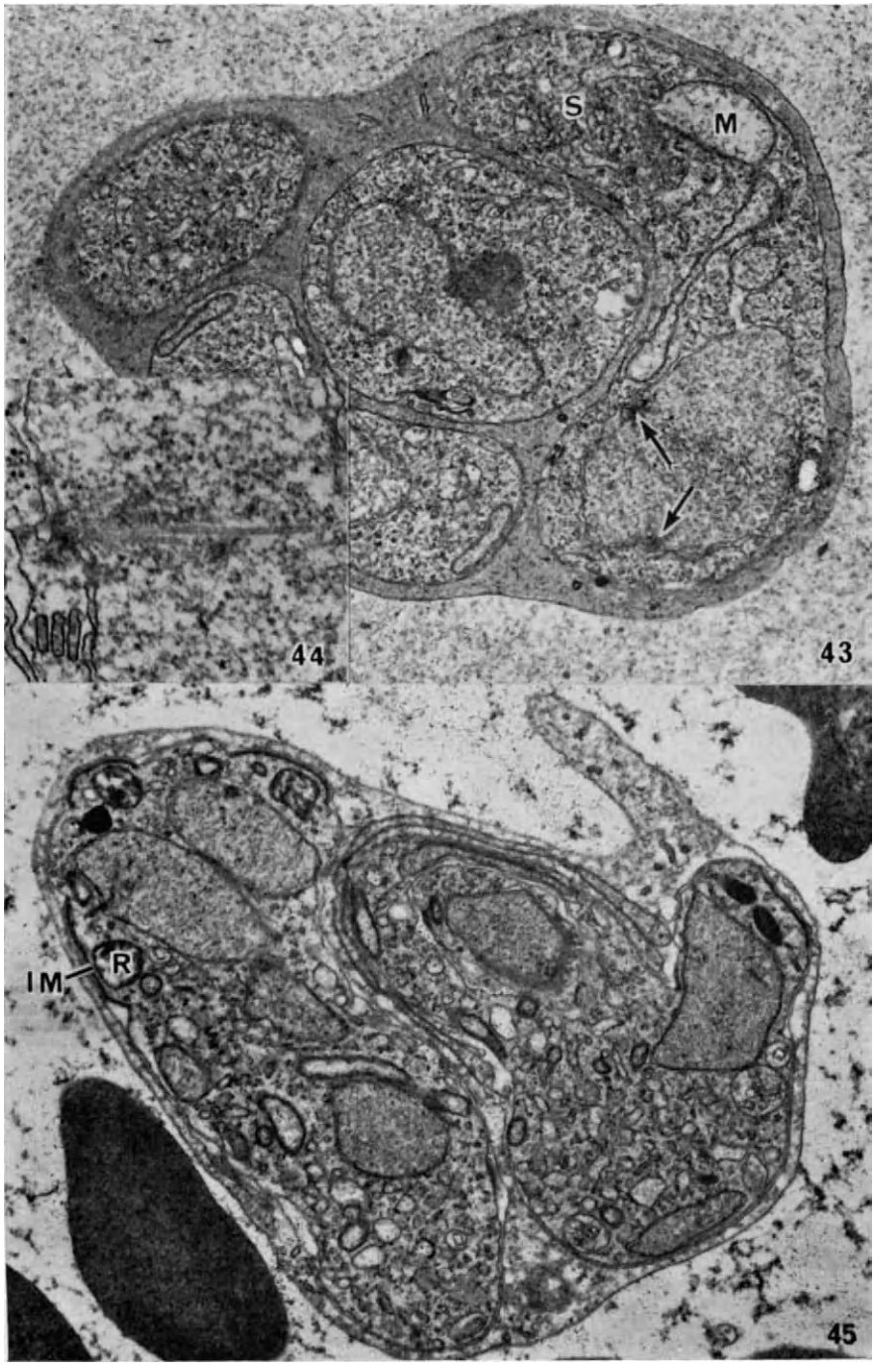
Figure 50. Mature intraerythrocytic macrogametocyte with numerous peripheral acristate mitochondria, dense monosome population, extensive smooth endoplasmic reticulum and small nucleus. Pellicle contains a single parasite unit membrane. Food vacuole? (FV), osmiophilic bodies (OB), pigment (P) (×12 300).

Figure 51. Mature intraerythrocytic microgametocyte devoid of mitochondria, with sparse ribosome distribution and large nucleus. Numerous osmiophilic bodies (OB) and pigment vesicles (P). Parasite pellicle predominantly a single membrane but with segments of double inner membrane present (arrows) (×10 900).

Figure 52. Microtubule organizing centre (MTOC) in diverticulum of nuclear envelope of microgametocyte, closely applied to electron-dense intranuclear body (INB) (×80 600).

Figure 54. MTOC in microgametocyte 1 min after activation, showing newly formed orthogonal tetrad of kinetosomes (×75 400).

Figure 55. LS of first endomitotic division of microgametocyte nucleus 1 min after activation. Spindle enters electron-dense centriolar plaque in nuclear pore attached to the cytoplasmic side of which are the kinetosomes and axonemes (arrows) (×42 500).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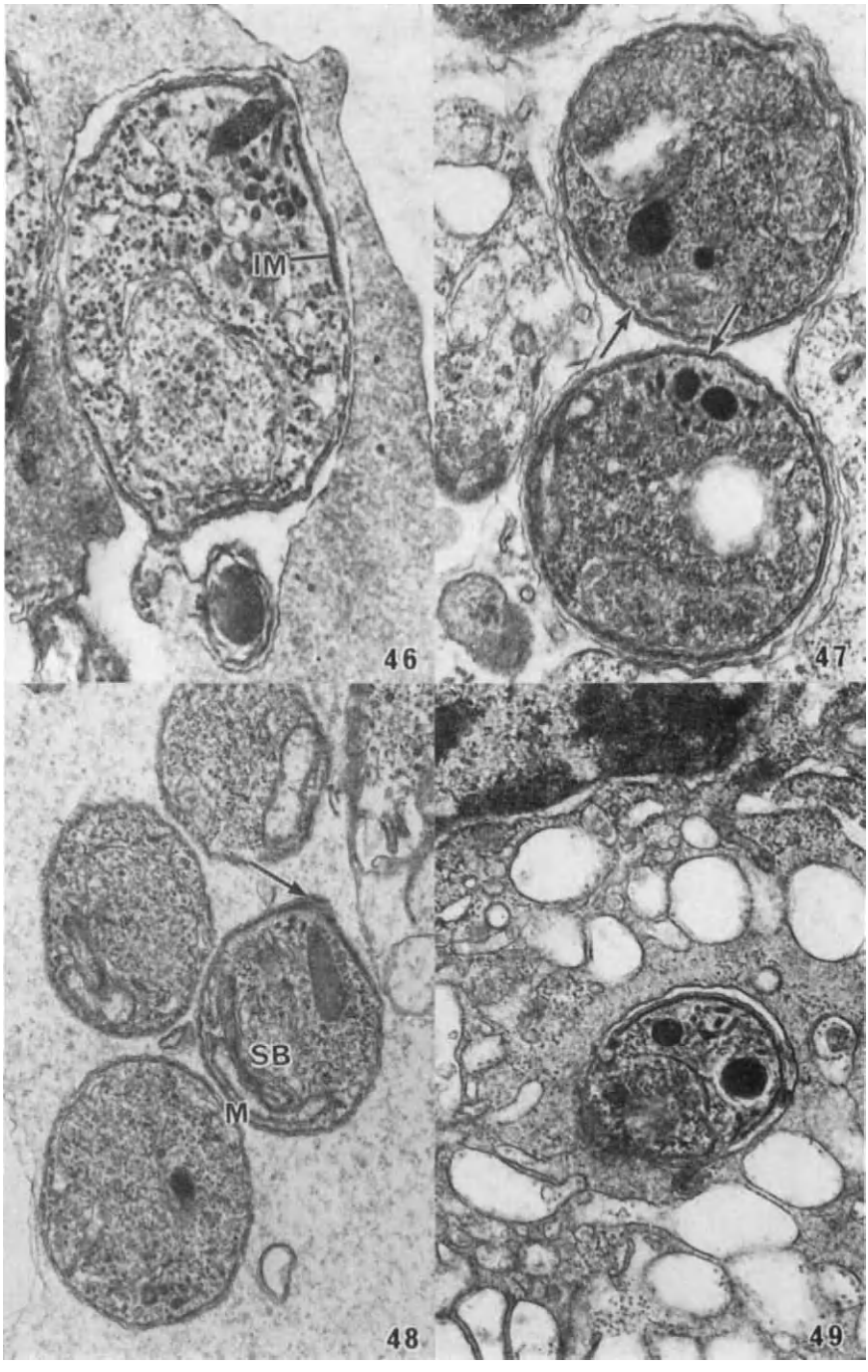
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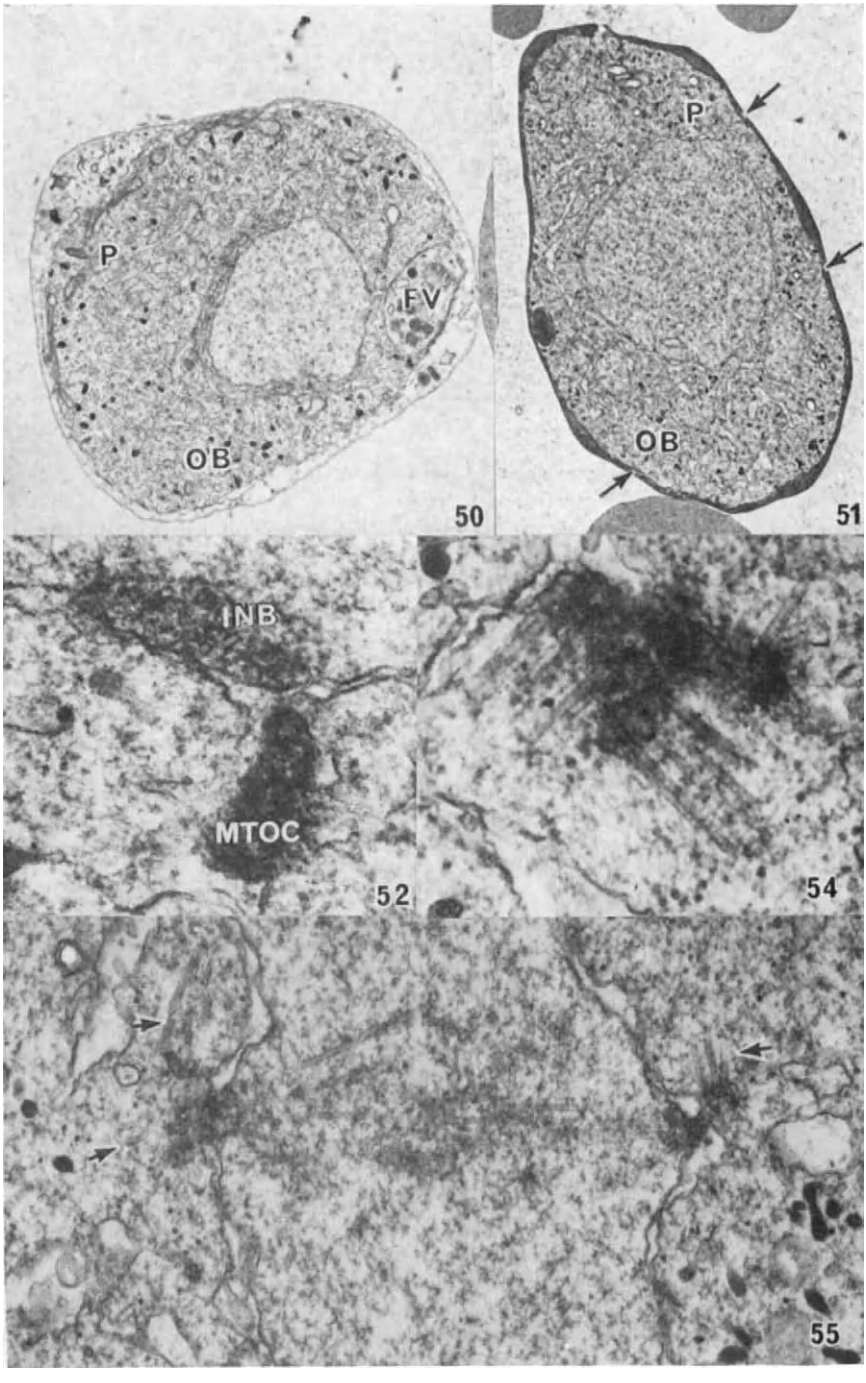
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Unless otherwise stated all figures are of *P. yoelii nigeriensis*.

Figure 56. Second microgametic nuclear division, showing poles of two spindles (arrows). Numerous sections of fully and partially formed axonemes present in the cytoplasm of the still intracellular parasite ($\times$16 100).

Figure 57. Section of a single pole of third division spindle of microgametocyte. Kinetosome has developed a juxtakinetosomal sphere and granule. The basket tubules have extended from their subpellicular location to surround the kinetosomal organelles. Chromatin is seen condensing on the nucleoplasmic face of the kinetochores ($\times$78 400).

Figure 58. SEM micrograph illustrating the axoneme organization about both poles of a single third division spindle ($\times$49 800).

Figure 59. Microgamete nucleus budding from the parental nucleus. Chromosomes are seen as electron-dense strands with attached fine loops ($\times$52 800).

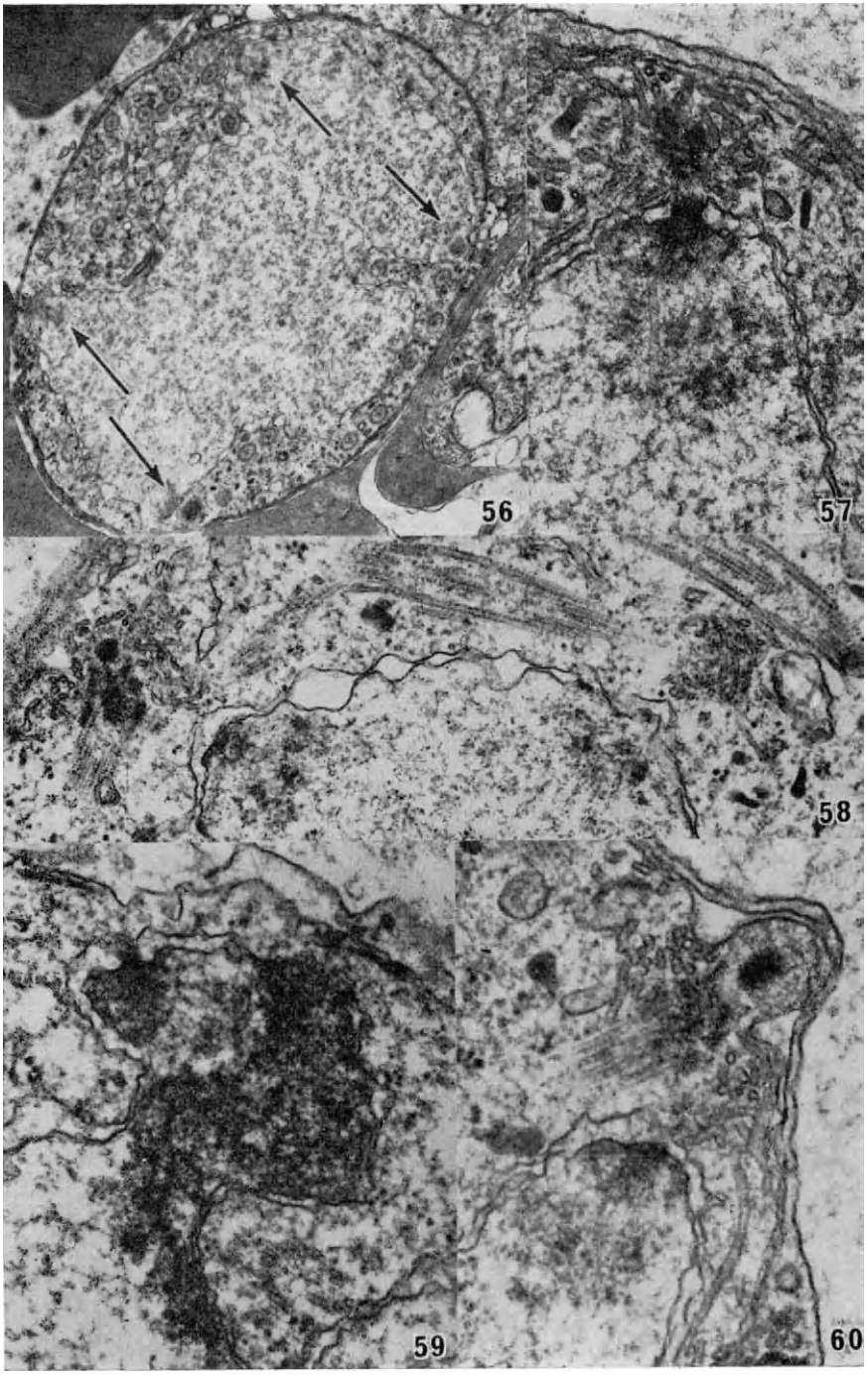
Figure 60. Exflagellation, the kinetosomal pole of the gamete axoneme is forced through the basket tubules and drags the nuclear spindle into the gamete. The plasmalemma and persistent host membranes are distended by the emergent gamete ($\times$68 400).

Figure 61. SEM micrograph of exflagellating microgametocyte, the distal pole of the microgametes containing the juxtakinetosomal sphere and granule are clearly rounded. The contained nucleus (N) is seen as a swelling on the side of the axoneme. The terminal expansion (t) is aberrant and probably contains fragments of gametocyte cytoplasm ($\times$7400).

Figure 62. SEM micrograph of anucleate microgamete showing the structural polarity of the gamete with rounded emergent pole and tapered "trailing" pole ($\times$10 700).

Figure 64. Recently fertilized extracellular macrogamete with electron-dense microgamete nucleus still associated with the intracellular axoneme. Note large female nucleus, and the absence of the osmiophilic bodies ($\times$7000).

Figure 65. Fertilized macrogamete, male nucleus now seen to be decondensing prior to fusion with macrogamete nucleus ($\times$9300).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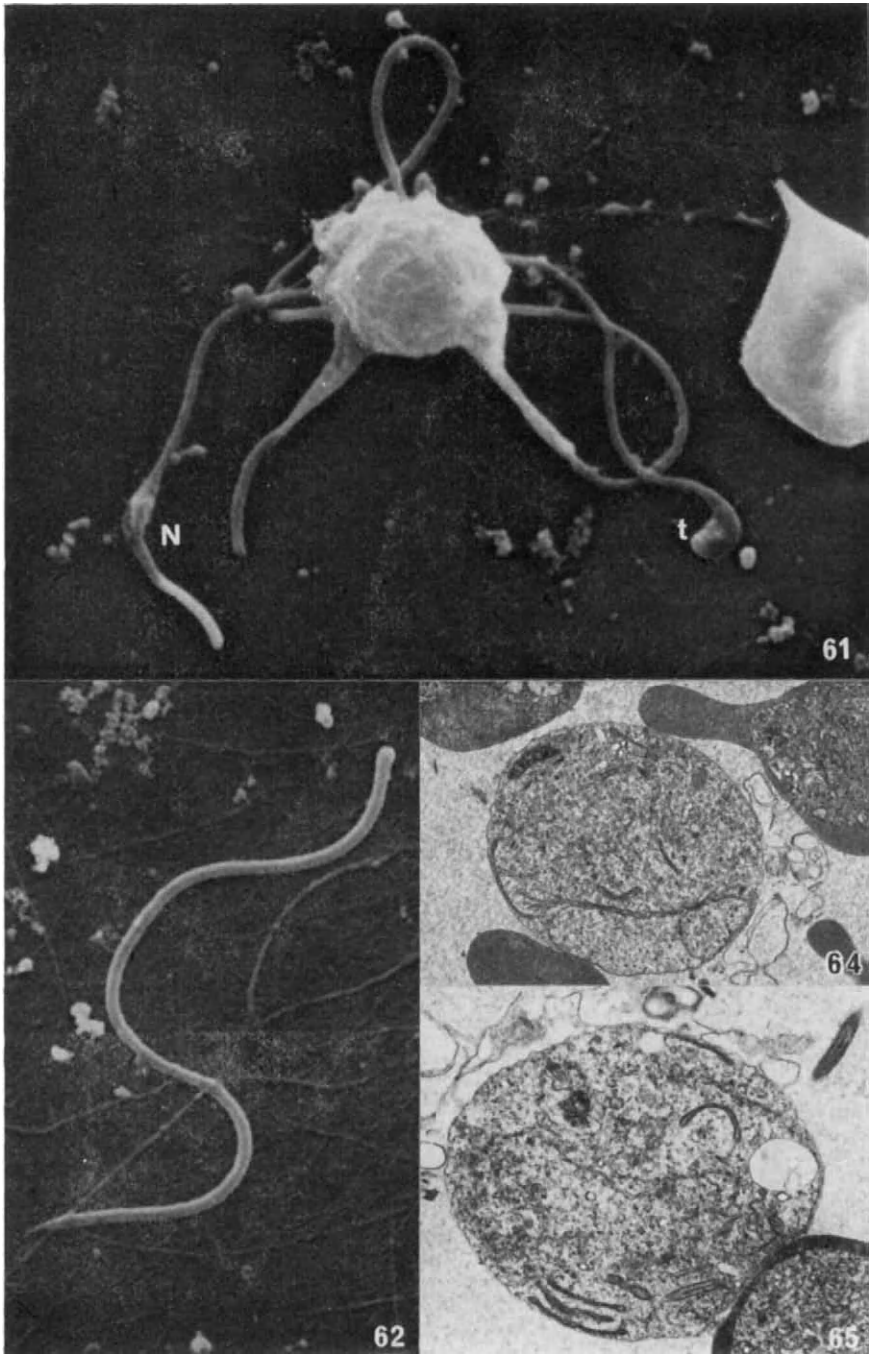
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parasites inside normocytes the size of the pigment grains was reduced in the RC compared to the drug-sensitive strain. Conversely, in a primaquine-resistant line the size of the pigment grains was increased (Howells *et al.*, 1968b). Neither primaquine nor chloroquine altered the distribution of cytochrome oxidase activity (Howells *et al.*, 1969).

One of the most interesting modifications of the trophozoite plasmalemma is the "metabolic window" (Figure 40). This structure is often situated at the bottom of a deep plate-like depression in the erythrocyte, 0·5–1·0 μm in diameter, where the parasite is overlayed by a 30–60 nm deep layer of dense erythrocyte cytoplasm through which the parasite plasmalemma is evaginated (Arnold *et al.*, 1971; Bodammer and Bahr, 1973). Within this membranous evagination are numerous vesicles or concentric membranous whorls which may be continuous with the nuclear envelope—which is frequently found in close apposition to the "metabolic window". Bodammer and Bahr (1973) speculate that this thin membranous expansion may be involved in either the transport of nucleotides into the parasite or the release of antigens, cytotoxin or humoral agents. Similarly Arnold *et al.* (1971) assume transport is the main function of the organelle and further suggest that the parasitized red cell may become attached to reticuloendothelial cells at this point. Rinehart *et al.* (1971) described a similar surface structure and have demonstrated by means of latex labelled antibodies that parasite antigen is found over the whole of the erythrocyte plasmalemma including the "metabolic window". In a study of the feeding mechanism of *B.microti*, Rudzinska (1976) concludes that a membranous organelle, homologous with the "metabolic window" of *Plasmodium*, is responsible for either the digestion of host cell cytoplasm or the excretion of waste products. Similarly Langreth (1976) proposes that the multilamellate organelle (see below) is extruded during excretion, this process forming the "metabolic window". However, the structure and role of this organelle during the normal development of *Plasmodium* should be examined with considerable care since it is known that: the osmotic fragility of malaria-infected erythrocytes is increased and that exposure to primaquine can induce the extrusion of the multilamellate organelle.

The multilamellate organelle (Figure 38) (Rudzinska and Trager, 1959), also termed the multilamellate body (Bodammer and Bahr, 1970); whorled membrane (Ladda and Steere, 1969), myelin forms (Cox and Vickerman, 1966) or concentric membraned organelle (Theakston *et al.*, 1968a; Aikawa and Antonovych, 1964), varies

greatly in size and position. It is most often connected to another membranous system of the parasite, e.g. the plasmalemma or "metabolic window" (Aikawa and Antonovych, 1964; Howells *et al.*, 1968b; Ladda, 1969; Bodammer and Bahr, 1970); the nuclear envelope (Howells *et al.*, 1968b; Ladda, 1969); a food vacuole (Cox and Vickerman, 1966); the internal membrane system of the mitochondrion (Ladda, 1969; Theakston *et al.*, 1969) or the point of apposition of adjacent parasites (Killby and Silverman, 1969a). The multilamellate organelle has been associated with cytochrome oxidase activity (Theakston *et al.*, 1969; Howells *et al.*, 1968c, 1969), NADH- and NADPH- dehydrogenase activity (Theakston *et al.*, 1967b, 1970b) and 6PGD activity (Theakston and Fletcher, 1973a, b). Cytochrome oxidase is considered a specific marker for mitochondria (Wallach and Lin, 1973) which thus suggests a mitochondrial function for the multilamellate organelle as proposed by Rudzinska and Trager (1959). However, the simultaneous localization of many of these enzyme activities to numerous other parasite membranes, such as the nuclear envelope and plasmalemma, casts doubt on the specificity of the cytochemical techniques used. A possible artefactual (Cox and Vickerman, 1966) or degenerative origin of the multilamellate organelle is suggested by an increased prevalence of the structure in cells exposed to chloroquine (Macomber *et al.*, 1967; Howells *et al.*, 1969), and by their occasional intramitochondrial location where myelin figures are induced by oxygen deprivation or change on post-mortem examination (Rhodin, 1974). In a study on the morphology of *P.gallinaceum* in the abnormal environment of the embryonic chick, Lushabaugh *et al.* (1976) suggest that certain changes (including multilamellate organelle formation) in these parasites could be a general response to less than optimal growth conditions. Notwithstanding the suggestion that the multilamellate organelle is an excretory organelle or lysosome (Langreth, 1976; Rudzinska, 1976) it is difficult to exclude the possibility that in the trophozoite the multilamellate organelle is formed from quantities of labile phospholipid which may be deposited locally during the fixation process.

The mitochondrion (Figure 43), variously termed the double concentric membraned organelle (Rudzinska and Trager, 1959); Rudzinska's organelle (Jerusalem and Heinen, 1965); double membraned organelle (Theakston *et al.*, 1968); sausage-shaped vesicle (Killby and Silverman, 1968); and membraned organelle (Killby and

Silverman, 1968, 1969a; Scalzi and Bahr, 1968), was originally described as cristate (Fulton and Flewitt, 1956) but subsequently found to be acristate or partially cristate (Ladda, 1966, 1969; Blackburn and Vinijchaikul, 1970). The suggested derivation of the mitochondrion from the plasmalemma (Ladda, 1969) and speculations on its possible role as an osmotic regulator (Theakston *et al.*, 1968a) are no longer believed. The threads of DNA which make up the mitochondrial genome have been described by Blackburn and Vinijchaikul (1970). Enzyme activities localized to the mitochondrion include cytochrome oxidase (Howells *et al.*, 1968c; Theakston *et al.*, 1969) and 6-phosphogluconate dehydrogenase (Theakston and Fletcher, 1973a, b). The latter is, however, normally regarded as an extramitochondrial enzyme. Enzymes not found in the mitochondrion include succinic dehydrogenase (Howells, 1970) and glucose-6-phosphate dehydrogenase (Theakston and Fletcher, 1973a, b, c); this absence suggests a cyclical development of mitochondrial activity in the invertebrate stages and inactivity in the vertebrate phase (Howells, 1970). If SDH is coded for by the nuclear genome, as in *Saccharomyces* (Clark-Walker and Linane, 1967), this cyclical activity is under nuclear control and would not reflect the individual response of each mitochondrial genome.

Throughout the cytoplasm there are numerous small vesicles which probably represent diffuse Golgi bodies, primary lysosomes and smooth endoplasmic reticulum (Ladda and Steere, 1969). There is no apparent polysaccharide reserve in the erythrocytic stages of *Plasmodium* (Schrevel, 1971). The young trophozoite contains a limited endoplasmic reticulum to which few of the numerous ribosomes are attached. The ribosomes are typically eukaryotic, i.e. 80 S comprised of 60 S and 40 S subunits (Sherwin *et al.*, 1975), the monosome measuring 36×25 nm and the subunits 23×18 nm and 11×18 nm, respectively (Aikawa and Cooke, 1971). Ribosomes are seen both in their inactive (monosome) and active (polysome) forms. Polysomes dissociate rapidly in parasites exposed to chloroquine (Macomber *et al.*, 1967; Ladda, 1966; Ladda and Arnold, 1965). This is presumably related to the inhibition of uptake of amino acids, e.g. methionine (Theakston *et al.*, 1971, 1972), and results in the formation of condensed ribonucleoprotein (RNP) chains.

The nucleus of the growing trophozoite enlarges rapidly (Ladda, 1969) suggesting an increase in the rates of nucleic acid and protein synthesis (Lewin, 1974). There is no compact nucleolus present,

ribosome synthesis occurring throughout the nucleus. Ladda (1966) and Ladda and Arnold (1965) showed that the drugs sontoquine and chloroquine induced condensation of nuclear RNP into a nucleolus-like structure which Arnold *et al.* (1969) suggested had the antigenic characteristics of a nucleolus. Howells *et al.* (1969) were unable to show these nuclear changes when primaquine and chloroquine-resistant lines of *P.berghei* were exposed to chloroquine. Primaquine did, however, induce nucleolar-like aggregates in the chloroquine-resistant line. The diffuse nucleolar organization may inhibit the cytochemical detection of nuclear RNA (Cuiča *et al.*, 1963). DNA and histones, by contrast, are readily detected by the Feulgen reaction and alkaline fast green reactions respectively (Bahr, 1965). The ultrastructural organization of the nucleus has been extensively studied by Aikawa *et al.* (1972). Loose clumps of 4 nm electron-dense particles are interspersed around the periphery of the nucleus with less dense zones containing 18–22 nm granules and scattered perichromatin fibrils. With nuclear enlargement the 4 nm particles become evenly distributed beneath the nuclear envelope. DNAase digestion removed these particles and other dense material from the heterochromatic regions. In the euchromatic regions, DNAase extraction revealed the 18–22 nm particles to be linked by fine fibrils, which probably represent the sites of transcription. Pyrimethamine reduced the density of 18–22 nm particles, fine fibrils, and the size of the euchromatic regions of the nucleus, and simultaneously induced small DNAase-sensitive regions within the nucleus. Ladda (1965) demonstrated that a number of drugs including amodiaquine, quinacrine and acridine orange all induced varied nuclear changes, as did actinomycin D (Ladda and Arnold, 1965). In a series of autoradiographic studies, Theakston and Fletcher (1968, 1970a) revealed the incorporation of ^{3}H-leucine, -glycine and -8 aminolaevulinic acid into nuclear proteins. Theakston *et al.* (1971, 1972) demonstrated a reduction of incorporation of ^{3}H-adenosine into the parasite nucleus following chloroquine treatment; cytoplasmic incorporation of ^{3}H-adenosine, and the overall incorporation of ^{3}H-uridine was, however, unaltered. They interpret these results to mean that the nuclear envelope and plasmalemma have different susceptibilities to chloroquine. Such treatment results in the expansion of the perinuclear space (Ladda and Arnold, 1965), which clearly reveals the few nuclear pores. Freeze-fracture studies have elegantly demonstrated the paucity of nuclear pores (Ladda and Steere, 1969). The nuclear pores are of the complex type

(Scalzi and Bahr, 1968) and contain a hollow electron-dense cylinder within the walls of which lie 8 small tubules (Vivier, 1967).

Observations on extracellular trophozoites are confined to those on artificially released parasites and on aberrant forms naturally escaping from the erythrocyte. Trophozoite migration has been recorded *in vivo* (Ferguson, 1958) and in ultrastructural studies (Peters, 1963; Ladda *et al.*, 1965; Jerusalem and Heinen, 1968). The emergent trophozoite is invariably invested by the membrane of the parasitophorous vacuole and occasionally by a layer of erythrocyte cytoplasm, micrographs of which may bear a striking resemblance to the "metabolic window" described on p. 131. While the significance of this breakdown of parasite–erythrocyte organization is not yet understood, Ladda *et al.* (1965) draw attention to the possible artefacts involved.

Artificially released murine malaria parasites have been examined by Ladda (1969), Killby and Silverman (1969b), Schneider (1970), Seed *et al.* (1973a, b) and Seed *et al.* (1974). Ladda, and Killby and Silverman, found that saponin lysis of the erythrocyte was more efficient than immune lysis, although neither method completely removed the enveloping host membranes. The parasites, while largely undamaged, contained swollen mitochondria and multilamellate organelles. French press and distilled-water lysis were considered the least satisfactory isolation techniques, both resulting in considerable disruption of the parasites.

F. The Intraerythrocytic Schizont

In the growing trophozoite the endoplasmic reticulum becomes markedly expanded and the cisternae fill with a dense matrix. The cytoplasm becomes noticeably denser, in part due to an increased ribosome density. The single mitochondrion becomes enlarged and buds daughter organelles (Aikawa, 1971; Blackburn and Vinijchaikul, 1970). The first sign of schizogony, however, is the appearance of an intranuclear mitotic spindle (Figures 43 and 44) beneath the persistent nuclear envelope (Vickerman and Cox, 1967a; Scalzi and Bahr, 1968). Each spindle pole is based in an electron-dense centriolar plaque situated in a nuclear pore. The plaque may extend 0·2 μm into the cytoplasm where, in face view, Aikawa *et al.* (1972) describe a circular organization. It is now clear that there is no cytoplasmic microtubule organizing centre (MTOC), i.e. a centriole, as described in the schizont

of *Eimeria* (Dubremetz, 1971). The spindle microtubules which radiate from the centriolar plaque are approximately 10 in number, up to 0·75 μm long and 19–22 nm in external and 11–13 nm in internal diameter. Most microtubules appear to be of the kinetochore type, although interzonal tubules have been recorded (Scalzi and Bahr, 1968). Up to 4 kinetochores are seen in individual sections as triple electron-dense bars 75 nm long and 74 nm wide. Condensed DNA is not visible around the kinetochore, although DNAase digestion does reduce the electron density of the adjacent nucleoplasm (Aikawa *et al.*, 1972). Interestingly, Aikawa (1971) notes the appearance of more than one spindle in a single nucleus. However, Aikawa *et al.* (1972) suggest that at telophase the nucleus is attenuated and separates into daughter nuclei which subsequently divide. While the majority of published studies support the latter hypothesis, the observation of multiple spindles in schizont nuclei, together with the established syncytial nature of the oocyst nucleus suggest that a similar nuclear organization (i.e. an accelerated mitosis) might occur within the schizont, nuclear fission being delayed until segmentation (Yamada *et al.*, 1972).

Within the segmenting schizont (Figure 45) small discs of continuous double membrane develop beneath the plasmalemma. Beneath these membranes are radiating subpellicular microtubules. In turn, beneath the microtubules lies a single membrane-bound vesicle approximately 0·3 μm in diameter with a fibrous electron-dense matrix; this is the first stage in the development of the rhoptry–microneme complex of the merozoite (Scalzi and Bahr, 1968). It must be assumed that both the labyrinthine membrane and the rhoptry vesicle are derived from the numerous Golgi vesicles of the schizont as described in *Eimeria* by Dubremetz (1975). As in formation of sporozoites and ookinetes, merozoite organogenesis is spatially determined by the location of a nuclear spindle, within a nuclear bud, beneath the plasmalemma of the schizont (Figure 45). Subsequently the edges of the labyrinthine membrane become retracted into the schizont cytoplasm, although Yamada *et al.* (1972) suggest that the merozoite is actively extruded. In the anterior part of the merozoite bud, the rhoptries rapidly become electron dense and lobed. Ductules develop extending through the newly differentiated apical rings until, in the mature merozoite, rhoptry proliferation progresses to the point where micronemes are formed (Figure 46). After the incorporation of the nucleus into the merozoite, a mitochondrion and its associated spherical body are enclosed in the

bud. The precise manner by which some organelles are incorporated into the merozoite and others are excluded is not understood. Those organelles excluded which form the residual body, include pigment containing vacuoles, membranous vesicles, endoplasmic reticulum and ribosomes.

Whilst still inside the expanded parasitophorous vacuole the merozoite plasmalemma becomes coated in a fibrous material (Miller *et al.*, 1974; Bannister, 1977) which is outwardly similar in appearance to material found in the vacuole (Figure 46). The surface of the intraerythrocytic merozoite released by sonication has two distinct patterns: a fine stippling similar to that of the trophozoite plasmalemma (Seed *et al.*, 1973a, b), described as "orange peel like" (Prior and Kreier, 1972) and a hexagonal "table-lace" pattern each ring of which is 0·25–0·3 μm long and 0·15–0·17 μm wide (Seed *et al.*, 1973). The hexagonal patterning is perhaps produced by the underlying labyrinthine network or may be a technical artefact. The surface of the plasmalemma is composed of a patchwork of hydrophobic lipid plaques interspersed with acid mucopolysaccharide which contains a low density of sialic acid groups—a molecular organization which would enhance the adhesive properties of the parasite (Seed *et al.*, 1974).

Following an asynchronous schizogony (Ladda, 1969), merozoites penetrate the restraining erythrocyte and most are liberated into the plasma (Figure 48), although Howells (1970b) has suggested that a "parental" erythrocyte may occasionally become parasitized again. Rupture of schizonts is accompanied by localized vesiculation of the erythrocyte plasmalemma and a 20% increase in erythrocyte volume, events similar to those recorded in erythrocyte invasion (Dvorak *et al.*, 1975) suggesting that a common mechanism is used to enter and leave erythrocytes. Bannister (1977), however, has suggested the erythrocyte is ruptured by osmotic forces caused by the release of osmotically active molecules into the parasitophorous vacuole. Free merozoites may become ingested by cells of the reticuloendothelial system (Figure 49). Similarly the residual body and its pigment is readily phagocytosed by the reticuloendothelial system. Pigment ingested by Küppfer cells within the liver is transferred by endocytosis to lysosomes. Later in an infection, however, the lysosomes become enlarged and pigment remains; this suggests the lysosome is unable to break down the haematin (Aikawa and Antonovych, 1964; Miwa and Tanikawa, 1965).

Pathological changes described in the kidney include hypertrophy

and microvillar extension of the endothelial cells, and cytoplasmic extension of mesangial cells associated with malarial antigen and IgM, IgG_1, IgA and C3 in the glomerulus (Suzuki, 1974; George *et al.*, 1976). Weise *et al.* (1975) found subendothelial and mesangial deposits and splitting of the basement membrane which they interpret as evidence for a transient immunocomplex glomerulonephritis.

Recently Yoeli (1976) made an ultrastructural examination of the brain of mice infected with a virulent line of *P.y.yoelii*. He described large schizonts completely occluding terminal capillaries and the choroid plexus. This occlusion was not accompanied by platelet aggregation or fibrin production, but swelling of the astrocyte processes probably resulted in a reduction in the cross-section of the vessels. Small haemorrhages though present in the cerebral cortex before death were more pronounced on post-mortem examination.

G. The Merozoite

The merozoite is almost spherical and measures approximately 1·5 μm in length and 1·0 μm in diameter (Figures 47 and 48). In the extracellular form the sparse surface coat becomes a 30 nm thick homogeneous layer over the parasite (Miller *et al.*, 1975). This granular coat (Ladda, 1969) is not bound by Ca^{2+} ions and contains gammaglobulins and little sialic acid. The coat, which is induced by contact with the plasma, is considerably thicker when merozoites are exposed to immune serum (Miller *et al.*, 1975). These authors suggest that the coating inhibits re-invasion (but not erythrocyte recognition) by agglutination of the merozoites, but it is difficult to understand how the receptor molecules in the merozoite coat are capable of recognizing the erythrocyte through the additional thick surface layers unless, as suggested by Brown (1976), antibodies against these molecules are of such low avidity that they may be competitively displaced by erythrocyte receptors. The work of Bannister *et al.* (1975) and Bannister *et al.* (1976) on *P.knowlesi* and *P.yoelii* suggests that the merozoite plasmalemma is covered by T-shaped pegs 20 nm high and 4 nm in diameter, the extensible arms of which are protein and are responsible for the non-specific binding of merozoites to other cells. Specific binding, they suggest, is achieved by anionic polysaccharide groups extending laterally from the stem of the peg. In contrast, Ladda *et al.* (1969) suggest there are no specific receptor sites and Sherman (1966) found

that trypsin, chymotrypsin and neuraminidase digestion of erythrocytes fail to inhibit invasion by merozoites. However, the work of Miller *et al.* (1975) has clearly established that specific binding sites associated with the Duffy group antigen exist for *P.knowlesi*. The tenuous attachment of the surface coat to the plasmalemma is broken as the coat is sloughed off during invasion of the erythrocyte (Ladda *et al.*, 1969; Bannister *et al.*, 1975). This action would remove any antibodies attached to the merozoite which might inhibit subsequent development of the trophozoite.

Beneath the plasmalemma lies the labyrinthine inner membrane. These membranes cover the whole of the surface of the parasite except for the apical region, and possibly a region at the posterior end of the cell. The function of this membrane—found in all the invasive stages of development—must be considered as structural, either as preserving the shape of the parasite in environments of differing osmotic potentials (Seed *et al.*, 1973) or acting as a skeletal system upon which the individual subpellicular microtubules or a system of microfilaments may move. Wong and Desser (1976) have suggested that such an interaction between the subpellicular microtubules and inner pellicular membranes may be responsible for movement of sporozoites in *Leucocytozoon*. In contrast to the prominent and numerous (26) subpellicular microtubules of merozoites of avian, saurian and primate malaria parasites, there is scant evidence for such a microtubular system in murine malaria parasites. While some authors briefly mention the presence of tubules (Scalzi and Bahr, 1968; Ladda, 1969) others fail to record their presence (Garnham *et al.*, 1969; Theakston *et al.*, 1968a; Killby and Silverman, 1969a; Yamada *et al.*, 1972). In my experience only a few microtubules are ever present, irrespective of the fixative used (Figure 47). Garnham *et al.* (1969) described a system of subpellicular microfilaments which might be involved in movements of the merozoite. The activity of microfilaments in the merozoite during invasion could be investigated by means of metabolic inhibitors, e.g. cytochalasin B (Jensen and Edgar, 1976a) or the actin like properties of the microfilaments could be demonstrated cytochemically. Observations of Huff (quoted by Garnham, 1966b) that the merozoite draws itself along a probing anterior filament are remarkably similar to observations on the ookinete, but remain unsubstantiated.

The concentric apical rings lie at the anterior margins of the inner pellicular membranes (Figure 48). These rings were erroneously termed

the conoid in early studies (Aikawa, 1967; Hepler *et al.*, 1966; Ladda *et al.*, 1965; Garnham *et al.*, 1969; Yamada *et al.*, 1972), but a true conoid is not found in the genera *Plasmodium*, *Babesia* and *Theileria* (Scholtyseck *et al.*, 1970).

The ducts of the rhoptries and micronemes pass through the apical rings (Figure 46). The rhoptries are flask shaped, approximately 0·5 μm in length and 0·12 μm in diameter, electron dense and membrane limited. The smaller micronemes are interconnected to the rhoptries by pore-like structures (Sterling *et al.*, 1972). Some authors have suggested that micronemes are precursors to the rhoptries (Ladda *et al.*, 1969; Rudzinska and Vickerman, 1968) but the opposite is true (see p. 136). Garnham *et al.* (1969) suggest these two structures represent different activity states of the same organelle. Unlike the observations on the sporozoite, there is no evidence for this. All workers are agreed that the rhoptries and micronemes are involved in penetration of the erythrocyte, since they rapidly disappear after invasion. If the erythrocyte containing the schizont is ruptured by the same activity as that used during re-invasion of the erythrocyte (Howells, 1970b; Dvorak *et al.*, 1975), the rhoptries of some merozoites would disappear during escape and such merozoites would be unable to invade new erythrocytes. Such altruistic behaviour has not been described. The chemical basis of rhoptry and microneme activity has been the subject of both considerable speculation and, recently, interesting experiment. Unlike the micronemes of *Toxoplasma*, which are known to contain mucopolysaccharides and acid phosphatase (Vivier and Petitprez, 1972), this enzyme is not present in the apical organelles of malaria parasites (Aikawa, 1971). Howells (1970b) was unable to demonstrate protease or lipase activity in the rhoptries, but suggested that they contained mucoproteins. While many authors assumed these structures secreted proteolytic enzymes (Garnham *et al.*, 1961; Aikawa, 1967; Theakston *et al.*, 1968a), others point out that the merozoite invaginates the erythrocyte membrane and does not therefore require hydrolytic enzymes (Ladda, 1969; Aikawa and Sterling, 1974; Bannister *et al.*, 1975), but secretes a surface active compound. Bannister *et al.* (1976), using negative-staining techniques, showed that the rhoptries contained a granular substance which, upon lysis of the organelle, could be released as a multilamellar material. Bannister (1977) suggests that this phospholipid or amphiphilic substance is incorporated into the membrane of the parasitophorous vacuole (which is known to differ in

structure from the erythrocyte plasmalemma; p. 108). Unquestionably the most telling results to date, suggesting that the rhoptries secrete a surfactant compound, are those of Kilejian (1976, 1977). She demonstrates the localization of a histidine-rich acidic protein in these organelles which, upon isolation, is capable of producing large invaginations in erythrocytes exactly as the merozoite does upon contact with the red blood cell (Dvorak *et al.*, 1975).

Lying between the rhoptries and the nucleus are numerous Golgi-like vesicles which Sterling *et al.* (1972) assume are part of the rhoptry–microneme complex. The organization of the nucleus of the merozoite is very similar to that of the sporozoite. Inactive heterochromatin is condensed against the nuclear envelope, there is no nucleolus and the nuclear envelope is perforated by few nuclear pores. The cytoplasmic face of the outer nuclear membrane is studded with ribosomes. Occasionally a multilamellate organelle is found in the nuclear envelope. The acristate mitochondrion and the closely associated spherical body lie posterior to the nucleus. Aikawa (1970) postulated that the spherical body may provide energy for merozoite movement. However, the prokaryotic organization of this organelle suggests it may simply represent one stage of mitochondriogenesis.

After an extracellular life of less than 30 min (Dennis *et al.*, 1975), the merozoite re-invades an erythrocyte, although platelets may rarely be invaded by merozoites of *P.berghei* (Fajardo, 1973, 1974; Fajardo and Tailent, 1974). Erythrocyte invasion has been studied *in vivo* by the light microscope (Dvorak *et al.*, 1975) and at the ultrastructural level (Ladda, 1969; Ladda *et al.*, 1969; Bannister *et al.*, 1975). Immediately after attachment of the merozoite to an erythrocyte, a large depression forms in the plasmalemma. Subsequently the small parasitophorous vacuole forms over a period of 10–20 sec. The merozoite may be seen rotating within the vacuole. The functional significance of this spinning activity is difficult to understand. Dvorak *et al.* (1975) suggest that a flow of medium through the vacuole into the swelling erythrocyte, induced by membrane disorganization, causes the merozoite to spin; however, it might well result from the asymmetrical forces exerted upon the parasite by the surface-active substances secreted by the parasite following its contact with the erythrocyte. The virtual absence of locomotory organelles within the merozoite makes an endogenous origin of such movement most unlikely. Ultrastructural studies reveal that the merozoite may attach initially in a random manner, but

subsequently becomes re-orientated with the apical complex applied to the erythrocyte plasmalemma. Garnham (1966b) did not consider reorientation a locomotory response of the parasite and, if this is so, the anterior end of the merozoite presumably has a higher affinity than other regions of the merozoite's surface for binding sites on the erythrocyte plasmalemma (Bannister *et al.*, 1975; Ladda *et al.*, 1969). Parasitophorous vacuole formation is clearly induced by the products of the rhoptry–microneme complex. The merozoite which is attached to the membrane is drawn into the vacuole as the invaginating membrane proliferates. The tight constriction of the erythrocyte plasmalemma rubs the surface coat from the invading merozoite, and presumably the membranes fuse together behind the now intraerythrocytic parasite. The parasitophorous vacuole is rapidly reduced in volume resulting in the close apposition of host and parasite membranes. There is no evidence that murine malaria parasites have the vacuole elaboration associated with the activity of dense microspheres in the merozoite of *P.knowlesi* (Bannister *et al.*, 1975). This could explain the relative scarcity of intraerythrocytic membranous clefts in cells infected with rodent malaria parasites (Killby and Silverman, 1969a; Aikawa and Thompson, 1971). The only other reported changes in host cell structure is the induction of vacuoles containing ferritin in the erythrocyte cytoplasm (Rudzinska, 1969).

H. The Gametocytes

The merozoite when invading an erythrocyte may develop in one of three ways: it may form an asexual schizont; a macrogametocyte or a microgametocyte. The exact timing of the commitment to any one developmental pathway is unknown in *Plasmodium*, although considering the results of Klimes *et al.* (1972) on sexual differentiation of merozoites of *Eimeria tenella*, it would not be surprising to find the merozoites, or even the preceding schizonts, already determined as to the sex of the gametocyte that will be produced upon re-invasion. However, Ladda (1969) and Killby and Silverman (1971) assume that gametocytes develop from trophozoites, i.e. differentiation of gametocytes and schizonts does not occur until after the merozoite has commenced feeding. Unfortunately the nature of the natural "trigger" toward sexual development still remains a complete mystery.

Ultrastructural details of the differentiation of a merozoite into

gametocytes are poorly understood in the Plasmodiidae for two reasons. Firstly, in those parasites where maturation of gametocytes is recognizable, e.g. *P.falciparum* this differentiation occurs in inaccessible tissues (although the recently developed ability to culture gametocytes of this species from asexual stages *in vitro* (Smalley, 1976) may soon circumvent this problem) and secondly, where maturation occurs in the peripheral circulation as with the murine malaria parasites, infections are, in experimental conditions, usually asynchronous making identification of parasites difficult. Studies on the Haemoproteidae, however, provide some insight on the early development of gametocytes *in vivo* (Bradbury and Roberts, 1970; Canning *et al.*, 1976). Aikawa *et al.* (1969) proposed that the gametocyte of *Plasmodium* was derived from a merozoite which had "failed" to undergo dissolution of subpellicular organelles, although it has been argued that gametocyte formation should perhaps be regarded as the normal route of development and that differentiation of trophozoite and schizont represent the unusual pattern of morphogenesis (Killick-Kendrick, 1973). Ladda (1969) suggests gametocytogenesis begins with the proliferation of the endoplasmic reticulum and the accumulation of electron-dense material within its matrix. The material then becomes condensed into small membranous vesicles, the osmiophilic bodies, which bud off from the reticulum. The osmiophilic bodies (Figures 50 and 51) are 75×250 nm in diameter and are unique to the gametocytes. They become randomly distributed throughout the cytoplasm and are rarely attached to the plasmalemma. These organelles bear a superficial resemblance to the rhoptry–microneme complex and, like them, are functional during "migration" of the parasite (see p. 140).

Other cytoplasmic organelles common to both male and female gametocytes are numerous randomly distributed membrane-bound pigment granules, which in *P.v.vinckei* are found as doublet or triplet crystals in each vesicle (Bafort, 1971). No cytostome has yet been described in the mature gametocytes, although double membrane-bound profiles which enclose host cell cytoplasm, and may therefore be food vacuoles, are common; alternatively these profiles represent digitate intuckings of plasmalemma. In contrast to the gametocytes of malaria parasites of birds (Aikawa and Sterling, 1973), neither Golgi body nor lysosomes (dense spherules) have been found in the mature gametocytes of murine malaria parasites.

Ladda (1969) believes the first unequivocal sign of gametocyto-

genesis is an enlargement of the nucleus in the young cell. Such enlargement would be expected during genome replication (Lewin, 1974), but it is only in the microgametocyte that such replication occurs, the mature microgametocyte being octaploid. The macrogametocyte, like the merozoite, is probably haploid (Sinden *et al.*, 1976). Ladda (1969) describes the immature gametocyte nucleus as having a granular and fibrous nucleoplasm with large aggregates at one pole. Killby and Silverman (1971) describe the immature gametocyte as having a dense cytoplasm and compact nucleus, but recognize that this appearance may be a result of degenerative changes. Similarly Sinden *et al.* (1976) conclude compact and dense mature gametocytes are aberrant forms.

The mature gametocyte fills the enveloping erythrocyte. The membrane of the parasitophorous vacuole may be involuted into flattened vesicles or clefts, and is closely applied to the plasmalemma of the parasite (Figure 51). Beneath the plasmalemma are discontinuous flattened vesicular expansions of smooth endoplasmic reticulum forming the inner pellicular layers (Bafort, 1971; Sinden *et al.*, 1976). The inner membrane layers of the pellicle are far less pronounced than those of avian or reptilian malaria parasites (Aikawa *et al.*, 1969; Sterling and Aikawa, 1973) and may vary in distribution during gametocytogenesis (Killby and Silverman, 1971). Tubules 18–31 nm in diameter (basket tubules) are occasionally found scattered beneath the pellicle.

The mature macrogametocyte (Figure 50) and microgametocyte (Figure 51) are readily differentiated. The cytoplasm of the female cell is packed with monoribosomes and has a well developed endoplasmic reticulum the cisternae of which are dilated by an electron-dense matrix and characteristically stacked against the nucleus. Numerous partially cristate mitochondria are distributed throughout the cytoplasm. The nucleus of the female is small compared to that of the male gametocyte. By contrast the microgametocyte contains few ribosomes in polysome clumps, is devoid of mitochondria, has a limited ER, and the large nucleus is frequently lobed. Although neither male nor female gametocytes contain a condensed nucleolus, by analogy with avian forms (Sterling and Aikawa, 1973) a diffuse nucleolus would be expected in the macrogametocyte, but not in the microgametocyte where a decline in density of cytoplasmic ribosomes during maturation is paralleled by nucleolor atrophy. The nuclei of both gametocytes are Feulgen negative suggesting that the DNA has become masked and inactivated

possibly by histones (Canning and Sinden, 1975). However, Toyé (unpublished) has been unable to demonstrate the presence of histones in gametocytes, using bromophenol blue or alkaline fast green techniques, suggesting the DNA in gametocytes is masked by something other than histones. The mature microgametocyte is clearly a terminal cell (Sterling and Aikawa, 1973; Sinden *et al.*, 1976) in which all essential processes of transcription and translation are reduced to the bare minimum for survival, and the lifespan of which will largely depend on the life of its component macromolecules. During microgametogenesis there is some evidence for a limited amount of *de novo* protein synthesis (Toyé *et al.*, 1977). By contrast, the macrogametocyte, although temporarily inactivated, is clearly prepared for renewed synthetic activity as the zygote after fertilization.

The nuclei of both micro- and macrogametocytes contain a diffuse electron-dense core which is DNAase sensitive. In the microgametocyte, an electron-dense intranuclear body (INB) (Figure 52) measuring $0{\cdot}5 \times 0{\cdot}2$ μm lies against the nuclear envelope, which contains both simple and complex nuclear pores. Within the INB are numerous paired electron-dense bars which Sterling and Aikawa (1973) equate with kinetochores, although their estimated number (20–40) is below that expected of 8 n chromosomes (i.e. 64–80); this suggests they may be present as doublets (Schrevel *et al.*, 1977) which will separate at the first gametic division. The nuclear envelope adjacent to the INB is expanded as a diverticulum (Figure 52) which is continuous with a cytoplasmic electron-dense structure, the microtubule organizingcent re (MTOC). In the avian parasite *P.gallinaceum* and the chelonian haemoproteid *Haemoproteus metchnikovi* a centriolar pinwheel is found embedded in the electron-dense MTOC (Sterling and Aikawa, 1973). A centriolar pinwheel is not present in the MTOC of murine malaria parasites. Either this represents a fundamental difference between these parasites, or fixation of the non-murine parasites was delayed long enough (60 sec) to allow gametogenesis and kinetosome development to begin before immobilization of the tissues occurred.

I. Gametogenesis

The nature of the physiological trigger for gametogenesis has attracted considerable attention for many years and has been reviewed at length by Bishop and McConnachie (1956, 1960). Factors implicated include

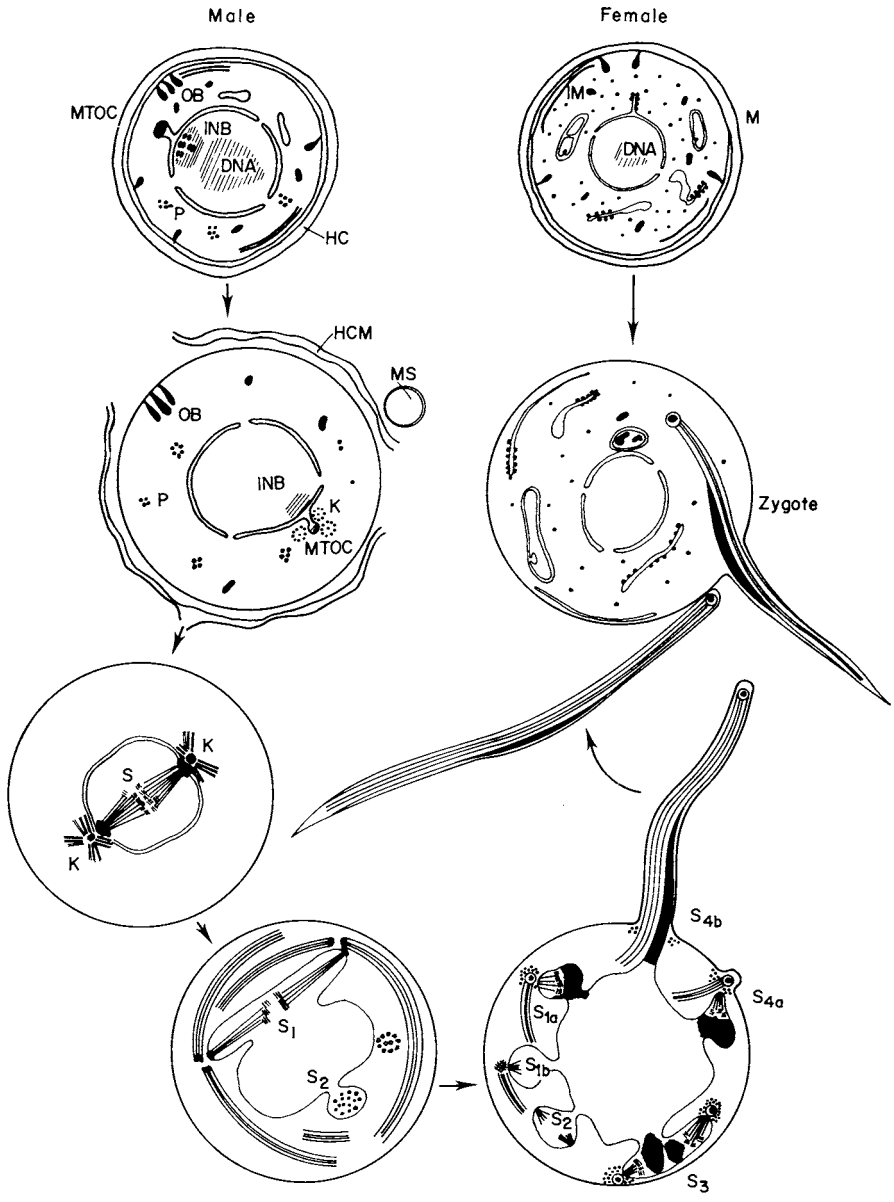

Figure 53. (*not to scale*) Gametogenesis and fertilization. *Abbreviations*. HC, Host cell; HCM, Host cell membrane; IM, inner pellicular membrane; INB, intranuclear body; K, kinetosome; M, mitochondria; MS, membranous spheres; MTOC, microtubule organizing centre; OB, osmiophilic bodies; S, intranuclear spindle.

pH, loss of CO_2, bicarbonate ions and plasma factors. Recently Carter and Nijhout (1976) have demonstrated that, in the presence of NaCl and glucose, an interrelationship between sodium bicarbonate concentration (15–100 mM) and pH (7·8–8·2) is one stimulus to exflagellation. The role of additional factors such as temperature, which in *P.y.nigeriensis* must drop to, or below, 30°C (Sinden and Croll, 1974) and mosquito factors (Micks *et al.*, 1948), clearly suggests that all the above factors are simply environmental stimuli which interact either directly or indirectly with a single class of receptor probably bound to the plasmalemma of the gametocyte. It is this receptor/effector mechanism which still requires identification.

The ultrastructural events of gametogenesis (Figure 53) has been studied *in vitro* in two murine malaria parasites, *P.berghei* (Garnham *et al.*, 1967a) and *P.y.nigeriensis* (Sinden and Croll, 1974; Canning and Sinden, 1975; Sinden, 1975b; Sinden *et al.*, 1976). Sinden and Croll (1974) subdivided gametogenesis into three distinct phases; maturation (subsequently termed activation, Sinden *et al.*, 1976), exflagellation and finally dispersal leading to fertilization.

At 20°C activation lasts approximately 7 min and involves the rounding up of both gametocytes, escape from the host cells, and subdivision of the microgametocyte's genome. Disruption of the host cell is probably mediated by the fusion of the osmiophilic bodies with the plasmalemma of the gametocyte. The secreted products disorganize the host erythrocyte which frequently ruptures releasing the spherical gamete. Alternatively the osmiophilic bodies become activated within the cytoplasm causing the imbibition of fluids which swell the gametocyte thereby rupturing the erythrocyte (Sinden *et al.*, 1976). Failure to break down the erythrocyte is frequent, and the gametes produced by the imprisoned parasite, despite their vigorous activity, fail to escape into the bloodmeal. However, after the successful lysis of the erythrocyte the extracellular gametocyte, which may be partially covered by membranous spheres and other fragments of erythrocyte plasmalemma, is exposed to the immunological response of the vertebrate host, even within the bloodmeal of the mosquito. This response occurs both *in vivo* and *in vitro* (Sinden and Smalley, 1976) although it is most marked *in vitro* where the phagocytic cells readily locate and ingest the extracellular parasites. This activity persists *in vivo* but is markedly reduced as the phagocytic cells themselves are killed by the digestive enzymes of the mosquito midgut. The effect of humoral antibodies directed

against microgametes, however, has a dramatic effect in limiting the transmission of the parasite to the mosquito *in vivo* (Carter and Chen, 1976), probably by the inactivation and agglutination of the microgametes (Gwadz, 1976).

Nuclear division in the microgametocyte begins with incredible rapidity, i.e. 60 sec after withdrawal of blood from the mouse. This fact alone precludes the possibility that genome replication can have occurred during gametogenesis. This conclusion is supported by the failure of the DNA replication inhibitors, mitomycin C and chloroquine, to prevent exflagellation (Toyé *et al.*, 1977). Simultaneously with the onset of genome segregation the DNA is reorganized and becomes Feulgen positive (Canning and Sinden, 1975). The significance of this unmasking of the DNA is unknown but may signal the renewal of transcription for a similar change occurs in the macrogamete where nuclear division does not occur. During the 7 min of activation the microgametocyte genome undergoes three successive divisions whilst surrounded by a persistent nuclear envelope, seen with the light microscope as a peripheral redistribution of masses of densely staining chromatin. At the ultrastructural level, nuclear division is achieved by the most fascinating chain of events, whereby genome segregation and axoneme synthesis and distribution are physically linked. Initially kinetosomes, composed of 9 single A tubules around a single central tubule, appear as orthogonally arranged planar tetrads (Figure 54) within the MTOC. It is assumed that genome segregation is basically similar to that of the oocyst and that two tetrads arise from the single MTOC; then division and apposition of the MTOC and the associated INB give rise to the first complete intranuclear spindle (Figure 55) (cryptomitosis of Hollande, 1972). This spindle is about 1·35 μm long, contains approximately 30 microtubules which are embedded in electron-dense centriolar plaques situated in expanded nuclear pores. The kinetosome tetrads, arranged tangentially to the nuclear envelope, are joined to the centriolar plaque by electron-dense material. At the second genome division each of the 2 spindle poles splits and the resulting spindles extend 3 μm across peripheral pockets in the nuclear envelope (Figure 56). Each spindle now has 15–30 microtubules and two kinetosomes at each pole. Kinetochores are clearly present on these spindles, but condensed chromosomes are not found. At the third and final segregation each of the 4 short (1·6 μm) spindles contains 6–12 microtubules, the majority of which bear kinetochores. At this division,

however, long fine threads surrounded by electron-dense material aggregate on the "nuclear" face of the kinetochore (Figure 57). As the spindle is drawn further into the nuclear pocket (Figure 59), this chromosomal condensation becomes marked until each pocket is filled with condensed chromatin. This unequivocal chromosomal condensation illustrates the unusual pattern of chromosomal organization in malaria parasites, namely the absence of condensation throughout the division of vegetative stages. The absence of condensation of individual chromosomes precludes the estimation of chromosome numbers by light microscopy (Wolcott, 1954, 1957; Canning and Anwar, 1968).

Concurrent with the chromosome segregation, dramatic changes in kinetosome organization occur. Each short 0·3 μm kinetosome extends into a complete 14 μm long axoneme by the continued assembly of the A tubules and polymerization of B tubules upon these. The rapidity of axoneme synthesis commonly produces configurations other than the normal 9 doublets and 2 central microtubules, e.g. (1–9)+0, (0–9)+1 and (0–9)+2. Aikawa *et al.* (1970) similarly recorded a series of aberrant forms (0–9)+2 in *Leucocytozoon* but concluded that they represented a novel sequence of axoneme assembly. At the final division each single kinetosome is situated between the nuclear envelope and the plasmalemma and is attached to a completed axoneme which is wrapped around the nucleus. At this point the branched subpellicular basket tubules, which have rapidly proliferated, extend down from the surface and envelope each kinetosome in a helical basket (Figures 57 and 58) inside of which develop an electron-dense juxtakinetosomal sphere and granule (Figure 58). At this moment exflagellation begins.

Exflagellation lasts 15–45 sec, during which the axonemes are fully extended through the gametocyte plasmalemma, and a nuclear bud is incorporated into each gamete (Figures 60 and 61). The axonemes, which have been completely inactive during synthesis, explode into action. The kinetosomal end is held against the plasmalemma by the basket tubules and the kinetosome with the electron-dense sphere is forced through the basket and distends the plasmalemma. The unidirectional movement of the axoneme is probably achieved by the interlocking of the axoneme microtubules and the basket tubules. The sphere at the distal end of the emerging gamete produces a typical rounded end to the cell (Figures 61 and 62). The kinetosome, which is still attached to the nuclear spindle pole drags the haploid genome into the gamete, although the violence of this process frequently breaks this

liaison and results in the formation of anucleate "sterile" gametes (Figure 62) which may represent up to 60% of the total population. The trailing end of the gamete is torn away from the plasmalemma by the incessant activity of the flagellar axoneme, this resulting in a tapered appearance to the pole of the gamete (Figure 62). The remaining microgametocyte residuum contains a large nucleus with Feulgen positive chromatin, and all the gametocyte cytoplasm.

The normal microgamete when measured in the scanning microscope is 14 μm ±3 μm long and 0·21 ±0·02 μm in diameter (Sinden, 1975b), (although in Giemsa-stained preparations gametes measured 16·8 ±0·29 μm in length—Killick-Kendrick, 1973b), and contains a single axoneme. The Feulgen positive nucleus which is long and cigar shaped (Figure 62) expands the gamete to 0·4 μm diameter; it contains a nucleoplasm occupied wholly by condensed chromatin and is enveloped by a double-membraned nuclear envelope. There is no mitochondrion in the microgamete, and no significant cytoplasm except where pockets of the gametocyte have accidentally been incorporated (Figure 62). Abnormal gametes may have more than the normal single axoneme, no nucleus at all, multiple nuclear fragments or a nucleus in which chromatin condensation has been only partially achieved. The microgamete is initially highly and constantly active, an activity which declines continuously over a period of 40 min (at 20°C) after which it is immobile. The sinusoidal waves passing down the gamete are alternately fast (10 waves sec^{-1}) and slow (1 wave sec^{-1}) (Figure 63) and may pass along the gamete in either direction. The resulting pattern of microgamete movement is erratic. Sinden and Croll (1974) were unable to detect any directed movement of the microgamete toward the macrogamete. However, the presence of recognizable gradients around the female cell is clearly demonstrated by the purposeful migration of phagocytes to the extracellular parasites (Sinden and Smalley, 1976). The source of the considerable energy expended by the microgamete is enigmatic for, unlike microgametes of the Eimeridae, there is no mitochondrion in the microgamete of *Plasmodium* (Scholtyseck *et al.*, 1972). While it is possible that the energy is derived from the surrounding medium, an intrinsic supply is suggested by the unchanged activity of the naked microgamete axoneme within the macrogamete cytoplasm (see p. 151).

The macrogamete of murine malaria parasites is the extracellular macrogametocyte. Unlike the avian parasite *H.columbae* (Gallucci,

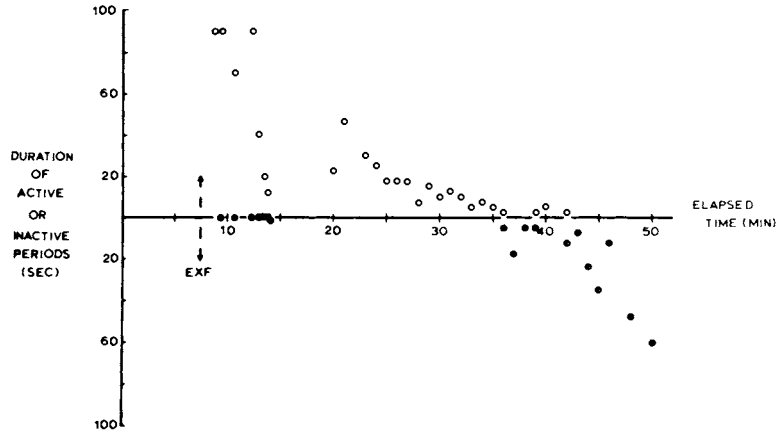

Figure 63. Microgamete activity: showing the duration of rapid, slow and immobile periods for all gametes (in the first 15 min period) and a single retained gamete for the remaining period (in which slow periods are not illustrated for clarity). ○, rapid movement; ●, slow movement; ◑, immobility (from Sinden and Croll, 1975).

1974a) there is no evidence of nuclear division during gametogenesis of the female cell and the macrogametocyte is therefore haploid.

Following contact of the micro- and macrogametes, the male cell slides gently back and forth over the surface of the macrogamete. During this process the amorphous layer covering the plasmalemma of each gamete become continuous. The microgamete then orientates itself perpendicularly to the surface of the female and the plasmalemmas of the two gametes fuse. Then with a characteristic burst of fast vibratory waves lasting 14–45 sec the axoneme and the attached nucleus move into the cytoplasm of the female cell. Unfortunately it is not known if the kinetosomal end of the microgamete is responsible for the fusion of the gamete membranes, thereby equating the juxtakinetosomal sphere and granule with the perforatorium of the Eimeriine microgamete and the acrosome of mammalian sperm. The axoneme continues to exhibit the characteristic microgamete activity pattern of alternate fast and slow movement, considerably agitating the macrogamete cytoplasm, but activity rapidly declines and ceases within approximately 5 min. The cessation of activity in all probability results from the depolymerization of the axoneme. The microgamete nucleus is readily recognized in the fertilized macrogamete by its small profile, dense nucleoplasm and close association with the axoneme (Figure 64). This nucleus rapidly becomes separated from the axoneme

and increases in volume, the nucleoplasm becoming less condensed (Figure 65). Nuclear fusion and zygote formation have not been detected in *Plasmodium* but are presumably similar to that of the avian haemoproteid *H.columbae* (Gallucci, 1974a).

IV. CYTOGENETICS

A. Karyotype

Nuclear organization of malaria parasites has been studied in living specimens by phase microscopy (Wolcott, 1954, 1955, 1957) and in fixed, stained preparations of oocysts (Bano, 1959; Dasgupta, 1954; Canning and Anwar, 1966, 1968, 1969) and gametes (MacDougal, 1947). These studies suggested that the merozoites and gametes contained two chromosomes and that the zygote (young oocyst) had a diploid complement of 4 which was reduced to 2 in a zygotic reduction division. Recent electron microscope studies, however, indicate the above work was in fact describing the distribution of cytoplasmic organelles (rhoptries) and lobes of the digitate oocyst nucleus.

In the absence of "normal" condensed chromosomes (see p. 153) three methods remain to determine the number of chromosomes in malaria parasites. First to calculate the number of linkage groups by genetic analysis, second to count the number of kinetochores (each of which is attached to a diffuse chromosome) on a mitotic hemispindle in thin sections (Table I) or on the intact spindle in thick sections by HVEM (Sinden, unpublished) or third to count the numbers of kinetochore microtubules in serial sections of spindles (Peterson and Ris, 1976). The limited cytological data collected to date suggest a haploid number of 10 chromosomes, Schrevel *et al.* (1977) favouring the estimate of 8. There are no sex chromosomes in *Plasmodium* since both macro- and microgametocytes may be obtained from cloned haploid blood stages.

B. Mitosis

Mitosis occurs during erythrocytic and exoerythrocytic schizogony, sporogony and microgametogenesis. In the last two cases nuclear fission does not occur until the sporozoite and microgamete are budded off the

parent cell, the intervening nucleus containing numerous spindles in a single polyploid nucleus. In contrast during erythrocytic and exo-erythrocytic schizogony a normal mitotic sequence of genome segregation and nuclear fission is assumed to occur. This interesting difference in nuclear organization is inadequately examined and is worthy of clarification.

The most complete description of mitosis is that found during sporogony. Schrevel *et al.* (1976) show that each spindle originates in a single centriolar plaque, which fills an expanded nuclear pore. No centriole is present at any stage in the life cycle of *P.y.nigeriensis* although, not unexpectedly, kinetosomes associated with microgamete axoneme production are found in a "centriolar" situation during microgametogenesis (Sinden *et al.*, 1976). Approximately 30 spindle microtubules terminate in the centriolar plaque, of which, theoretically 16–20 are attached to the kinetochores. Simultaneously the centriolar plaque, kinetochores and chromatids divide and the resulting hemi-spindles separate. The separation is achieved either by the expansion of the nuclear envelope, or by the elongation of an electron-dense "couche fibrillaire" between the centriolar plaques. Following apposition of the spindle poles the couche fibrillaire disappears, and the interzonal microtubules, which are continuous between poles, are formed. In the oocyst the nuclear envelope is involuted into characteristic gastrula-like invaginations at each spindle pole; these invaginations are, however, not found in any other stage of the life cycle. Kinetochore and chromosome separation are probably achieved by shortening and depolymerization of the spindle microtubules since kinetochores have been found in an anaphase position touching the centriolar plaque (Canning and Sinden, 1973).

The absence of condensed chromatin except during telophase of the final microgametic division (Sinden *et al.*, 1976) is similar to the chromosome condensation cycle described in *Aggregata* (Belar, 1926) where the chromosomes are in leptomenal form until they condense during telophase. *Plasmodium* differs from *Aggregata* only in that during the accelerated mitosis even the condensation at telophase is omitted, although why chromosomal condensation should occur during the final microgametic division, but not the final nuclear divisions of sporogony and schizogony (merogony), is not known.

C. Meiosis

The position of meiosis in the life cycle of the protozoa is highly variable. There is a gametic reduction in the Heliozoa, Ciliates, Opalinina and some Polymastigophora; an intermediate reduction in the Foraminifera and a zygotic reduction in some Polymastigophora, the Phytomonadina, Gregarines and intestinal Coccidia (Grell, 1973). Meiosis in the Eucoccidia is remarkable in that in those suborders studied (i.e. Adeleina and Eimeriina) there is both morphological (Grell, 1973) and quantitative cytochemical evidence (Canning and Morgan, 1975) that meiosis is a one-step division and that no chiasmata are formed between chromosomes.

Table I

Kinetochore counts on single thin sections of intranuclear spindles

Nuclear Division	Number of kinetochores in a single thin section of one hemispindle
Ookinete (first zygotic division)	3·22 ±s.d. 1·20
Oocyst	3·85 ±s.d. 1·79
Budding sporozoite	3·00 ±s.d. 1·73
Budding merozoite (haploid)	2·73 ±s.d. 0·65
Budding microgamete (haploid)	2·56 ±s.d. 1·08

It is estimated there are three thin sections cut within each hemispindle. The haploid chromosome number is therefore about 10.

The position of meiosis in the life cycle of *Haemosporina* remains unknown for a variety of reasons, including: the inability to count chromosomes as a result of the unusual chromatin condensation cycle; the nuclei are too small to measure quantitative cytochemical staining to the accuracy required; the variability of staining of the gametocyte nuclei with Feulgen stain raises doubt as to the reproducibility of cytochemical techniques and the probable absence of chiasmata and therefore synaptinemal complexes precludes the positive ultrastructural identification of meiosis. However, should the zygote (ookinete) divide mitotically it would be expected that 2 n kinetochores would be present on each hemispindle, whereas if it divides meiotically only n kinetochores would be expected. The data available (Table I) suggest that the number of kinetochores is constant throughout the life cycle and that

meiosis is a zygotic division. Proof will only be obtained by a more intensive cytological study or the genetic analysis of recombinants derived from single oocyst infections (Canning and Sinden, 1973).

Should *Plasmodium* like other Coccidia undergo a one-step zygotic reduction division, crossing over would not be expected and genetic recombination will be limited to chromosome reassortment or the rare event of translocation of broken chromosomes within the zygote.

V. FUTURE DEVELOPMENTS IN ULTRASTRUCTURAL STUDIES

The fine structure of murine malaria parasites has been extensively studied throughout most of their complex life cycles. There remain, however, three phases of development which are inadequately understood. First the transition of a newly invaded intraerythrocytic merozoite into a mature macro- or microgametocyte. In this period one anticipates that there are unique predetermined periods of genome replication and protein synthesis, processes which are then inhibited, awaiting the activation of an unknown trigger to initiate the violent activities of gametogenesis. Second, details of the organization of the pre-erythrocytic schizont are sadly lacking. Finally, the greatest single omission is a total absence of knowledge as to the fate of the sporozoite following its injection into the vertebrate host. Whilst it is simple to assume that the sporozoite actively penetrates the liver parenchymal cell directly, we have no evidence for this. Perhaps we should reconsider the possible intervention of cells of the reticuloendothelial system prior to liver invasion and the possible passive ingestion of the sporozoite by such cells. The answer to this question is clearly required before the process of infection can be brought into true perspective.

Even in the phases of development where the morphology has been extensively described, there remains an embarrassing wealth of organelles the functions of which remain the subject of continued speculation. It is to be hoped that the rapid proliferation in histochemical and cytochemical techniques applied to studies on malaria parasites over the past decade will continue, and that the activities of these structures will thereby be explained. The most significant technological advance in electron microscopy in this field is that of energy dispersive X-ray analysis. The ability of this technique to

localize small concentrations of individual elements in both thin sections and scanning microscope preparations may complement and enhance more conventional cytochemical investigations.

While the protozoa are renowned for their diverse and complex nuclear organization, murine malaria parasites present an unusual combination of variations including the absence of a discrete nucleolus; a mitotic cycle which does not usually involve chromosome condensation and a unique cycle of genome replication, repression and reactivation during gametocyte and gamete formation. These problems are rapidly moving into the arena of the biochemist, but their solution will be of importance to cell biologists and geneticists alike.

All workers on malaria have, at some time, been intrigued by the weaving movement of the ookinete, the spinning of the merozoite, the gentle gliding of the sporozoite or the violent lashing of the microgamete. Unfortunately the ultrastructural foundation for the observed activity has only been forthcoming for the microgamete. In the merozoite, ookinete and sporozoite it has been speculated here that the subpellicular microtubules interact with the pellicular membranes, or that the activity of a subpellicular microfilament array cause the parasite to change in shape and therefore move. Such speculations, however, only expose our ignorance of the true mechanisms involved, and hopefully may therefore stimulate studies in these areas. Other intriguing problems directly related to movement are the nature of the tactile responses of the parasites to their target cells. The specific receptor sites for the merozoite are demonstrable on the erythrocyte, and gradients are found around the macrogametes, but the tactile behaviour of the ookinete and sporozoite remain unstudied. The identification of specific binding sites or tactile gradients for any of the invasive stages would subsequently allow the life cycle to be interrupted at these vulnerable phases.

If murine malaria parasites are to continue to be useful models for studies on the ultrastructural organization of human malaria parasites we can no longer afford to ask the simple question—"What is the subcellular morphology of the parasite?" We must ask the more fundamental questions—"What *processes* are taking place at each stage of development, and how are these processes organized?"

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4. Biochemistry

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I. INTRODUCTION

Since the discovery of *Plasmodium berghei* Vincke & Lips, 1948, many papers have been published on the biochemistry of this and other murine malaria parasites. Almost all of these papers report investigations on the intraerythrocytic stages, and it might therefore be expected that these stages at least would be understood in some detail. However, after more than a quarter of a century of research, neither the biochemistry of the parasite nor the nature of its dependence on the host red cell is more than sketchily understood. There are many reasons for this, but the main one is the frequent failure to distinguish between the metabolic processes of the parasite and those of the host, with the consequence that many of the so-called "facts" of parasite biochemistry are of doubtful value.

It is often assumed that to investigate a particular biochemical activity of parasites, it is only necessary to compare that activity in normal and infected blood. However, as well as parasites, parasitized blood contains plasma, platelets, white cells and red cells, each of which may change in number or metabolism during the infection. Before an enzyme or metabolic process can be attributed to the parasite, there must be a clear and precise demonstration that it does not originate in one of these altered host systems.

If plasma is not removed, the alterations in several of its enzyme activities during the infection (Sadun *et al.*, 1966) must be considered.

Platelets are usually ignored, but since their metabolic activities are more complex than those of erythrocytes (Marcus and Zucker, 1965), a rise in the platelet count would correspondingly alter the metabolic activities of the blood. Fortunately, in the case of *P.berghei* in the white mouse at least, their numbers fall during the infection (Fabiani *et al.*, 1958).

A. Interference by White Cells

It is surprising that white cells too are sometimes ignored, although their numbers in the blood stream rise markedly during the infection (Broun, 1961; Kretschmar, 1961; Whitfeld, 1953). Leucocytes contain many enzymes which are not present in mature erythrocytes (compare Cline, 1965 with Brewer, 1974), so that if leucocytes are not removed, their metabolic activities could easily be attributed to the parasites.

Investigators who choose not to remove white cells from parasite preparations must therefore demonstrate conclusively that they do not interfere at all in the experiments they describe. If they fail to do this, then the results cannot be accepted as evidence of the biochemical activities of the parasite.

Even a statement that white cells have been removed from parasitized blood must be treated with caution, since some of the methods used are remarkably inefficient (Homewood and Neame, 1976). The only method which has been shown to remove almost all of the white cells from *P.berghei*-infected blood (Williams and Richards, 1973; Homewood and Neame, 1976) is passage of the blood through a column of cellulose powder (Fulton and Grant, 1956). Before any other method can be accepted, it must be thoroughly tested under the experimental conditions to be used, that is, leucocytes and parasitized red cells must be counted by standard methods before and after treatment. Vague statements such as "most of the white cells were removed" are not acceptable. Nor can it simply be assumed without testing that a method shown to remove white cells from the blood of other species will be just as effective for the blood of rodents infected with malaria parasites. As stressed by Carson and Tarlov (1962) in connection with investigations on human erythrocytes: ". . . contamination of red cell preparations with even a few nucleated cells, especially leucocytes, can give false results."

B. Interference by Immature Red Cells

Immature red cells also differ metabolically from the mature erythrocyte (Lowenstein, 1959; Rapoport, 1961; Bunn, 1972), but their effects in parasitized blood are more difficult to evaluate and overcome. Some strains of *P.berghei* in the white mouse have a requirement for reticulocytes (Ladda and Lalli, 1966; Ott, 1968) so that the parasitaemia and the reticulocyte count will rise together. However, with some other strains of *P.berghei* the percentage of immature red cells, as judged by histological staining, does not rise at all (Hanson and Thompson, 1972) or not until late in the infection (Tella and Maegraith, 1965; Kreier *et al.*, 1972), in spite of the obvious anaemia of the infected animal (Broun, 1961; Tella and Maegraith, 1965; Wellde *et al.*, 1966). Nevertheless, it cannot be assumed that during infection with these strains there is no change in the properties of the red cell population. There is little direct correlation between the levels of enzymes charac-

teristic of immature red cells and the percentage of reticulocytes detected histologically (Goetze and Rapoport, 1954; Schröter *et al.*, 1967). Only biochemical, not histological, tests can give information on metabolic changes in the red cell population. For example, such biochemical tests show that an isoenzyme of malate dehydrogenase which is characteristic of reticulocytes can be detected in the red cells of mice infected with a strain of *P.berghei* which causes high parasitaemias without accompanying reticulocytosis (Momen *et al.*, 1975). In addition, when animals previously made anaemic with phenylhydrazine were infected with this strain, it increased the activity of the isocitrate dehydrogenase of the reticulocytes (Howells and Maxwell, 1973a).

Even the preparation of free parasites from a mixture of parasitized cells and leucocytes may not separate the metabolic processes of parasite and host. The serious extent to which free parasites are contaminated with host membranes and other material has been demonstrated by electron microscopy (Killby and Silverman, 1969; Aikawa and Cook, 1972; Seed *et al.*, 1973), and host enzymes may also be present (Tsukamoto, 1974). The effects on white cells of the various methods of preparing free parasites have not been investigated, but presumably fragments of these cells as well as of mature and immature red cells contaminate the supposedly "free" parasites. In addition, the free parasites are usually collected by centrifugation and, unless great care is taken, any unlysed subcellular structures of host origin, such as mitochondria and nuclei, will be collected with the parasites. A surprising number of enzymes have been found in association with isolated nuclei (Allfrey and Mirsky, 1959; Roodyn, 1959, 1963), mitochondria (Hesselbach and DuBuy, 1953) and membranes (Schrier, 1963; Green *et al.*, 1965), as well as those normally considered to be characteristic of such organelles. Some of the enzymes can be released merely by changing the buffer in which the particles are suspended (Green *et al.*, 1965), and could thus be released, for example, during electrophoresis.

C. Precautions in Investigations of Intraerythrocytic Stages

Investigation of the metabolism of the intraerythrocytic stages of rodent malaria parasites is thus considerably more difficult than might initially be supposed. By far the greatest difficulty lies in making a clear

distinction between the metabolic activities of the parasite and those of the host, the importance of which has been stressed many times, most recently by Fletcher and Maegraith (1972) and by Oelshlegel and Brewer (1975). Without such distinction, it is obvious that the biochemistry of the parasites will never satisfactorily be understood.

In conclusion, it is clear that all investigations into the metabolism of the intraerythrocytic stages of rodent malaria parasites should fulfil the following conditions:

1. the blood cells should be washed free of plasma or the changes in the plasma enzymes should be determined;
2. either white cells should be removed by a method shown to be effective by standard methods of cell counting or it should be clearly shown that there is no possibility of confusion between the metabolism of parasites and white cells;
3. it should be shown that the activity measured could not be due to metabolic changes in the red cell population.

The results of experiments in which these conditions were not observed must be treated with caution if not distrust; although the metabolic activity attributed to the parasites may be of parasite origin, some or all of it may originate in contaminating host material. In cases of doubt, it is safer to disregard the results of such experiments on the intraerythrocytic stages of rodent malaria parasites.

Little is known of the biochemistry of the other stages of the life cycle of the parasites. Most of the information which is available has been obtained by histochemical methods, which have the advantage of identifying enzymes in the parasite itself; nevertheless, these methods too can give misleading results if adequate controls are not used.

Because of the many possibilities of error in studying the metabolism of malaria parasites, only papers which give complete experimental details will be reviewed. Abstracts, communications at meetings, and laboratory demonstrations will therefore not be included.*

* In the following pages certain names are abbreviated as follows: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; DNA, deoxyribonucleic acid; FMN, flavin mononucleotide; IMP, inosine monophosphate; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PABA, para-aminobenzoic acid; PAGA, para-aminobenzoylglutamic acid; RNA, ribonucleic acid.

II. CARBOHYDRATE METABOLISM

Table I

Utilization of glucose and production of lactate by intraerythrocytic stages of murine malaria parasites

Cell type	White cells present?	Glucose used ($\mu g\ 10^9$ cells^{-1} h^{-1})	Conversion to lactate (%)	Reference
Infected cells				
P.c.chabaudi in rat cells	Few		85	Coombs and Gutteridge (1975)
P.berghei in rat cells	?		~70	Fulton and Spooner (1956)
P.berghei in rat cells	Yes	898		Cenedella and Saxe (1971)
P.berghei in rat cells	Yes	1954	83	Cenedella *et al.* (1970)
P.berghei in mouse cells	No	1640–18 194	41	Bowman *et al.* (1960, 1961)
P.berghei in mouse cells	Yes	2112	83	Cenedella and Jarrell (1970)
P.berghei in mouse cells	Yes	145		Cenedella (1968)
Free parasites				
P.berghei		1067–1170	59–73	Bowman *et al.* (1960, 1961)
P.berghei		936	88	Cenedella and Jarrell (1970)
P.berghei		62		Cenedella (1968)
P.berghei		930	94	Cenedella *et al.* (1970)

A. Glycolysis

The intraerythrocytic stages of rodent malaria parasites do not store glycogen or other polysaccharides (Sen Gupta *et al.*, 1955; Ciucă *et al.*, 1963). The amount of glucose used h^{-1} by 10^9 parasitized cells *in vitro* varies from 145 μg to over 18 000 μg, and by 10^9 free parasites from about 60 μg to nearly 1200 μg (Table I), although part of the great differences in the amount of glucose used may be simply a consequence of differences in experimental conditions. For example, the utilization of glucose by a relatively dense suspension of cells decreases with time

(Fulton and Spooner, 1956; Bowman *et al.*, 1960), probably due to reduction in the pH of the medium or to shortage of glucose. Also, the amount of glucose used may depend on its concentration in the medium, as it does with *P.knowlesi* (Scheibel and Miller, 1969).

The amount of glucose used by free parasites of *P.berghei* is less than that used by the same number of parasitized cells in the same conditions, either because of inevitable damage sustained by the parasites during the process of freeing them from the red cell or because lysis of the red cells reduces the extent of interfering metabolism. Mature erythrocytes use very little glucose, but immature red cells generally have a much higher rate of glycolysis (Bernstein, 1959; Rapoport, 1961).

The reported extent to which the glucose used by parasites is converted to lactate varies from about 40 to 90%. Probably one of the most reasonable figures is that of 85% reported by Coombs and Gutteridge (1975), as in this case glucose utilization and lactate production were linear for 18–19 h of incubation of blood of low parasitaemia (less than 12%) taken from a rat infected less than 24 h previously with *P.c.chabaudi*, although the glucose used by the red cells would be a relatively high proportion of the total.

Other than lactate, no significant amounts of any end products of the metabolism of glucose by *P.berghei* have been identified. Fulton and Spooner (1956) showed that the small amounts of acetate and succinate found were probably produced by host cells. Bryant *et al.* (1964) found that over 70% of the glucose used by *P.berghei* in mouse cells was lost as volatile compounds, but these were not investigated further. No volatile compounds were produced by free parasites. Nor can the discrepancy between glucose utilization and lactate production be accounted for by production of 2,3-diphosphoglycerate, as Ali and Fletcher (1974) showed that this compound falls in infected blood.

On the whole, it would seem that in the intraerythrocytic stages rodent malaria parasites metabolize glucose by glycolysis, converting most but not all of it to lactate. The enzymes involved have not been well studied. The increased activity of hexokinase measured in *P.berghei*-infected blood by Fraser and Kermack (1957) may have been partly due to the white cells present and to immature red cells, which contain higher activities of this enzyme than do mature red cells (Hutton, 1972). The electrophoretic investigations of Carter (1970, 1973) on various strains of *P.berghei* and *P.vinckei* did not include controls for white cells and immature red cells, but one of the bands of activity due to hexo-

kinase had an electrophoretic mobility which depended on the strain of parasite; the same was true of glucosephosphate isomerase, and these two enzymes were therefore present in the parasite as well as in the host cell.

Another enzyme of the glycolytic sequence, pyruvate kinase, was investigated by Oelshlegel *et al.* (1975). White cells were removed by a method which is inadequate for separating leucocytes from parasitized mouse cells (Homewood and Neame, 1976), and immature red cells were not included as controls. The isoenzyme of pyruvate kinase which was detected in parasitized but not in normal blood cannot without further evidence be attributed to the parasite, as it has been reported that pyruvate kinase of human leucocytes has a different electrophoretic mobility from that of red cells (Koler *et al.*, 1964).

The presence of lactate dehydrogenase in *P.berghei* was reported by Sherman (1962), but as white cells and immature red cells were not considered, the enzyme detected electrophoretically cannot with certainty be attributed to the parasite. Carter (1973) showed that lactate dehydrogenases of several strains of *P.berghei* and *P.vinckei* had different electrophoretic mobilities, and Tsukamoto (1974) detected all the possible isoenzymes of mouse lactate dehydrogenase plus an additional isoenzyme in rodent malaria parasites. An earlier report (Phisphumvidhi and Langer, 1969) claimed that the lactate dehydrogenase of *P.berghei* had a much higher affinity for pyruvate than did that of the host cell. The enzyme attributed to the parasite may have been partly of host origin, but in any case, its apparently higher affinity for pyruvate is based on an arithmetical error rather than on a true difference between the enzymes of free parasites and erythrocytes. The *Km* for pyruvate of the enzyme obtained from free parasites was 0·36 of that of the host enzyme, not 1/36 as stated. Even if there had been a significant difference in *Km*, it is difficult to see how *P.berghei* could benefit from pyruvate acquired from the host.

B. Pentose Phosphate Pathway

Investigations of the pentose phosphate pathway in malaria parasites are complicated by the fact that much higher glucose-6-phosphate dehydrogenase activity is present in reticulocytes than in mature erythrocytes (Hutton, 1972; Rozenszajn *et al.*, 1972). It has been reported also that the enzymes of immature and mature red cells behave

differently on electrophoresis (Walter *et al.*, 1965). The pentose phosphate pathway is also present in leucocytes (Beck, 1958). It appears to be little used by malaria parasites, as Bowman *et al.* (1961) found that less than 2% of the glucose used by free parasites of *P.berghei* or by parasitized mouse cells was metabolized by this pathway, and Bryant *et al.* (1964) could detect no radioactivity in 6-phosphogluconate after incubation of free parasites with labelled glucose.

Electrophoretic studies of glucose-6-phosphate dehydrogenase have given conflicting results. Carter (1973) found the same additional enzyme in several strains of *P.berghei* but as white cells were not removed nor reticulocyte controls used, it is possible that this isoenzyme was of host origin; this doubt was later raised by Carter himself (see footnote, Killick-Kendrick, 1974). Langer *et al.* (1967) similarly detected in free parasites of *P.berghei* an isoenzyme of glucose-6-phosphate dehydrogenase which was distinct from that of normal red cells; most but not all of the white cells were removed but reticulocyte controls were not used. Tsukamoto (1974) could not detect this enzyme in *P.berghei.*

Jung *et al.* (1975) and Picard-Maureau *et al.* (1975) showed that during growth of *P.vinckei* in the red cell, the activity of the glucose-6-phosphate dehydrogenase of infected cells did not rise as would be expected of a constituent of the parasite, but decreased, as would be expected of a constituent of the red cell undergoing destruction during the growth of the parasite. The different stages were separated from whole blood on a density gradient, with the possibility that white cells might have confused the results by becoming concentrated in some fractions, but the authors reported that this did not happen.

Theakston and Fletcher (1971, 1972), in an electron microscopical study, detected glucose-6-phosphate dehydrogenase in mouse red cells but not in *P.berghei*, except in the so-called "food vacuole", now considered to be an extension of the red cell cytoplasm within the parasite (Howells *et al.*, 1968; Ladda, 1969). In contrast, Theakston and Fletcher (1973) showed activity due to phosphogluconate dehydrogenase in *P.berghei* as well as in the red cell. In addition, Carter (1973, less convincingly in 1970) found isoenzymes of phosphogluconate dehydrogenase which had different electrophoretic mobilities in different strains of *P.berghei* and *P.vinckei*. Langer *et al.* (1967) reported the presence of this and other enzymes of the pentose phosphate pathway in free parasites of *P.berghei*, but the extent of contamination with host material is difficult to determine.

Sherman (1965) concluded that any decrease in the glucose-6-phosphate dehydrogenase activity of the parasitized cell was not sufficient to cause a fall in the amount of reduced glutathione. However, the test used was designed for the relatively simple system of normal red cells, and it may be more difficult to interpret the results obtained with a mixture of normal and immature red cells, parasites and white cells. Picard-Maureau *et al.* (1975) reported that the reduced glutathione content of parasitized cells increased markedly during the intra-erythrocytic cycle of *P.vinckei*. Fletcher and Maegraith (1970) also detected a rise in the level of reduced glutathione towards the end of an infection with *P.berghei*, but white cells were not removed, and their glutathione content, which is about seven times that of red cells (Hardin *et al.*, 1954), may have contributed to the increase.

Pollack *et al.* (1966) showed that *P.berghei*, like the glucose-6-phosphate dehydrogenase-deficient red cell, is sensitive to oxidative agents, suggesting a similar deficiency of this enzyme in the parasite. However, since the oxidative agents used also have other actions, their effect on the growth of *P.berghei*, although suggestive, cannot be taken as proof of pentose phosphate pathway activity in the parasite.

C. Citric Acid Cycle

The intraerythrocytic stages of the rodent malaria parasites do not possess a functioning citric acid cycle. Infected mouse cells or free parasites of *P.berghei* converted only 0·3–0·4% of the metabolized 6-^{14}C-glucose to labelled carbon dioxide (Bowman *et al.*, 1961), and although Bryant *et al.* (1964) felt that *P.berghei* within the red cell (but not free parasites) metabolized glucose by means of the citric acid cycle, the end products of the metabolism were not identified nor was the extent of host cell interference determined.

Additional isoenzymes of malate dehydrogenase appear in mouse blood on infection with *P.berghei*, but the presence of this enzyme does not indicate activity of the citric acid cycle, as it is present in the mature erythrocyte which lacks most of the enzymes of the cycle (Brewer, 1974). The investigation of malate dehydrogenase in parasitized cells is complicated by its presence in white cells (Borel *et al.*, 1959) and by the presence in immature red cells of an isoenzyme with an electrophoretic mobility different from that of mature erythrocytes (Momen *et al.*, 1975). Sherman (1966) showed that a new isoenzyme of malate

dehydrogenase appeared in free parasites of *P.berghei*, but white cells were not removed and reticulocyte controls were not used. The same enzyme was reported by Tsukamoto (1974) who removed white cells, but again the enzyme of immature red cells was not investigated. Carter (1970, 1973) attributed to *P.berghei* an isoenzyme of malate dehydrogenase which was later shown by Momen *et al.* (1975) to originate in immature red cells. Another isoenzyme was considered by both Carter (1970, 1973) and Momen *et al.* (1975) to be of parasite origin. Momen *et al.* (1975) could not detect malate dehydrogenase in some strains of *P.berghei*, but this did not prove it was absent.

Nagarajan (1968a) detected malate dehydrogenase and fumarase in free parasites of *P.berghei*, although reticulocyte stroma also showed malate dehydrogenase activity. Several other enzymes of the cycle, including succinate dehydrogenase, could not be detected in free parasites, although succinate stimulated their respiration. Howells (1970b) could not detect succinate dehydrogenase by electron microscopy in intraerythrocytic *P.berghei*, nor could Howells and Maxwell (1973b) detect isocitrate dehydrogenase after electrophoresis of extracts of *P.berghei*.

D. Carbon Dioxide Fixation

Nagarajan (1968c) reported that *P.berghei*, either free or within the rat reticulocyte, could fix labelled carbon dioxide into organic acids, mainly malate, but some of the radioactivity was also found in glutamate and aspartate. The results could only be explained by the operation of the citric acid cycle, although the enzymes of this cycle could not be detected (Nagarajan, 1968a). The low activities of the enzymes which must have been present were quite possibly due to host cell contamination, although fixation of carbon dioxide into malate or oxaloacetate may have been carried out by the parasite.

Neither Tsukamoto (1974) nor Nagarajan (1968a) could detect malic enzyme in free parasites of *P.berghei*, but Siu (1967), Forrester and Siu (1971) and McDaniel and Siu (1972) demonstrated the presence of phosphoenolpyruvate carboxylase. This enzyme is widely distributed in plants and bacteria, but has never been found in animal tissues (Lane *et al.*, 1969). Siu (1967) also found phosphoenolpyruvate carboxykinase in *P.berghei*; this enzyme is found in mitochondria of animal cells, although it is not known if it is present in white cells or reticulocytes.

The intraerythrocytic stages of murine malaria parasites thus metabolize glucose almost entirely to lactate by the Embden-Meyerhof pathway, with perhaps a small amount of carbon dioxide fixation, while the contribution of the pentose phosphate pathway is very small and that of the citric acid cycle is virtually non-existent.

E. Non-erythrocytic Stages

The biochemistry of the other stages of the parasite has hardly been studied at all. The mitochondria of pre-erythrocytic *P.berghei* have been described as both cristate (Terzakis *et al.*, 1974) and acristate (Desser *et al.*, 1972) (see Chapter 3), but whatever their morphology, they do not contain detectable succinate dehydrogenase activity (Howells and Bafort, 1970). From this slight evidence, it would appear that the pre-erythrocytic stages of *P.berghei* resemble the erythrocytic ones in lacking a functional citric acid cycle, but differ from them in possessing glucose-6-phosphate dehydrogenase.

Mature gametocytes of *P.berghei* have cristate mitochondria but no succinate dehydrogenase activity could be detected in them (Howells, 1970b). The oocyst and sporozoites, on the other hand, have both cristate mitochondria and demonstrable succinate dehydrogenase activity (Howells, 1970b). In addition, NAD- and NADP-dependent isocitrate dehydrogenase activities of parasite origin were detected after electrophoresis of infected mosquito stomachs (Howells and Maxwell, 1973b). It is therefore possible, but by no means proved, that the oocyst and the sporozoites, unlike the other stages of *P.berghei*, have a functional citric acid cycle.

III. RESPIRATION AND ELECTRON TRANSPORT

As the intraerythrocytic stages of rodent malaria parasites lack a functional citric acid cycle, it might be expected that mitochondrial electron transport would also be absent. These stages of *P.berghei* do have mitochondria, as shown by staining with Janus Green (Sen Gupta *et al.*, 1955), but with very few cristae (Ladda, 1969; Howells, 1970b). It is, however, possible for mitochondria without cristae to have a complete set of cytochromes (Ritter and André, 1975), and there have been some indications that the intraerythrocytic stages of rodent

malaria parasites do have a functioning electron transport chain. Thus it has been reported that *P.berghei* uses oxygen, contains cytochrome oxidase, NADH dehydrogenase and coenzyme Q, and that energy production by the parasite is reduced by recognized inhibitors of electron transport (see Table II for references).

Table II

Reports of electron transport mechanisms in intraerythrocytic stages of *P.berghei*

Oxygen utilization	Jones *et al.* (1951)
	Fulton and Spooner (1956)
	Bowman *et al.* (1960)
	Cho and Aviado (1968)
	Nagarajan (1968a)
Cytochrome oxidase	Nagarajan (1968a)
	Howells *et al.* (1969)
	Scheibel and Miller (1969)
	Theakston *et al.* (1969)
Coenzyme Q	Skelton *et al.* (1970)
NADH dehydrogenase	Theakston *et al.* (1970a)
Sensitivity to inhibitors of electron transport	Nagarajan (1968b)
	Homewood *et al.* (1972b)

A. Oxygen Utilization

Measurements of oxygen utilization by parasitized cells from which white cells have not been completely and certainly removed cannot give information on the respiration of parasites (see Cline, 1965, for references on oxygen utilization by white cells). Even when white cells have been removed, immature red cells with their high respiratory activity (Jones *et al.*, 1953; Fulton and Spooner, 1956; Lowenstein, 1959) may remain. The preparation of free parasites does not necessarily overcome the interference by host cells unless it is shown that the mitochondria of such cells do not contaminate the parasite preparation. In almost all the recorded cases of oxygen utilization by *P.berghei*, interference by host material was not ruled out, and in these cases it cannot be assumed that the parasite itself used oxygen. Indeed Jones *et al.* (1951) showed that maximum utilization of oxygen by infected rat cells was obtained when the animal was recovering from infection with *P.berghei* and the number of parasites was so low that they could have

used only an insignificant amount of oxygen. The one case in which the parasite itself appeared to use oxygen was reported by Nagarajan (1968a). White cells were removed and reticulocyte stroma were shown not to use oxygen, but free parasites of *P.berghei* still respired. Succinate was the only effective substrate, but succinate dehydrogenase could not be detected; the mechanism of the respiration therefore remains a mystery.

B. Electron Transport Chain

Cytochrome oxidase was demonstrated in *P.berghei* by a cytochemical technique which does not appear to be completely specific, as it did not detect cytochrome oxidase in the mitochondria of rat liver, but did detect a great deal of activity at other, cytoplasmic, sites (Seligman *et al.*, 1967). Both this extramitochondrial activity and the activity detected in *P.berghei* were inhibited by cyanide at a concentration of 10^{-2}M, which is far too high to be specific for cytochrome oxidase (Dixon and Webb, 1964). There is thus a certain amount of doubt that the activity detected cytochemically in *P.berghei* was due to cytochrome oxidase.

The careful experiments of Scheibel and Miller (1969) seem to demonstrate by biochemical means that free parasites of *P.berghei* contain cytochrome oxidase. Platelets as well as white cells were removed, but it is possible that part of the activity was due to immature red cells. Rabbit reticulocytes contain mitochondria (Gasko and Danon, 1972) and, in addition, an enzyme apparently originating in host-cell mitochondria has been found in *P.berghei*-infected mouse cells (Momen *et al.*, 1975). The free parasites of *P.berghei* prepared by Nagarajan (1968a) were able to transfer electrons from ascorbic acid to oxygen indicating cytochrome oxidase activity.

The NADH dehydrogenase detected cytochemically in *P.berghei* (Theakston *et al.*, 1970a) could not be detected in free parasites (Nagarajan, 1968a). The presence of this enzyme would not necessarily imply that an electron transport chain is also present.

Blood from normal mice as well as that from mice infected with *P.berghei* contains coenzymes Q_7, Q_8 and Q_9, and the changes in their relative proportions on infection could be caused by changes in the host cells.

Inhibitors of electron transport also inhibit chloroquine-induced pigment clumping, which is an energy-requiring process as synthesis of

protein and nucleic acids is involved (Warhurst *et al.*, 1971). However, the complications introduced by the use of chloroquine, a drug whose mode of action is far from clear, as well as the high concentrations of inhibitors used, mean that caution must be used in interpreting this observation. Nagarajan (1968b) showed that the incorporation of ^{32}P-phosphate by free parasites of *P.berghei* was completely inhibited by dinitrophenol and by sodium azide, although he was unable to demonstrate the synthesis of ATP. Carter *et al.* (1972) measured the incorporation of adenosine into ATP, but did not test the effects of inhibitors.

Although there are several intriguing hints that *P.berghei* has an electron transport chain which possibly uses oxygen as the final acceptor, this has yet to be proved conclusively. At least one energy-requiring process, that of chloroquine-induced pigment clumping, can take place in the absence of oxygen (Homewood *et al.*, 1972b).

Cytochrome oxidase activity was also detected cytochemically in the oocyst of *P.berghei* (Howells, 1970a), but the same doubts apply to this as to the cytochrome oxidase of the intraerythrocytic stage.

IV. AMINO ACID AND PROTEIN METABOLISM

A. Protein Digestion

Intraerythrocytic *P.berghei* degrades the haemoglobin of the mouse erythrocyte, incorporating amino acids into its own protein and leaving the unused haem in pigment (Theakston *et al.*, 1970b). It is possible that in obtaining sufficient quantities of the least common amino acids of haemoglobin, the parasite will obtain more of the plentiful ones than it can use. It has been reported that the concentration of free amino acids rises during incubation of parasitized rat reticulocytes (Cenedella *et al.*, 1968), but the pH at the end of 2–4 h incubation of a rather dense suspension of cells was not recorded, and white cells, with their proteolytic enzymes (see Cline, 1965), were not removed.

The proteolytic enzymes which the parasite must use to digest haemoglobin were investigated by Cook *et al.* (1961) in extracts of *P.berghei* released from mouse red cells. White cells were probably removed, but immature red cells, which contain higher levels of protease activity than do mature erythrocytes (Goetze and Rapoport, 1954; Ellis *et al.*, 1956) were not considered. Extracts had two pH

optima for the digestion of haemoglobin, one at a pH of about 4, and the other at a pH of about 8. Cook *et al.* (1969) later showed that the method used for assay of proteolytic activity (increase in optical density at 280 nm) could be unreliable in certain circumstances, particularly in crude extracts in which nuclease activity could be mistaken for proteolytic activity (perhaps accounting for the apparent preference for reticulocyte cytoplasm of the alkaline protease of extracts of *P.berghei*).

Chan and Lee (1974) reported that the alkaline protease of *P.berghei*-infected mouse cells could be chromatographically separated into three fractions, two of which were active against mouse, but not human, haemoglobin, while the third acted equally well on both types of haemoglobin. Normal mouse red cells had low proteolytic activity but immature red cells were not investigated, and white cells were removed by a method tested only on normal chicken and human blood, so that it is difficult to determine which of the proteases originated in the parasite.

Other investigators have been more interested in the acid proteases of the parasite, on the assumption that the parasite digestive vacuole is analogous to a lysosome and therefore contains proteolytic enzymes which have acid pH optima. True lysosomes contain acid phosphatase (De Duve, 1963), which is also found in the digestive vacuoles of many protozoa (Müller *et al.*, 1963; Maguadda and Pennisi, 1969), although its function is unknown. Sen Gupta *et al.* (1955) and Aikawa and Thompson (1971) found acid phosphatase in the digestive vacuoles of *P.berghei*, but it could not be detected in the parasite by Scorza *et al.* (1972) who did detect aryl sulphatase, another enzyme usually found in lysosomes. Acid phosphatase appears to be absent from the sporogonic stages of *P.berghei*, although aryl sulphatase is present (Davies, 1974).

Levy and Chou (1973) showed that red cells from mice infected with *P.berghei* had two to three times more activity of a cathepsin D-like protease than did cells from normal mice. White cells were removed by a method which was inefficient in other hands (Homewood and Neame, 1976), but acid phosphatase, which should have increased if white cells or reticulocytes had been present, showed little increase over the levels of normal blood, even after 8–9 days of infection. Further studies (Levy and Chou, 1974) showed that the enzymes from normal and parasitized blood were similar with respect to molecular weight, *Km* for haemoglobin and sensitivity to inhibitors, but the pH optima were slightly

different. Both enzymes could be inhibited by pepstatin (an inhibitor of carboxyl proteases, such as cathepsins D and E), chymostatin (an inhibitor of chymotrypsin) and antipain and leupeptin (both inhibitors of cathepsin B). It is unusual for one enzyme to be affected by all these inhibitors, which are usually considered to be specific, but a mixture of different enzymes is apparently ruled out as each inhibitor was able to inhibit the proteolytic activity completely. In addition, pepstatin, chymostatin and phenylmethane sulphonyl fluoride (which inhibits serine proteases) all reduced the incorporation of isoleucine into protein by parasitized mouse cells (Levy and Chou, 1975). The ability of pepstatin, a pentapeptide, to reach the parasite suggests a drastic alteration in the membrane of the parasitized cell, as this is the only membrane which the inhibitor has been shown to cross (Dean, 1975).

B. Malaria Pigment

The end-product of the digestion of haemoglobin by malaria parasites is pigment. Early studies on several species including *P.berghei* (Fulton and Rimington, 1953) suggested that this pigment was merely haemin or haematin, but the methods used to extract and analyse it were harsh. Deegan and Maegraith (1956) suggested that the pigment of primate malarias did indeed contain haematin but that this was combined with some other, probably nitrogenous, substance. Sherman *et al.* (1965) reported that the nitrogenous part of the pigment of *P.berghei* was protein, produced by the partial digestion of haemoglobin, but as pointed out by Homewood *et al.* (1972a), the pigment preparations analysed were contaminated with cell membranes. Nevertheless, as only about 10% of it appears to be haemin (Homewood *et al.*, 1975), pigment must contain some protein or other substance. The autoradiographic studies of Theakston *et al.* (1970b, 1972) showed leucine but not methionine associated with pigment within the digestive vacuole. It is doubtful that the high molecular weight reported for pigment (Homewood *et al.*, 1972a) is meaningful, as sodium hydroxide solution was used as solvent. In fact, the size of the pigment "molecule" may well vary, as the individual grains of pigment in a parasite have different absorption spectra, perhaps representing different transition states between haemoglobin and the final end-product (Morselt *et al.*, 1973).

C. Amino Acid Metabolism

Langer and Phisphumvidhi (1971) detected in *P.berghei* an L-amino acid oxidase which used FMN as cofactor. No D-amino acid oxidase was found. It is not known whether white cells contain amino acid oxidases.

The glutamate dehydrogenase purified by Walter *et al.* (1974) from *P.chabaudi* resembled a microbial enzyme in being specific for NADP; no NAD-specific enzyme was found. The glutamate dehydrogenase detected by Tsukamoto (1974) after electrophoresis of extracts of *P.berghei* was similarly NADP-specific, but Langer *et al.* (1970) and Picard-Maureau *et al.* (1975) found that glutamate dehydrogenase of *P.berghei* and *P.vinckei* had about one third the activity with NAD as cofactor that it had with NADP. Langer *et al.* (1970) found no effect of ADP or ATP on the activity of the enzyme when NADP was the cofactor, suggesting that it was therefore not of animal origin, but part of the NAD-dependent activity may have been due to white cells. Picard-Maureau *et al.* (1975) found that the activity of glutamate dehydrogenase increased during the intraerythrocytic cycle of the parasite, suggesting that it originated in the parasite itself.

The additional isoenzymes of aspartate aminotransferase that Tsukamoto (1974) found when he compared free parasites of *P.berghei* with normal mouse blood may have been of parasite origin, but human reticulocytes contain an isoenzyme of aspartate aminotransferase which is not present in mature erythrocytes (Fiorelli *et al.*, 1969).

Some malaria parasites are dependent on exogenous supplies of amino acids (Polet and Conrad, 1969), but it is not known to what extent this is true of the rodent malaria parasites. It has been suggested (Langer *et al.*, 1969) that *P.berghei* will need a constant supply of methionine from the host, not only for protein synthesis but also as a source of methyl groups, as the parasite is unable to form a reserve of these groups in phosphatidylcholine. In addition, even if supplied with choline (or betaine) it cannot transfer the methyl groups to homocysteine to form methionine, the donor of methyl groups in most reactions. As little methionine is present in mouse haemoglobin (Rifkin *et al.*, 1966), the parasite may need to obtain this amino acid from the plasma. *P.berghei* can, however, synthesize methionine by adding a methyl group derived from serine to homocysteine.

The incorporation of amino acids such as leucine (Richards and

Williams, 1973; Coombs and Gutteridge, 1975) into parasite protein has sometimes been used as an indication of parasite growth *in vitro*. In this type of experiment, interference by relatively small numbers of white cells or immature red cells can be great, as both these cells synthesize protein (Williams and Richards, 1973). Autoradiographic studies can distinguish between incorporation by parasites and by host cells, but the results are not as easily quantitated. It has been shown by this means that intraerythrocytic *P.berghei* incorporates exogenous lysine and leucine (Büngener and Nielsen, 1967), and methionine and leucine (Theakston *et al.*, 1970b, 1972).

The above discussion suggests that remarkably little is known of the means by which intraerythrocytic stages of rodent malaria parasites obtain amino acids, either from haemoglobin or from plasma, or of the mechanism of formation and structure of pigment and the synthesis of proteins.

V. LIPID METABOLISM

Wallace *et al.* (1965) examined the lipids of *P.berghei* freed from rat cells by saponin, and showed the presence of phospholipid (the class of lipid present in largest amounts, as in most cells), sterols, triglycerides, sterol esters and free fatty acids. It is difficult to know how much the lipids of the membranes of the host cells contributed to these analyses, since white cells were not removed, and reticulocytes contain 4–5 times as much lipid as do mature erythrocytes (Van Deenen and de Gier, 1964).

Lawrence and Cenedella (1969) and Rao *et al.* (1970) found not only that rat reticulocytes contained more lipid than did mature erythrocytes, but that the lipid content of *P.berghei*-infected cells was even higher. Both authors found that phosphatidylethanolamine levels rose in infected cells and those of sphyngomyelins and lysolecithins fell. Rao *et al.* (1970) calculated, however, that the lipids of the parasites were less than 6% of the total lipids of infected cells.

The synthesis of lipids by malaria parasites may also be difficult to separate from that of reticulocytes (Marks *et al.*, 1960; Sloviter and Tanaka, 1967; Ballas and Burka, 1974) and of white cells (see Cline, 1965) if they are not removed. Cenedella (1968) found that cells from mice infected with *P.berghei* incorporated more glucose carbon into lipid than did cells from normal mice, but no allowance was made for

incorporation by white cells or by immature red cells. Nearly all of the incorporated glucose carbon (0·5% of the glucose used) was found in phospholipids. This is also true of white cells (Sbarra and Karnovsky, 1960) and reticulocytes (Sloviter and Tanaka, 1967), but the same type of incorporation was also found with free parasites.

After infusion of ^{14}C-labelled glucose into *P.berghei*-infected rats (Cenedella *et al.*, 1969a), the lipids of free parasites were labelled mostly in lecithins, with smaller amounts in phosphatidylethanolamines, phosphatidic acid and polyglycerolphosphatides. Incorporation of oleic acid followed a similar pattern, suggesting that *P.berghei* could obtain at least part of its fatty acids from the plasma, but the ability of white cells to incorporate fatty acids into lipids (Elsbach, 1959, 1962) may account for an unknown proportion of the incorporation of oleic acid.

It has been reported that *P.berghei* also contributes fatty acids to the plasma by breakdown of host lipids (Cenedella, 1968; Cenedella *et al.*, 1969b). Some of the comments made earlier with regard to the production of amino acids by the parasites may apply here, although in the absence of glucose (and presumably therefore a less drastic reduction in pH), the production of fatty acids was reduced by only about 20%. Free parasites contained phospholipase A activity, which could not be detected in the stroma of reticulocytes, although white cells (which contain lipases—Elsbach and Rizack, 1963) were not tested.

It can be seen that little is known of the composition or synthesis of the lipids of murine malaria parasites. A very small part, if any, of the glucose used by the parasites is used to synthesize lipid, but the parasite may obtain fatty acids both from the plasma of the host and by breakdown of host lipids.

VI. NUCLEIC ACIDS

A. DNA and RNA

P.berghei, in common with other organisms, contains DNA, RNA and DNA-bound histones (Sen Gupta *et al.*, 1955; Ciucă *et al.*, 1963; Bahr, 1966). By electron microscopy, Bahr (1966) estimated that an intra-erythrocytic schizont contains about 10^{-13} g of DNA, while Whitfeld (1952, 1953) by direct measurement of extracted nucleic acids found about 5×10^{-8} g of DNA trophozoite^{-1}. The difference of several

orders of magnitude between these two estimations cannot be entirely due to white cells in Whitfeld's preparations (a human white cell contains about 10^{-11} g of DNA—Davidson *et al.*, 1951) nor to growth of the parasite. The estimate of Bahr (1966), although it is difficult to see how it was obtained, compares more closely with the DNA content of *P.knowlesi* (Gutteridge and Trigg, 1972), and with that of other organisms, which contain between about 10^{-13} and 10^{-10} g of DNA cell^{-1} (Davidson, 1972).

The content of guanine plus cytosine (G+C content) of the DNA prepared by Whitfeld (1953) was about 40%, which is the same as that found for mouse DNA by Gutteridge *et al.* (1971). The latter authors found that the G+C content of the DNA of *P.vinckei* and three strains of *P.berghei* was 24%, reinforcing the suggestion of Wolcott (1957) that *P.berghei* is more closely related to the avian malarias (G+C content about 20%) than to the primate ones (G+C content 37%). The data of Gutteridge *et al.* (1971) further showed that the DNA of malaria parasites is double-stranded and likely to be linear rather than circular.

The RNA of *P.berghei* has been little investigated. The 40S ribosomal subunit was found to contain 15S RNA, while the 60S subunit contained 25S RNA (Tokuyasu *et al.*, 1969) and was thus intermediate between the bacterial value of 23S and the mammalian one of 28S (Davidson, 1972). The smaller ribosomal subunit was easily labelled with radioactive orotic acid or ^{32}P-phosphate, but no significant labelling was found in the larger subunit, leaving its origin a mystery. It was suggested (Ilan *et al.*, 1969) that the lack of specificity of the parasite aminoacyl-tRNA synthetases might enable it to use host ribosomes.

The synthesis of DNA and RNA was shown to be discontinuous in *P.vinckei* by Jung *et al.* (1975). The different intraerythrocytic stages were separated on a Ficoll gradient, and although white cells were not removed, their metabolic activities were not detected in the parasite fractions. Both DNA and RNA synthesis were highest in old ring forms and young trophozoites. Although the rate of thymidylate synthetase activity remained high in the schizont (Walter and Königk, 1971a), the rate of DNA synthesis fell (Jung *et al.*, 1975).

Mature red cells and reticulocytes contain little or no DNA, and although reticulocytes contain RNA (Lowenstein, 1959) they cannot synthesize it from exogenous uridine (Borsook, 1964) or adenosine (Van Dyke *et al.*, 1969).

White cells contain both RNA and DNA and these can be synthesized from exogenous purine and pyrimidine bases and nucleosides (see Cline, 1965). Büngener and Nielsen (1967) did not, as frequently stated, report that adenosine is not incorporated by leucocytes, but they did show autoradiographically that for every five silver grains found in a cell of *P.berghei* after incubation with ^{3}H-adenosine, three were found in each white cell. If this is an accurate indication of the relative capacities of the two types of cells for the incorporation of adenosine, then the total uptake by the white cells in infected blood would be a very small fraction of that incorporated by the parasites which outnumber white cells by about a thousand times (e.g. Whitfeld, 1953; Homewood and Neame, 1976). This does, however, seem a rather insecure foundation on which to base experiments involving the incorporation of adenosine and other purines into the nucleic acids of parasites.

B. Metabolism of Purines

Intraerythrocytic stages of rodent malaria parasites can use exogenous purines for nucleic acid synthesis (Büngener and Nielsen, 1967, 1968, 1969; Van Dyke and Szustkiewicz, 1969; Van Dyke *et al.*, 1969, 1970a, b; Lukow *et al.*, 1973; Theakston *et al.*, 1972; Neame *et al.*, 1974; Van Dyke, 1975). In cells which synthesize purines *de novo*, the phosphate and ribose portions of the molecule are already present when the purine is formed. Cells which can use exogenous purines for nucleic acid synthesis must add the ribose and phosphate moieties to the preformed purine, and to do this must possess the enzymes of the "salvage pathway" to form AMP from adenine or adenosine (Figure 1).

Several of these enzymes are present in human erythrocytes and lymphocytes (see Parks *et al.*, 1975) but species differences occur. For example, red cells of humans cannot convert IMP to AMP (Lowy *et al.*, 1962) but those of rabbits (Lowy *et al.*, 1961) and mice (Miyazaki and Minaki, 1972) can do so.

All the enzymes listed above have been detected in *P.chabaudi* freed from mouse red cells after removal of leucocytes (Lukow *et al.*, 1973). Further purification of the parasite adenosine deaminase (3) by Schmidt *et al.* (1974), and of hypoxanthine-guanine phosphoribosyltransferase (5) and adenine phosphoribosyltransferase (1) by Walter and Königk (1974a) showed that these enzymes had similar characteristics to those from mammalian sources. Büngener (1967) found that mouse cells

infected with *P.berghei* or *P.vinckei* contained isoenzymes of adenosine deaminase (3) which had different electrophoretic mobilities in the two species of parasite; the same was true of purine nucleoside phosphorylase (4). It seems therefore that murine malaria parasites possess enzymes of the salvage pathway for purines. They also possess adenylate kinase (Carter, 1973).

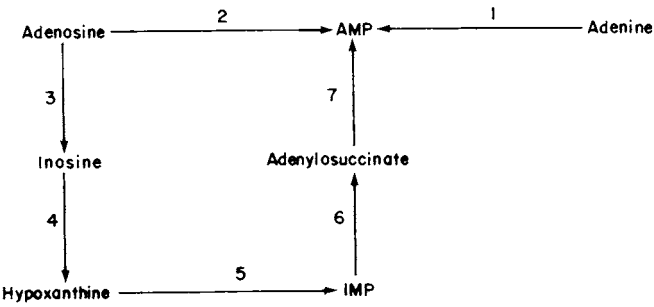

Figure 1. The salvage pathway for purines; 1: adenine phosphoribosyltransferase, 2: adenosine kinase, 3: adenosine deaminase, 4: inosine phosphorylase, 5: hypoxanthine-guanine phosphoribosyltransferase, 6: adenylosuccinate synthetase, 7: adenylosuccinase, IMP: inosine monophosphate.

Parasites can use the purines of the red cell pool, as shown by allowing *P.berghei* to infect red cells previously loaded with radioactive adenosine (Büngener and Nielsen, 1969). Mouse erythrocytes supplied with adenosine *in vitro* phosphorylate part of it to the nucleotides and deaminate part of it to inosine, some of which leaves the cell. Part of the hypoxanthine formed from the inosine also leaves the red cell, but some of it is converted to adenine nucleotides (Miyazaki and Minaki, 1972). It would therefore be an advantage for an intraerythrocytic parasite to be able to use a variety of purines and their derivatives. Lantz *et al.* (1971) found that *P.berghei*, free or within the erythrocyte, incorporated more label from radioactive AMP than from adenosine. It was not suggested that the parasite used AMP directly, but that it took up adenosine released by phosphorylase in the serum of the incubation medium; less added adenosine was incorporated because it was rapidly deaminated to inosine. It was later shown by Manandhar and Van Dyke (1975) that after incubation of free parasites with labelled adenosine, radioactivity outside the parasites was found in adenosine itself and also in inosine and hypoxanthine, while inside the parasites,

radioactivity was mainly in the phosphorylated derivatives IMP, AMP, ADP and ATP. Addition to the medium not only of unlabelled adenosine but also of unlabelled hypoxanthine and inosine reduced the amount of radioactivity found in the parasites. The authors suggested that metabolism of adenosine to hypoxanthine via inosine took place on the outside of the parasite; hypoxanthine then entered the cell and was converted to IMP and hence to AMP and the other adenosine phosphates. However, before the extracellular conversion of adenosine to hypoxanthine could be attributed entirely to the parasite, it would be necessary to ensure that host enzymes were not involved, as the necessary enzymes are present in mouse erythrocytes (Miyazaki and Minaki, 1972), and human lymphocytes (Parks *et al.*, 1975). White cells were not removed and the concentration of saponin used in the preparation of free parasites was about one-tenth of that needed for complete disintegration of the red cell membrane (Dourmashkin *et al.*, 1962), while white cells, with a lower cholesterol content (Rouser *et al.*, 1968) would be even less susceptible to saponin lysis. In addition, free parasites were apparently not washed thoroughly to remove host enzymes. A scanning electron micrograph showed little host membrane, but sections would be better indicators of contamination.

However, the parasite can use hypoxanthine. Van Dyke (1975) showed that the amount of radioactivity in the nucleic acids of parasitized red cells was higher after incubation with labelled hypoxanthine than with adenosine or inosine, although with free parasites, hypoxanthine and adenosine were incorporated to nearly the same extent. Adenine was more poorly incorporated by both free parasites and parasitized red cells.

Additional support for the suggestion that hypoxanthine is the purine most readily taken up by malaria parasites was provided by Büngener (1974a, b) who showed that treating infected mice with allopurinol increased the growth rate of *P.berghei*. Allopurinol also increased slightly the incorporation of radioactivity into the nucleic acids of free parasites when the labelled precursor was adenosine, adenine or hypoxanthine (Van Dyke, 1975). Allopurinol inhibits xanthine oxidase, the enzyme which degrades hypoxanthine to xanthine and thence to uric acid; it was assumed that this inhibition would raise the concentration of hypoxanthine in the red cell. However, preventing loss of purines from the pool via hypoxanthine may raise the levels of other purines as well as hypoxanthine.

C. Metabolism of Pyrimidines

Pyrimidines, which are incorporated into the nucleic acids of white cells (see Cline, 1965), are not used by rodent malaria parasites, as shown autoradiographically for thymidine (Büngener and Nielsen, 1967; Walter *et al.*, 1970) and for uridine (Büngener and Nielsen, 1967). Theakston *et al.* (1972) confirmed the lack of incorporation of thymidine, but found that *P.berghei* did incorporate uridine. The absence of radioactivity in nucleic acids extracted from *P.berghei* after incubation with labelled pyrimidine bases or nucleosides was shown by Van Dyke *et al.* (1970b) and Neame *et al.* (1974).

It has been suggested that pyrimidines are not incorporated into the nucleic acid of the parasite because they are unable to cross the red cell membrane (Gutteridge and Trigg, 1970; Oelshlegel and Brewer, 1975). However, free parasites are also unable to use exogenous thymidine (Walter *et al.*, 1970), and in fact, pyrimidines cross the membranes of human red cells (Lieu *et al.*, 1971; Oliver and Paterson, 1971), and thymidine can enter immature red cells of mice (Büngener and Nielsen, 1967). In addition, cytosine and thymidine enter not only the red cells of normal mice but also of those infected with *P.berghei* (Neame *et al.*, 1974).

Van Dyke *et al.* (1970b) felt that it was the membrane of the parasite, not of the erythrocyte, which was impermeable to pyrimidines, as radioactivity could be detected in free parasites of *P.berghei* after incubation with labelled adenosine but not with labelled thymidine. However, if both purines and pyrimidines entered the parasites but only purines were phosphorylated, the non-phosphorylated and therefore freely diffusible pyrimidines would be more easily lost during washing of the cells. As the rather lengthy washing procedures of Van Dyke *et al.* (1970b) were not checked for such losses, the suggestion that the parasite membrane is impermeable to pyrimidines is not completely proved. Indeed, *P.chabaudi* is unable to phosphorylate thymidine as it lacks the necessary kinase (Walter *et al.*, 1970). Whether the parasite lacks a salvage pathway for pyrimidines or whether its membrane is impermeable to them, the result would be the same, that is, the parasites would fail to incorporate exogenous pyrimidines into their nucleic acids.

If murine malaria parasites cannot use pyrimidines supplied by the host, they must have the necessary enzymes to synthesize their own.

Aspartate transcarbamylase was found to increase with increasing parasitaemia in whole blood of rats infected with *P.berghei* (Van Dyke *et al.*, 1970b). As the authors noted, this enzyme is found in reticulocytes, and it is also found in the particulate fraction of human white cells (Smith and Baker, 1959). If the same is true of rat white cells (which were apparently not removed), the host enzyme could become concentrated in free parasite preparations. Dihydroorotate dehydrogenase had a much higher activity in red cells infected with *P.berghei* or *P.vinckei*, and in free parasites, than in uninfected erythrocytes (Krooth *et al.*, 1969). Again, the presence of this enzyme in rabbit reticulocytes (Lotz and Smith, 1962) and in white cells (Smith and Baker, 1959) complicates the interpretation of these results, especially as dihydroorotate dehydrogenase was found to be associated with the particulate fraction of white cells.

The final step in the synthesis of thymidine (as the monophosphate) is the transfer of a methyl group from methylene tetrahydrofolate to deoxyuridine monophosphate. The enzyme which catalyses this reaction, thymidylate synthetase, was investigated in *P.chabaudi* by Walter *et al.* (1970). White cells were apparently not removed, but this enzyme cannot be detected in human leucocytes (Silber *et al.*, 1963) although it is present in mouse reticulocytes (Reid and Friedkin, 1973a). However, the activity measured in *P.chabaudi* was of parasite origin because its activity was higher in younger than in older intraerythrocytic stages, rising steeply due to new synthesis of enzyme, in the later part of the cycle, just before the first nuclear division (Walter and Königk, 1971a). Thymidylate synthetase has also been detected in *P.berghei*, and in this case the enzymes of parasites and host could be distinguished on the basis of molecular weight. The parasite enzyme had an apparent molecular weight of more than 100 000, while the molecular weight of the enzyme from mouse reticulocytes was only about 68 000 (Reid and Friedkin, 1973a).

In summary, the intraerythrocytic stages of rodent malaria parasites are unable to use pyrimidines supplied by the host, either because they do not cross the parasite membrane or because the parasite does not have a salvage pathway for them. Only one of the enzymes involved in the synthesis of pyrimidines by the parasite has been detected with certainty in spite of their important role in the metabolism of the parasite. In contrast to the pyrimidines, the parasite incorporates exogenous purines into its nucleic acids and several enzymes of the

purine salvage pathway have been detected in the parasite. The purine most readily taken up by the parasite may be hypoxanthine, which may also be the form in which purines are supplied to the red cell (Pritchard *et al.*, 1970).

VII. FOLATE METABOLISM

The inhibition of the growth of *P.berghei* caused by feeding the host on milk (Maegraith *et al.*, 1952) was later shown to be due to a lack of para-aminobenzoic acid (PABA) in the diet (Hawking, 1954). Sulphonamides, known antagonists of PABA in bacterial systems, also inhibit the growth of the parasite, and this inhibition is overcome more effectively by PABA than by folic acid (Thurston, 1954; Rollo, 1955). In addition, pyrimethamine, a potent antimalarial, inhibits the synthesis of folate cofactors in bacterial cells (Hitchings and Burchall, 1965). These observations suggest that *P.berghei*, unlike its mammalian host, must synthesize folate cofactors *de novo*.

The reactions involved in the synthesis of tetrahydrofolate are shown in Figure 2.

Ferone (1973) demonstrated that *P.berghei* was capable of carrying out all the reactions from 2-amino-4-hydroxy-6-methyl-7,8-dihydropteridine to dihydrofolate, by either pathway. The activities of hydroxymethyldihydropteridine pyrophosphate kinase (1) and of dihydropteroate synthetase (2) were located in two different proteins as they are in bacteria (Richey and Brown, 1969; Shiota *et al.*, 1969), rather than in the same one as in pea seedlings (Okinaka and Iwai, 1970a, b). The molecular weights of both parasite enzymes were in the range 200 000–250 000, much higher than those previously reported for bacterial enzymes (see Ferone, 1973), although no attempt was made to determine whether these were true molecular weights or associations of molecules. The enzymic synthesis of dihydrofolate by the plasmodial enzyme was inhibited by several sulphonamides, with 50% inhibition being caused by concentrations ranging from 10^{-5} to 10^{-3}M with different sulphonamides. In *P.chabaudi*, however, the two activities were found in two different enzymes, although the dihydropteroate synthetase of *P.chabaudi*, like that of *P.berghei*, had a high molecular weight (Walter and Königk, 1974b). The enzyme of *P.berghei* was also competitively inhibited by sulphanilamide and sulphaguanidine (Walter

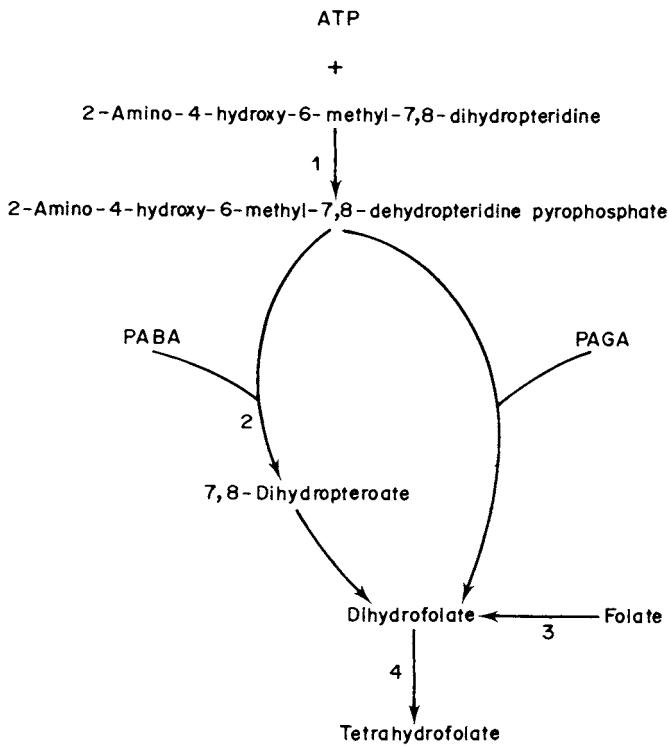

Figure 2. The synthesis of tetrahydrofolate; 1: hydroxymethyldihydropteridine pyrophosphate kinase, 2: dihydropteroate synthetase, 3: folate reductase, 4: dihydrofolate reductase, PAGA: para-aminobenzoylglutamic acid.

and Königk, 1971b), and by dapsone and sulphadiazine (McCullough and Maren, 1974).

The enzymes discussed above are clearly of parasite origin because mammals do not possess them. Mammals do, however, possess dihydrofolate reductase (4), but in this case confusion between enzymes of host and parasite can be avoided because of the great differences in their molecular weights. Dihydrofolate reductase from almost all mammalian and bacterial sources has a molecular weight of about 20 000, while that of *P.berghei* has a molecular weight about ten times higher, in the region of 190 000 (Ferone *et al.*, 1969). In some respects, such as the *Km* for dihydrofolate and stimulation of activity by salts and urea, the malarial enzyme resembled the mammalian one, but it differed markedly in its sensitivity to certain inhibitors. The concentration of

pyrimethamine needed for 50% inhibition of dihydrofolate reductase from mouse cells was about 10^{-6}M, while the plasmodial enzyme was 50% inhibited at a concentration of about 5×10^{-10}M.

The synthesis of dihydrofolate reductase by *P.chabaudi* was investigated by Walter and Königk (1971a). No attempt was made to separate host and parasite enzymes, but the enzyme was found to increase its specific activity during the cell cycle, the rise in activity occurring at the same time as the increase in thymidylate synthetase mentioned earlier, that is, just before the first nuclear division. Dihydrofolate reductase has also been detected cytochemically in *P.berghei* (Gerzeli and Polver, 1969).

Plasmodial synthesis of folate cofactors from dihydrofolate was shown by Ferone and Hitchings (1966). Extracts of free parasites were able to produce folinic acid (substances promoting the growth of *Pediococcus cerevisiae*, and presumably consisting of cofactor forms of tetrahydrofolate) from added dihydrofolate, but the very small production of dihydrofolate from folate (perhaps due to contaminating host material) suggests that the parasites lack a folate reductase (3). The production of folinic acid from dihydrofolate was greatly reduced by pyrimethamine at a concentration of $7{\cdot}5 \times 10^{-6}$M, although the authors point out that this is about 100 times the estimated serum concentration in humans after the minimum effective dose. In many bacteria, dihydrofolate reductase can also reduce folate (see Brown, 1970, for discussion) but this does not seem to be the case with the enzyme of *P.berghei*, whose lack of a folate reductase helps to explain why folate is less effective than PABA in overcoming parasite growth by sulphonamides. The level of folate cofactors in mouse blood was shown to increase during infection with *P.berghei*, although there was more change in the form of folate cofactors than in the total amount (Reid and Friedkin, 1973b).

The enzymes involved in the further metabolism of tetrahydrofolate have not been investigated in the rodent malaria parasites. Presumably, in common with *P.lophurae* (Platzer, 1970), they can convert tetrahydrofolate to 5,10-methylenetetrahydrofolate, the cofactor used by thymidylate synthetase for the production of thymidine monophosphate. Inhibition of dihydrofolate reductase would prevent the regeneration of this cofactor, and therefore would stop nucleic acid synthesis. Reduction of DNA synthesis by *P.berghei* after treatment with pyrimethamine has been observed (Schellenberg and Coatney, 1961).

The folate cofactors used in the synthesis of purines have not been

investigated, but it would be expected that such factors are not produced by murine malaria parasites.

VIII. DISCUSSION

This review reveals how little is known with certainty of the biochemistry of the murine malaria parasites. To a large extent, what is known is simply whether or not an enzyme can be detected in the parasite; only a few of the enzymes have been studied in detail, and virtually nothing is known of the control of any metabolic processes.

The intraerythrocytic stages of the parasites appear to rely on glycolysis for energy production, but the enzymes of the glycolytic sequence have been little studied, in spite of their importance to the parasite. It is possible that the parasite also uses some form of electron transport, but further investigation is required before this can be accepted. The pentose phosphate pathway, if present at all, is used to metabolize only a small proportion of the glucose. However, the parasitized cell utilizes so much glucose that even a small part of it would be sufficient to produce the pentose sugars needed for nucleic acid synthesis. Lacking glucose-6-phosphate dehydrogenase, the parasite could perhaps rely on the pentose phosphate pathway of the host cell, as suggested by Motulsky (1964), or it could perhaps produce ribose from fructose-6-phosphate by means of transketolase and transaldolase, as the absence of glucose-6-phosphate dehydrogenase does not necessarily imply the absence of the other enzymes of the pathway. For example, *Tetrahymena pyriformis* possesses several enzymes of the pentose phosphate pathway, including transketolase and transaldolase, although glucose-6-phosphate dehydrogenase cannot be detected (Eldan and Blum, 1975). Malaria parasites would presumably not need as much reduced NADP as most growing cells, as they are able to obtain many precursors ready formed from either the red cell or the plasma. In order to obtain these substances from the plasma, however, the parasite must change the permeability of the red cell, as suggested by Herman *et al.* (1966). Normal erythrocytes of mice and several other animals are surprisingly impermeable even to glucose (Ulrich, 1934), but the membrane of the parasitized cell is freely permeable to this sugar (Homewood and Neame, 1974; Neame and Homewood, 1975) and presumably to other molecules. The membrane of the red cell must

therefore be considerably altered by the parasite. Differences between the proteins of blood cells from normal and infected mice have been demonstrated (Weidekamm *et al.*, 1973) but it was not shown whether these differences were due to specific changes in the membranes of the red cells caused by the parasites, or were due to the presence of membranes of white cells, immature red cells produced as a response to anaemia, or even parasites themselves. If it could not change the permeability of the mouse erythrocyte to glucose, the intraerythrocytic parasite could not survive, but it is not known how the change is produced, nor whether similar changes are caused in the human erythrocyte which is already permeable to glucose.

As well as the enzymes producing folate cofactors, the enzymes involved in the synthesis of nucleic acids seem to provide possible points of chemotherapeutic attack, as some of them have been shown to differ from those of the host, sometimes having properties in common with those of both mammals and bacteria. The parasite's inability to use exogenous pyrimidines is well documented, but little is known of the enzymes involved in their synthesis. The parasite is able to use exogenous purines, although it is not certain that it is unable to synthesize them. As the red cells are used to transport purines from the liver to other tissues of the body (Pritchard *et al.*, 1970), a plentiful supply of these precursors will always be available to the intraerythrocytic parasite and, unfortunately, the enzymes of the plasmodial salvage pathway seem on the whole to be similar in their properties to those of mammals.

The biochemistry of the other stages of malaria parasites is even more difficult to study than that of the intraerythrocytic stages. One indication of a change in metabolism which takes place on transfer from the mammalian to the insect host is the appearance of enzymes of the citric acid cycle, suggesting a "metabolic switch" similar to that described in the African trypanosomes (Vickerman, 1965). The citric acid cycle may be necessary in the sporogonic stages to allow more efficient use of the available carbohydrate, or it may permit the parasite to use amino acids which are present in high concentration in insect haemolymph as a source of energy. The stages of the murine malaria parasites which are most easily investigated biochemically are the intraerythrocytic stages but, even here, the difficulties are great. Because of these difficulties, experiments on malaria parasites should be performed and interpreted with even more care than experiments on

other organisms. Many of the criticisms made in this review have been severe, in some cases perhaps over-severe, but the present state of confusion will continue until the biochemistry of malaria parasites is investigated in such a way that such criticisms can no longer be made.

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5. Genetics

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I. INTRODUCTION

Genetic studies are likely to yield much information concerning the basic biology of malaria parasites. It can be expected that some features of the life cycle which are only partially understood, such as fertilization and meiosis, will be clarified, and many aspects previously totally obscure will be illuminated, e.g. the ability of clones of haploid

organisms to produce both male and female gametocytes, and the relative frequencies of self- and cross-fertilization. In a more general way, confirmation could be obtained that malaria parasites undergo a regular eukaryotic life cycle, with typical regular sexual processes, as distinct from the more irregular processes of genetic exchange in prokaryotes.

Genetic analysis also makes possible a firm demarcation of the various taxonomic categories (species, subspecies, etc.) which are often difficult to establish in micro-organisms showing relatively little morphological variation. Use of precise genetic markers is particularly important, as will be demonstrated from results with enzyme variation. In addition, the ability of groups of organisms to undergo hybridization and genetic exchange is an important indication of taxonomic identity. Identification of strains in the laboratory, where the only guide is usually a label on an animal cage or a specimen in a deep-freeze compartment, is also greatly aided by the use of precise genetic markers.

Study of the genetic composition of isolates and wild populations has both practical and theoretical value. It can be shown, for example, that a given isolate of a parasite derived from a single blood sample may comprise a mixture of two or more genotypes, the different behaviour of which may give confusing results in laboratory work. Knowledge of the genetic variation within wild populations of parasites, due to various processes such as mutation, recombination, selection, migration, etc. should help us to understand the capacity of these organisms to avoid various natural and artificial obstacles (drugs, antibodies, etc.).

Little genetic work on any malaria parasites was carried out before the rodent species had been established in laboratory animals, and details of the life cycle had been worked out. The techniques commonly used in genetic work with free-living organisms are difficult to apply to parasites with a complex life cycle involving two hosts. At the moment, work with human malaria parasites is obviously impracticable since, until recently, no *in vitro* culture system was available. Primate species are also unsuitable due to the large numbers of costly animals which are required for genetic studies. Until the work to be described here was started, few suitable genetic characteristics were known in any *Plasmodium* species.

The avian parasite *P.gallinaceum* is relatively easy to maintain in chicks in the laboratory. However, little natural variation is known in this species, since until recently only a single wild isolate obtained many

years ago was available (Brumpt, 1935). After a long series of laboratory passages, Greenberg and Trembley (1954a, b) obtained two variants which differed in their resistance to pyrimethamine and their ability to produce erythrocytic and secondary exoerythrocytic forms. By infecting chicks with a mixture of the two lines, transmitting the mixture through mosquitoes and re-infecting chicks from the sporozoites thus produced, these workers obtained indications of recombination between the characters that distinguished the parent lines. This interpretation was, however, open to question since one of the distinguishing characters (production of secondary exoerythrocytic forms) was known to be unstable.

Rodent malaria parasites have the advantage for genetic work of being available as numerous wild isolates which are likely to be genetically diverse. Moreover, it is relatively simple to maintain large numbers of mice as hosts for the numerous progeny clones required. Finally, details of the life cycle are sufficiently well known, and all stages of the life cycle can be obtained in the laboratory.

The plan of the work described here was based on the idea of studying first the enzyme characteristics, whereby the main genetic features of the organism could be established. Subsequently the studies were extended to more variable characteristics, such as drug resistance, antigenic variation and virulence, which are of considerable importance in the study of human malaria. Investigations on the cytogenetics of the malaria parasite are discussed in Chapter 3.

II. MATERIAL, DEFINITIONS AND METHODS

A. Material

Numerous isolates have been made of the four species *P.berghei*, *P.yoelii*, *P.chabaudi* and *P.vinckei*, all obtained from wild rodents in Central Africa. Of these species, three are subdivided into subspecies, as indicated in Table I. Studies on enzyme variation in all species have been carried out; other work concerning drug resistance, virulence and antigenic variation has been restricted to *P.yoelii* and *P.chabaudi*. The last mentioned is particularly suitable for genetic work in view of its extensive enzyme polymorphism.

Table I

Isolates of rodent malaria species and subspecies available in the laboratory; the isozyme composition and details of origin of each isolate are given

Plasmodium species and subspecies	Isolate	Origin				Enzyme composition			
		Host species	Host specimen	Region of capture	Date of capture	GPI	6PGD	LDH	GDH
P.berghei	K173	*G.surdaster*	K173	Katanga	1948	3	1	1	3
	SP11	*A.d.millecampsi*	—	Katanga	1961	3	1	1	3
	ANKA	*A.d.millecampsi*	—	Katanga	1966	3	1	1	3
	LUKA	*A.d.millecampsi*	—	Katanga	1966	3	1	1	3
	NK65	*A.d.millecampsi*	—	Katanga	1964	3	1	1	3
P.y.yoelii	17 X	*T.rutilans*	17 X	Central African Republic	1965	1	4	1	4
	32 X	*T.rutilans*	32 X	Central African Republic	1965	1	4	1	4
	33 X	*T.rutilans*	33 X	Central African Republic	1965	2	4	1	4
	55 X	*T.rutilans*	55 X	Central African Republic	1965	1	4	1	4
	86 X	*T.rutilans*	86 X	Central African Republic	1965	1	4	1	4
	146 X	*T.rutilans*	146 X	Central African Republic	1965	1	4	1	4
	5AD	*T.rutilans*	AD	Central African Republic	1969	1,2	4	1	4
	3AE	*T.rutilans*	AE	Central African Republic	1969	1	4	1	4

	3AF	*T.rutilans*	AF	Central African Republic	1969	1	4	1	4
	1AK	*T.rutilans*	AK	Central African Republic	1969	1	4	1	4
	1AR	*T.rutilans*	AR	Central African Republic	1969	2	4	1	4
	2AZ	*T.rutilans*	AZ	Central African Republic	1969	1	4	1	4
	14BE	*T.rutilans*	BE	Central African Republic	1969	1	4	1	4
	1BF	*T.rutilans*	BF	Central African Republic	1969	2	4	1	4
	1BG	*T.rutilans*	BG	Central African Republic	1969	1	4	1	4
	2BR	*T.rutilans*	BR	Central African Republic	1970	2	4	1	4
	2CF	*T.rutilans*	CF	Central African Republic	1970	2	4	1	4
	2CL	*T.rutilans*	CL	Central African Republic	1970	1	4	1	4
	2CN	*T.rutilans*	CN	Central African Republic	1970	1,10	4	1	4
	5CP	*T.rutilans*	CP	Central African Republic	1970	1	4	1	4
	2CU	*T.rutilans*	CU	Central African Republic	1970	1	4	1	4
	2CX	*T.rutilans*	CX	Central African Republic	1970	1	4	1	4
P.y.killicki	193L	*T.rutilans*	193L	Brazzaville	1966	1	4	1	1
	194ZZ	*T.rutilans*	194ZZ	Brazzaville	1968	1	4	1	1
P.y.nigeriensis	N67	*T.rutilans*	N67	Nigeria	1967	2	4	1	2

Table I

Plasmodium species and subspecies	Isolate	Origin				Enzyme composition			
		Host species	Host specimen	Region of capture	Date of capture	GPI	6PGD	LDH	GDH
P.c.chabaudi	54 X	*T.rutilans*	54 X	Central African Republic	1965	4	3	3	5
	864VD	*T.rutilans*	864VD	Central African Republic	1970	4	3	4	5
	3AC	*T.rutilans*	AC	Central African Republic	1969	4	2,3	2,4	5
	2AD	*T.rutilans*	AD	Central African Republic	1969	4	2	3,5	5
	16AF	*T.rutilans*	AF	Central African Republic	1969	4	2	5	5
	AJ	*T.rutilans*	AJ	Central African Republic	1969	4	3	2	5
	1AL	*T.rutilans*	AL	Central African Republic	1969	4	2	2	5
	1AM	*T.rutilans*	AM	Central African Republic	1969	4	2	3	5
	1AQ	*T.rutilans*	AQ	Central African Republic	1969	4	3	2	5
	1AS	*T.rutilans*	AS	Central African Republic	1969	4	2	3	5
	4AT	*T.rutilans*	AT	Central African Republic	1969	4	3	2,3	5
	1BC	*T.rutilans*	BC	Central African Republic	1969	4	3	4	NT
	40BE	*T.rutilans*	BE	Central African Republic	1969	4	3	2	5
	BJ	*T.rutilans*	BJ	Central African Republic	1969	4	2	4	5

	1BK	*T.rutilans*	BK	Central African Republic	1969	4	7	2	5
	1BS	*T.rutilans*	BS	Central African Republic	1970	4	2	5	5
	2CB	*T.rutilans*	CB	Central African Republic	1970	4	3	4	5
	2CE	*T.rutilans*	CE	Central African Republic	1970	4	3	3,4	5
	2CP	*T.rutilans*	CP	Central African Republic	1970	4	2	4,5	5
	2CQ	*T.rutilans*	CQ	Central African Republic	1970	4	3	5	5
	4CR	*T.rutilans*	CR	Central African Republic	1970	4	2	3	5
	2CW	*T.rutilans*	CW	Central African Republic	1970	4	3	4	5
P.c.adami	556KA	*T.rutilans*	556KA	Brazzaville	1970	8	2	8	5
	408XZ	*T.rutilans*	408XZ	Brazzaville	1972	8	2	10	5
P.v.vinckei	v–52	*A.d.millecampsi*	—	Katanga	1952	7	6	6	6
	v–67	*A.d.millecampsi*	—	Katanga	1967	7	6	6	6
P.v.petteri	1BS	*T.rutilans*	BS	Central African Republic	1970	9	5	7	6
	2BZ	*T.rutilans*	BZ	Central African Republic	1970	9	5	7	6
	2CR	*T.rutilans*	CR	Central African Republic	1970	5,9	5	7	6
	2CE	*T.rutilans*	CE	Central African Republic	1970	5	5	7	NT
P.v.lentum	170L	*T.rutilans*	170L	Brazzaville	1966	6	5	7	6
	483L	*T.rutilans*	483L	Brazzaville	1966	6	5	7	6
	194ZZ	*T.rutilans*	194ZZ	Brazzaville	1968	6	5	7	6
	408XZ	*T.rutilans*	408XZ	Brazzaville	1972	11	5	9	6
P.v.brucechwatti	1–69	*T.rutilans*	1–69	Nigeria	1969	6	6	9	6
	N48	*T.rutilans*	N48	Nigeria	1967	6	6	9	6

G.surdaster, Grammomys surdaster; *T.rutilans, Thamnomys rutilans*; *A.d.millecampsi, Anopheles dureni millecampsi*; NT, enzyme not tested.

B. Definitions

Before proceeding to describe the genetics of these organisms, it is essential to define the terms isolate, line and clone, which will be used frequently.

1. Isolate

This refers to a sample of parasites collected on a single occasion from a wild rodent or mosquito and preserved either by passaging through laboratory animals or kept as deep-frozen material. An isolate is not necessarily genetically homogeneous and may even contain representatives of more than one species. For example, "isolate 2CE" (see Table I) refers to parasites from a wild thicket rat denoted 2CE, and comprises organisms of the two species *P.c.chabaudi* and *P.v.petteri*, which are referred to as "*P.c.chabaudi* isolate 2CE" and "*P.v.petteri* isolate 2CE".

2. Line

This refers to a collection of parasites which have undergone a particular laboratory passage. By strict definition every laboratory manipulation of parasites creates a new line, but usually parasites are described as belonging to a line only after a special treatment, such as selection for drug resistance. All the parasites in a line have certain characteristics in common but they need not be genetically identical.

3. Clone

A clone is a group of genetically identical organisms derived from a single cell by asexual reproduction. Cloning is a procedure of particular importance in genetic work with unicellular organisms, and should always be carried out, if feasible, prior to determining the characteristics of a new isolate, before using an isolate in genetic experiments, and again before classifying the progeny.

C. Methods

1. Cloning

Cloning of blood forms by micro-manipulation has been achieved with avian malaria parasites by Demidowa (1934), Coulston and Manwell

(1941), Downs (1947) and Bishop (1958); and with rodent parasites by Diggens (1970). However, in genetic work a dilution method has to be used because of the large numbers of clones required in a short time.

As source of material, a donor mouse is chosen in which the parasitaemia is rising and in which infected cells contain only single parasites. The rare occurrence of multiple infections in red cells is neglected in subsequent calculations. The blood is diluted with cold serum Ringer (50% calf serum, 50% mammalian Ringer) to yield a concentration of one or less parasitized cells 0·1 ml^{-1} of diluent. 0·1 ml aliquots are then injected intravenously into mice, which are examined for the presence of parasites after seven days for *P.yoelii* and after ten days for *P.chabaudi*. From the proportion of mice which become infected, one can estimate the numbers established from 1, 2, 3, etc. parasites by means of the Poisson distribution. Thus if, for example, each inoculum contains an average of one parasite, it is expected that approximately 63% of mice injected will become infected, and that approximately 58% of these infections will be single clones. If each inoculum contains only 0·1 parasite, only 10% of mice injected will become infected, but 96% of these will contain single clones. These calculations are subject to various errors but are adequate for most purposes; they are based on the assumption that a single parasite is capable of establishing an infection, as was demonstrated in a dilution assay by Diggens (1970) and confirmed in our own laboratory.

2. *Hybridization and analysis of progeny*

The ideal method of hybridization of malaria parasites would be to fertilize isolated macrogametes of one line with microgametes of another. The zygotes would then be permitted to develop in mosquitoes, producing oocysts, and sporozoites allowed to develop from single oocysts. Single sporozoites would then be introduced into individual mice. This procedure is at present impracticable. Although there are a few reports of infections from sporozoites derived from single oocysts (Trembley *et al.*, 1951, in *P.gallinaceum*; Walliker, 1972, in *P.y.nigeriensis*), the numbers of successful infections produced in this way have been insufficient for genetic work.

In practice, the following procedure, similar in essentials to that used by Greenberg and Trembley (1954a) for *P.gallinaceum*, has been used for rodent plasmodia. The method is illustrated in Figure 1. Two

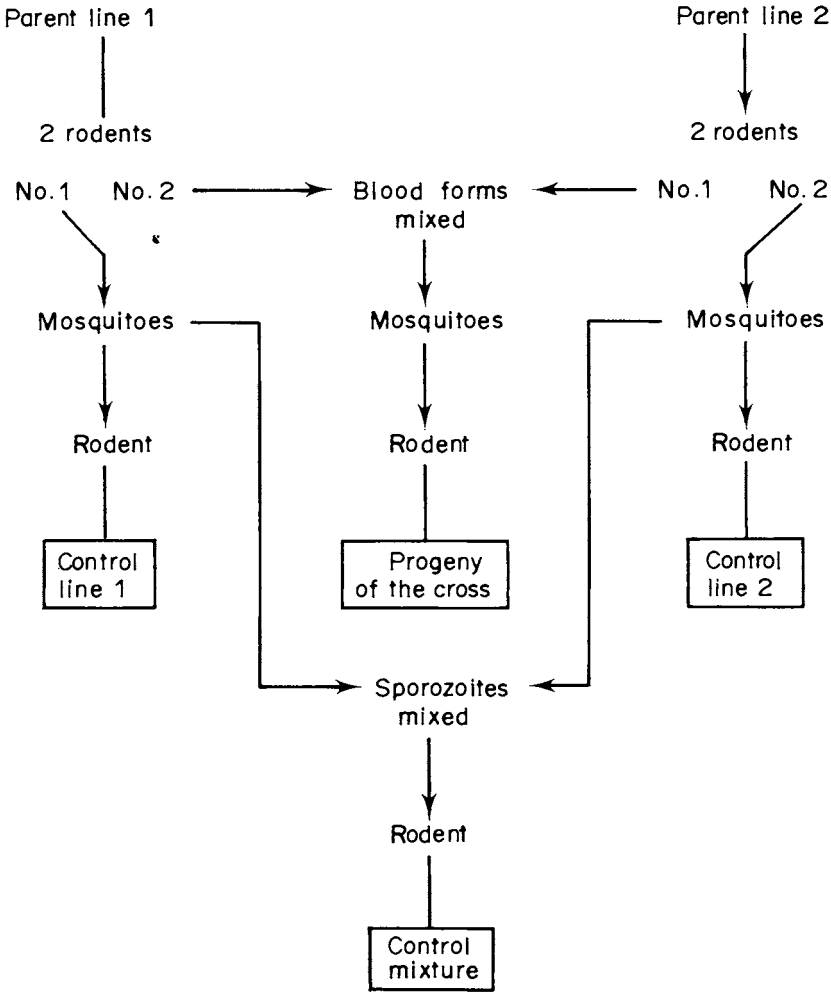

Figure 1. Procedure used in making cross-preparing and control samples.

parasite lines are taken, differing in respect of at least two pairs of hereditary characters. Gametocytes of the two lines are mixed in equal proportions and the mixture injected intravenously into a rodent or introduced into the chamber of a mosquito membrane feeding apparatus. Mosquitoes are allowed to feed on the mixture. Gametes from both lines should then be formed in the mosquitoes and, assuming self- and cross-fertilization occurs equally frequently, 50% of the resulting zygotes will be hybrids and the remaining 50% will consist of equal proportions of the two parental lines. From the hybrid zygotes, recombination of genetic factors will take place at meiosis (assumed to take place during early divisions of the zygote—see Chapter 3).

The zygotes are allowed to develop into oocysts, and the latter to produce sporozoites which are used to infect new rodents. The blood forms which develop are cloned and classified with regard to the characters distinguishing the parental lines. Presence of recombinants is a certain indication that cross-fertilization has occurred, while non-recombinants may result from either self- or cross-fertilization.

Control infections of each parent line are transmitted through mosquitoes and examined in the same way as the parasite mixture. An additional control consists of mixing sporozoites of the two parent lines in equal proportions and establishing new infections in mice (see Figure 1). The absence of non-parental forms among these parasites shows that recombination occurs only after cross-fertilization of gametes, and not by a gene transfer mechanism involving other stages of the parasite life cycle. A mechanism of this kind, termed "synpholia", was postulated by Yoeli *et al.* (1969), but no evidence for such a process has been found in subsequent work (see p. 231).

3. *Types of variant*

Two main classes of variant have been used in genetic work: those occurring in natural populations which are polymorphic for such characters as enzyme variants and antigens and those produced by induction and/or selection of variations in the laboratory, such as drug resistance and virulence. The methods used to study each character are described in the appropriate sections.

III. ENZYME VARIATION

A. Introduction

Enzyme variation in rodent malaria parasites can be readily studied by starch gel electrophoresis of extracts of partially purified parasite samples. This technique involves the migration of the extracted proteins through a slab of starch gel, caused by the application of an electric field across the gel. After electrophoresis for a set time, the position to which a particular enzyme has migrated is identified by applying to the gel a stain specific for the enzyme being studied. This results in a band of stain being deposited at the position of the enzyme.

The rate of migration of a protein, and thus its final position in the gel, depends primarily on its charge which is determined mainly by the charged groups of its constituent amino acids. As there is a direct relationship between genes and polypeptides, variations in the mobility of enzymes revealed by this method nearly always reflect gene differences. The enzyme characteristics of a genetically homogeneous line, as observed by electrophoresis, are exceedingly stable and change only if a mutation occurs; environmental factors are relatively unimportant.

In protozoan species, there is usually little morphological variation, and hence the existence of stable enzyme variants is exceptionally useful for the characterization of species and subspecies. In *Paramecium aurelia*, Tait (1970 and unpublished) succeeded in identifying each of the 14 syngens (sibling species) by the mobility of four enzymes on starch gel electrophoresis. The only way of distinguishing these species previously had been by making use of laborious mating tests. With many other protozoa, such mating tests are impracticable or, of course, impossible where mating does not occur at all.

For these reasons, studies on enzyme variation, especially by gel electrophoresis, are of central importance in differentiating genetically distinct micro-organisms, and should be placed foremost in genetic research on protozoa.

B. Methods

The blood forms of malaria parasites are the most useful stages for studies of enzyme variation. A sample of infected blood (0·5–5 ml,

depending on the parasitaemia and the activity of the enzyme under investigation) is centrifuged, and the parasites released from their host cells by saponin lysis. This separates the parasites from most of the host material; some of the latter remains, however, so it is necessary to run control samples of uninfected blood to identify host enzyme bands. Usually, host and parasite enzymes are readily distinguishable (see Figure 2).

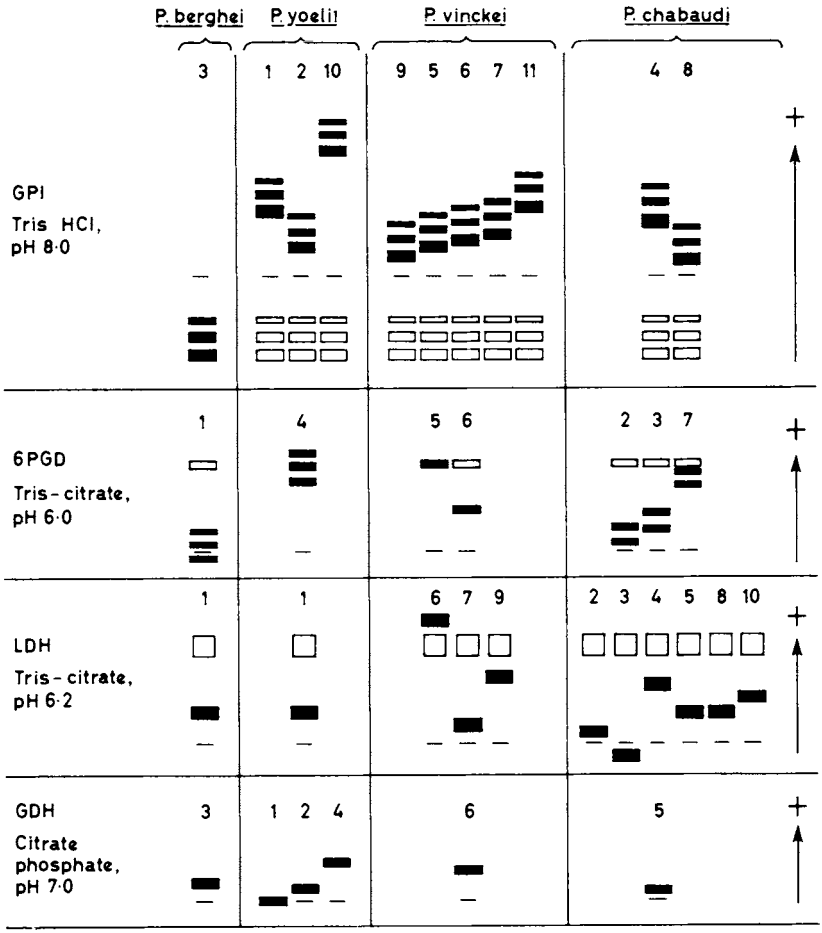

Figure 2. Electrophoretic forms of glucose phosphate isomerase (GPI), 6-phosphogluconate dehydrogenase (6PGD), lactate dehydrogenase (LDH) and NADP-dependent glutamate dehydrogenase (GDH), using selected systems of electrophoresis (modified from Carter, 1973 and unpublished). Parasite bands are shown in black; those of the rodent host (mouse) in white. Not all enzyme variants can be distinguished by the electrophoresis systems illustrated here.

The following four enzymes have been most studied:

1. glucose phosphate isomerase (GPI);
2. 6-phosphogluconate dehydrogenase (6PGD);
3. lactate dehydrogenase (LDH);
4. NADP-dependent glutamate dehydrogenase (GDH).

Some other enzymes (e.g. malate dehydrogenase, adenylate kinase, hexokinase) have also been studied, but less than the others (Carter, 1973).

Several electrophoretic forms of each of the above enzymes have been identified from the rodent malaria parasites studied, each variant being denoted by a number, e.g. GPI-1, GPI-2, etc. The actual numbers signify only the order of discovery.

C. Survey of Enzyme Variants in Four Murine Malaria Parasites

Table I summarizes the data on all variants of four enzymes (GPI, 6PGD, LDH, GDH) in the four rodent malaria species *P.berghei*, *P.yoelii*, *P.chabaudi* and *P.vinckei*. Each species is distinguishable by reference to any of the enzymes except LDH, which appears to be alike in *P.berghei* and *P.yoelii*. However, it is not excluded that further work, using different conditions for electrophoresis, might separate these two also. All the subspecies can readily be distinguished by one or more variants. Finally, different isolates of a given subspecies show numerous variants of one or more enzymes. Thus, at each level of taxonomic diversity there is enzyme variation, which is more pronounced at the species than at the lower levels. It should be noted also that the variation between different isolates of a subspecies is quite distinguishable from that between subspecies or species. For example, *P.c.chabaudi* isolates may contain one of four LDH variants (LDH-2, -3, -4, -5), but none of these forms occurs in any other subspecies, even though further variants (LDH-1, -6, -7, -8, -9, -10) are found.

Although occasionally a given isolate may produce two bands corresponding to a particular enzyme (e.g. isolate 5AD of *P.y.yoelii* shows GPI-1 and GPI-2), this is due to the presence of a mixture of two parasite clones in a single host, as can be shown by dilution experiments by which the two clones can be separated. Only a single variant of a given enzyme is produced by a genetically homogeneous parasite line.

D. Genetic Analysis of Enzyme Variation

It is to be expected that all the variation displayed in Table I is caused by the presence of different genes in the different isolates. This statement is based on much work with other organisms and on general theoretical considerations concerning gene expression. However, we can prove the point directly only in situations where hybridization and Mendelian analysis is feasible. So far this has been done at the subspecific and lower levels in the case of malaria parasites.

As an example, we give the effects of crossing two lines of *P.c.chabaudi*, one (411AS) containing 6PGD-2 and LDH-3, the other (96AJ) 6PGD-3 and LDH-2 (Rosario, 1976). The results are shown in Table II. As

Table II

Recombination between forms of 6PGD and LDH following cross between *P.c.chabaudi* lines 411AS and 96AJ

Parents	Characteristics	
Line 411AS	6PGD-2, LDH-3	
Line 96AJ	6PGD-3, LDH-2	
Progeny		Number of clones isolated
Parental types	6PGD-2, LDH-3	43
	6PGD-3, LDH-2	12
Recombinant types	6PGD-2, LDH-2	9
	6PGD-3, LDH-3	6

From Rosario (1976).

expected, there are four enzyme classes among the progeny clones, two like the original parent classes and two recombinants. Out of 70 clones, there are 15 recombinants and 55 non-recombinants. Assuming equal proportions of cross- and self-fertilization, one would expect 35 of the clones to be derived from hybrid zygotes. With independent assortment of the genes controlling the two enzymes, 50% of the clones derived from hybrid zygotes (i.e. 17·5) should give rise to recombinant clones. The actual number obtained was 15. Clearly these results do not deviate from what would be expected, even though the relative numbers of the two parental types are very unequal. The reason for this is not clear, but may be connected with other characters of the parental lines.

Numerous results of a similar nature have been obtained, showing segregation and recombination involving enzyme forms with various other genetic characteristics (Walliker *et al.*, 1973, 1975).

It is noteworthy that these results show that the blood forms of the parasites are haploid. This is clear from the finding that individual clones produced from crosses between enzymically distinct lines show but a single band on a gel after staining for a particular enzyme. If the blood forms were diploid, band patterns characteristic of heterozygotes would be found: either there would be simultaneous presence of the two parental bands or more complex patterns where the enzyme molecules consist of several polypeptides. The exact stage at which meiosis takes place cannot be specified from genetic results at present. All that can be concluded is that recombination (and therefore meiosis) occurs between zygote formation in the mosquito and the emergence of parasites into the blood of the vertebrate host. Further work involving studies of the progeny of single oocysts should clarify this question (see Chapter 3).

E. Genetic Structure of Wild Populations

Populations of malaria parasites may be considered at various levels of complexity: within single vertebrate or insect hosts; within a geographical region occupied by many hosts or finally within a larger area of country comprising a number of separate regions. A limited amount of data is now available on enzyme variation in populations at these different levels.

With restricted parasite populations within individual rodents, there is evidence for the presence of more than one species or subspecies, and more than one variant of a given subspecies. Thus single individuals of thicket rats (*T.rutilans*) caught in the Central African Republic have been found to contain mixtures of *P.y.yoelii*, *P.c.chabaudi* and *P.v.petteri* (Carter and Walliker, 1975). At different times (ranging over months or years) there are fluctuations in the frequencies of different species, but it is clear that several species can co-exist in a given animal for a protracted time under laboratory conditions, without additional infection (see Chapter 2). Moreover, within isolates of a given subspecies, more than one enzyme variant may occur (see Table I: isolates 5AD, 2CN of *P.y.yoelii*, isolates 3AC, 2AD, 4AT, 2CE, 2CP of *P.c.chabaudi*, isolate 2CR of *P.v.petteri*). This implies that infection of a

rodent by two or more clones of parasites belonging to the same subspecies may take place, either simultaneously from a mosquito containing a mixed population or on different occasions from different mosquitoes. Thus isolates are not necessarily pure clones.

The breeding behaviour of a population of organisms can be assessed to some extent from the occurrence and frequency of different combinations of genes in different wild individuals. This is particularly clear from our data on *P.c.chabaudi* (see Table I). Three forms of the enzyme 6PGD occur and four of LDH. Theoretically, with random mating and equal frequencies of the various alleles, twelve recombinant types should occur with equal frequencies. In practice, out of the rather small number of twenty isolates from different rodents, all isolated in the Central African Republic, no fewer than nine combinations were found. Only three combinations involving 6PGD-7 were lacking, and this is not surprising in view of the rarity of this form. These results imply an extensive degree of random mating (panmixia) in the parasite population of *P.c.chabaudi* studied.

By contrast, the four species *P.berghei*, *P.yoelii*, *P.chabaudi* and *P.vinckei* show no overlap in their enzyme variants. We consider that no gene exchange between these species is taking place. The subspecies also appear to be reproductively isolated since they are all distinguishable in regard to at least one enzyme, although in the laboratory it has been possible to cross-fertilize the two subspecies *P.y.yoelii* and *P.y.nigeriensis*. Recombination between enzyme, drug resistance and strain-specific immunity markers was detected (Oxbrow, 1973; Morgan, 1974). The conditions for this experiment, however, were artificial, and it is unlikely that such crossing takes place in nature.

IV. DRUG RESISTANCE

A. Introduction

Resistance to drugs is known in general to arise by a number of mechanisms, such as by spontaneous mutation of nuclear genes and subsequent selection of mutants, by mutation of mitochondrial genes, by transfer of plasmids (resistance transfer factors) in bacteria and finally by various epigenetic transformations or physiological adaptations. Malaria parasites develop drug resistant forms which may have

serious clinical implications, e.g. the spread of chloroquine-resistant forms of *P.falciparum* in South-East Asia and South America. Knowledge of the genetic basis of drug resistance is important in connection with drug-treatment procedures, and especially with attempts to restrict the development of new drug-resistant strains. By the genetic methods we have developed it is now possible to analyse the mechanisms controlling the appearance of drug-resistant parasites in rodent malaria species, and it is hoped that the findings can be applied also to the problem of malaria of man.

Our genetic work on drug resistance has been largely restricted to studies with pyrimethamine and chloroquine; a little work has also been done with sulphadiazine.

B. Genetics of Pyrimethamine Resistance

1. Origin

As discussed elsewhere (Chapter 8), pyrimethamine, an inhibitor of dihydrofolate reductase, is an excellent agent for eliminating at least the blood stages of malaria parasites. Most strains of rodent malaria parasites are eliminated by a dose of 1 mg kg^{-1} given intraperitoneally for four days (Peters, 1970). The biochemical action of the drug is discussed in Chapter 4.

Parasites which are able to grow in the presence of pyrimethamine can readily be obtained in the laboratory. Two methods have been used: a single-step method, using a single course of treatment with a high dose (e.g. 50 mg kg^{-1} for four days) and continuous drug pressure, starting with a low concentration and gradually increasing the dose over a number of blood passages. The first method has been used to obtain pyrimethamine resistance in *P.yoelii* by Diggens (1970), Walliker *et al.* (1973) and Morgan (1974) and in *P.chabaudi* by Walliker *et al.* (1975). The second method has been used for *P.berghei* by Rollo (1952), Thurston (1953), Rabinovich (1965) and Jacobs (1965), and for *P.vinckei* by Yoeli *et al.* (1969).

The resistant forms obtained by such methods are remarkable for their stability in the absence of drug pressure. For example, a resistant clone of *P.yoelii* obtained by Morgan (1974) was unaltered in its pyrimethamine response after 55 blood passages, 18 mosquito transmissions and five months' storage in liquid nitrogen. Other workers have

obtained similar results (e.g. Greenberg and Bond, 1954, with *P.gallinaceum*; Diggens, 1970, with *P.berghei*). Morgan (1974) also showed that resistant forms of *P.yoelii* could be obtained by using clones of sensitive parasites as starting material, thus excluding the possibility that a few resistant organisms were initially present and were merely selected by the drug treatment. These results imply that the change from sensitivity to resistance is probably truly genetic, and that reverse mutation is rare. The results of selection experiments by Bishop (1962) and Morgan (1974) suggest that pyrimethamine-resistant mutants arise at a low frequency (probably less than one in 10^9 parasites), although because of the nature of *in vivo* selection tests it is difficult to estimate mutation rates in malaria parasites with accuracy.

2. *Inheritance*

Hybrids have been made between pyrimethamine-resistant and sensitive lines marked with various other genetic factors, and recombination demonstrated, indicating Mendelian behaviour of the resistance character. Some data showing this are given in Table III.

The ratios of sensitive to resistant clones in the progeny vary in different experiments. This may be connected with the selective advantage or disadvantage of these and other characters which may be present in the parent lines, with the length of time between the cross and cloning of the progeny or with other (e.g. nutritional) factors. This matter requires further study.

This work has also shown that recombination between pyrimethamine resistance and the other markers takes place only after cross-fertilization of gametes. No recombinants are found when simple mixtures of sporozoites or of blood forms of the two parent lines are examined (Walliker *et al.*, 1971, 1973, 1976). In earlier work, Yoeli *et al.* (1969) claimed that genetic exchange involving resistance to pyrimethamine could take place between trophozoites of two parasite lines during their simultaneous development in the blood of the same host. A pyrimethamine-resistant *P.vinckei* line which was not infective to hamsters and a drug-sensitive *P.berghei* line, infective to hamsters, were first mixed together in mice, before being injected into pyrimethamine-treated hamsters. From some of these animals, pyrimethamine-resistant *P.berghei* was recovered. The explanation given by Yoeli *et al.* for this result was that transfer of resistance from the *P.vinckei* to the

P.berghei line had occurred in the mice, probably between trophozoites of the two species when simultaneous invasion of the same erythrocyte had taken place. No confirmatory evidence for this process ("syn-pholia") has been reported, either in the work of Walliker *et al.* quoted above, or in that of Schoenfeld *et al.* (1974), although no attempts have been made to repeat the exact procedures used by Yoeli and his co-workers. The most likely alternative explanation is that a mutation conferring resistance to pyrimethamine had occurred in the parent *P.berghei* line during its passage with *P.vinckei* (Diggens *et al.*, 1970).

Table III

Recombination between pyrimethamine resistance and enzyme markers in three crosses between *P.y.yoelii* lines and between *P.c.chabaudi* lines

P.y.yoelii	(Walliker *et al.*, 1973)	
Parents	Characteristics	
Line A	Resistant, GPI-1	
Line C	Sensitive, GPI-2	
Progeny		Number of clones isolated
Parental types	Resistant, GPI-1	21
	Sensitive, GPI-2	30
Recombinant types	Resistant, GPI-2	13
	Sensitive, GPI-1	7
P.c.chabaudi	(Walliker *et al.*, 1975)	
Parents	Characteristics	
Line 47AS	Resistant, 6PGD-2	
Line 10AJ	Sensitive, 6PGD-3	
Progeny		Number of clones isolated
Parental types	Resistant, 6PGD-2	22
	Sensitive, 6PGD-3	14
Recombinant types	Resistant, 6PGD-3	6
	Sensitive, 6PGD-2	2
P.c.chabaudi	(Rosario, 1976)	
Parents	Characteristics	
Line 411AS	Resistant, 6PGD-2	
Line 96AJ	Sensitive, 6PGD-3	
Progeny		Number of clones isolated
Parental types	Resistant, 6PGD-2	4
	Sensitive, 6PGD-3	46
Recombinant types	Resistant, 6PGD-3	3
	Sensitive, 6PGD-2	17

3. Characteristics of pyrimethamine-resistant mutants

The categories "resistant" and "sensitive" in progeny of crosses are quite distinct, and no clear indications of different degrees of resistance among the resistant lines have yet been found. It is probable, however, that the various independently obtained pyrimethamine-resistant mutants display some variation in their tolerance to the drug, and also in their patterns of cross-resistance to other drugs. Numerous workers have shown that resistance to pyrimethamine is usually associated with cross-resistance to other antifolates such as proguanil and cycloguanil (e.g. Bishop, 1962; Thompson and Bayles, 1968) but it is not yet known whether separate genetic factors are involved.

A few genetic studies have been carried out on the relationship between pyrimethamine resistance and resistance to the sulphonamide drug sulphadiazine. Parasites which have developed resistance to pyrimethamine in the laboratory usually develop an increased requirement for para-aminobenzoic acid (PABA) and thus show an increased sensitivity to sulphadiazine (which competes with PABA). Parasites made resistant to sulphadiazine, however, frequently show an increased tolerance to pyrimethamine (e.g. Thurston, 1953). MacLeod (1977) investigated a number of *P.chabaudi* lines resistant to these drugs and concluded that at least two genetic loci were involved. This was shown in a cross between a line resistant to both pyrimethamine and sulphadiazine and a line sensitive to both drugs. Recombinant forms showing resistance to each drug separately were obtained among the progeny (Table IV). The characteristics of each recombinant were

Table IV

Analysis of 29 clones derived from cross between *P.c.chabaudi* lines differing in pyrimethamine (pyr) and sulphadiazine (sulpha) response

Parents	Characteristics	
Line 340AS	Pyr-res, sulpha-res	
Line 10AJ	Pyr-sens, sulpha-sens	
Progeny		Number of clones isolated
Parental types	Pyr-res, sulpha-res	13
	Pyr-sens, sulpha-sens	7
Recombinant types	Pyr-res, sulpha-sens	2
	Pyr-sens, sulpha-res	7

Pyr, pyrimethamine; sulpha, sulphadiazine; res, resistant; sens, sensitive. From McLeod (1977).

similar to those of mutants obtained independently by treatment of sensitive parasites with each drug. It appeared that mutation at one locus could produce a 20-fold increase in resistance to pyrimethamine accompanied by a 27-fold increase in sensitivity to sulphadiazine, and mutation at a second locus could produce a five-fold increase in resistance to sulphadiazine associated with a slight (three-fold) increase in resistance to pyrimethamine.

In further work, MacLeod found evidence for a third locus, conferring resistance to sulphadiazine with little or no cross-resistance to pyrimethamine. It seems likely that the type of mutant obtained depends on the conditions under which drug pressure is applied, e.g. the quantity of PABA in the diet of the host.

4. Selective disadvantage of pyrimethamine-resistant mutants

To test whether pyrimethamine-resistant mutants possess a selective disadvantage when in competition with sensitive forms, an experiment was carried out in which mice were infected with a mixture of sensitive and resistant forms of *P.c.chabaudi* (Rosario *et al.*, 1978). A resistant line was mixed with the parent-sensitive line from which the resistant line had been derived, each line thus being genetically similar except for the resistance factor. An initial proportion of 50% sensitive-: 50% resistant-blood forms (in a total of 10^6 parasites) was injected into mice, and clones established at various times after injection were examined for drug response. After 30 days of the mixed infection, the majority of clones derived from the mixture were pyrimethamine sensitive; in a total of 67 clones examined at this stage, 56 were sensitive to the drug and 11 were resistant.

Pyrimethamine-sensitive parasites appeared, therefore, to possess an advantage over resistant forms in the absence of drug-selection pressure. The basis for this selection is not clear, but it may be the increased PABA requirement of the resistant form (see p. 233).

C. Genetics of Chloroquine Resistance

1. Origins

By comparison with their apparently uniform sensitivity to pyrimethamine, different wild species and subspecies of rodent malaria

parasites display considerable variation in their response to chloroquine. Thus *P.yoelii* isolates are innately resistant to high doses (e.g. 50 mg kg^{-1} for four days), even in the absence of previous contact with the drug (Warhurst and Killick-Kendrick, 1967; Peters, 1968), while *P.berghei* and *P.chabaudi* are naturally sensitive (Peters, 1970).

There are many records of the development of *P.berghei* lines resistant to chloroquine (e.g. Ramakrishnan *et al.*, 1957; Sautet *et al.*, 1959; Peters, 1965; Hawking, 1966; Peters *et al.*, 1970; Schoenfeld *et al.*, 1974). Most studies have shown that the chloroquine resistance produced in this species is unstable in the absence of the drug (e.g. Peters, 1965). Reports of stable chloroquine resistance may be due to selection by the drug of an innately resistant *P.yoelii*-type organism from infections mixed with *P.berghei* and *P.yoelii* (Peters *et al.*, 1978).

Stable resistance to chloroquine has been obtained in *P.vinckei* by Powers *et al.* (1969) and in *P.chabaudi* by Rosario (1976), by subjecting sensitive parasites to gradual increases in drug pressure. In both these studies, stable chloroquine resistance was produced from parasite lines which were already resistant to pyrimethamine; attempts to develop stable chloroquine resistance in pyrimethamine-sensitive lines have not yet been successful, for reasons which are unknown. In the experiments of Rosario, sensitive organisms were exposed to a constant drug pressure at a low level (2 mg kg^{-1}), and surviving parasites were then exposed to a slightly higher level of drug (3 mg kg^{-1}) for a number of passages. The resistant line thus obtained was found to be stable after 25 passages without drug and after mosquito transmission. Although the level of resistance attained was low, treatment of 3 mg kg^{-1} for six days was sufficient to distinguish clearly between resistant and sensitive parasites.

2. Inheritance

Rosario (1976) made a genetic analysis of chloroquine resistance in *P.chabaudi*. The results are summarized in Table V, from which it will be seen that approximately equal numbers of resistant and sensitive clones were produced in the progeny of the cross. Moreover, recombination occurred between the factors for chloroquine resistance and three other markers (pyrimethamine resistance, 6PGD and LDH). Thus, chloroquine resistance here shows Mendelian inheritance.

A result of particular interest is that chloroquine resistance is

independent of pyrimethamine resistance. This makes it difficult to understand the failure, mentioned above, to obtain stable chloroquine-resistant mutants from pyrimethamine-sensitive lines, since it is now clear that such forms can be produced, at least following recombination (see also p. 374).

Table V

Analysis of 70 clones derived from a cross between *P.c.chabaudi* lines differing in pyrimethamine response, chloroquine response, LDH type and 6PGD type.

Parents	LDH form	6PGD form	Pyrimethamine response	Chloroquine response	
Line 411AS	3	2	R	R	
Line 96AJ	2	3	S	S	
Progeny					Number of clones isolated
Parental types	3	2	R	R	4
	2	3	S	S	32
Recombinant types	2	3	S	R	11
	2	3	R	S	0
	2	3	R	R	0
	2	2	S	S	2
	2	2	S	R	7
	2	2	R	S	0
	2	2	R	R	0
	3	3	S	S	1
	3	3	S	R	2
	3	3	R	S	2
	3	3	R	R	1
	3	2	S	S	1
	3	2	S	R	7
	3	2	R	S	0

R, resistant to drug pressure; S, sensitive to drug pressure. From Rosario (1976).

3. *Selective advantage of chloroquine-resistant mutants*

A competition experiment involving mixtures of chloroquine-sensitive and chloroquine-resistant forms of *P.c.chabaudi* was carried out, which yielded the surprising result that the resistant parasites seemed to possess a selective advantage, at least in the experimental conditions (Rosario *et al.*, 1978 and unpublished). The experiment was similar to that described for pyrimethamine-resistant mutants (p. 234). In addition

to 50% resistant:50% sensitive parasite mixtures, initial inocula containing resistant:sensitive forms in the proportions 10:90 and 90:10 were used. Clones were established from the resulting infections after various intervals of time and tested for drug response. Mosquitoes were also permitted to feed on each mixture 11 days after inoculation, and the resulting sporozoites used to infect mice; clones derived from these mice were tested for chloroquine response.

The numbers of resistant and sensitive clones derived in these experiments are shown in Table VI. The results indicate an apparent

Table VI

Drug response of clones derived from mixed infections of chloroquine-resistant and sensitive lines of *P.chabaudi*

Initial inoculum (10^6 blood) forms	Blood-induced infection at 30 days			Sporozoite-induced infection at 7 days		
	Total clones	Sensitive	Resistant	Total clones	Sensitive	Resistant
50% Resistant / 50% Sensitive	9	0	9	8	2	6
90% Resistant / 10% Sensitive	8	0	8	11	0	11
10% Resistant / 90% Sensitive	4	0	4	6	2	4

selective advantage of the resistant form in both blood- and mosquito-transmitted mixtures. These results, if confirmed, would appear to indicate that chloroquine-resistant parasites, once they have arisen in populations subjected to drug-selection pressure, would then supplant the sensitive organisms in mixed populations, even in the absence of drug pressure. This finding has obvious implications for the human parasite *P.falciparum*, in which it is well known that chloroquine resistance occurs widely in South-East Asia but not yet in Africa (see p. 368).

V. VIRULENCE

Considerable variation occurs in the virulence of rodent malaria parasites. Part of this variation may be due to non-genetic causes, such as the age and strain of the rodent host, diet, concomitant infections by other organisms (see Chapter 7) and the passage history of the parasite

line. Genetic factors also play a part which, by using the techniques described above, it is now possible to study (Walliker *et al.*, 1976).

In *P.y.yoelii*, the parasites normally give rise to a mild infection in the blood of mice and thicket rats. The blood forms are usually restricted to reticulocytes throughout the infection, except that sometimes a limited invasion of mature erythrocytes occurs at an early stage (3–5 days). The infection rises slowly to a peak parasitaemia of up to 60% within 15 days, after which the animals recover rapidly.

In 1971 a parasite line derived from isolate 17 X of *P.y.yoelii* was found to have undergone a sudden enhancement of virulence (Yoeli *et al.*, 1975). Reticulocytes were invaded during the first two days of infection, as in typical *P.y.yoelii*, but thereafter extensive invasion of mature erythrocytes took place, a 90% parasitaemia being attained within five days and death usually ensuing within seven days.

The newly acquired virulence was stable following passage in blood and mosquitoes. Host and dietary influences were excluded from being the primary cause of increased virulence, because the difference between the virulent line (denoted YM) and a mild line of different origin (denoted A/C) was maintained in mice of the same age and strain, given similar diets. By reducing the quantity of PABA in the diet, the blood forms of the virulent line YM could be restricted to reticulocytes, thus reducing the virulence, but this change was not permanent as the parasites re-invaded mature erythrocytes when normal PABA levels were restored.

A cross was made between line YM which, as well as being highly virulent, contains enzyme-type GPI-1 and is sensitive to pyrimethamine, and line A/C, which is not virulent, contains enzyme-type GPI-2 and is resistant to pyrimethamine. The results are shown in Table VII. All possible recombinants were obtained, showing clearly the genetic nature of the virulence, and suggesting independent segregation of the three characters. Three progeny clones showed atypical development (being neither typically "virulent" nor typically "mild"). The basis for this is not clear; possibly additional genetic factors are involved.

When injected into mice, simple mixtures of sporozoites of the two lines YM and A/C produced infections which, on cloning, were shown to consist of only the two parental types, with no recombination. Virulence could only be transferred by genetic exchange during the sexual stages. Thus, a mechanism based on a concomitant infective agent was excluded.

Table VII

Analysis of 56 clones derived from crosses between *P.y.yoelii* lines differing in infection type, pyrimethamine response and GPI type

Parents	GPI form	Pyrimethamine response	Infection type	
Line YM	1	S	V	
Line A/C	2	R	M	
Progeny				Number of clones isolated
Parental types	1	S	V	15
	2	R	M	5
Recombinant types	2	R	V	10
	1	S	M	1
	2	S	V	3
	1	R	M	5
	1	R	V	9
	2	S	M	5
Clones showing atypical infections	1	R		1
	1	S		1
	2	R		1

V, virulent; M, mild; R, resistant; S, sensitive.

These results show clearly the genetic basis of virulence, which presumably arose as a spontaneous mutation in the original strain of *P.y.yoelii* from which line YM was derived. Once such a mutation occurred, it would presumably be selectively advantageous under laboratory conditions, although not necessarily in nature as the host animals might be killed.

More recent work on an independently occurring virulent line has shown that mutation had occurred in this line at a different locus from that involved in line YM. On crossing the two virulent lines, a proportion of non-virulent progeny clones were produced by genetic recombination (Walliker *et al.*, 1977).

Perhaps the most striking characteristic distinguishing the virulent line YM from other lines of *P.y.yoelii* is its capacity to multiply in mature erythrocytes. Whether this is due to an alteration in the ability of the merozoites to penetrate the erythrocyte membranes, or to an increased ability to multiply once inside, is unknown. However, the phenomenon emphasizes the role of the parasite genotype in the host–parasite interactions necessary for development of the infection. In man, the

influence of host genes such as those for sickle cell anaemia, glucose-6-phosphate dehydrogenase (Luzzatto, 1974) and the Duffy antigens (Miller *et al.*, 1975) have been demonstrated. We now have an example illustrating the role of the parasite genome.

VI. ANTIGENIC DIFFERENCES

The genetics of antigenic variation in protozoa has been studied so far only in free-living organisms such as *Paramecium* and *Tetrahymena* (Beale, 1974). An understanding of the mechanisms involved in the genetic control of antigens in malaria parasites has been hampered by the difficulties of detecting clearly defined variants among the rodent species. The schizont-agglutination test has distinguished antigenically distinct parasites in infections of the monkey parasite *P.knowlesi* (Brown and Brown, 1965), and immunodiffusion has been used to demonstrate antigenic diversity in isolates of *P.falciparum* in man (Wilson *et al.*, 1969). In species infecting rodents, however, evidence for antigenic differences has been found only when less sensitive tests, such as cross-protection, have been used.

Oxbrow (1973) investigated the genetic basis of cross-protection differences between lines of the two subspecies *P.y.yoelii* and *P.y.nigeriensis*. The *P.y.yoelii* line, termed line A, was unable to infect mice which had recovered from infection with a line of *P.y.nigeriensis* termed line D. Line D, on the other hand, could infect mice which had recovered from infection with line A. Neither line could infect mice previously infected with the homologous line. Lines A and D also differed by enzyme-type and pyrimethamine-sensitivity markers (Table VIII).

A cross was made between lines A and D from which 16 clones were derived and examined for each parental character (Table VIII). Two clones showed the enzyme and drug-sensitivity characteristics of line A, but were able to grow in mice which had recovered from line A, and one possessed line D markers but was unable to grow in these mice. Three further clones were recombinant for the enzyme and drug-sensitivity characters, the remaining ten being parental types.

The results indicated, therefore, that the ability of parasites to grow in immunized mice was controlled by genetic factors in the parasites, the factors involved being able to undergo recombination with other markers. The subject has not been studied further mainly because of

practical difficulties in using cross-protection tests to examine large numbers of cloned parasites. When more simple and sensitive immunological techniques become available it is hoped that a more precise genetic analysis can be made.

Table VIII

Analysis of 16 clones derived from crosses between *P.y.yoelii* line A and *P.y.nigeriensis* line D

Parents	GPI form	Pyrimethamine response	Survival in mice immune to line A	
Line A	1	R	No	
Line D	2	S	Yes	
Progeny				Number of clones isolated
Parental types	1	R	No	3
	2	S	Yes	7
Recombinant types	1	R	Yes	2
	1	S	No	3
	1	S	Yes	0
	2	R	No	0
	2	R	Yes	0
	2	S	No	1

VII. CONCLUSION

The results presented in this chapter show that the variations studied—whether concerned with enzyme characteristics, drug resistance, virulence or antigenic differences—are all inherited according to normal Mendelian rules. So far as can be decided with present techniques, rodent malaria parasites show a regular eukaryotic type of life cycle, with a regular alternation of diploid and haploid phases. The blood forms are haploid, but it is not at present possible to state, from genetical data, the precise stage in the life cycle when meiosis takes place.

Clones of haploid cells give rise to both micro- and macrogametes, the differentiation of which must therefore be non-genetic. When gametes of different isolates of the same subspecies are mixed, self- and cross-fertilization occur with approximately equal frequencies. In one

case, a cross has been made between two subspecies (*P.y.yoelii* and *P.y.nigeriensis*) but this is considered to be exceptional, and to take place only in laboratory conditions. Parasites from different species do not, so far as can be observed, hybridize. On the other hand, there is random mating of diverse genotypes in wild populations of the same subspecies, a process which would be expected to generate a good deal of variation in natural populations.

The demonstration that resistance to pyrimethamine and chloroquine is due to the occurrence of gene mutations, which once arisen may spread through a population, is important in relation to the problem of drug resistance in malaria parasites of man. Of particular interest is the rather surprising finding that chloroquine-resistant parasites seem to have an advantage over chloroquine-sensitive forms, even in the absence of drug pressure—at least in the experimental conditions of this work. A similar process in human malaria might be expected to produce a rapid dissemination of chloroquine-resistant parasites.

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6. Immunological Responses

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I. RODENT MALARIA AS AN EXPERIMENTAL MODEL FOR IMMUNOLOGICAL STUDIES

Various species and strains of rodent plasmodia provide, in their different host associations, ample opportunity to investigate the most diverse aspects of the immune response to malaria. This host–parasite relationship will be considered here in its broadest sense, encompassing the various manifestations of innate resistance, as well as the immunity acquired following exposure of the host to the various parasite stages and/or products. Host resistance will be analysed in the light of recent advances in the understanding of basic immune mechanisms operating in relation to parasites, other infectious agents and tumour cells. Investigation of the immune response to rodent malaria will certainly continue to benefit greatly from the fact that much of the expanding knowledge of cellular immunology, immunogenetics, receptors and other cell surface properties, has been obtained through the use of various species of rodents.

We will review the parameters used to measure the various types of immune responses and the results obtained in rodent malaria. The main emphasis, in the past, on serological methods, has brought forth considerable data on humoral responses to these parasites. Because of the availability of excellent recent reviews on this subject (Brown, 1969; Zuckerman, 1970) we will focus mainly on more recent work on the humoral antimalarial response.

The emphasis here will be two-fold: to correlate, whenever feasible, the different antibody responses with their possible role in protection and to differentiate among the responses induced by the successive developmental stages of plasmodia. Since most of the antibody-mediated protective immune responses appear to be stage specific and directed against the "free" extracellular parasite stages, namely merozoites and sporozoites, these will be treated separately, focusing on their analogies and differences.

Considerably less is known about cell-mediated immune responses and their role in host resistance to malaria. Several of the methods used to investigate these aspects have only recently been introduced to studies on malaria parasites, so that the available information is still very fragmentary. Without question, this is an area in need of extensive further experimental work. The possibility of considerable immuno-

logical manipulation of the rodent hosts and the vast amount of available information on the various immunocompetent cells and their multiple interactions, makes the rodent malarial model particularly suited for this type of research (for review, see Raff, 1973).

Significant progress has been made within the last ten years in achieving protection against both rodent and simian malaria by means of active immunization, using either erythrocytic stages or sporozoites as the immunogen. The fact that limited pilot vaccination attempts in man, using irradiated sporozoites, have induced protection against *Plasmodium falciparum* and *P.vivax* malaria (Clyde *et al.*, 1973, Rieckmann *et al.*, 1974, Clyde *et al.*, 1975) clearly emphasizes the potential of this approach and the need for further experimentation in this area.

Another very promising field of investigation, largely unexplored but of potential medical relevance, is the immunopathology of malaria. Again the rodent model appears to provide optimal conditions for experimental manipulation and investigation of this aspect, as well as for the virtually unexplored role of the genetic determination of the immune response to this infection.

II. EVIDENCE FOR IMMUNITY TO MALARIAL INFECTIONS

It has become increasingly obvious that significant host resistance to infection with malaria does occur. The evidence for this includes innate resistance to infection, actively acquired immunity and the feasibility of passive transfer of immunity. The immune response, in particular the protective response, to *Plasmodium* is strictly stage specific. For this reason, the stage of the parasite used to assay the response must be homologous to the stage of the parasite which induced immunity.

A. Innate Resistance

The susceptibility of a particular host to infection with certain species of *Plasmodium*, while resisting others, indicates that there are host factors which control infection. These are ultimately governed by the genetic constitution of the host.

Studies performed on 6 strains of inbred mice (Greenberg and Kendrick, 1957a) revealed considerable differences in the susceptibility

of these animals to blood-induced *P.berghei* infections. C57BL mice developed moderate parasitaemia during the first week of infection and had the longest survival time with this invariably lethal infection. Swiss mice developed the highest parasitaemia during the first week of infection, while STR mice had the lowest curves of parasitaemia. These differences were apparently not attributable to adaptation of the parasite in Swiss mice, since passage of the parasite strain from Swiss mice to other strains did not change these results (Greenberg and Kendrick, 1957b). Studies on the F_1 and backcross generations of these strains of mice indicated that multiple genes govern the differences in the course of infection (Nadel *et al.*, 1955; Greenberg and Kendrick, 1958). In addition, the disease is most probably modulated by immune mechanisms which may vary in mice of different genetic backgrounds. (See Chapter 7 for discussion on the role of concomitant infections.)

Wéry (1968) studied the susceptibility of rodents to *P. yoelii yoelii*, *P.chabaudi* and *P.berghei*. He found that "Theiler's Original" mice, 4–6 weeks old, were susceptible to all three strains. Wistar rats were susceptible to *P.y.yoelii* while hamsters (*Mesocricetus auratus*) were not. The natural host of *P.chabaudi* is *Thamnomys rutilans* and some other sylvatic rats are also susceptible such as *Mastomys coucha* and *Hybomys univittatus*. Albino rats, hamsters and guinea pigs were not susceptible to *P.chabaudi*. Among the mice ("Theiler's Original"), Wéry noted that some mice were "susceptible" and others "resistant". In susceptible mice parasitaemias increased until 50% of the red cells were infected and severe anaemia developed. The infection became chronic and then latent within 20 days. In the "resistant" mice, parasitaemia reached about 20% at the time of crisis and anaemia was less severe. The parasitaemia declined slowly and the infection became latent. *P.berghei* induced a chronic infection in "Theiler's Original" strain. 80% of mice infected with sporozoites developed a patent infection.

Bafort's (1971) studies on susceptibility of rodents to *P.vinckei vinckei* showed that some species were susceptible to infection, some developed low-grade or subpatent infections, while others were not susceptible. Among those species tested that were susceptible were *Apodemus sylvaticus*, *Microtus agrestis* and *Clethrionomys glareolus*. Species showing some degree of resistance, in that only low-grade or subpatent infections developed, were: *Mastomys* (*Praomys*) *natalensis*, *Mesocricetus auratus*, *Peromyscus maniculatus*, *Sigmodon hispidus* and *Acomys cahirinus*.

Albino rats, *Cricetulus griseus*, *Meriones shawi*, *M.unguiculatus*, *M.pyramidium*, *Lagurus lagurus*, *Lepus canicubes* and *Cavia cobaya* were not susceptible to *P.v.vinckei*.

Whether the differences in the course of infection of malaria-infected rodents are determined by characteristics of the red blood cell membranes or intraerythrocytic factors is unclear. In primates there is evidence indicating that susceptibility to plasmodial species is controlled, in part, at the level of the erythrocyte membrane. Butcher *et al.* (1973) have shown that erythrocytes of host species susceptible to *P.knowlesi* infection bind merozoites of *P.knowlesi* in an *in vitro* system, whereas these merozoites do not adhere to erythrocytes of species resistant to this infection.

There is evidence that specific receptors on red cells are responsible for invasion by merozoites (Miller *et al.*, 1975b). Human erythrocytes that were Duffy blood group positive, either Fy^{a+} or Fy^{b+}, were susceptible to *in vitro* invasion by *P.knowlesi*, whereas FyFy cells were not. These antigens may actually be the receptor(s) for invasion or closely associated with such a receptor. Genetic studies in man (Sanger *et al.*, 1955) indicate that Negroes have a low frequency of Duffy positive blood groups and are resistant to infection with *P.vivax* (Young *et al.*, 1955). It is possible, therefore, that the Duffy blood group antigens may also be associated with susceptibility to *P.vivax*.

There are indications that survival of the parasite within the host erythrocyte may also be genetically controlled. For example, bush-baby (galago) erythrocytes were invaded by *P.knowlesi*, but the parasites failed to survive in culture (Butcher *et al.*, 1973). Certain rodent strains of *Plasmodium* preferentially invade immature erythrocytes, whereas other strains prefer mature red blood cells. The basis for the preference is unknown. A number of genetically controlled intraerythrocytic metabolic disorders, including structural and quantitative changes in haemoglobin, and glucose-6-phosphate dehydrogenase deficiency, are known to interfere with susceptibility to malarial infection in man (Luzzatto, 1974).

Considerably less is known with regard to genetic or other factors controlling the fate of the sporozoites which initiate plasmodial infections. Factors which govern the susceptibility to invasion of the parenchymal liver cells by sporozoites, parasite survival and development in hepatocytes and merozoite release from these cells, are not understood. The observation that an equal number of sporozoites of

P.berghei, from the same preparation, produces approximately nine times fewer exoerythrocytic (EEF) forms in A/J mice than in thicket rats, *Thamnomys surdaster*, their natural host, and that young rats develop about three times as many EEF as do A/J mice (Vanderberg *et al.*, 1968) documents the importance of these factors. Furthermore, Most *et al.* (1966) observed that a series of strains of inbred mice varied considerably in their susceptibility to sporozoite-induced *P.berghei* infection. In this study, the susceptibility to infection varied from 100% patency in C57 L/J, A/J and STb/J mice, to 0% patency in DBA 1/J mice. The basis for this difference in susceptibility and the genetic control mechanism remain to be clarified.

An important factor in the innate immunity of rodents to plasmodial infection is the host's age (Zuckerman, 1970). Young rats are more susceptible to *P.berghei* and to *P.vinckei* infections. This age-dependent immunity to *P.berghei* infections is either less pronounced or not evident in mice.

B. Actively Acquired Immunity

An immune response to the inoculation of parasitized erythrocytes or sporozoites of rodent malaria can best be demonstrated upon administration of antimalarial drugs or modification of the host diet, during the infection. Such treatment, by depressing parasitaemia and the subsequent development of a fulminating infection, allows the host to acquire a certain degree of immunity.

1. Immunity to blood-induced infection

a. Use of antimalarial drugs. Mice which have been allowed to recover from blood-induced *P.berghei* infections by administration of chloroquine (Lapierre, 1954), atabrine (mepacrine) (Cox, 1957, 1958, 1959, 1962, 1964) or primaquine diphosphate (Box and Gingrich, 1958), develop resistance to challenge. Similarly, mice which have been treated with chloroquine phosphate to depress *P.vinckei* infections, acquire a lifetime immunity to subsequent challenge (Cox, 1966; Cox *et al.*, 1969).

Establishment of the immune state depends on the duration of parasitaemia, and the longer this period, the greater the degree of immunity that develops. Thus, timing of the administration of the

antimalarial drug is critical. Treatment with sulphadiazine of rats (Satya Prakash, 1959, 1960b) or mice (Satya Prakash, 1960a) early in the course of their infections with *P.berghei*, interfered with the acquisition of immunity.

b. Modification of the host's diet. By withholding an essential growth factor, it is possible to attenuate the virulence of the parasite. Mice maintained on a para-aminobenzoic acid-deficient milk diet during the infection develop a strong and long-lasting resistance to subsequent challenge with *P.berghei*-parasitized erythrocytes (Kretschmar, 1965; Jerusalem, 1965, 1966, 1968; Schindler and Mehlitz, 1965). Similarly, immunity in mice against the virulent B strain of *P.vinckei* can be induced by placing the animals on a meat diet deficient in para-aminobenzoic acid (Adler and Gunders, 1965). As with drug treatment, survival following challenge was dependent on the extent and duration of parasitaemia during the immunization period.

2. *Immunity to sporozoite-induced infection*

Five- to six-week-old rats, which usually survive a sporozoite-induced *P.berghei* infection without drug treatment, produce species-specific antisporozoite antibodies (Spitalny and Nussenzweig, 1973). Antibodies, present two weeks after the intravenous inoculation of the rats with viable sporozoites, were detected by the circumsporozoite (CSP) reaction (Vanderberg *et al.*, 1969), and persisted for approximately one week. A squirrel monkey (*Saimiri sciureus*) which had acquired a *P.brasilianum* infection in nature also had detectable antisporozoite (CSP) antibodies (Nussenzweig *et al.*, 1970).

As with active immunity to erythrocytic stages, the immune response to viable sporozoite inoculation can be detected more easily when host survival is prolonged through drug treatment. Verhave (1975) administered non-attenuated sporozoites of *P.berghei* to rats, which were given chloroquine to suppress parasitaemia, in order to determine whether the parasites would induce a protective response to a subsequent sporozoite challenge. He observed that these sporozoite-infected rats developed a significantly smaller number of liver stages than did control animals upon sporozoite challenge. This protective effect could be amplified by repeatedly infecting the animals with sporozoites.

C. Passively Acquired Immunity

The importance of humoral factors in the control of malaria infection is also demonstrated by the protection resulting from the passive transfer of protective factors either congenitally or by serum (reviewed by Brown, 1969).

1. Congenital transfer of immunity

Blacklock and Gordon (1925) first observed that in areas endemic for malaria, neonates and infants up to one month of age showed a low incidence of infection, and it was suggested that this immunity was passively acquired from the mother. Experimental confirmation of this phenomenon was first provided by a study of Bruce-Chwatt (1954). This study suggested that immunity to *P.berghei* infection could be transmitted through the milk of immune mother rats to their offspring, mainly during the later stages of the nursing period. More detailed investigations followed on the subject (Terry, 1955, 1956; Bruce-Chwatt and Gibson, 1956; Serguiev and Demina, 1957; Demina, 1958) and were reviewed by Bruce-Chwatt (1963). In these studies rats were immunized either before or during pregnancy with increasing doses of parasitized erythrocytes. Rats born to these mothers were subsequently challenged with *P.berghei*-infected red blood cells at various stages after birth. Immunity was transmitted chiefly through the milk of the immune mother, and to a lesser degree, through the placenta. The degree of transmitted immunity was directly related to the degree of immunity of the mother, and waned rapidly as the rats matured. Immunity of NMRI mice against *P.berghei* was not transmitted to their offspring either via the placenta or via the milk (Kretschmar, 1962; Gail and Kretschmar, 1965).

Adler and Foner (1965) immunized mice against an avirulent strain of *P.vinckei* and showed that transfer of protection to their offspring occurred chiefly through the milk of immune animals. However, mice radically cured of *P.vinckei* by the use of mepacrine or chloroquine, while themselves resistant to challenge, were unable to transmit this sterile immunity to their offspring (Cox, 1965).

The role of antibodies in congenital transfer of immunity has been studied. Isfan and Ianco (1964) and Gail *et al.* (1967) found that the gamma-globulin levels were higher than normal in the sera of newborn uninfected rats or mice born to mothers repeatedly infected with

P.berghei. Zuckerman *et al.* (1969a), demonstrated the occurrence of antiplasmodial precipitins which had been passively transferred to their litters by rats infected with *P.berghei*. The precipitin titres could be positively correlated with the number of infections of the mothers and the degree of protection of the young rat to challenge. However, no direct correlation could be made between the presence of a specific precipitin and protection. Often more than one precipitin was present in the milk, and protection might be associated with only one of these multiple antibodies (Zuckerman *et al.*, 1969b). More recently, Palmer (1975), using the indirect fluorescent antibody test, demonstrated passive transfer of maternal *P.berghei* antiplasmodial antibodies through the milk to weanling rats. No *in utero* transfer of antibodies could be demonstrated after the primary infection.

2. *Passive transfer of immunity with immune serum*

Passive transfer of immune serum can induce some degree of protection against plasmodial infections. Thus, in 1961, Cohen *et al.* demonstrated that administration of hyperimmune serum to *P.falciparum*-infected children effected a considerable reduction in parasitaemia. Similarly, studies with the rodent malarias have shown that hyperimmune sera may be effective against either blood-induced or sporozoite-induced infections (WHO, 1975).

a. Passive transfer of immunity to blood-induced infection. The laboratory model used most extensively in passive transfer studies has been *P.berghei* infections in rats, since in this system, the host develops a demonstrable parasitaemia, eventually recovers, and has ample opportunity to develop protective antibodies.

Numerous investigators have reported on the protective effect of rat anti-*P.berghei* serum (Fabiani and Fulchiron, 1953; Fabiani and Orfila, 1956; Bruce-Chwatt and Gibson, 1956; Isfan, 1966). Sera collected after the initial parasitaemia were only slightly protective when given to rats at the time of challenge, whereas sera from multiple-infected rats had a substantial suppressive effect in the recipients. Patency was delayed by several days under these conditions, but parasitaemia eventually developed.

The study of Diggs and Osler (1969) indicated that strikingly small volumes of rat anti-*P.berghei* serum, less than 0·05% of the recipient's plasma volume, significantly reduced parasitaemia when given to

normal rats at the time of challenge. The protective activity in the serum was in the 7S immunoglobulin fraction. A study by Stechschulte *et al.* (1969) confirmed that the 7S fraction of rat anti-*P.berghei* serum, containing IgG and IgA, was protective. Zuckerman and Golenser (1970) also confirmed these observations. In addition, they observed that the IgM-rich fraction of the same serum was totally non-protective for inbred Lewis rats. Recently, Golenser *et al.* (1975) demonstrated that sterile immunity was obtained in rats given concomitant inoculations of *P.berghei*-parasitized erythrocytes and immune serum, rich in IgG, obtained from outbred Sabra rats.

Diggs and Osler (1975) studied the suppressive effect of immune serum on pre-existing parasitaemia. On day three of *P.berghei* infection, rats were given varying amounts of immune serum. Results indicated a dose-dependent clearance of parasites.

Mice are also protected, to some degree, against *P.berghei* infection by transfer of hyperimmune rat serum. Thus, Martin *et al.* (1966) found a slight delay in mortality of infected mice injected with immune rat serum. Briggs *et al.* (1966, 1968) showed that the protective effect of rat serum was a function of the number of infections to which the serum donor had been exposed and of the amount of antiserum used. Treated mice showed a significant delay in the development of patency, but all eventually died with high parasitaemias. Similar observations were made by Golenser *et al.* (1975).

Jerusalem (1968) found that the serum of *P.berghei*-infected Swiss mice, maintained on a para-aminobenzoic acid-deficient diet, was protective upon transfer. Briggs and Wellde (1969) found that antiserum from immunized mice, when concomitantly injected with viable *P.berghei* parasites, caused a delayed course of infection.

b. Passive transfer of immunity to sporozoite-induced infection. Transfer of antisporozoite sera alters the course of sporozoite-induced infection. Concomitant administration of sporozoites and large volumes of immune serum to normal mice caused considerable acceleration of the clearance rate of sporozoites from the peripheral circulation. In fact, the rate of clearance of infective sporozoites from these mice was similar to that observed in actively immunized animals. Passive serum transfer also resulted in the formation of a significantly reduced number of exoerythrocytic liver forms. However, in contrast to mice actively immunized with irradiated sporozoites, the malaria infection of immune

serum recipients was never totally suppressed, and the infections were invariably lethal (Nussenzweig *et al.*, 1972a).

III. HUMORAL IMMUNITY TO RODENT MALARIA

The recovery of rats from *P.berghei* infection is associated with a rise in serum euglobulins (Fabiani *et al.*, 1952; de Smet, 1955). Corradetti *et al.* (1954, 1955) reported that in rats, the gamma-globulin level was increased relative to the total serum nitrogen. These observations were confirmed by Ciuča *et al.* (1964) and by Isfan and Ianco (1964). Woodruff (1957) noted that the gamma-globulin level in the sera of rats with *P.berghei* was further increased by re-inoculation, and concluded that this was, at least in part, due to the formation of antiplasmodial antibody. Re-inoculation also enhanced the antibody response to *P.berghei* in mice (Box and Gingrich, 1958).

Sadun *et al.* (1965) found that there was little change in the serum protein pattern of mice succumbing to *P.berghei* infection. However, the amount of gamma-globulins was increased in the serum of *P.berghei*-infected mice which survived a drug-treated infection (Briggs *et al.*, 1960; Sadun *et al.*, 1965). Gail and Kretschmar (1965) found that both beta- and gamma-globulin levels rose in NMRI mice surviving infection with *P.berghei*. Diggs and Osler (1969) showed that the protective activity of rat anti-*P.berghei* serum could be removed by precipitation with a specific rabbit anti-rat gamma-globulin. This activity was present mainly in the 7S immunoglobulin eluate obtained from a Sephadex G-200 column.

Thus there is general agreement that gamma-globulins rise in the course of recovery of rodents from infection with *P.berghei*. However, it is not known what proportion of the increased gamma-globulin represents antiplasmodial antibody, nor what proportion is protective (Zuckerman, 1970).

A. Antibodies to Blood Stages of Plasmodia

1. Methods of detection

A variety of methods are used for detection of rodent antiplasmodial antibodies. Complement-fixing antibody was found in the sera of

P.berghei-infected rats (Vargues *et al.*, 1951), as well as in animals injected with frozen and thawed preparations of parasites, liberated from red blood cells (Pautrizel and Nguyen Vinh Nien, 1953). However, as Schindler (1965) and Schindler and Mehlitz (1965) have pointed out, the complement-fixing antibody detected in *P.berghei*-infected NMRI mice is not directly correlated with protection.

Antiplasmodial antibody has also been demonstrated in rodent malaria by double diffusion in gel (Zuckerman *et al.*, 1965b). Precipitins were found in the sera of rats throughout the initial *P.berghei* infection and during latency. Titres rose following re-infection, particularly following the immunization of rats with a cell-free extract of *P.berghei*. These precipitins were species specific, and reacted only with *P.berghei* and not with extracts of *P.vinckei* (Goberman and Zuckerman, 1966).

The tanned red blood cell haemagglutination test (Boyden, 1951) has been widely used to detect antibody developed against various parasites (Brown, 1969). Desowitz and Stein (1962), Desowitz (1965) and Desowitz *et al.* (1966), utilized this technique for the detection of antibodies in *P.berghei*-infected rats. Antibody titres rose during the course of the infection and remained at a high level for three months or more. Uninfected rat sera often gave false positive agglutination reactions, with much lower titres. Rabbits immunized with extracts of *P.berghei*-infected red blood cells also produced antiplasmodial antibody, although titres were much lower than those recorded in rats given multiple infective doses.

Tanned red blood cells coated with *P.berghei*, *P.cynomolgi*, *P.coatneyi* or *P.vivax* antigens cross-reacted with antisera from patients with vivax and falciparum malaria (Stein and Desowitz, 1964; Desowitz and Saave, 1965). Cross-reactions, generally of low titre, occurred among all these antigens, particularly when the antiserum contained high levels of antibody. Thus, *P.berghei* appears to share antigenic specificities with both simian and human plasmodia.

Kreier and Ristic (1964) labelled sera from *P.berghei*-infected rats with fluorescein and showed direct binding of this antiserum to *P.berghei*-infected red cells. In addition, using an indirect fluorescence technique, mouse gamma-globulin to *P.berghei* was detected on infected red blood cells of mice. Using immunofluorescence, Sodeman and Jeffery (1965) demonstrated low titres of natural antibody reacting with *P.berghei*-infected red blood cells in the sera of a certain percentage of normal mice.

Cox *et al.* (1969) employed the indirect fluorescent antibody technique, using specific anti-mouse IgM and IgG, and demonstrated the presence of both classes of antibody. Antibody levels rose rapidly during the first 8 to 12 days after infection of mice with *P.vinckei* (chloroquine treated) and remained elevated for several weeks.

Finerty *et al.* (1972) compared the humoral response of germ-free (GF) and conventionally reared (CV) *P.berghei*-infected mice. Individual serum samples were assayed for immunoglobulin levels by a modification of the radial gel diffusion technique (Mancini *et al.*, 1963). Serum anti-*P.berghei* titres were determined by the indirect fluorescent antibody technique. They found that the antibody response of GF mice infected with *P.berghei* was more rapid, the antibody titres were higher, and these mice survived longer than did infected CV mice. This suggested that serum antibody played a role in the increased survival time of the GF mice. The anti-*P.berghei* response preceded any significant rise in total 7S immunoglobulin. GF mice also showed a greater 7S immunoglobulin response than did CV mice.

In a later study, Finerty *et al.* (1973) evaluated the immune response to *Eperythrozoon coccoides* and *P.berghei* in germ-free and conventionally reared mice infected with both parasites. IgM levels closely paralleled the antibody responses to *P.berghei*, suggesting that most of the antibody to the parasite was present in this immunoglobulin class. In comparison to the large immunoglobulin response, relatively low levels of antibody to both parasites were detected in GF and CV mice.

Voller *et al.* (1975) described a new method for the detection of antimalarial antibodies, which thus far has been used only to detect anti-*P.vivax* and anti-*P.falciparum* immunoglobulin. This method, the enzyme-linked immunoabsorbent assay (ELISA) described by Engvall and Perlmann (1971, 1972), permits a quantitative, objective determination of antibody titres. Antigen, in this instance a soluble extract of the erythrocytic stages of *P.knowlesi*, is used to coat a plastic tube or plate. This is treated with the antibody-containing serum, washed, and then incubated with an anti-immunoglobulin-containing antiserum to which the appropriate enzyme has been linked. Finally, the amount of bound enzyme, and thus of immunoglobulin, is determined spectrophotometrically, upon addition of the corresponding substrate. This technique has so far proven to be sensitive, and to give some low titre, false positive reactions, possibly due to "autoimmune" antibodies and anti-immunoglobulin commonly found in the tropics.

2. Role of antibodies in protection

Cohen *et al.* (1961) suggested that protective antibodies may act on free malaria parasites rather than on infected erythrocytes. Transfer of hyperimmune serum to children infected with *Plasmodium falciparum* did not decrease the numbers of circulating parasites until completion of schizogony. Cohen and Butcher (1970) later demonstrated that addition of hyperimmune serum to short-term *in vitro* cultures of *Plasmodium knowlesi*, a synchronous parasite of monkeys, did not interfere with the intraerythrocytic development of this parasite, but interfered with development after schizogony. The authors suggested that the inhibitory action of the antibody, which was shown to be complement independent, was directed against the merozoites.

Hamburger and Kreier (1975), working with a *P.berghei*–rat system, recently investigated antibody-mediated *in vivo* elimination of free parasites and infected erythrocytes. Free parasites were isolated from infected blood by continuous flow sonication. Using indirect fluorescence, they found that, *in vitro*, antiplasmodial antibodies bound to free parasites within 5 minutes, whereas infected and normal erythrocytes did not bind antibodies. Pre-treatment of free parasites with immune serum did not increase the rate of their *in vivo* elimination if the parasites were washed before injection; the same result was also obtained with infected red blood cells. If, following washing, the free parasites were resuspended in various dilutions of immune serum, they were efficiently eliminated *in vivo*. However, this was not true for the infected erythrocytes. The parasites themselves, therefore, had to be associated with a critical amount of antibody in order to be eliminated *in vivo*. The authors further suggested that the binding of protective antibodies to the parasites was reversible, and attributed this to a low avidity of the protective antibodies or to a shedding of the immune complexes from the parasites' surface.

B. Antiplasmodial Antibodies to Sporozoites

1. Circumsporozoite Precipitation reaction

Antisporozoite antibodies were first detected by Russell *et al.* (1941) who observed that the serum of sporozoite-immunized birds agglutinated this parasite stage, but not parasitized red blood cells. In rodent

malaria, Vanderberg *et al.* (1969) described a reaction of immune serum with sporozoites which was designated the circumsporozoite precipitation (CSP) reaction. The reaction is characterized by the formation of a terminal thread-like precipitate, often more than twice the length of the sporozoite, when these parasites are incubated with immune serum (Figure 1). The CSP reaction is easily seen by phase contrast microscopy

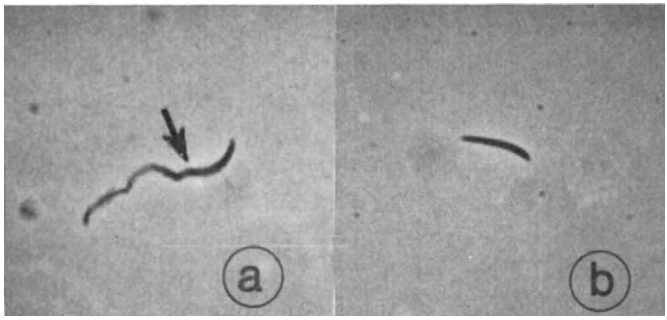

Figure 1. Phase contrast microscopy of sporozoites of *P.berghei* (× 1000). Effects of incubation in immune serum. (*a*) Sporozoite incubated in immune mouse serum (30 min at 37°C). Note the very long thread-like precipitate (positive CSP reaction), to the left of the arrow. (*b*) Parasite incubated in normal mouse serum.

after ten or more minutes of incubation at room temperature or at 37°C, and is complement independent. This reaction does not occur at 4°C or upon formalin treatment of the sporozoites (Cochrane *et al.*, 1976). Frozen-and-thawed sporozoites produce, when incubated with immune serum, a peculiar type of CSP reaction consisting of a granulated deposit along most of the parasite surface (Vanderberg *et al.*, 1969).

CSP antibodies are produced by mice and rats, not only upon their intravenous immunization with irradiated sporozoites of *P.berghei*, but also upon the bite of infected irradiated mosquitoes (Vanderberg *et al.*, 1970) as well as after injection of viable sporozoites (Spitalny and Nussenzweig, 1973; Spitalny *et al.*, in press). The CSP antibody response in rats given a single dose of irradiated sporozoites was detectable for a considerably longer period than in rats injected with viable sporozoites (Spitalny and Nussenzweig, 1973). Rats also produce CSP antibodies when injected with sporozoites of simian and human plasmodia, to which these animals are not susceptible (Nussenzweig

et al., 1973; Nussenzweig and Chen, 1974). The 7S gamma-globulin fraction of immune serum reacts with the sporozoites to produce the CSP reaction, and antibody titres increase after administration of multiple sporozoite doses (Spitalny and Nussenzweig, 1973).

CSP antibodies strongly cross-react with sporozoites of heterologous species of murine malaria parasites. Thus, the serum of mice immunized with X-irradiated sporozoites of *P.berghei* produces a positive CSP reaction with sporozoites of *P.vinckei* and *P.chabaudi* and the reverse is also true (Nussenzweig *et al.*, 1972b). Cross-protection to sporozoite challenge parallels these serological findings (Nussenzweig *et al.*, 1969b).

CSP antibodies are strictly stage specific. The sera of animals immunized with sporozoites of *P.berghei* have no detectable effect on either the asexual blood stages, gametocytes, ookinetes or exoerythrocytic forms of the same parasite strain (Vanderberg *et al.*, 1972; Vanderberg, 1973). Furthermore, the antigen(s) responsible for the CSP reaction differ during sporozoite maturation. Immunization with oocyst sporozoites produces an antiserum which reacts with both oocyst and salivary gland sporozoites. However, antisera produced by immunization with salivary gland sporozoites react primarily with this developmental stage, and not with oocyst sporozoites (Vanderberg *et al.*, 1972). Salivary gland sporozoites are also considerably more infective and immunogenic than oocyst sporozoites. This phenomenon of "antigenic maturation" has also been documented and investigated in more detail in relation to sporozoites of *P.cynomolgi* (Nussenzweig and Chen, 1974).

2. Surface coat formation

a. Detection by electron microscopy. Recently, Cochrane *et al.* (1976) investigated antibody-induced, ultrastructural changes of malarial sporozoites. When sporozoites of *P.berghei* and *P.cynomolgi* were incubated in their respective antisera, they became covered by a very prominent and loosely packed surface coat of fine fibrillar material (Figure 2). The results of an additional incubation of the sporozoites with rabbit anti-mouse IgG conjugated to hemocyanin, suggested that the surface coat resulted from an interaction of immunoglobulin with the sporozoite's surface. Pre-fixation of sporozoites with formalin, which destroyed infectivity of the parasites, did not appreciably alter coat formation.

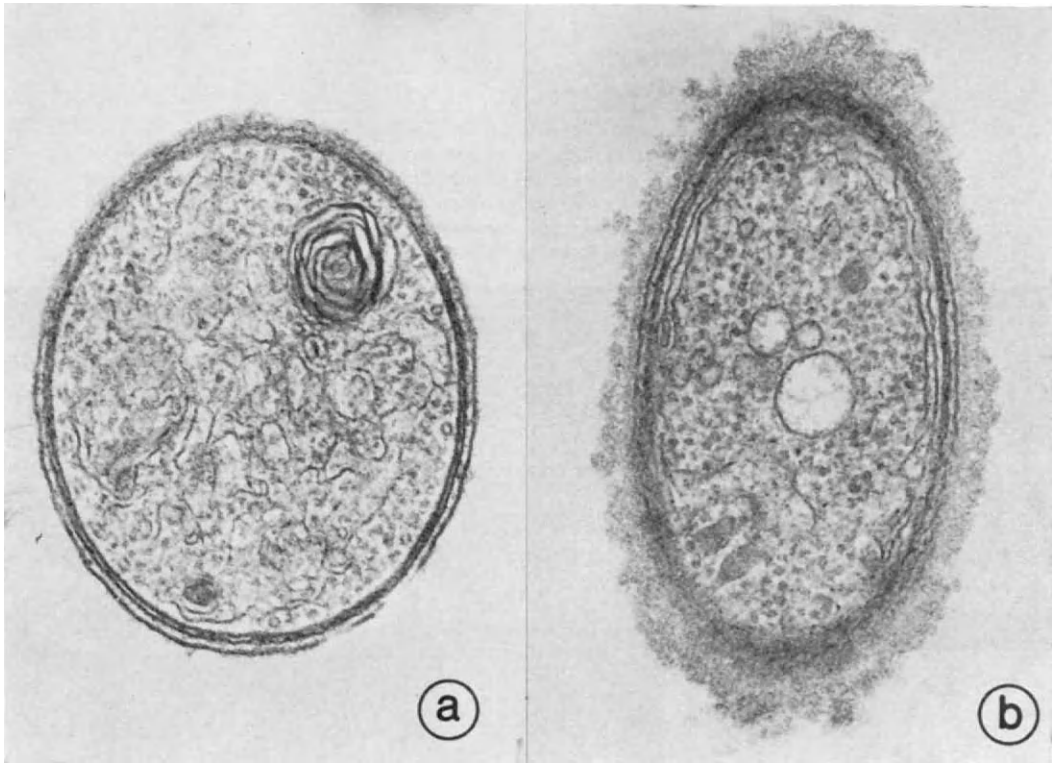

Figure 2. Electron micrographs of sporozoites of *P.berghei* (×50 000). Effects of incubation in immune serum. (*a*) Sporozoite incubated in serum of normal mouse (30 min at 37°C). (*b*) Sporozoite incubated in serum of immunized mouse. A prominent coat of fine fibrillar material covers the entire surface of the parasite (courtesy of Dr Masamichi Aikawa, The Department of Pathology, Case Western Reserve University, Cleveland, Ohio, USA).

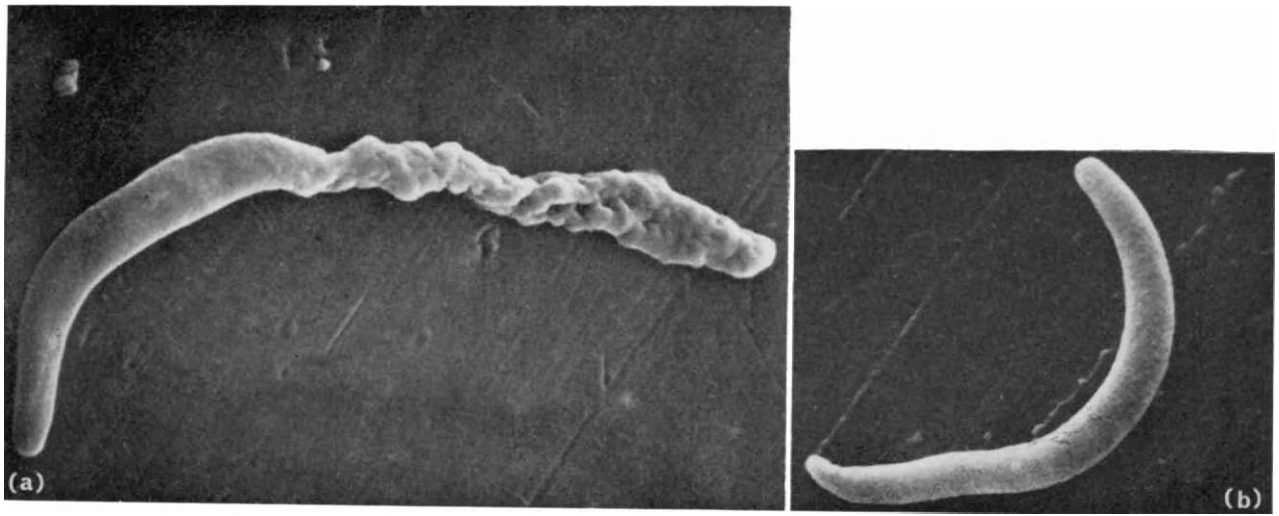

Figure 3. Alteration of sporozoites of *P.berghei* following immune serum incubation. Scanning electron micrographs ($\times 9000$). (*a*) Sporozoite incubated in immune serum. The body of the parasite appears smooth. The irregular configuration of the CSP reaction extends a considerable distance posteriorly. (*b*) Sporozoite incubated in normal serum. Note the smooth surface of the parasite. The anterior end of the sporozoite is narrow, and can be clearly distinguished from the rounder posterior end.

Coat thickness was variable, being minimal at the anterior end, and maximal at the posterior end of the parasites (Figure 3). Cochrane and co-workers suggested that a posteriorly directed movement of the immune complexes, analogous perhaps to capping, could result in the formation of the circumsporozoite precipitate.

b. Detection by fluorescent antibody technique. Immunoglobulin deposits on the surface of sporozoites incubated with immune serum can also be detected by indirect immunofluorescence (S. Nardin and R. S. Nussenzweig, in press). Because it does not require viable parasites, this technique has a considerable advantage over other methods used to detect antisporozoite antibodies. In fact, both short-term formalin-fixed sporozoites and sporozoites maintained at −70°C produce as good a reaction as a fresh parasite preparation. The reaction is very sensitive since it detects antisporozoite antibodies before they become demonstrable by the CSP reaction. The specificity of this technique is identical to that of the CSP reaction.

3. Sporozoite neutralizing activity

When sporozoites of *P.berghei* are incubated in immune serum for 45 min at room temperature, there is a considerable loss of infectivity, as revealed by the significantly lower percentage of patency which these parasites induce in normal recipients (Nussenzweig *et al.*, 1969a). This sporozoite-neutralizing activity (SNA) appears in mice after two immunizing doses, and increases in titre with further immunization. The reaction is complement independent and does not occur at 4°C. Complete loss of sporozoite infectivity can be obtained with rather dilute immune serum samples (Spitalny, 1973).

Sporozoite neutralization does not affect other developmental stages of the same strain of parasite. However, SNA, produced after immunization of mice with sporozoites of *P.berghei*, causes the loss of infectivity of sporozoites of all species of murine malaria parasites. Sporozoite neutralization does not represent a peculiarity of the rodent malaria system, since neutralizing activity was detected in the sera of rhesus monkeys which had been immunized against sporozoites of *P.cynomolgi* (Chen, 1974).

4. Correlation between the presence of anti-sporozoite antibodies and protective immunity

There is considerable correlation between the occurrence of anti-sporozoite antibodies and protective immunity. However, there are a variety of conditions in which humoral and protective antisporozoite responses are dissociated. Thus, protection against challenge with sporozoites of *P.berghei* occurs in the absence of detectable anti-sporozoite antibodies, both during the early stage of immunization (Spitalny and Nussenzweig, 1973) and in mice splenectomized prior to their immunization (Spitalny *et al.*, 1976). The presence of anti-sporozoite antibodies in the absence of protection has also been observed after immunization of mice with homogenized, sonicated or otherwise disrupted sporozoites (Spitalny and Nussenzweig, 1972).

The relationships among the different manifestations of anti-sporozoite antibodies, namely CSP, coat formation and SNA, and the question of whether these represent antibodies against the same or different antigens remain to be clarified.

IV. CELL-MEDIATED RESPONSES

It is certain that cellular components play a role in acquired immunity to malaria. In addition, humoral responses do not account for all the observed protection. Humoral and cell-mediated immune mechanisms (CMI) should certainly not be considered completely independent of one another, since it is unlikely that one mechanism operates exclusively.

A. Evidence for CMI Responses to Erythrocytic Stages of Rodent Malaria

1. Role of the thymus on the course of malarial infection

Thymectomy of neonatal rats, at an age at which these animals are normally quite resistant to this parasite, followed by their challenge with *P.berghei*, caused marked exacerbation of the infection. This was reflected in increased mortality rates, higher parasitaemias and more pronounced anaemia (Brown *et al.*, 1968; Stechschulte, 1969a). The

latter author measured the antiplasmodial antibody response of these thymectomized infected rats, both by immunofluorescence and passive haemagglutination, and found them to be similar to that of controls.

The administration of antithymocyte serum (ATS) to rats, prior to and after their inoculation with *P.berghei*, mimicked the effect of thymectomy (Spira *et al.*, 1970). All of these animals (including young adult rats) developed a continually ascending parasitaemia, and died. The level of reticulocytes in the ATS-treated animals was similar to that of control rats, and their humoral response appeared to be unaltered.

Congenitally athymic, (*nu/nu*) mice developed a lethal infection upon inoculation with an otherwise mild strain of *P.y.yoelii* (17 X) (Clark and Allison, 1974). The parasitaemia reached 80% in these homozygous *nu/nu* mice, and practically every reticulocyte was parasitized. In contrast, the *nu/+* littermates had a maximal parasitaemia of 10% and parasites were undetectable at the end of the second week of infection. The administration of ATS to mice, throughout the course of their infection with *P.y.yoelii* (17 X) produced similar, although somewhat less dramatic, results. A number of ATS-treated mice had a considerably longer course of infection and reached very high levels of parasitaemia, but did survive (Barker and Powers, 1971a). These authors reported a delay in the occurrence of antiplasmodial antibodies, and the timing of the antibody response appeared to be correlated with recovery from the infection. Barker *et al.* (1971b) reported comparable results after either ATS or hydrocortisone treatment of the inoculated mice. A third type of manipulation of their immune system, splenectomy, had considerably more drastic effects, resulting in a 100% lethal outcome of the infection.

These results differ from those obtained by Sheagren and Monaco (1969), who thymectomized and then administered rabbit AMLS (anti-mouse lymphocyte serum) to adult *P.berghei*-infected mice. Whereas thymectomy *per se* appeared to have very little effect, the administration of rabbit AMLS significantly delayed mortality, and decreased parasitaemia. The authors felt that a direct antiparasitic effect of the rabbit AMLS could not be excluded in these experiments.

In hamsters, thymectomy, or administration of ATS, has been found to be highly beneficial, apparently by decreasing the autoallergic reactions and the immunopathology resulting from the *P.berghei* infection (Wright, 1968; Wright *et al.*, 1971).

2. *Malarial-induced alterations of T and B cell numbers and function*

Marked involution of the thymus was observed in lethal *P.berghei* infections in mice (Krettli and Nussenzweig, 1974) but not in mild *P.y.yoelii* infections. During the fourth week of infection, lymph nodes of *P.berghei*-infected mice had approximately one-third the number of cells compared with those in lymph nodes of control animals. Complement receptor lymphocytes (CRL) were found to be reduced early during the infection, whereas theta-bearing (T) cells remained unaltered until the third week of infection and then declined. The "null cell" population was considerably increased in the lymph nodes of these *P.berghei*-infected mice. This is similar to observations in malaria-infected patients (Wyler, unpublished).

Comparative studies of the spleens in mice with either fatal or non-fatal *P.yoelii* infections have shown that recovery is associated with a vigorous response of the germinal centres. Displacement or replacement of T lymphocytes by pyroninophylic cells was observed in the T cell-dependent, peri-arteriolar regions, in both fatal and resolving infections (Jayawardena *et al.*, 1975a).

Jayawardena *et al.* (1975b) examined both T cell numbers and T cell function of cells obtained from the spleens of *P.berghei*, as well as *P.yoelii*-infected mice. The results were quite different in these two malarial infections. *P.berghei* induced a transient increase in T cell numbers, which fell to subnormal levels during the late stage of the infection. *In vitro*, the mitotic activity induced by PHA, which is primarily a T cell mitogen, was decreased in the spleens of the *P.berghei*-infected animals. *In vivo*, the response to oxazolone, a skin-sensitizing agent, was also considerably reduced. Infection with *P.yoelii*, on the contrary, caused a considerable, sustained increase in the number of dividing cells present in the spleens of these infected animals; there was an increase in PHA response, and no alteration in sensitization of the mice to oxazolone. Jayawardena *et al.* (1975a) also investigated the response of unfractionated or T cell-depleted spleen cells of *P.berghei* and *P.yoelii*-infected CBA mice to LPS, a bacterial polysaccharide. Both infections caused a significant decrease in the LPS-responsive cells. Differently from the PHA response, the mitotic activity induced by LPS did not return to normal levels upon resolution of the infection.

Weinbaum *et al.* (1976) have demonstrated that Balb/c spleen cells of mice that had recovered from a *P.yoelii* (17 X non-lethal) infection could be stimulated *in vitro* by syngeneic erythrocytes infected with the lethal strain of this parasite (*P.yoelii* 17 XL), or a soluble antigen preparation of schizonts of this parasite. Anti-theta treatment of the spleen cells markedly reduced the proliferative response, indicating that T cells were largely responsible for proliferation. Normal spleen cells also had a significant response, although the kinetics suggested that this was either a primary response to antigen or due to mitogenic factors in the antigen preparation.

A delayed type hypersensitivity reaction was demonstrated by Finerty (1975), in mice immunized with killed blood forms of *P.berghei*. These reactions were elicited by injection of frozen-and-thawed parasites into the foot pads. Positive reactions (foot pad swelling) correlated well with protection in infected, cyclophosphamide-treated mice.

3. Adoptive transfer of immunity by cells

The capacity of lymphoid cells of animals which had recovered from a *P.berghei* infection, to transfer resistance to a subsequent malarial challenge, has been the focus of several investigations. Stechschulte (1969b) transferred cells obtained from the thoracic duct, lymph nodes, spleens and peritoneal cavity, from inbred rats which had recovered from a *P.berghei* infection, to normal rats of the same strain. Protection to challenge, seven days after the cell transfer, was pronounced in the animals which had received spleen cells, and slight in the rats receiving immune lymph node cells. No protection was conferred by thoracic duct lymphocytes, obtained from the same immune donors, or by the cells obtained from the peritoneal washings of these animals. Similar results were obtained by Roberts and Tracey-Patte (1969), and also by Phillips (1970), in the same host–parasite system. In all of these experiments, plasmocytes, lymphocytes, macrophages, as well as malarial antigen were transferred.

Data are still relatively scarce on the transfer of purified cell populations. Brown (1971) reported that treatment of *P.berghei*-infected rats with antithymocyte serum considerably reduced the ability of the lymphoid cells of these animals to transfer protection. He also found that removal of the immunoglobulin-bearing cells from the cell

suspension, by means of an anti-Ig coated column, did not abolish their capacity to confer protection (Brown, 1974).

Using a different host–parasite association, *P.yoelii*-immunized mice, Jayawardena *et al.* (1975c) reported that the passive transfer of serum obtained from these animals protected recipient mice against a *P.yoelii* infection. This serum transfer produced only a very transient inhibitory effect in T cell-deprived recipients. The transfer of immune spleen cells conferred a significant degree of protection, even when the cell suspension had been pre-treated with anti-T serum, or when the cells were given to T cell-deprived recipients.

The mechanism whereby the various lymphocyte subpopulations interact with macrophages, and possibly with immune serum, in order to interfere with the malaria parasites, is still far from understood. Coleman *et al.* (1975) described an *in vitro* cytotoxic effect resulting in lysis and ^{51}Cr release from malaria-infected red blood cells. This effect was mediated by macrophage-depleted immune spleen cells and was antibody dependent. The authors suggested that activated thymus-dependent cells or "null cells" might be involved in this cytotoxic effect.

B. Cell-mediated Responses in Sporozoite-induced Immunity

1. Role of the thymus

When thymectomized, irradiated, bone marrow-reconstituted mice were immunized by the repeated administration of irradiated sporozoites of *P.berghei*, these animals completely failed to develop protection against a subsequent challenge (Spitalny *et al.*, 1977). These mice also failed to develop antisporozoite antibodies, indicating that this response was thymus dependent. Reconstitution with thymocytes restored both the capacity to develop protection as well as an anti-sporozoite antibody response. Similar results were found using nude (*nu/nu*) mice, that is, absence of protection and CSP antibodies. When exposed to an identical schedule of immunization *nu/+* littermates developed both these responses.

2. Adoptive transfer of immunity by cells

Preliminary results obtained by J. P. Verhave (personal communication) indicate that the protection of sporozoite-immunized mice can be

transferred by the administration of immune spleen cells to recipients. In order to achieve transfer of protection, the recipients must be irradiated and the cell transfer followed by a single sporozoite inoculation, which *per se*, fails to induce significant protection. Incubation of immune spleen cells with an anti-theta serum abolished their capacity to confer protection against sporozoite challenge, indicating that T cells play an essential role in sporozoite-induced protection. Chen *et al.* (1977) arrived at a similar conclusion by observing that B cell immunocompetence is not an absolute requirement for the development of sporozoite-induced protection. A considerable number of mice injected from birth with goat antiserum to mouse μ-chain (μ-suppressed) became protected after repeated immunization with irradiated sporozoites. These animals did not develop detectable antisporozoite antibody levels.

V. IMMUNIZATION OF RODENTS AGAINST MALARIA

A variety of methods of immunization has been employed in order to establish optimal conditions for obtaining protection against malaria. In addition, immunization studies have allowed the mechanisms of acquired immunity to rodent malaria to be investigated. Experimental approaches have utilized immunization with repeated or long-lasting infection with viable parasites, attenuated or killed parasites or parasite fractions. In addition, immunization procedures have sometimes included the use of adjuvants with the parasite preparation. Non-specific factors also confer some protection against infection.

A. Immunization with Blood Stages of Malaria

1. Repeated or long-lasting infection

This is discussed on pp. 252–254.

2. Attenuated parasites

Effective immunization of rodents can be achieved through the use of attenuated parasites. Parasites with decreased virulence sometimes arise spontaneously, or can be induced by either tissue culture passage or irradiation.

a. Tissue culture passage of parasitized erythrocytes. Weiss and de Giusti (1964a, b, 1966) and Weiss (1965) reported that the virulence of a strain of *P.berghei* had been altered by maintaining infected rat bone marrow or liver, *in vitro*, in tissue culture medium containing normal lamb, calf or hamster serum. The explants induced normal infections when implanted into rats, but had a reduced virulence in mice. Culture of infected explants in homologous rat serum did not decrease the virulence of the strain. After a number of passages from rat to culture to rat, most of the recipient mice recovered and were resistant to re-inoculation both with the attenuated and virulent, parent strains.

b. Irradiation of parasitized red blood cells. Immunization with irradiated parasitized red blood cells was first reported by Ceithaml and Evans (1946) in an avian malaria system. Chicken erythrocytes, parasitized with *Plasmodium gallinaceum*, were exposed to 30 000 rad and injected into normal chickens. A degree of resistance to challenge with *P.gallinaceum* was obtained.

Corradetti *et al.* (1966) reported the immunization of rodents with irradiated blood forms. Rats were immunized with a single dose of infected red blood cells which had been irradiated at 20 000 rad or more. After challenge with viable parasitized erythrocytes, they developed a lower parasitaemia of shorter duration than that of control animals. Rats immunized with infected red blood cells, irradiated at 18 000 rad or less, had a prolonged prepatent period upon challenge, but parasitaemia levels and mortality rates were unaltered compared to control animals. These observations were confirmed and extended in mice and rats. Wellde and Sadun (1967) and Wellde *et al.* (1969) found that the degree of acquired resistance was proportional to the number of immunizing doses and the number of parasites used for challenge.

Mouse or rat hyperimmune sera, obtained using irradiated red blood cells as the immunogen, effectively suppressed early infections in recipient mice when injected concomitantly with parent parasites. These sera had minimal effect when injected concomitantly with a "variant" strain recovered from immunized mice following challenge. The "variant" strain remained refractory to the immune anti-"parent" sera even after 18 passages through normal mice (Briggs and Wellde, 1969). This finding of "variant" populations of *P.berghei* corroborated the earlier observations of Cox (1959, 1962) on drug-treated *P.berghei*-infected mice. Relapsing populations of *P.berghei*, obtained from these

mice, differed from parent strains in terms of virulence, immunogenicity and susceptibility to latency-inducing treatments.

3. Heat-inactivated parasitized red blood cells

Heat-inactivated parasitized blood has been used to induce immunity to *P.berghei* infections in mice (D'Antonio *et al.*, 1969a; D'Antonio, 1972). Preparations of parasitized red blood cells were incubated at different temperatures (41–56°C) for varying periods of time, and then used as the immunogen. The immunization schedule consisted of either a single inoculum or multiple injections. No patent or subpatent infections resulted from the injection of heat-inactivated parasitized erythrocytes. Immunized mice, upon challenge with viable *P.berghei* parasites, developed mild anaemias and low levels of parasitaemia which became undetectable after one to three weeks.

4. Parasite fractions

Limited success has been obtained in the immunization of animals with parasite fractions. Zuckerman *et al.* (1965a, 1967) found that young rats developed a significant measure of protection after immunization with a cell-free extract of *P.berghei*. Maximum resistance to challenge was achieved with three immunizing doses and was demonstrated by an increase of the prepatent period, lower parasitaemias which were shorter in duration, and decreased mortality rates. Sterile protection was not achieved. However, Cox (1965) and Schindler (1965) failed to detect any protective response in mice immunized with extracts of *P.berghei*.

D'Antonio *et al.* (1969b, 1970) attempted immunization of A/J mice by intraperitoneal injection of either solubilized or non-solubilized material extracted from blood stages of *P.berghei*. Upon challenge, the recipients experienced transient, low grade parasitaemias, and most of the mice recovered completely. The course of infection was similar in all protected groups independent of the route of challenge, intravenous or intraperitoneal, or the type of plasmodial preparation used for immunization. The protective fraction was present in the partially purified plasmodial material contained in two void volume eluates of a Sephadex G-200 column (D'Antonio *et al.*, 1970).

B. Cross-protection in Blood-Induced Infections

Numerous data are available on cross-protection among blood-induced infections of various rodent malaria species or strains. Mice with a chronic *P.chabaudi* infection survived an otherwise lethal *P.vinckei* infection but were not protected against *P.berghei* (Nussenzweig *et al.*, 1966; Yoeli, 1966; Yoeli *et al.*, 1966).

Cox and Voller (1966) found that rats which had recovered from *P.berghei* infections were immune to challenge with *P.yoelii*. In reciprocal experiments, rats immune to *P.yoelii* showed only a partial immunity to *P.berghei*. The authors suggested that the greater virulence of *P.berghei* was responsible for this difference. The *P.berghei*-immunized animals were susceptible to *P.chabaudi* and *P.vinckei* challenge. Absence of cross-protection between *P.berghei* and *P.vinckei* was earlier demonstrated by Rodhain (1953, 1954), Fabiani and Orfila (1959) and Cox (1966). Cox (1970) and Barker (1971) extended these studies to mice and obtained results similar to those reported for rats. More recently, Hargreaves *et al.* (1975) showed partial protection against *P.berghei* and minimal protection against *P.vinckei* after immunization with a mild strain of *P.yoelii* (17 X).

Protection can be induced by a mild strain to a virulent strain of the same species. Thus, Adler and Foner (1961) demonstrated protection of mice against the virulent B strain of *P.vinckei* following previous infection with the relatively benign hamster-adapted H strain. Barker (1971) noted that mice, after recovery from a *P.yoelii* (17 X) mild strain infection, showed solid protection against a virulent isolate of the parent strain. He attributed the protection to sharing of "functional antigens" between the two strains. Oxbrow (1973) showed that when the virulence of *P.y.nigeriensis* was reduced to a level resembling that of the mild strain *P.y.yoelii*, it still protected against normally virulent *P.y.nigeriensis*. Hargreaves *et al.* (1975) observed protective immunity induced by two mild strains of *P.yoelii* (17 X and 33 X) against a virulent strain of this *Plasmodium*.

This cross-protection parallels to some degree findings of serological cross-reactivity. Thus, Zuckerman (1964b) and Zuckerman and Spira (1965), using immunoelectrophoresis, showed that *P.berghei* and *P.vinckei* had several common and several specific antigens. Using the fluorescent antibody technique, Voller (1965) demonstrated a slight cross-reaction between *P.berghei* and *P.vinckei*. It is not known if the

response to any of these common antigens is responsible for protection.

Bray and El-Nahal (1966), using the indirect hemagglutination test, and El-Nahal (1967), and Cox and Turner (1970), using the indirect immunofluorescent technique, found that the four malaria parasites of murine rodents could be classified into two antigenically distinct groups: (i) *P.chabaudi* and *P.vinckei* and (ii) *P.berghei* and *P.yoelii*.

C. Immunization with Sporozoites

Initial studies using malarial sporozoites as immunogens were those of Mulligan *et al.* (1941) and Russell *et al.* (1942). A considerable proportion of fowls vaccinated with sporozoites of *Plasmodium gallinaceum*, inactivated by ultraviolet light or by grinding or drying and subsequent reconstitution were resistant to sporozoite challenge. Richards (1966) confirmed and extended these results using the same host–parasite system. Sporozoites inactivated with formalin or by freeze-thawing also conferred partial protection to sporozoite challenge. These studies were followed by investigations on the immunogenicity of sporozoites of rodent malaria.

1. Living sporozoites

Verhave (1975) carried out extensive studies to determine whether immunization of rats with non-attenuated sporozoites of *P.berghei* could induce a protective response to a subsequent sporozoite challenge. To prevent red blood cell parasitaemia during immunization, chloroquine was administered in the drinking water of these animals. Responses were evaluated by the number of exoerythrocytic forms developing after challenge. Results were variable, but in general, 3 or 4 immunizing doses were sufficient to reduce the number of exoerythrocytic forms to undetectable levels. This protection, effected by either intravenous inoculation or mosquito bite, was evident for several months. When the animals were challenged after one year, a considerable reduction in the number of developing exoerythrocytic forms was still observed. Similar observations were made by Beaudoin *et al.* (1975) who were also able to immunize rats against the ANKA strain of *P.berghei*, by placing them on a suppressive regimen of chloroquine during immunization.

2. *Irradiated sporozoites*

Sporozoite-induced protection was obtained in a rodent malaria system upon immunization of mice with X-irradiated sporozoites of *P.berghei* (Nussenzweig *et al.*, 1967). In these initial experiments, in which the animals received a single immunizing dose, the percentage of animals protected against an otherwise lethal sporozoite challenge varied considerably. Protection of 90% or more of the animals was obtained by increasing the number of immunizing doses to three or more (Nussenzweig *et al.*, 1969a). Sterile immunity was obtained under these conditions, since no parasites were detectable directly after challenge, or indirectly, by subinoculation of blood of the immunized mice. Mice splenectomized after immunization also showed sterile immunity. Protection remained unaltered for approximately two months after the last immunizing dose and then declined progressively.

Initially, sporozoites used for immunization were obtained by dissection of the salivary glands of *P.berghei*-infected mosquitoes. In subsequent experiments, the use of gradient-purified sporozoites permitted the harvesting of larger numbers of parasites. Sporozoites of *P.berghei*, as well as *P.cynomolgi*, recovered from a linear Renografin/BSA gradient, were shown to retain fully their immunogenicity and infectivity (Krettli *et al.*, 1973). Furthermore, contamination of these parasites by bacteria, fungi and mosquito tissue, was considerably reduced.

Non-infected salivary glands failed to induce protection against sporozoite challenge when administered according to the same schedule and in comparable amounts to those of sporozoite-infected salivary glands (Nussenzweig *et al.*, 1969b; Spitalny and Nussenzweig, 1972). In contrast, Alger *et al.* (1972) reported that a certain percentage of mice which had been repeatedly injected intraperitoneally with non-infected mosquito salivary glands, did not become infected upon intraperitoneal sporozoite challenge. As these authors suggested, this might be due to a non-specific stimulation of cells in the peritoneal cavity, which could then interfere with the further development of the sporozoites used for challenge. In fact, the intraperitoneal route of infection provides considerably less consistent results than the intravenous injection of sporozoites of *P.berghei* (Vanderberg *et al.*, 1968).

Immunization with irradiated sporozoites injected intravenously produced a significantly greater percentage of protected animals and a minimal degree of variation from experiment to experiment (Spitalny

and Nussenzweig, 1972). Mice immunized by other routes (intramuscularly, intraperitoneally or intradermally) exhibited much lower levels of protection.

Immunization with irradiated sporozoites of *P.berghei* protected extensively against challenge with sporozoites of *P.vinckei* and *P.chabaudi* and the reverse was also true (Nussenzweig *et al.*, 1972). By contrast, in human malaria there is strict species specificity between the immunizing and the challenging sporozoite species. Cross-protection is observed between different geographic strains of the same species (Clyde *et al.*, 1975). CSP and indirect fluorescent antibody tests also demonstrate species specificity among the simian malarias (Chen *et al.*, 1976; J. McNamara, unpublished).

This immunity is strictly stage specific since it does not affect the development or viability of the erythrocytic stages in mice immunized with *P.berghei* sporozoites (Nussenzweig *et al.*, 1969b). Furthermore, oocyst sporozoites of *P.berghei* fail to induce protection (Vanderberg *et al.*, 1972).

3. Killed sporozoites

Alger *et al.* (1972) and Spitalny and Nussenzweig (1972) reported that the repeated injection of heat-inactivated sporozoites of *P.berghei* induced a variable degree of protection in mice. These authors obtained minimal or no protection upon immunization with frozen-and-thawed sporozoites.

Beaudoin *et al.* (1975) reported a significant degree of protection in mice repeatedly injected with both irradiated and non-irradiated *P.berghei* sporozoites killed in formalin. Our own observations indicate that formalinized or lyophilized sporozoites of *P.berghei* are poorly immunogenic since mice immunized with these sporozoite preparations showed minimal or no protection upon challenge (A. H. Cochrane *et al.*, unpublished).

All immunization attempts using sporozoites disrupted by sonication or homogenization have failed to induce a significant degree of protection. Considerable protection has been achieved, therefore, only when the sporozoites used for immunization were intact. Perhaps intact sporozoites retain their capacity to undergo some further partial development within the rodent host or they elicit a different immune response from that produced by disrupted sporozoites.

D. Comparison of the Basic Characteristics of Blood Stage and Sporozoite-induced Protection

One basic difference in the protective response induced by immunization with these different developmental stages is the extent of the protection and the response to challenge. Animals effectively immunized with sporozoites, at least in the rodent system, acquire "sterile immunity", i.e. no patent or subpatent parasitaemias occur upon challenge. However, those immunized animals which do become patent upon challenge invariably die of the infection after a prolonged prepatent period.

Protection induced by immunization with blood stages, on the contrary, is usually partial. The immunized animals present upon challenge a very mild, usually short-lived infection, from which they recover.

Another rather important difference is the range of the specificity of protection. Animals immunized with blood forms of rodent malaria are usually protected against the plasmodia species used for immunization, but remain fully susceptible to challenge with the other rodent malaria species. In the case of sporozoite immunization, protection is also directed against sporozoites of the other murine malaria parasites.

The understanding of these and other basic differences in the immune response to erythrocytic parasites and sporozoites will depend on greater knowledge of the mechanisms of resistance and the antigens involved.

E. Use of Adjuvants in Rodent Malarial Immunization

Adjuvants have been employed widely in potentiating the immune response of hosts to various parasite antigens. Freund's complete adjuvant (FCA) has been used with inactivated parasites or fractions of avian plasmodia (Freund *et al.*, 1945a; Coffin, 1951) and primate plasmodia (Freund *et al.*, 1945b, 1948; Targett and Fulton, 1965; Brown *et al.*, 1970; Phillips *et al.*, 1970; Schenkel *et al.*, 1973; Mitchell *et al.*, 1974, 1975; Simpson *et al.*, 1974; Brown and Tanaka, 1975). Results of these studies, although variable, show that FCA can enhance the protective effect of the malaria antigens.

However, relatively little work has been done with rodent malaria antigens in conjunction with adjuvants. Desowitz (1975) found that protective immunity as demonstrated by lower mortality rates, reduced

parasitaemias, and a shortened course of infection was induced by soluble, erythrocytic stage, *P.berghei* antigens administered with a series of adjuvants. These adjuvants included saponin, hexylamine, *Bordetella pertussis* vaccine, levamisole and polyinosinic-poly-cytidylic acid (poly I:C). The same antigens, combined with Freund's complete adjuvant, bacterial endotoxin, vitamin A, polyadenylic-polyuridilic acid (poly A:U) failed to induce any significant degree of protection.

Administration of *Corynebacterium parvum*, a potent RES stimulant, prior to a single dose of irradiated sporozoites protected a considerable number of A/J mice against challenge with viable sporozoites of *P.berghei* (S. Nardin, unpublished). Most animals failed to develop parasitaemia, and those which did showed a significantly increased prepatent period. In control animals, the single injection of *C.parvum* alone or irradiated sporozoites alone provided minimal levels of protection. The animals which had been effectively immunized by administration of *C.parvum* and a single dose of irradiated sporozoites, did not have detectable sporozoite-neutralizing activity in their sera at the time of challenge.

F. Non-specific Protection Against Malarial Infection

A variety of substances has been shown to induce a degree of protection against blood-induced and/or sporozoite-induced rodent malaria infections. Thus, Martin *et al.* (1967) and MacGregor *et al.* (1969) found that pre-treatment with *Escherichia coli* endotoxin had a moderately suppressive effect on the course of blood-induced *P.berghei* infections of rats and mice.

Mice injected intraperitoneally with Freund's complete adjuvant and then challenged two weeks later with *P.berghei*-parasitized erythrocytes, showed a low parasitaemia during the first week following challenge. In those animals which survived the infection, peak parasitaemia was reached late in infection and then sharply declined (Jerusalem, 1968).

There is some evidence that interferon may play a non-specific role in protection against infections of rodent malaria. Schultz *et al.* (1968) used Newcastle disease virus as an interferon inducer. They found the mean survival time of mice treated and then challenged with *P.berghei*-infected red blood cells to be significantly greater than that of control animals.

Recently, Clark *et al.* (1976) used live BCG and obtained marked

protection in CBA mice against the mild strain of *P.yoelii* (17 X) challenge and a somewhat reduced protection against *P.vinckei*.

Mice pre-treated with heat-inactivated *C.parvum*, were significantly protected against a subsequent challenge with sporozoites of *P.berghei* (Nussenzweig, 1967). A variable percentage of these mice failed to develop parasitaemia and, in others, the length of the prepatent period was considerably increased. *C.parvum* also affected the course of the blood infection, since some animals which became patent survived. In a series of papers, Jahiel *et al.* (1968a, b, 1969, 1970) showed that three interferon inducers, Newcastle disease virus, statolon and a double-stranded copolymer of polyriboinosinic acid and polyribocytidylic acid (poly I:C), protected mice either totally or partially against *P.berghei* sporozoite challenge. The data indicated that the stage most sensitive to the protective effect of the interferon inducers occurred late in exoerythrocytic development.

VI. IMMUNOPATHOLOGY

Much of the immunopathology associated with malaria infection in rodents appears to derive from the presence of soluble malaria antigens and the synthesis of antibody to these antigens, followed by the formation of immune complexes. The subsequent activity of these complexes and their persistence in the circulation and tissues may be responsible for many of the pathological sequelae of this infection. In addition to soluble parasitic antigens, the possibility that host antigens are altered by the parasite or the disease process, and that an immune response to these altered host components ensues, must be considered.

The fact that many of the phenomena discussed below are also found in other host–malaria associations supports the hypothesis that a common pathological process is involved. Differences in the pathology observed in various infected hosts may be attributable to differences in the course of infection. These may depend both on the plasmodial and the host species, the age of the host, its genetic constitution and environmental factors.

A. Plasmodial Antigens

The presence of soluble circulating malaria antigens in mice was demonstrated by Seitz (1972) by double diffusion in agar of sera from

P.berghei-infected mice tested against sera of mice which had recovered from this infection. The occurrence of two or three different plasmodial antigens was demonstrated in this way. Multiple antigens were also demonstrated by countercurrent electrophoresis, a faster and more sensitive technique (Seitz, 1975). Similarly, soluble malaria antigens have been demonstrated in monkeys infected with *P.knowlesi* (Eaton, 1939; Cox, 1966) and with *P.malariae* and *P.falciparum* (Lambert and Houba, 1974), and in humans with *P.falciparum* infections (McGregor *et al.*, 1968; Wilson *et al.*, 1969, 1973, 1976a, b).

The importance of these antigens resides not merely in their occurrence in the serum, but in their persistence during the course of infection. While this persistence has not been directly demonstrated in the rodent system, it occurs, for example, in human infection with *P.malariae*, in which considerable antigen is released, which elicits a strong immunopathological response (Cohen, 1973) evidenced by deposition of immune complexes in the kidney.

B. Antibodies

The occurrence of serum antibodies during malaria infections has been amply documented and discussed on pp. 257–260. In relation to immunopathology, it should be pointed out that the antibody response is usually of greater magnitude than might be expected on the basis of the observed protection and the amount of antibody known to be specific to malaria antigens. Cohen (1974) has pointed out that in chronic malaria high levels of IgG are produced which are not anti-malarial and that this may account for the occurrence of cold agglutinins and immunoconglutinins during infection. The presence of cold agglutinins, for example, has been documented during the course of infection in rodents and will be discussed in more detail.

C. Circulating Immune Complexes

The occurrence of circulating immune complexes is very likely the consequence of the presence of malaria antigen and antibody in the circulation. This has been demonstrated in humans (Houba and Lambert, 1973) by precipitation of ^{125}I-labelled antibody to *P.malariae* in sera of Nigerian nephrotics by 7·5% polyethylene glycol. This method was described for precipitation of Clq-bound immune com-

plexes (Clq is a component of complement) in 2·5% polyethylene glycol (Nydegger *et al.*, 1974).

Circulating immune complexes have not been demonstrated directly in rodents, but the changes in complement levels, the deposition of immune complexes in the kidney, and the occurrence of erythrocyte-bound antibody and anaemia all suggest that circulating immune complexes probably occur during the infection.

D. Changes in Complement Levels

Alterations in complement levels of rodents infected with *P.berghei* were first observed by Fogel *et al.* (1966). Recently Krettli *et al.* (1976) measured complement levels as a function of the solubilization of immune complexes (complement release activity or CRA), an activity of complement which depends on the alternative pathway of complement fixation (Miller and Nussenzweig, 1975; Takahashi *et al.*, 1976). After an initial brief increase, a progressive decrease in CRA was observed which could be positively correlated with degree of parasitaemia. In addition, when C3 levels were measured directly by immunodiffusion, they were observed to follow the same pattern, a transient initial increase and then a progressive decline. The authors suggested that immune complexes may consume complement in these mice and, indeed, these sera had an anti-complementary effect on normal sera. Complement-containing immune complexes may subsequently be deposited in the tissues. Observations on the kidneys of *P.berghei*-infected animals suggest this happens.

Similarities and differences between the changes in the levels of individual complement components in malaria-infected mice and other host systems may be of importance. Fogel *et al.* (1966) observed a drop in total complement levels of monkeys, hamsters and chickens infected with *P.knowlesi*, *P.berghei* and *P.gallinaceum*, respectively, indicating that components of the classical pathway of complement activation were altered as well. More specifically, Cooper and Fogel (1966) noted a drop in Cl, 2, 4 and 3 following merozoite release in monkeys infected with *P.knowlesi*. Recently, levels of C4 were found to be diminished in monkeys infected with *P.coatneyi*, and this diminution was dependent on both release of merozoites and the presence of antibody (Glew *et al.*, 1975). Similarly, in *P.vivax* infections of man, complement was depleted during paroxysms when parasitaemia was high and antibody was

present (Neva *et al.*, 1974), and C3 levels were depleted in humans infected with *P.falciparum* (Greenwood and Brueton, 1974; Srichaikul *et al.*, 1975). Interestingly, in infections of another blood protozoon, *Babesia rodhaini* in rats, Chapman and Ward (1976) observed a sharp decline in C3 due to an increased catabolic rate and a decreased synthetic rate of this complement component.

In contrast to findings in the mouse and man, C3 and C6 are not depleted in monkeys infected with *P.coatneyi* (Atkinson *et al.*, 1975). While activation of the alternative pathway (certainly) occurs in mice (Krettli *et al.*, 1976), this does not exclude the possibility that the classical pathway is also activated. It is of interest that human autologous red blood cell stroma can activate the alternative pathway, and it has been suggested that this may occur intravascularly when erythrocytes are damaged (Poskitt *et al.*, 1973). In addition, the alternative pathway is thought to be activated during chronic glomerulonephritis (Hunsicker *et al.*, 1972), and malaria nephropathy appears to be characterized by this type of kidney lesion.

In monkeys and humans it seems clear that immune complexes consisting of newly released merozoites and antibody are in part responsible for complement fixation, and this may also be so in rodents since CRA was related to the degree of parasitaemia. Whether there are alterations in synthetic and catabolic rates of complement components during malaria is still not known.

E. Deposition of Immune Complexes in the Kidney

Malaria antigen has been detected in the liver, spleen and kidneys of Swiss albino mice following infection with *P.berghei* using fluoresceinated antibody to *P.berghei* (Boonpucknavig *et al.*, 1973a). More detailed observations of the kidney (Boonpucknavig *et al.*, 1972) showed that during the second week of infection, granular deposits of antigen were found along the walls of the capillaries of the glomeruli and in the mesangial areas. Sometimes the lumens of the capillaries were occluded by antigen or erythrocytes containing antigen. Three weeks after infection the amount of antigen deposited in the kidneys seemed somewhat diminished.

Mouse globulin and C3 were detected in the glomeruli of the kidney by immunofluorescence seven days after infection, in a similar pattern to that of antigen deposition (Boonpucknavig *et al.*, 1972). The deposits

were increased in amount two and three weeks after infection. Antibody, eluted from sections of kidney, could bind to *P.berghei*-infected cells, demonstrating its specificity.

Our own observations corroborated the presence of deposits of gamma-globulin in the glomeruli of *P.berghei*-infected A/J mice (H. J. Lustig *et al.*, unpublished). Using fluorescein-conjugated rabbit anti-mouse globulin, these granular deposits were detectable after the first week of infection, and increased progressively as the parasitaemia rose during the two subsequent weeks.

These observations are similar to findings in the development of glomerulonephritis of NZB/NZW mice. In this syndrome, antinuclear immune complexes and C3 are deposited in capillary walls and in the mesangium in a granular pattern (Lambert and Dixon, 1967).

Immunofluorescence studies of kidneys of patients with malaria show deposits of immune complexes in the kidney, again suggesting a similar pathogenesis. Ward and Kibukamusoke (1969) observed deposits of IgM, IgG, IgA, complement and fibrin in the kidneys of East Africans with nephrotic syndrome following infection with *P.malariae*. Malaria antigen was detected in three of ten cases. Kidney biopsies of nephrotic Nigerian children, showed that virtually all had immunoglobulin deposits; many also had C3, and one-third had demonstrable *P.malariae* antigens in the glomeruli. A coarse, granular immunofluorescence pattern was usually associated with the deposition of IgG_3 and complement, while a finer granular deposit was noted with IgG_2 and absence of complement (Houba and Lambert, 1973).

Experimentally, sera of patients with *P.malariae*, which had been labelled with ^{125}I, bound to kidneys of nephrotics, while normal sera did not (Houba and Lambert, 1973). In addition, labelled sera of *P.malariae* patients bound preferentially to kidneys of monkeys infected with *P.malariae* rather than *P.falciparum*, demonstrating the specificity of the binding (Lambert and Houba, 1974).

Immunopathological findings in the kidney, associated with malaria, are characterized by deposition of immune complexes leading to chronic glomerulonephritis, rather than changes associated with deposition of antibody to glomerular basement membrane (Dixon, 1972). However, in some instances deposits of IgG and complement have been found in proximal tubular cells (Houba and Lambert, 1973) suggesting that part of the response in humans may be autoimmune. The question of whether the deposition of immune complexes causes

kidney damage and then initiates an autoimmune response to kidney antigens or other "self" antigens has not been resolved.

The morphological changes associated with the deposition of immune complexes in mice (Boonpucknavig *et al.*, 1972, 1973b) and in humans (WHO, 1972) have been described in detail. The implications of these findings are that the injury to the basement membrane is mediated by immune reactions between parasitized erythrocytes, and macrophages and polymorphonuclear cells sequestered against the glomerular epithelial cells, as well as by the accumulation of soluble immune complexes.

F. Alterations of Kidney Function

Abnormalities associated with kidney function have been studied in malaria-infected mice (Miller *et al.*, 1968) and hamsters (Sesta *et al.*, 1968). In mice there were progressive changes beginning with haemoglobinuria, then elevated blood urea nitrogen (BUN), and a drop in the percentage excretion of phenosulphonephthalein. The cortex had a diminished blood content suggesting a decrease in blood flow due to lower solute excretion or intrarenal shunting. In hamsters uremia and haemoglobinuria were observed. The kidneys showed haemosiderosis and accumulation of fatty acids. An increased BUN has also been observed in monkeys infected with *P.knowlesi*. These animals also showed haemoglobin reabsorption droplets in the proximal tubules (Rosen *et al.*, 1968).

G. Malaria-associated Anaemia

There are probably several causes for the anaemia associated with malaria infections. Clearly one factor is the lysis of infected erythrocytes during release of merozoites. However, as was suggested by Zuckerman (reviewed in 1964a) there are other possible causes: antimalarial drugs may cause haemolysis of red cells; malaria antigens may coat the erythrocytes, followed by absorption of antibodies to these antigens; heterophile or autoantibodies to red cells might be present and the host cells may become more fragile or altered in some other way by plasmodia. In addition, there may be partial inhibition or an inadequate level of haemopoiesis. Observations in rodents have suggested that heterophile or autoantibodies against red cells are produced,

and/or that antigen–antibody complexes may coat red cells during infection. This, plus the malaria-induced stimulation of the reticulo-endothelial system could explain the increased ingestion of red blood cells. In addition, there is evidence that red blood cells are more fragile during infection with *P.berghei* (Seed *et al.*, 1976).

Zuckerman (1960) found that in rats infected with *P.berghei* the loss of erythrocytes exceeded the percentage of infected cells, particularly following the parasitic crisis. Using an antiserum against rat serum, peripheral blood cells from infected rats were agglutinated. Treatment of rats with phenylhydrazine-HCl, cardiac bleedings or infection with *P.vinckei*, all of which produced reticulocytosis in rats, induced a Coombs positive reaction with the anti-rat serum (Zuckerman and Spira, 1961). However, it was noted that this test could not distinguish between the possibilities that autoantibody was present or that a globulin-like site was exposed on these cells (as suggested by Jandl, 1960), or that there was a different basis for the Coombs positive reaction in infected and uninfected animals. Cox *et al.* (1966) observed that during infection of rats with *P.berghei*, uninfected as well as infected erythrocytes were phagocytosed in the spleen and bone marrow. In addition, agglutinins for trypsinized rat and human erythrocytes were present in the sera of these rats. Kreier *et al.* (1966) also found antibodies to trypsinized erythrocytes in rats infected with *P.berghei* and noted that these antibodies were cold-reactive. These findings were not corroborated by George *et al.* (1966).

We have detected cold-reacting antibodies consistently during *P.berghei* infections of A/J mice. Using a rabbit antiserum to gamma-1 and gamma-2 chains of mouse immunoglobulin, agglutination of peripheral blood cells of infected mice can be detected from the twelfth day of the infection. The titre of agglutination increases as the parasitaemia rises. Purified ^{125}I-labelled antibody to mouse gamma chains was observed to bind to the blood cells of these mice. This binding could be detected as early as the third day after infection. Autoradiographs revealed label over both infected and uninfected reticulocytes, but not on mature uninfected erythrocytes (Lustig *et al.*, 1977). These findings do not permit one to distinguish between the possibilities of autoantibodies to reticulocytes, the exposure of a globulin-like moiety on reticulocytes or the passive binding of immune complexes to these cells. Determination of the binding specificity of these antibodies may answer this question.

The development of anaemia in NZB mice presents some apparent similarities to malaria anaemia. An antigen associated with murine leukaemia virus appears in the circulation when the mice are about three months old. Red cells of these mice become Coombs positive at the time that the antigen appears. Later this antigen is eliminated from the circulation and glomerulonephritis ensues (Mellors *et al.*, 1968).

As with the other immunopathologic phenomena described in rodent malaria, anaemia and heterophile or autoantibodies and immune complexes have been observed in other plasmodia–host associations. McGhee (1960) observed destruction of polychromatophil erythrocytes in ducklings when parasitaemia was low. In *P.lophurae* infection of chickens, a haemagglutinin for aged chicken erythrocytes, and normal or trypsinized human erythrocytes, was detected (Barrett *et al.*, 1970). Soni and Cox (1974, 1975a, b) have described factors in the serum of malaria-infected chickens which produce anaemia and kidney damage, when injected into normal chickens. Certain of these factors could be eluted from infected cells, and one factor was a cold-reacting haemagglutinin.

Coombs positive anaemia has also been described in a case of human *P.vivax* infection (Barrett-Conner, 1967). In addition, Rosenberg *et al.* (1973) observed IgM antibodies to red blood cells, in patients with *P.falciparum* infections, which were associated with anaemia and reduced survival time of normal red blood cells. Heterophile antibodies to guinea pig, rat and human erythrocytes were found in the sera of Gambians having a high incidence of malaria infection (Kano *et al.*, 1968). Topley *et al.* (1973) suggested that malarious red cells might be coated with C3, since they observed that these red cells were occasionally agglutinated by antisera to C3, and some patients had elevated immunoconglutinin titres. A perhaps analogous finding has been made in humans with rheumatoid arthritis and systemic lupus erythrematosus. These patients were shown to have cold-reacting antibodies present on circulating lymphocytes (Winchester *et al.*, 1974).

Taken together, these findings strongly suggest that either immune complexes, autoantibodies or both, are present on peripheral blood cells, and that these contribute to the pathogenesis of malarial anaemia.

Anaemia might also enhance infection with malaria by providing immature red cells preferred by certain strains of murine plasmodia. Ladda and Lalli (1966) demonstrated that mice which were made polycythaemic by hypertransfusion, developed lower grade infections of

P.berghei which were often rapidly cleared. In addition, hypertransfusion during the course of an otherwise lethal infection allowed 90% survival of mice infected with *P.berghei* (Hejna *et al.*, 1974).

H. Cerebral Malaria

Rodents provide several experimental models for the investigation of cerebral involvement in malaria, often associated with human *P.falciparum* infections. Cerebral lesions can be prevented in *P.berghei* infections of hamsters by neonatal thymectomy of the animals prior to infection (Wright, 1968) or by treatment of hamsters with antithymocyte antiserum (Wright *et al.*, 1971). It was suggested that in the presence of an intact thymus, antibodies were produced which caused agglutination of infected cells, particularly in the capillaries of the cerebrum.

Yoeli and Hargreaves (1974) reported on the enhancement in virulence of a mild strain, *Plasmodium yoelii* (17 X) after deep-freeze storage. The strain causes fulminating infections leading to the death of infected mice in 6 to 7 days. The brains of these mice showed petechial haemorrhages, and brain squash preparations revealed blockage of capillaries by infected erythrocytes. In addition, Mercado (1965) observed that a strain of *P.berghei* (KBG 173), after passage in mice, produced a paralytic effect in rats. The brains of these rats were also haemorrhagic.

It is still unclear what change in the host or parasite leads to the involvement of the brain. Electron microscopic studies indicate changes in the infected erythrocyte membrane which may enhance trapping of these red blood cells in the capillaries of the brain (Aikawa *et al.*, 1972). In humans it has also been shown that involvement of the brain during *P.falciparum* infection is associated with intravascular fibrin deposition, evidenced by the presence of fibrin degradation products (Reid and Nkrumah, 1972; Jaroonvesama, 1972).

VII. IMMUNOSUPPRESSION

The immunosuppression which occurs during the course of rodent malaria infection has recently been comprehensively reviewed by Wedderburn (1974). Much of the information on this subject has been obtained using *P.yoelii*, which in mice normally produces a self-limiting

infection. Humoral responses were found to be depressed upon administration of a series of unrelated antigens, including tetanus toxoid, aggregated human gamma-globulin (Barker, 1971; Greenwood *et al.*, 1971; Voller *et al.*, 1972) and sheep erythrocytes (SRBC) (Salaman *et al.*, 1969). A decreased number of haemolytic plaque-forming cells to SRBC was observed in the spleens of malaria-infected mice (Salaman *et al.*, 1969).

The humoral immune response to some other antigens, however, such as bacteriophage φX 174, keyhole limpet haemocyanin or human serum albumin given in conjunction with pertussis vaccine, is unaltered by the plasmodial infection. Immunization of *P.yoelii*-infected mice with human transferrin resulted in a normal rate of antibody formation (Stewart and Voller, 1973) but the antibody was of lower affinity than in normal mice.

Bomford and Wedderburn (1973) investigated the reciprocal effects of a concomitant chronic malaria and Moloney lymphomagenic virus (MLV) infection. The simultaneous injection of both agents into young adult mice produced an increased incidence of lymphomas after a considerably shortened prepatent period (Wedderburn, 1970). The malaria infection was exacerbated in the double-infected group, which had a longer lasting parasitaemia from which some of the animals failed to recover. Furthermore, the double-infected animals produced less virus-neutralizing antibodies than mice injected with MLV alone. This was shown to be due mainly to a selective suppression of IgG virus-neutralizing antibody (see Chapter 7).

Varying the schedule of inoculation of these two agents, MLV and *P.yoelii*, clearly revealed the importance of the relative timing of these infections in relation to tumour development. The incidence of lymphomas was maximal when the malaria inoculation preceded by a few days, or was given simultaneously with the MLV administration. Reactivation of the malaria process by reinoculation of the mice with another plasmodial species, *P.berghei*, plus simultaneous administration of MLV, also resulted in marked enhancement of lymphomagenesis (Wedderburn, 1974).

Results obtained after immunization of malaria-infected mice with SRBC were also reported (Wedderburn, 1974). In the self-limiting *P.yoelii* infection, the timing of the inoculation of the SRBC was of paramount importance in determining the level of the immune response, measured by the number of plaque-forming cells (PFC).

Compared to control animals, the number of PFC was maximally reduced when the RBCs were injected at the height of infection, and returned to normal levels during the later stages of the disease. This contrasted with the results obtained in *P.berghei*-infected animals, in which the anti-SRBC response remained low until their death. Again, it was mainly the IgG response which was considerably depressed.

The question of the possible effects of experimental malaria infections on skin graft rejection and skin sensitization to unrelated antigens is more controversial. Greenwood and colleagues (1971) found that contact sensitivity to picryl chloride or oxazolone was not depressed in *P.yoelii*-infected mice, and they also failed to observe any significant alteration of the time of rejection of allogeneic skin graft by these mice. However, Sengers *et al.* (1971) reported a significantly delayed rejection of rat skin by mice chronically infected with *P.berghei*.

More recently Jayawardena *et al.* (1975b) investigated the possible alterations of thymus-derived lymphocytes (T cells) during experimental rodent malaria infections and this has been discussed on pp. 000–00. In summary, *P.yoelii* infections were observed to induce massive T cell activation, development of protective immunity to malaria, and normal levels of response to PHA and oxazolone. *P.berghei*, in contrast, induced at first a limited degree of T cell activation, followed by generalized failure of T cell function.

Malaria infections have long been known to be associated with an increased activity of the reticuloendothelial system. In rodent malaria, in particular, this has been evaluated by the rate of colloidal carbon clearance which was increased in different rodent hosts and upon infection with different plasmodial species (Cox *et al.*, 1964; Lucia and Nussenzweig, 1969; McGregor *et al.*, 1969; Cantrell and Elko, 1970; Kitchen and Di Luzio, 1970).

Loose *et al.* (1972) investigated macrophage function in malaria-infected animals with regard to the inductive phase of the immune response. Sheep red blood cells (SRBCs) were injected into the peritoneal cavity of malarious and normal animals and then transferred to normal recipients. The macrophages obtained from malaria-infected mice induced a much lower number of plaque-forming cells (PFC) in the recipient. The authors postulated that this might indicate that malaria induces a defect in antigen digestion and/or processing by macrophages and that this might be, in part, the basis for malaria-induced immunosuppression. An additional macrophage alteration

observed in malaria-infected animals is the inability to detoxify endotoxin, so that endotoxin sensitivity is increased 41-fold in *P.berghei*-infected mice (Loose *et al.*, 1971).

Another possible mechanism for malaria immunosuppression is the occurrence of suppressor T cells. A. N. Jayawardena (personal communication) has recently obtained some *in vitro* results which could be interpreted on this basis. He observed that the *in vitro* response to SRBC, which was decreased in malaria-infected mice, was not depressed in cell suspensions obtained from malaria-infected nude mice. Furthermore, when spleen cells from malaria-infected mice were added to normal spleen cells, this resulted in inhibition of the normal cell response to SRBC.

Malaria also has a suppressive effect on the autoimmune syndrome exhibited by female NZB/NZW mice. Mice of this strain, infected with *P.berghei*, had lower mortality, less proteinuria and less severe kidney damage than uninfected controls (Greenwood and Voller, 1970a). The development of Coombs' positive anaemia, associated with NZB mice, was delayed in mice of this strain infected with *P.berghei*, but damage to the kidneys was as severe or more severe in infected mice (Greenwood and Voller, 1970b). It is thought that NZB/NZW mice are deficient in suppressor T cells and malaria infection may alter this defect.

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7. Concomitant Infections

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I. INTRODUCTION

It is rare for an animal to be completely free from any infection and almost as rare for it to harbour only one. Most rodents in the wild are hosts to a wide variety of infectious agents ranging from viruses to tapeworms; among laboratory mice and rats only those bred specifically "germ free" are without the most common infections and even they may

harbour occult viruses. Some of these infectious agents are patent and easily diagnosed, while others may be subpatent and require sophisticated techniques for their recognition. Most infections, especially those that are difficult to recognize, tend to be ignored by parasitologists working with a particular parasite and are regarded as some kind of background, similar to variations in the age, sex or diet of the host, which perhaps needs to be known about but is better ignored. It is only when a concomitant infection interferes with the one under investigation that it receives any attention and, because this has happened with malaria in rodents, the possibility of important interactions between malaria and other agents has been recognized. The range of interactions between infectious agents is considerable and it is probably true to say that there is no pathogen which cannot affect a malaria infection in some way and, conversely, be affected by the presence of a malaria parasite.

It is important to understand from the outset that an animal infected with a parasite is a different kind of animal from an uninfected one. This means that when a rodent is infected with malaria in the wild, or in the laboratory, the course of the malaria infection will, to a greater or lesser extent, be governed by the presence of other infectious agents. Practically nothing is known about the parasitic flora and fauna of the wild rodent hosts of the malaria parasites. What is known about the interactions between malaria and other infections has been derived largely from laboratory studies in mice and rats and, to a lesser extent, hamsters and a few laboratory-bred wild rodents. There have been relatively few studies on the interactions between malaria parasites and other organisms *per se* and most have been peripheral to some other kind of investigation. For this reason, many of the results obtained are difficult to interpret, but what does emerge is a pattern which is compatible with the hypothesis that other agents can alter the immunological responses to malaria and the physiological conditions necessary for the survival of malaria parasites and vice versa. Where the other agent adversely affects the immune response the malaria parasite is at an advantage, but when the other agent stimulates the response the malaria parasite may suffer. Similarly, an infectious agent that stimulates the production of the kind of blood cells for which a malaria parasite has a predilection causes an enhancement of the malaria, whereas the production of the wrong kind of blood cells depresses it. Most of the known interactions can be explained in terms of these

phenomena and, while it is possible that this is not the whole story, it is appropriate at the present juncture to restrict discussion to these areas of immunity and the availability of suitable substrate. Although immunity to rodent malaria is discussed in Chapter 6, it is necessary to touch on some aspects here, albeit superficially, as the discussion of the interactions between malaria parasites and other organisms will be based partly on current immunological ideas.

The infectious agents that interact with malaria parasites will be described group by group and observations relating to these interactions will be presented. General conclusions, together with an assessment of the underlying mechanisms of these interactions, will be left until the end of the chapter. The nomenclature of the malaria parasites will be as outlined in Chapter 1, and the species named in the original papers will be amended to bring them into line with the current classification. Subspecific names will only be used when essential, and *P.yoelii* will be used for *Plasmodium y.yoelii*, *P.chabaudi* for *P.c.chabaudi* and *P.vinckei* for *P.v.vinckei*. *P.berghei* is considered to be monotypic.

It is important at this stage to note that, until recently, it has been possible to work with a mixed infection of malaria parasites without actually being aware of it. Carter and Walliker (1975) have pointed out that one of the widely used strains of *P.chabaudi*, originating from the Central African Republic, actually consists of two species, *P.chabaudi* and *P.v.petteri*; thus some investigators may have been working with *P.v.petteri* or a mixture of *P.chabaudi* and *P.v.petteri* thinking that they were using *P.chabaudi*. The description of *P.vinckei* given by Bafort (1971) indicates contamination with *P.berghei*, a situation which others, including myself, have discovered independently. Again in my laboratory a strain thought to be *P.atheruri* in fact contains a *P.v.petteri* type parasite (D. Walliker, personal communication). While the use of clones and enzyme analyses now makes work with such contaminated strains inexcusable, it is possible that some of the interactions reported in the past resulted from using mixed species. It is now too late to interpret these results in any way other than that proposed by the original author and, except in the most obvious cases, no attempt will be made to do so. Similarly, although most workers are now aware of the effects on malaria of contaminating infections of *Eperythrozoon coccoides* and *Haemobartonella muris*, this has not always been so and even now some workers, usually those using *P.berghei* and *P.vinckei* on a casual basis, are unaware of the existence of these contaminants and

their effects. Unless the presence or absence of *E.coccoides* or *H.muris* is recorded there is little that can be done to determine whether or not one of these agents was present at the time of the experiment and, as with mixed species, no attempt will be made to read more into a paper than is actually written there.

II. ORGANISMS INVOLVED IN CONCOMITANT INFECTIONS

A. Viruses

1. Effects of viruses on malaria

The investigation of the effects of viruses on malaria has been concentrated on two aspects; the protective effects of viruses against malaria, based on the assumption that this may be due to the production of interferon or some similar substance, and the enhancement of malaria due to the immunodepressive effects of certain viruses.

The possibility that interferon may be protective against malaria has not been investigated using natural virus infections of rodents but, nevertheless, the observations that have been made are relevant to such infections. The first indications that viruses may suppress malaria came from the work of Yoeli *et al.* (1955) who found that blood-induced infections of *P.berghei* in mice were reduced or suppressed in animals exposed to West Nile virus, but were unaffected by Influenza A. This work was not followed up until 1968 when Jahiel *et al.* and Schultz *et al.* independently showed that Newcastle Disease virus protected mice against *P.berghei*. Jahiel *et al.* (1968a) used sporozoite- and blood-induced infections and found that protection was most marked when the virus was given 16–24 h after the sporozoites; parasitaemias then developed in only 13 out of 40 mice compared with all of the controls. After 48 h this protection was less marked and limited to an increase in the prepatent period. Blood-induced infections were not affected by the virus, and the authors suggested that protection was due to the production of interferon which acted on late primary exoerythrocytic stages. The evidence for interferon being the mediator of this protection is given in a separate paper (Jahiel *et al.*, 1968b). Schultz *et al.* (1968) also used Newcastle Disease virus as an interferon inducer and demonstrated protection against blood-induced *P.berghei* infections in mice

given the virus 2 h before or 12–15 h after the malaria parasites. Protection was limited to an increase in survival time from 7 to 9·2 days and was attributed to a direct effect of interferon on the erythrocytic stages. Evidence which suggests the contrary comes from the work of Gobert *et al.* (1971) who gave mice Newcastle Disease virus 7, 24, 48 or 100 h before a blood-induced infection with *P.berghei*. Survival time (but not survival rate) was increased, but this could not be correlated with interferon activity which reached a peak 8·5 h after the injections of the virus and could not be detected after 48–100 h.

When mice are exposed to murine oncogenic viruses which actually cause infections, protection against malaria is not seen and, instead, concomitant malaria infections are considerably enhanced. Most of the investigations into the nature of this phenomenon have been based on the use of naturally self-limiting infections with *P.yoelii*. Enhanced and often fatal infections with this parasite occur in mice infected with the Moloney leukaemogenic virus (MLV) (Wedderburn, 1970), the Rowson-Parr virus (RPV) (Salaman and Wedderburn, 1969; Cox *et al.*, 1974) and the urethane leukaemia virus (ULV) (Salaman *et al.*, 1969). ULV is vertically transmitted and therefore must exert its effect over a long period, but with MLV and RPV the greatest exacerbation occurs when the two agents are given at the same time or within a few days of one another. The interactions between RPV and *P.yoelii* have been extensively studied and the virus causes the greatest enhancement of malaria if given 4 to 5 days before it. No deaths occur in mice given RPV more than 8 days before, or 7 or more days after *P.yoelii*, and this period of enhancement of the malaria coincides exactly with the period of greatest immunodepression caused by the virus as determined by the depletion of plaque forming cells in the Jerne technique (Cox *et al.*, 1974). Similar results have been obtained with *P.chabaudi* (Figure 1). As the possibility that increased reticulocytosis might produce these results by favouring the development of *P.yoelii* appears to have been ruled out, it can be concluded that the enhancement of the malaria infection results from the immunodepression caused by the virus (Cox *et al.*, 1974; Wedderburn, 1974).

2. *Effects of malaria on viruses*

Although *P.berghei* in mice has been shown to induce the production of interferon which is effective against vesicular stomatitis virus and

Semliki Forest virus in L cells (Huang *et al.*, 1968), there is no evidence to suggest that malaria has an adverse effect on virus infections. On the other hand, the reverse is true. Congdon and Westcott (1972), following up the observations of Huang *et al.*, injected mice with influenza virus 48 h after *P.berghei* and found that antibody levels to the virus were depressed after 10 days; they were, however, unable to reproduce these

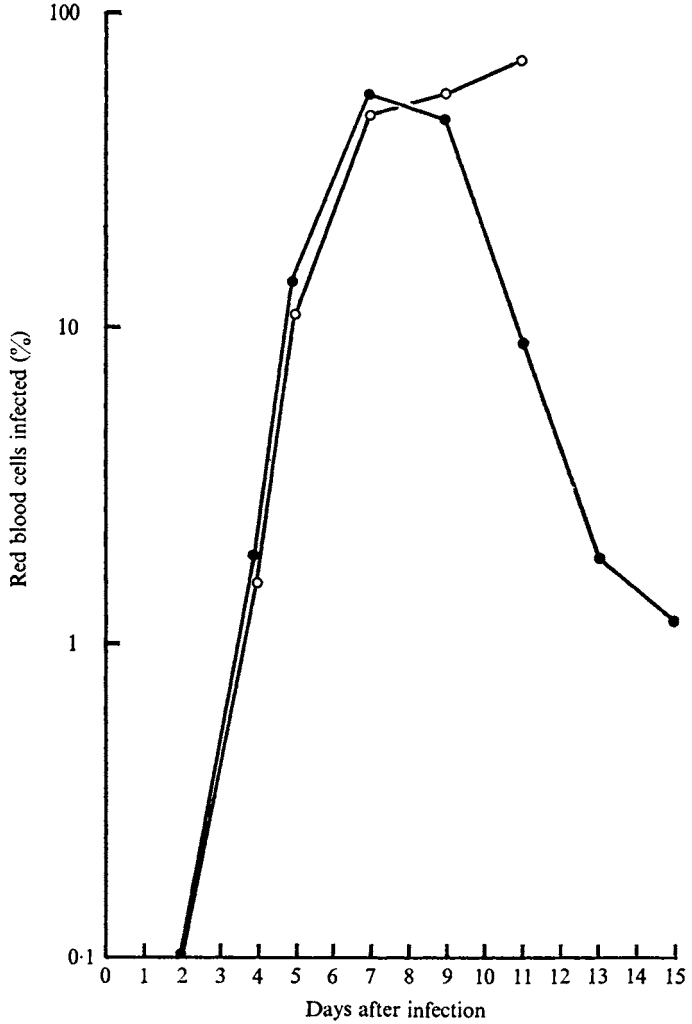

Figure 1. Parasitaemias in mice infected simultaneously with Rowson-Parr virus and *P.chabaudi* and *P.chabaudi* alone. ○———○, *P.chabaudi* and Rowson-Parr virus; ●———●, *P.chabaudi* alone (reproduced with permission, from Cox *et al.*, 1974).

results in a second experiment. Congdon and Westcott indirectly confirmed what had been known since 1969, that malaria parasites cause a generalized immunodepression (see Chapter 6), and mice with malaria are unable to mount a good immune response to viruses introduced during this period (Salaman *et al.*, 1969). The experimental evidence for this comes mainly from studies on mice infected with *P.yoelii* and MLV. In mice simultaneously infected with these two agents, the incidence and magnitude of lymphomas associated with the virus are considerably increased (Salaman *et al.*, 1969; Salaman and Wedderburn, 1970; Bomford and Wedderburn, 1973; Wedderburn, 1974) and antibody levels to the virus are decreased (Bomford and Wedderburn, 1973). In mice infected with *P.yoelii*, depression of the immune response to MLV is of limited duration. While mice given MLV 16 days before or 14 days after infection with malaria show few signs of lymphomagenesis (Wedderburn, 1974), animals infected with *P.yoelii* and challenged four weeks later with *P.berghei* and MLV do develop lymphomas (Wedderburn, 1974).

Increased spontaneous lymphomagenesis has also been recorded in mice with a *P.berghei* infection controlled with a para-aminobenzoic acid-free diet (Jerusalem, 1968). After 9 months, 18 out of 24 chronically infected mice had developed spontaneous lymphomas of the lymphoreticulocytic type. The agent causing the lymphomagenesis was not recognized but it was suggested that it was an oncogenic virus. As well as these aleukaemic lymphomas, Jerusalem (1968) has recorded the appearance of spontaneous murine leukaemias in mice chronically infected with *P.berghei*.

Mention must be made here of a latent "microorganism" that causes kidney damage in mice infected with *P.yoelii nigeriensis* (Suzuki, 1974). The agent has not been identified and it may be a virus, the effects of which are enhanced by the malaria infection, or it may be nothing at all to do with the parasite.

B. Bacteria Other Than Mycoplasmas and Rickettsiae

1. Effects of bacteria on malaria

There have been relatively few studies on the interactions between bacteria and rodent malarias although these must often co-exist in the wild. Much of what has been done has been concerned with the

possibility of protecting mice against malaria using bacteria or bacterial extracts to enhance phagocytic activity. Unfortunately this emphasis on phagocytic stimulation has tended to obscure other interactions which may be of more importance. These artificial situations are discussed briefly in this chapter because they probably do not differ in essence from the natural state and because of the light they throw on other interactions.

The interactions between the spirochaete *Borrelia duttoni*, which causes relapsing fever in shrews and rodents, and *P.berghei* in mice have been studied by Colas-Belcour and Vervent (1954). Mice were infected with the spirochaete and subsequently challenged with *P.berghei*. In general, the spirochaetes and the malaria had little effect on one another although a few of the doubly infected animals died earlier than expected. *P.berghei* sometimes brought about a recrudescence of the spirochaete infection and this was accompanied by a reduction in the *P.berghei* parasitaemia. Colas-Belcour and Vervent record similar findings for *Borrelia microti* and *B.merionesi* which are now regarded as synonyms for *B.duttoni*. Much the same kind of results were recorded by Sergent (1957) and Sergent and Poncet (1957) with *Borrelia hispanica* (which causes human Hispano-African relapsing fever) and *P.berghei* in rats. The spirochaetes had no effect on the malaria infection when the two organisms were given 1 to 17 weeks apart. When given together, 18 out of 22 rats suffered more severe infections and one died from *P.berghei*.

Intravenous injections of the living Bacillus Calmette-Guerin (BCG) strain of *Myobacterium bovis* protect mice against blood-induced *P.yoelii* or *P.vinckei* infections, 30 or more days later (Clark *et al.*, 1976). Protection was limited to a reduction of parasitaemia in 6 out of 8 mice infected with *P.yoelii*, and the survival of *P.vinckei*-infected mice after high parasitaemias that killed the control animals. The implications of these findings will be discussed later.

Bacterial endotoxins also confer some protection against *P.berghei* in rats and mice (Martin *et al.*, 1967). The lipopolysaccharide endotoxin of *Escherichia coli* was given to rats or mice one day before, or on the same day as infection with the blood stages of *P.berghei*. Parasitaemias were always slightly lower than in the controls and survival time was slightly longer. Although the results are statistically significant the protection recorded was minimal. Three injections of endotoxin were no better than one, intraperitoneal injections were better than intravenous and

the endotoxin had no effect if given 3 or 6 days before *P.berghei*. Michel (1975) obtained better results against sporozoite-induced infections of *P.yoelii* using a phospholipid bacterial extract given intravenously 24 h earlier. Parasitaemias failed to develop in most of the treated animals and this was attributed to enhanced phagocytosis. Nussenzweig (1967) also protected mice against sporozoite infections of *P.berghei* but used whole killed *Corynebacterium parvum* given intravenously 3 to 19 days earlier. Overall, 37 out of 71 mice did not succumb to infection and survival times were prolonged in some of those which did. This protection was attributed to phagocytic stimulation by *C.parvum*. It is unlikely that this is the whole explanation because Clark *et al.* (1977) were also able to protect mice against malaria parasites using *C.parvum* given intravenously or intraperitoneally 3 months or more earlier. After this time the effect of *C.parvum* on phagocytic activity is minimal. Mice were protected against blood-induced infections with *P.chabaudi* or *P.vinckei*, but not with *P.berghei*.

2. *Effects of malaria on bacteria*

Animals with malaria are more susceptible to infection with bacteria during the patent infection than are uninfected animals. Mice exposed to *Salmonella typhimurium* one day before or up to 5 days after infection with *P.berghei* die earlier than mice infected with either of these agents alone (Kaye *et al.*, 1965). Deaths were attributed to enhanced salmonella infections and the spleens of the mice contained 1×10^8 bacteria g^{-1} at death. Mice infected with *P.yoelii* exhibit a decreased antibody response to *Bordetella pertussi* (which causes whooping cough in man) on the seventh or thirteenth day of the malaria infection, and when such mice are challenged with living *B.pertussi* their survival rate is significantly reduced (Viens *et al.*, 1974). Similar results have been obtained by Voller *et al.* (1972) for tetanus toxoid in *P.yoelii*-infected mice. The antibody response to the toxoid is considerably reduced 4, 10 or 15 days after infection with *P.yoelii* and remains reduced even after a booster dose of the toxoid 28 days later.

C. Mycoplasmas

The mycoplasmas are separated from the other bacteria by the absence of a cell wall, and it is convenient to regard them separately in this

chapter because of the peculiar nature of the relationship which exists between one of them, *Mycoplasma neurolyticum*, and rats infected with *P.berghei*. Mercado (1965, 1973) noted that a paralysing syndrome associated with *P.berghei* in rats occurring 4 to 7 days after infection, and separated a filterable agent from the sera of these rats. This agent caused paralysis when given to rats with (non-paralysing) *P.berghei*, but not in other circumstances. Tully and Mercado (1972) assessed the available evidence and concluded that the agent must be *M.neurolyticum* although it could not actually be isolated and identified. The paralysing effect was most marked if the mycoplasmas were given to rats 7 to 8 days before infection with *P.berghei* and did not occur if the two agents were given together, or if the *P.berghei* were given first.

D. Rickettsiae: *Eperythrozoon and Haemobartonella*

The infectious agents interacting with malaria in rodents which have been the most intensively studied are the rickettsiae. This is because they commonly occur as subpatent infections in rats and mice and are only noticed when they interfere with the malaria. Rickettsiae are difficult to classify but they are now usually grouped with the bacteria (Kreier and Ristic, 1973). However, like the mycoplasmas, it is convenient to consider them separately. The most important species are *Eperythrozoon coccoides* in mice and *H.muris* in rats. *H.muris*, or organisms very similar to it, have occasionally been recorded from mice (see Figures 2 and 3). The biology of *E.coccoides* and *H.muris* has been described in detail by Kreier and Ristic (1968). Both organisms cause the same kind of disease characterized by anaemia with consequent reticulocytosis (Thurston, 1954), splenomegaly and erythrophagocytosis (Cox and Calaf-Iturri, 1976), and both remain dormant for months. Increased phagocytic activity is characteristic of *E.coccoides* infections (Gledhill *et al.*, 1965) and the rickettsiae induce a very good antibody response (Hyde *et al.*, 1972).

1. Effects of rickettsiae on malaria

Peters (1965) first recorded that mice simultaneously infected with *P.berghei* and *E.coccoides* survived longer (12 to 13 days) than mice with *P.berghei* alone (5 to 8 days). Lowered parasitaemias in mice with a strain of *P.berghei* contaminated with *E.coccoides* were observed by

Thompson and Bayles (1966). Similar results were obtained in more detailed studies by Ott *et al.* (1967) and Finerty *et al.* (1973) in conventional but not in germ-free mice. Thurston (1955) found that *E.coccoides* had no effect on *P.berghei* given 7 days later. Kretschmar (1963) also recorded negligible effects and Peters (1965) could detect no effects unless mice were infected with the two organisms at about the same time. Suntharasamai and Marsden (1969) found that prior infection with *E.coccoides* had no effect on *P.yoelii* in mice infected 18 h later.

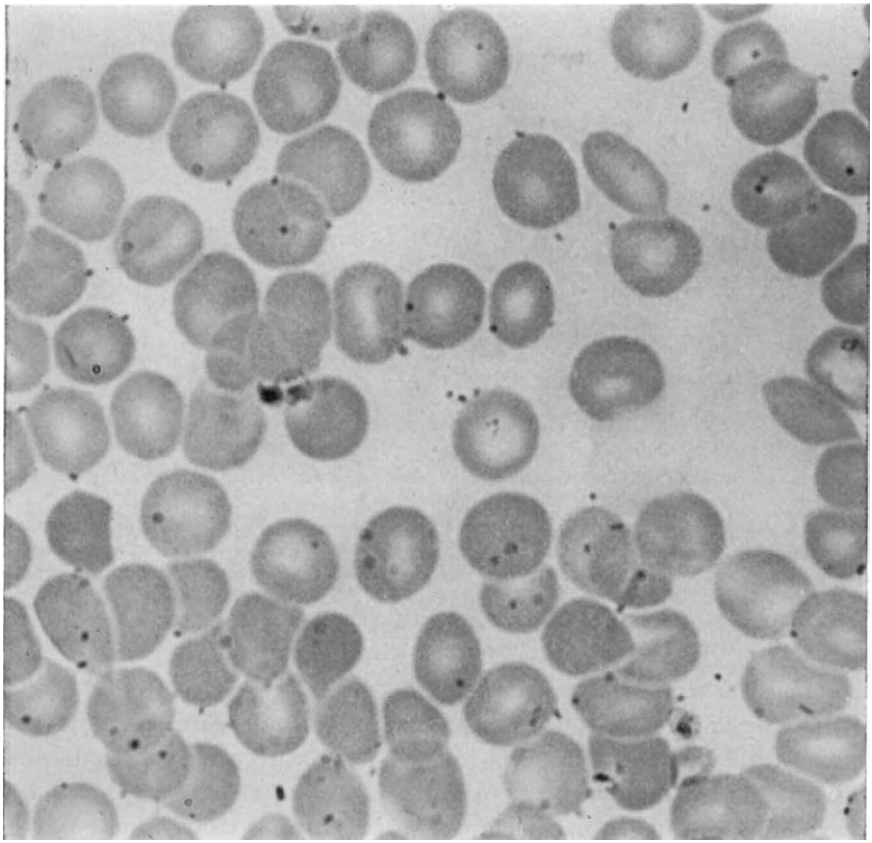

Figure 2. *E.coccoides* in the blood of a mouse. In Giemsa-stained thin films *E.coccoides* appears as red round- or rod-shaped bodies. The round forms often have clear centres. These disc-like bodies occur on red cells and also free in the plasma. *E.coccoides* appears early in mixed infections with malaria parasites and often disappears altogether within a few days.

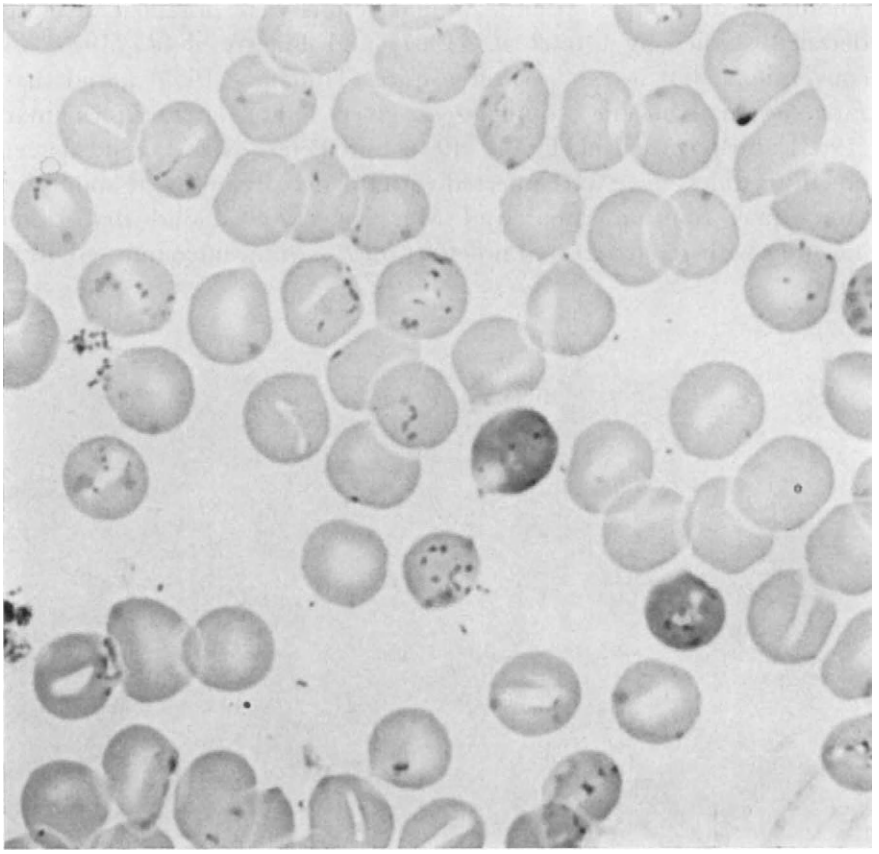

Figure 3. *H.muris* in the blood of a mouse. In Giemsa-stained thin films *H.muris* appears as red round, rod- or dumbbell-shaped bodies. The parasite is usually associated with red cells and is rarely seen free in the plasma. The round bodies never take on the disc-like appearance of *E.coccoides*. Like *E.coccoides*, *H.muris* appears early in mixed infections and can easily be missed. Latent infections of both *E.coccoides* and *H.muris* recrudesce after splenectomy.

P.chabaudi and *P.vinckei* are more affected by *E.coccoides* than *P.berghei* or *P.yoelii*, and *P.vinckei* infections which are usually fatal are milder in mice with *E.coccoides* (Cox, 1966). *P.chabaudi* has received a considerable amount of attention because much of the early work with this originally avirulent parasite was carried out with strains contaminated with *E.coccoides*. When the *E.coccoides* was eliminated the strains became more virulent (Peters, 1967), and the fatal patterns of infection resembled those caused by *P.vinckei* (Ott and Stauber, 1967). *E.coccoides* affected the outcome of *P.chabaudi* infections most when mice were infected

simultaneously but had little effect if given 3 or 6 days before (Ott *et al.*, 1967). Voller and Bidwell (1968) also noted the greatest effect when *E.coccoides* was given at the same time or one day before *P.chabaudi* but found some residual effects after 11 or 16 days. Virulence of *P.chabaudi* is not simply determined by the presence of *E.coccoides* for Cox (1970c) found that 85% of mice free from *Eperythrozoon* and *Haemobartonella* recovered spontaneously from infections with *P.chabaudi*, and that this recovery rate was not affected by anti-eperythrozoa drugs.

The effects of *H.muris* infections on malaria have received little attention. Hsu and Geiman (1952), in a paper that is very difficult to interpret, found that *P.berghei* infections were enhanced in rats with *H.muris* and this has been unambiguously confirmed by Smalley (1975). Mercado and von Brand (1962) found that metastatic calcification caused by high doses of dihydrotachysterol (Hytakerol) was reduced in rats with *P.berghei* and even more so in rats with *P.berghei* and *H.muris*.

In the only report of mixed infections of *E.coccoides* and *H.muris* in mice simultaneously infected with a malaria parasite, Peters (1971) found that in some mice both the *E.coccoides* and the *H.muris* infections were low and the *P.berghei* parasitaemias developed slowly, but that in others the two rickettsial infections reached early peaks and the *P.berghei* infection progressed rapidly.

It is tempting to try to explain the effects of *E.coccoides* and *H.muris* in terms of the availability of reticulocytes. Enhanced reticulocytosis would favour *P.berghei* and *P.yoelii*, most strains of which have a predilection for these cells, and retard infections with *P.vinckei* and *P.chabaudi* which require mature cells for their development (Ott *et al.*, 1967; Ott and Stauber, 1967; Cox, 1975). The importance of reticulocytosis on the outcome of *P.vinckei* and *P.chabaudi* infections has been clearly demonstrated in mice in which the output of reticulocytes has been enhanced with phenylhydrazine (Ott, 1968; Viens *et al.*, 1971) or depressed by beta-methasone (Cox, 1974). The effects of reticulocytosis on *P.berghei* infections are more difficult to understand. Increased reticulocytosis, resulting from infections with *E.coccoides* or *H.muris*, could account for the heightened infections recorded by Hsu and Geiman (1952) and Smalley (1975), but could hardly explain the milder infections recorded by Peters (1965), Thompson and Bayles (1966), Ott *et al.* (1967) and Finerty *et al.* (1973). Ott (1968) has suggested that an early lack of reticulocytes forces *P.berghei* to invade mature cells and this contributes to more intense infections than those

in mice with abundant reticulocytes. Thompson and Bayles (1966) noted that, when their strain of *P.berghei* was cleared of its contaminating *E.coccoides*, the malaria parasites occurred predominantly in mature cells. Peters (1965) was unable to find any correlation between reticulocytosis and the effects of *E.coccoides* on *P.berghei* and suggested that competition for metabolites might be a cause of the depressed malaria. The possibility that cross-immunity exists between *E.coccoides* and *Plasmodium* spp. is unlikely in view of the fact that Finerty *et al.* (1973) failed to identify any cross-reacting antigens using an indirect fluorescent antibody technique. It is equally unlikely that non-specific increased reticuloendothelial activity could account for the milder malaria infections in *E.coccoides*-infected mice as chemical stimulators of such activity have little or no effect on *P.berghei* infections (Jerusalem and Kretschmar, 1965; Bliznakov, 1971).

Contamination of strains of murine malaria parasites with *E.coccoides* or *H.muris* is relatively common and these organisms can ruin the most carefully planned experiments. *E.coccoides*, however, can easily be controlled by eliminating the vectors (Gledhill *et al.*, 1965), by using inocula containing less than 500 blood cells (Büngener, 1968) or by drug treatment (Thurston, 1955; Kretschmar, 1963; Thompson and Bales, 1966; Peters, 1967; McHardy, 1974).

2. *Effects of malaria on rickettsiae*

P.berghei infections cause recrudescences of latent *E.coccoides* infections in mice (Thurston, 1955) and *H.muris* infections in rats (Hsu and Geiman, 1952). This is presumably due to the immunodepressive effect of malaria.

3. *Bedsoniae*

The Bedsoniae are virus-like micro-organisms long regarded as viruses but now classified with the bacteria close to the rickettsiae. This group contains the so-called Ornithosis (Psittacosis) virus. Yoeli *et al.* (1955) regarded it as a virus and found that exposure to this organism reduced or suppressed *P.berghei* infections in mice in the same way as West Nile virus.

E. Other Species of Malaria

Until the middle of the 1960s it was assumed that recovery from rodent malaria protected the host against subsequent infection with the same species and that the immunity conferred was species specific. With the discovery of the relatively avirulent species, *P.chabaudi* and *P.yoelii*, more intensive investigations became possible and it was soon realized that a considerable degree of heterologous immunity exists between different species (Cox, 1975 and Table I).

Mice which have recovered from *P.chabaudi* infections are also immune to the more virulent *P.vinckei* (Cox and Voller, 1966; Nussenzweig *et al.*, 1966; Yoeli *et al.*, 1966; Cox, 1970a). Bafort (1969), however, found only limited protection against *P.vinckei*. *P.chabaudi* does not protect mice against *P.berghei* (Cox and Voller, 1966; Nussenzweig *et al.*, 1966; Yoeli *et al.*, 1966; Cox and Milar, 1968; Cox, 1970a) and this also applies to sporozoite-induced infections (Richards, 1968). There is no appreciable degree of protection between *P.chabaudi* and *P.yoelii* (Cox and Voller, 1966; Cox, 1970a) and this is also true for sporozoite challenge (Richards, 1968).

Mice which have been cured of infections with *P.vinckei* resist challenge with intraerythrocytic stages of *P.chabaudi* (Cox and Voller, 1966; Bafort, 1969; Cox, 1970a) but not of *P.berghei* (Cox and Voller, 1966; Cox, 1970a), although they are well protected against *P.yoelii* (Cox, 1970a). Rats which have recovered naturally from *P.vinckei* infections succumb to blood-induced *P.berghei* (Cox and Voller, 1966).

While *P.yoelii* partially protects mice against blood-induced *P.vinckei* (Bafort, 1969; Cox, 1970a; Hargreaves *et al.*, 1975) and provides a better protection against *P.chabaudi* (Cox, 1970a), this is not true when the challenge is sporozoite induced (Richards, 1968). Because of the close relationship between *P.yoelii* and *P.berghei* it might reasonably be assumed that there would be a considerable degree of protection between these species, but this is not so. Mice which have recovered from *P.yoelii* infections are usually susceptible to infection with blood-induced *P.berghei* (Demina *et al.*, 1969a; Barker, 1971; Oxbrow, 1973; Hargreaves *et al.*, 1975) although Cox (1970a) found some protection. Wedderburn (1974) and Wedderburn *et al.* (1975) obtained a degree of protection in the majority of animals in which the superimposed *P.berghei* infections assumed a chronic form. Richards (1968) showed

that mice immune to *P.yoelii* resist *P.berghei* sporozoite challenge. In rats, *P.yoelii* does not protect against *P.berghei* (Cox and Voller, 1966; Demina *et al.*, 1969a, b; Glazunova *et al.*, 1972).

Conflicting results have been reported from experiments in which mice cured of *P.berghei* have been challenged with *P.vinckei*. Cox and Voller (1966) and Bafort (1969) found little protection but, in a much more extensive study, Cox (1970a) found that the majority of mice immune to *P.berghei* did resist challenge with *P.vinckei*. Similarly, Cox and Voller (1966) found no protection against blood-induced *P.chabaudi* in mice immune to *P.berghei*, a result which was the same when the challenge was sporozoite induced (Richards, 1968). In later experiments, however, Cox (1970a) found that some animals were protected. *P.berghei* protects mice against *P.yoelii* (Cox, 1970a; Oxbrow, 1973), and this protection extends to sporozoite challenge (Richards, 1968) and is also seen in rats (Cox and Voller, 1966).

In addition to experiments with the four most frequently used rodent malarias, there have been a few observations with other species. Cox (1970a) found that *P.atheruri* protected mice against *P.chabaudi* and (to a lesser extent) *P.vinckei* and *P.yoelii*, but not against *P.berghei*. Protection against *P.atheruri* was conferred by *P.chabaudi* and, to some degree, by *P.vinckei*, *P.berghei* and *P.yoelii*. Oxbrow (1973), in addition to the observations discussed above, found that *P.berghei* protected against *P.y.killicki* and *P.y.nigeriensis*; that *P.y.yoelii* protected against *P.y.killicki* but not *P.y.nigeriensis*; that *P.y.killicki* protected against *P.y.yoelii* and *P.y.nigeriensis* but not *P.berghei* and that *P.y.nigeriensis* protected against *P.berghei* and all subspecies of *P.yoelii*.

From time to time, avirulent strains of virulent species, or virulent strains of normally avirulent species, have been obtained. The immunity which exists between the derived and parent strains is of the normal homologous type. Avirulent strains of *P.berghei* protect against virulent strains (Weiss, 1968; Kretschmar, 1969) and similar results have been obtained with *P.vinckei* and *P.chabaudi* (Kretschmar, 1969). Avirulent strains of *P.yoelii* protect rats and mice against virulent strains of the same species, but not against *P.berghei* (Demina *et al.*, 1969a; Glazunova *et al.*, 1972; Hargreaves *et al.*, 1975). The avirulent and virulent strains of *P.yoelii* can co-exist in rats (Glazunova *et al.*, 1972).

All these observations relate to the challenge of animals recovered from infection with another malaria parasite. It is difficult to determine which of these animals harboured latent infections of the original

Table I

Percentage of mice protected by prior infections with homologous or heterologous blood parasites

Immunizing infection	Challenged with							
	P.vinckei	*P.chabaudi*	*P.berghei*	*P.yoelii*	*P.atheruri*	*B.rodhaini*	*B.microti*	*A.garnhami*
P.vinckei	100	70	7	100	50	100	100	92
P.chabaudi	80	100	0	34	100	60	100	100
P.berghei	90	40	80	56	50	66	60	58
P.yoelii	56	34	34	100	17	75	56	58
P.atheruri	17	83	0	67	100	75	75	50
B.rodhaini	95	40	0	0	83	100	100	100
B.microti	90	75	7	0	67	100	100	100
A.garnhami	53	67	0	20	67	100	100	100

Reproduced with permission, from Cox (1975a).

Table II

Fluorescent antibody titres obtained using blood parasites and homologous and heterologous antisera

Antiserum to	Antigen						
	P.vinckei	*P.chabaudi*	*P.berghei*	*P.yoelii*	*P.atheruri*	*B.rodhaini*	*B.microti*
P.vinckei	2560	640	640	320		160	160
P.chabaudi	640	2560	320	160	80	10	10
P.berghei	320	160	2560	320		160	80
P.yoelii	40	80	160	2560		0	0
P.atheruri		160			2560		
B.rodhaini	20	20	40	20		2560	80
B.microti	40	40	40	20		40	2560

Reproduced with permission, from Cox (1975a).

parasite and which had totally eradicated the infection but, in practice, this does not affect the outcome of the challenge (unpublished).

P.chabaudi does not protect against *P.vinckei* given at the same time but does if given as little as one day before (Nussenzweig *et al.*, 1966). In mice simultaneously infected with *P.berghei* and *P.vinckei* (which do not usually cross-protect) the *P.berghei* infection is retarded but all the animals rapidly die (Yoeli and Sklarsh, 1970; Bafort, 1971).

It is difficult to explain all the patterns of cross-immunity recorded and conventionally it is convenient to look for cross-reacting antigens. These have been extensively studied using the indirect fluorescent antibody technique (Cox, 1970b; Cox and Turner, 1970; see Table II) and could account for most of the protection recorded, but are difficult to correlate with the general lack of protection against *P.berghei*. This subject will be discussed again on p. 335.

F. Piroplasms

1. Effects of piroplasms on malaria

A considerable degree of heterologous immunity exists between the malaria parasites and piroplasms of rodents (Cox, 1975). Mice which have recovered from infections with avirulent *Babesia microti* are resistant to infection with *P.vinckei*, *P.chabaudi* and *P.atheruri*, but not *P.berghei* or *P.yoelii* (Cox, 1968, 1970a, 1972a). Mice which have been cured of infections with *B.rodhaini* resist challenge with *P.atheruri*, *P.vinckei* and *P.chabaudi* but not *P.berghei* or *P.yoelii* (Cox, 1970a, 1972a). In rats, however, there is evidence of slight protection against *P.berghei* in animals which have recovered from *B.rodhaini* (Cox and Milar, 1968). *Anthemosoma garnhami* protects mice against *P.vinckei* and *P.chabaudi*, and provides some protection against *P.yoelii* but not against *P.berghei* (Cox, 1972b). Cross-reacting antigens common to malaria parasites and piroplasms in rodents have been detected using an indirect fluorescent antibody technique (Cox, 1970b; Cox and Turner, 1970; see Table II).

2. Effects of malaria on piroplasms

P.vinckei, *P.chabaudi*, *P.berghei*, *P.yoelii* and *P.atheruri* protect mice against *B.rodhaini* and *B.microti* (Cox, 1970a, 1972a). *P.chabaudi* protects rats or mice against *B.rodhaini* (Cox and Milar, 1968) and *P.vinckei* and

P.yoelii protect mice against *B.hylomysci* (Bafort *et al.*, 1970). *P.vinckei* and *P.chabaudi* protect mice against *A.garnhami* and so, to a lesser extent, do *P.berghei*, *P.yoelii* and *P.atheruri* (Cox, 1972a).

G. *Toxoplasma*

Because it is ubiquitous, *Toxoplasma gondii* must sometimes occur together with a malaria parasite but the only investigation into this interaction has been a laboratory study using *T.gondii* and *P.yoelii* in mice. Strickland *et al.* (1972) infected mice with *T.gondii* 12 days before to 7 days after infection with *P.yoelii*. In mice infected with *T.gondii* before *P.yoelii*, malaria infections lasted longer, were more severe and caused more deaths than in control animals, and the antibody response to both *P.yoelii* and *T.gondii* was depressed. These results are interesting because they indicate that interferon produced in response to the *Toxoplasma* infection (Rytel and Jones, 1966; Freshman *et al.*, 1966) exerts no protective effect against the malaria as was suggested by Jahiel *et al.* (1968) and Schultz *et al.* (1968). This conclusion appears to agree with the observations made by Gobert *et al.* (1971). It seems equally likely that if any interferon is produced in response to the malaria parasite (Huang *et al.*, 1968), it is either inadequate or inefficient against *T.gondii* which is otherwise susceptible to the action of interferon (Remington and Merigan, 1968).

H. Trypanosomes

The possibility that trypanosomiasis and malaria in rodents might affect one another was first investigated by Hughes and Tatum (1956). In rats infected with both *P.berghei* and *Trypanosoma lewisi*, the *P.berghei* parasitaemia was prolonged and enhanced. The *T.lewisi* infection was unaffected until the ninth day when the number of trypanosomes in the blood at first rose to twice the level seen in animals with *T.lewisi* alone and then declined. Shmuel *et al.* (1975) found that in rats infected simultaneously with *P.berghei* and *T.lewisi* the malaria infections were enhanced, and that in rats infected with *T.lewisi* 7 days after *P.berghei*, the number of trypanosomes was increased. Jackson (1959), using rats infected simultaneously with *P.berghei* and *T.lewisi*, found that the two parasites did not appear to affect one another, although 17 out of 25 rats died from unspecified causes.

In mice, Bafort (1971) found that *T.musculi* (= *T.duttoni*) given at the peak of a *P.vinckei* infection had no effect on either parasite but that the doubly infected animals died early with severe anaemia. Quite different results were obtained by Büngener (1975) and Cox (1975). Büngener (1975) infected mice with *P.berghei* and with *T.musculi* at the same time or 3, 6 or 9 days later. In the mice infected after 6 or 9 days the number of trypanosomes in the blood reached very high levels and killed some of the mice. Büngener attributed the increased trypanosome infections to malaria-induced changes in the liver sinusoids which favoured the multiplication of *T.musculi*. Cox (1975) infected mice with *P.yoelii* and

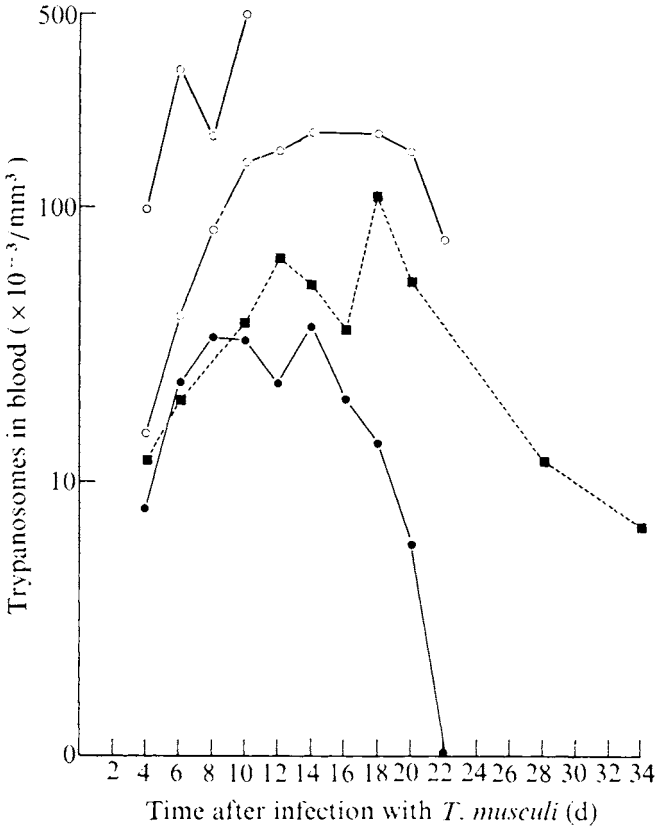

Figure 4. *T.musculi* infections in mice previously or simultaneously infected with *P.yoelii*. ●———●, Controls; ■ - - - ■, *P.yoelii* administered before infection; ○———○, *P.yoelii* administered on the seventh day of infection (in this group fatal and non-fatal infections have been separated) (reproduced with permission, from Cox, 1975b).

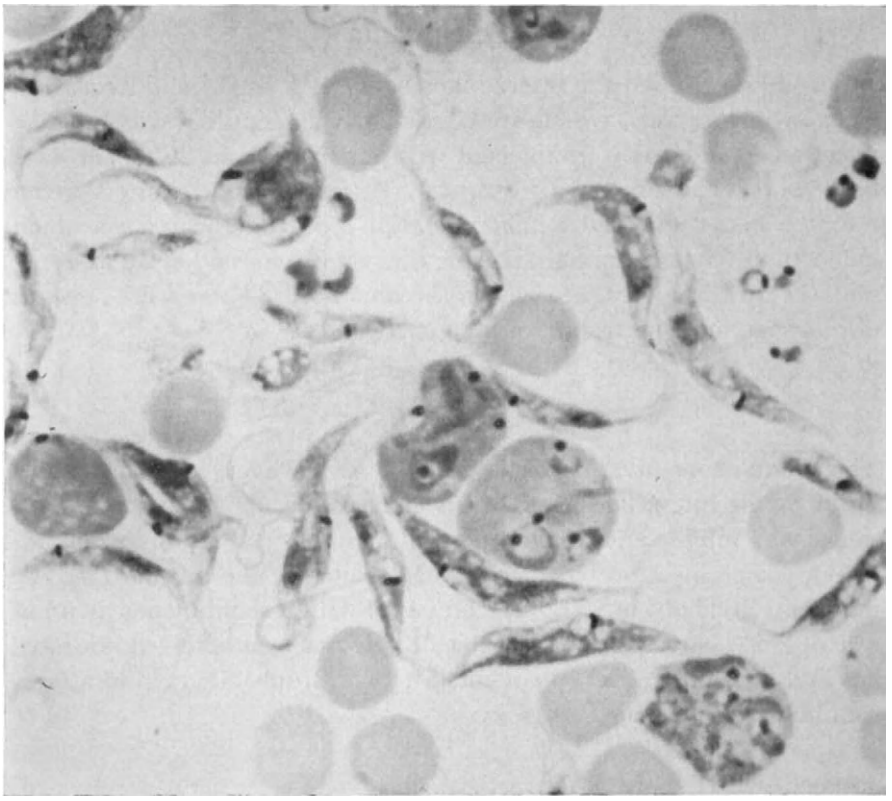

Figure 5. *T.musculi* infection in a mouse infected seven days earlier with *P.yoelii*. This photograph shows approximately one trypanosome to every red blood cell on the thirteenth day of the infection. In the controls there was one trypanosome for every 200 red blood cells.

T.musculi at the same time or 7 days later. The trypanosome infections were considerably enhanced and reached levels up to 10 times higher than those in mice infected with *T.musculi* alone, and some of the mice died (see Figures 4 and 5). The effect of *P.berghei* on *T.musculi* infections was most marked when the trypanosomes were given 7 days after *P.yoelii*. Cox (1976) suggests that the enhanced trypanosome infections are due to immunodepression by the malaria. There was no appreciable effect on the malaria infection.

Mice immune to *T.musculi* infections are not immune to *P.vinckei* (Bafort, 1971; Cox, 1972c), *P.chabaudi*, *P.berghei*, *P.yoelii* or *P.atheruri* (Cox, 1972c), nor is there any reciprocal immunity (Cox, 1972c).

I. *Leishmania*

Adler (1954) studied the interactions between *P.berghei* and *Leishmania infantum* in hamsters, which are particularly susceptible to both these infections. Hamsters were infected with *L.infantum* and 21 to 171 days later were challenged with *P.berghei*. A number of hamsters given *P.berghei* 30 to 81 days after *L.infantum* recovered from the malaria which killed all the control animals. During the recovery period, dead or dying malaria parasites were seen within the blood cells. There were no signs of blockage or hypertrophy of phagocytic cells.

J. *Giardia*

The spontaneous invasion of tissues such as the liver, lung, heart and brain by the intestinal flagellate *Giardia muris* occurs when the infestation is very intense and in mice infected with *P.berghei* (Radulescu *et al.*, 1971). Tissue invasion by *G.muris* was detected at the peak of *P.berghei* infections in 80 out of 100 mice compared with a spontaneous invasion rate of 20%. No explanation of this finding has been offerred nor have any reports of similar phenomena come from other laboratories routinely maintaining *P.berghei*.

K. Helminths

There have been several observations on the interactions between schistosome infections and malaria in rodents. Yoeli (1956), using *Schistosoma mansoni* and *P.berghei* in *Microtus guentheri*, found that in voles infected with *P.berghei* one or two weeks before or after *S.mansoni* the malaria infection was prolonged, and the animals had difficulty in ridding themselves of it. In voles infected with *P.berghei* four or seven weeks after *S.mansoni*, during the period when the most active tissue reaction to the worms was occurring, the malaria infections were milder than in normal infections, some animals spontaneously recovering and some never becoming infected. The outcome of the interaction between these two parasites therefore depends on the interval between the two infections. Lewinsohn (1975), using *S.mansoni* in mice infected three or five weeks later with blood-passaged *P.yoelii*, concluded that these parasites do not greatly affect one another.

One of the characteristics of *S.mansoni* infections is the formation of

granulomatous reactions of the delayed hypersensitivity type around the schistosome eggs. Abdel-Wahab *et al.* (1974) investigated the effect of *P.yoelii* on this reaction by injecting eggs intravenously into mice at the same time as the malaria parasites and found that the malaria suppressed the formation of granulomata during the dual infection, particularly after 16 days when the granulomatous reactions were only half as large as those in control mice. These authors also state that in mice harbouring *S.mansoni* adult worms and *P.yoelii* the granulomata are much smaller than in mice without the malaria.

One attempt has been made to examine the effects of malaria on the expulsion of the nematode worm *Trichuris muris* in mice. Phillips *et al.* (1974) infected mice with *T.muris* and with *P.berghei* at the same time or 5, 7 or 12 days later and examined the mice for worms after 23 or 25 days of nematode infection. They found some evidence which suggested that *P.berghei* inhibited the rejection of the worms.

Expulsion of the nematode *Nippostrongylus brasiliensis* from rats is not affected by *P.berghei*, but infections with the worm slightly increase the malaria parasitaemias (Golenser *et al.*, 1976).

L. Concomitant Infections in Mosquitoes

This chapter would be incomplete without some mention of the effects of concomitant infections on malaria parasites in mosquitoes. Mosquitoes harbour a wide range of infectious agents which include viruses, bacteria, protozoa and nematodes (Jenkins, 1964; Chapman *et al.*, 1970). Many of these agents are relatively non-specific and infect several species of mosquito so it is very difficult to select what is known about the effects of these concomitant infections on murine malaria from what is known of other malarias. This whole subject would require a major review in its own right, but what is of relevance here is the fact that some of these agents happen to infect *A.stephensi* which is the most widely used laboratory vector of murine malarias. Wéry (1968) summarized the main factors that could influence the development of oocysts of murine malarias in mosquitoes and drew attention to the fact that other infectious agents could have an adverse effect on their development. This was subsequently demonstrated to be true by Davies *et al.* (1971) and Bird *et al.* (1972) for a virus and by Vavra and Undeen (1970) and Hulls (1971, 1972) for a microsporidian. Davies *et al.* found two viruses and a rickettsia in the midgut epithelial cells of *A.stephensi*

and demonstrated that one of the viruses interfered with the development of *P.berghei* oocysts. What is apparently the same virus (a cytoplasmic polyhedral virus) was shown by Bird *et al.* (1972) to occur in the midgut cells of *A.stephensi* infected with *P.yoelii* and also within the oocysts and sporozoites themselves. The sporozoites were deformed but no experiments to determine any effects on transmission were performed.

There is a suggestion that the rickettsia seen by Davies *et al.* (1971) may render *A.stephensi* refractory to infection with *P.berghei*. Susceptibility to infection was lost while the mosquitoes were contaminated and returned when the rickettsial infection disappeared.

Microsporidians in *A.stephensi* have received considerable attention and the most important contaminant is *Nosema algerae* (Vavra and Undeen, 1970; Hulls, 1971, 1972; Canning and Sinden, 1973). The development of *P.berghei* oocysts is adversely affected by the presence of this microsporidian and the viability of the sporozoites is reduced (Hulls, 1971, 1972).

It is not at all clear at present how important concomitant infections with viruses and microsporidians are in the transmission of murine malarias. In the laboratory they are clearly a nuisance but in the wild their effects may be negligible as natural infections are rare (see Ward and Savage, 1972). Nor is it clear how microsporidians and viruses could affect the development of malaria parasites in ways other than by destroying the midgut walls or by shortening the life of the mosquito. This is a subject which requires much more work.

III. CONCLUSIONS

From the survey of the literature given above, it is apparent that there is hardly an infectious agent that cannot in some way affect, or be affected by, murine malaria. Fortunately, in the laboratory, concomitant infections either rarely cause any noticeable effect or cause overt disease the impact of which on the malaria is so marked that it cannot escape notice. In the laboratory, the two most important groups of contaminant infections are the rickettsiae and viruses. Most research workers are now continually on the look-out for rickettsiae, which can usually be seen in routine blood films, but virus infections are difficult to diagnose and present a different problem which has seldom been

recognized. Many of the features of virus infections can, however, be identified and, in general, unexpectedly enhanced or prolonged malaria infections may be attributable to virus contamination, while lowered infections probably result from contamination with rickettsiae. The ameliorating effects of virus-induced interferon are so marginal that they can probably be ignored, particularly as the effects tend to be very short lived. In the wild, both rickettsiae and viruses, as well as other agents, may markedly affect the outcome of malaria infections but as so little is known about natural infections it is unwise to speculate further.

The diverse interactions between malaria and other organisms have until recently defied the postulation of a synthetic hypothesis to account for them all, but current immunological theory has caught up with the phenomena observed and it is now possible to attempt an explanation of all the interactions so far described.

A. Effects of Malaria on Other Infections

The effects of malaria on infections with other agents are the easiest to explain. In all cases of enhanced infections the explanation probably lies in the phenomenon of immunodepression characteristic of malaria (see Chapter 6). Thus enhanced viral lymphomagenesis and leukaemias, depressed immunological responses to bacteria, manifestations of the presence of mycoplasmas, recrudescences of rickettsial infections, depressed antibody responses to *Toxoplasma*, enhanced trypanosomiasis, decreased delayed hypersensitivity to helminths and the possible adverse effects on worm expulsion can all be attributed to immunodepression during the malaria infection. Any exceptions to this general pattern are probably due to the timing of the two infections with respect to one another because the period of immunodepression is fairly short lived and, outside this period, another infection is unlikely to be affected by the malaria.

Malaria infections have adverse effects on subsequent infections with piroplasms or the other malaria parasites and the explanation of this is probably also immunological. From the evidence available it seems that these interactions are due to a normal homologous immune response which, for some reason or another, is also operative against heterologous parasites. The most obvious explanation is that these parasites have antigens in common with which the antibodies react.

Other immunological phenomena may, however, be involved and these are discussed below.

B. Effects of Other Infections on Malaria

The effects of other infectious agents on malaria can be explained under four headings, of which one probably has little significance. These are:

1. the availability of suitable red blood cells which either enhance or depress a malaria infection depending on the host cell preference of the species involved;
2. immunodepression by other agents causing enhancement of malaria infections;
3. an immune response elicited by another agent which also happens to be effective against malaria, thus causing depressed infections;
4. enhanced macrophage activity resulting from the first infection and possibly effective in depressing the malaria infection through non-specific phagocytosis.

The single most important factor which determines the outcome of a malaria infection in rodents is the availability of suitable red blood cells. In general, parasites of the *berghei* group prefer reticulocytes while those of the *vinckei* group prefer mature cells. It has been clearly demonstrated that a reduction in the availability of reticulocytes depresses infections with *berghei*-group parasites and that reticulocytosis depresses *vinckei*-group infections. The availability of a suitable milieu on the outcome of malaria is so great that large doses of the immunosuppressive drug beta-methasone actually cause a depression of *P.yoelii* infections because the drug also inhibits the formation of reticulocytes which the parasite preferentially invades. On the other hand *P.chabaudi* infections are enhanced under the same circumstances (Cox, 1974). The anaemia and reticulocytosis following rickettsiae infections are probably the main cause of the modified malaria infections which occur in doubly infected animals. Published observations do not all confirm this hypothesis but it must be remembered that the timing of the two infections, the sizes of the respective inocula and the cell preferences of particular strains of malaria modify the actual outcome of the malaria infection. Different strains of *P.berghei* exhibit different degrees of preference for reticulocytes and this preference may change during the course of an infection. At present, the availability of suitable cells seems

to offer the best explanation of the interactions which have been found to occur between rickettsiae and malaria in rodents. It is possible that viruses may affect malaria parasites in the same indirect way as rickettsiae since many viruses alter the red blood cell picture of their hosts. In double infections which have been examined, however, other hypotheses provide better explanations of the results observed.

Immunodepression is now regarded as a phenomenon common to many infectious diseases and is best documented in virus infections (Salaman, 1969). It is fairly clear that enhanced malaria in mice infected with viruses is due to viral immunodepression and, although the definitive experiments have not been performed, it is possible that enhanced malaria infections following infections with spirochaetes, *T.gondii* and *S.mansoni* can be similarly explained.

The possibility that malaria parasites affect and are affected by other agents through a common immune response needs some explanation. Extensive cross-immunity experiments have shown that heterologous protection against the piroplasms *B.microti* and *B.rodhaini* is the rule, that heterologous protection against *P.vinckei* and *P.chabaudi* is not as strong as it is against the piroplasms and that heterologous protection against *P.berghei* is almost impossible to achieve (Cox, 1970a, 1975). Immunization with BCG and *C.parvum* produces broadly similar results (Clark and Allison, 1976; Clark *et al.*, 1976, 1977) and it is tempting to suggest that the mechanisms of heterologous immunity are similar in all these infections and that they are essentially no different from those that bring about homologous immunity.

There is one explanation that fits all the known facts. BCG and *C.parvum* are known to stimulate the production of non-antibody mediators of immunity (Sher *et al.*, 1975) which can bring about or contribute to the death of both piroplasms and malaria parasites (Clark, 1976; Clark *et al.*, 1977). The mechanism seems to be the same for both parasites and is characterized by the intraerythrocytic death of the parasites. Intracellular death is characteristic of the recovery phase of homologous immunity to malaria parasites (Clark *et al.*, 1975), and also occurs in heterologous immunity (unpublished). It therefore seems reasonable to postulate the existence of a common mechanism in which a variety of organisms specifically trigger the release of a non-antibody mediator of immunity with a wide range of specificity. Such a mechanism could operate in conjunction with, in parallel with or as an alternative to, the normal antibody response. The production of such

a substance could account for: heterologous immunity between piroplasms and malaria; heterologous immunity between different species of malaria parasites and depressed malaria infections following the administration of bacterial antigens or *Leishmania.* Leishmaniasis is interesting because in *Leishmania*-infected animals, malaria parasites were seen to be dying inside red cells (Adler, 1954), an observation seen in BCG-immunized mice (Clark *et al.*, 1975). As specifically induced but non-specifically expressed resistance of this kind receives more attention, its importance in the explanation of the interactions between malaria parasites and other agents is likely to increase.

Non-specific stimulation of phagocytic activity has frequently been suggested as a mechanism explaining the depressed malaria infections which occur after exposure to rickettsiae or bacterial antigens. This hypothesis is not supported by experimental evidence and the availability of suitable red blood cells or the production of a non-specific protective substance provide better explanations. *C.parvum*, for example, is a potent stimulator of reticuloendothelial activity, but protection against malaria increases with time after immunization with *C.parvum* instead of decreasing which would be expected if reticuloendothelial activity depressed the malaria (unpublished).

C. Epidemiology of Concomitant Infections

The fact that many infections interfere with murine malaria and that such interference may be reciprocal presumably has a profound influence on the transmission of the infectious agents involved. This subject has received little attention and it is impossible to do it justice here. It is obvious, however, that any parasite that enhances another infection first increases the chances of the subsequent transmission of that infection but may eventually stop it completely by bringing about the death of the host. The example of *P.yoelii* and *T.musculi* illustrates this point. Neither of these parasites normally kills its host but in *P.yoelii*-infected mice the *T.musculi* infections are both enhanced and prolonged (Cox, 1975). Thus *P.yoelii* increases the chances of transmission of *T.musculi* but some mice die from the enhanced trypanosome infection and from such animals no further transmission is possible. Viruses also tend to enhance and prolong malaria infections and cryptic virus infections may play a major part in the transmission of murine malaria. On the other hand, rickettsiae and piroplasms depress

murine malaria and would normally decrease the possibility of transmission, although this possibility would be increased for an unusually virulent and frequently fatal malaria infection.

Most of what we know about murine malaria has been derived from careful experiments and observations of single infections in the laboratory. It is important to remember, however, that this information provides only a baseline against which field observations can be assessed. In the field, single infections are probably very rare, and for a realistic understanding of the epidemiology of murine malaria concomitant infections should be considered as the norm

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Chapter 8 is dedicated to the memory of the late Dr Leo Rane who, with his wife Dr Dora Rane, devised a novel technique for the mass screening of antimalarial drugs based on the use of rodent malaria. With this model they examined an unprecedented number of compounds, totalling more than one quarter of a million, and thus contributed to the development of a new generation of invaluable drugs.

8. Chemotherapy

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I. INTRODUCTION

Before the discovery of rodent malaria in 1948 the bulk of research relating to the chemotherapy of malaria had been carried out in avian models, employing in particular *Plasmodium gallinaceum* in young chicks (see review in Peters, 1970a). From 1941 to 1945 the US National Microbiological Institute (now part of the National Institutes of Health) examined more than 15 000 chemical substances, an unprecedentedly large number. The avian malarias and certain malaria parasites of primates also were used extensively for basic investigations on the biochemistry and physiology of *Plasmodium*. The ease of

manipulation and economy of rodent malaria in mice as compared with birds, permitted a rapid expansion of research that was, one hoped, likely to be of more direct relevance than the avian models to human malaria.

In the early 1960s chloroquine resistance was reported in the malignant tertian parasite *P.falciparum*. Coincidentally the US Armed Forces became engaged in action in South-East Asia where chloroquine resistance proved to be both widespread and of serious practical implication. Since then there has been an exponential growth of interest in malaria chemotherapy. The great bulk of new research has been carried out with rodent *Plasmodium* and the growing interest is reflected in the following figures shown in Table I.

Table I

Numbers of references included in bibliographies on rodent malarias

Author and (year)	Number of references	Number on chemotherapy and (% of total)	
Vincke and Lips (1952)	129	35	(27·1)
Thurston (1953)	103	26	(25·2)
Jadin (1965)	489	123	(25·2)
Aviado (1969)	787	254	(32·3)
Peters and Howells (unpublished)	1080	946	(87·5)

An analysis of papers in our collection yields the following interesting observations:* 946 of 1080 papers deal specifically with rodent malaria in chemotherapeutic research. The species referred to in these 946 papers were, *P.berghei* 903 (83·5%); *P.yoelii* 94 (8·6%); *P.vinckei* 85 (7·9%); *P.chabaudi* 65 (6·0%); others 14 (1·3%). Of the 1080 papers only 6·3% refer to experiments *in vitro*. The great majority of the references were limited to the asexual erythrocytic stages (92·1%), 6·3% dealing with gametocytes, 12·6% the sporogonic stages and 10·1% refer to the exoerythrocytic stages.

Over one-half of the papers relating to chemotherapy are concerned with pharmacology (478 or 50·5%), one-third deal specifically with new antimalarial drugs (330 or 34·9%) and the remainder refer to parasite

* The development of such statistics can provide an entertaining (if pointless) diversion. For example, all the experiments ever carried out on chemotherapy using rodent malaria have involved roughly one kiloton of pure *P.berghei*. This mass of parasites (not to mention their rodent hosts) converted into an edible form, would provide enough protein to feed 50 people for one year.

metabolism and biochemistry (232 or 24·5%). The majority (85%) of experiments in these papers were carried out in mice, the remainder being mainly in rats.

Most of these experiments have been necessitated by a massive screening programme designed to find new drugs with which to treat chloroquine-resistant human malaria, and to prevent relapses caused by *P.vivax* of man which yields only with difficulty to the very limited battery of antimalarials in current use. Since about 1963 over a quarter of a million chemical substances have passed through a screen which is based primarily on *in vivo* testing in mice. From 1948, when it was first realized how valuable *P.berghei* would be for studies on chemotherapy, probably something in the order of four to five million mice have been used, four million at least in the current screen. How much more humane it would all be if *in vitro* systems were available for this kind of work. Unfortunately, no adequate *in vitro* substitute has yet been evolved that can replace the mouse for the great majority of experiments that have been involved, and that will continue to be necessary in the search for new and more effective drugs for the prevention and treatment of malaria. Valiant attempts have been made but none, so far, has proven totally successful.

So far this account has dealt solely with the role of rodent malaria in chemotherapy research, and this theme is expanded upon in the following section. Antimalarial agents, in their turn, have proved invaluable in illuminating many features of the physiology and biochemistry of the parasites themselves, and this aspect of experimental chemotherapy is discussed in some detail on pp. 358–381.

II. THE VALUE OF RODENT MALARIA IN ANTIMALARIAL DRUG SCREENING

A. The Search for New Blood Schizontocides

In an earlier work Peters (1970a) summarized the techniques that had been developed on the basis of rodent malaria (mainly the old Katanga strain K173 of *P.berghei*) to evaluate chemicals as potential blood schizontocides. In the years immediately following the discovery of *P.berghei* there was relatively little interest in new drugs. Chloroquine, primaquine and proguanil had been discovered and developed largely

on the basis of their activity to *P.gallinaceum*, and little need was felt for yet other new antimalarials. Drug resistance although not, in 1948, a matter for serious concern, was beginning to appear with reports from both the laboratory and the field of difficulties with proguanil.

At this time workers in the Wellcome research laboratories were examining series of compounds from which was to emerge, in a year or two, the powerful prophylactic pyrimethamine. It was evident to those investigators that the potential of parasites to develop resistance to pyrimethamine should be examined without delay, and in this way the new "wonder drug" became one of the very first for which rodent malaria provided an ideal experimental model. Already a number of chemotherapists had seized on the new parasite, and a series of papers was published describing the activity of the then current antimalarials such as quinine, 8-aminoquinolines, mepacrine, chloroquine and other 4-aminoquinolines, sulphonamides, sulphones, and even the new antibiotics of the tetracycline group which did not, in fact, attain prominence as antimalarials until very recently.

From those early days *P.berghei* provided the basis for the search for new antimalarials in drug screening programmes conducted routinely by a number of pharmaceutical companies, and a few non-commercial research centres but, as stated previously, interest in this field was limited until the advent of chloroquine resistance in *P.falciparum*. Clearly the need for drugs that would be active against parasites resistant to chloroquine necessitated the use of an appropriate chloroquine-resistant screening model, in addition to the standard model which was based on the broadly drug-sensitive old laboratory strain of *P.berghei*, or one of the more recently isolated strains such as Yoeli's NK65. As it became apparent that many strains of *P.falciparum* were proving resistant not only to chloroquine, but also to unrelated compounds such as pyrimethamine, it was obvious that a battery of drug-resistant models would have to be developed.

The story of drug resistance in malaria and the efforts exerted by research workers around the world to study this problem has already been recounted at length (Peters, 1970a) and need not be repeated here. Suffice it to say that in *P.berghei* was found the first mammalian malaria parasite which could readily be manipulated in order to produce strains resistant to almost any available antimalarial drug. Indeed, in our own laboratory, there is so far no active antimalarial compound that we have tested to which we cannot produce resistance

in *P.berghei*. From this experience has arisen a battery of strains resistant to chloroquine, quinine, primaquine, cycloguanil, pyrimethamine, dapsone and sulphonamides, with various levels of resistance to those compounds that, today, form the basis of secondary drug screening *in vivo* for interesting compounds that emerge from the primary, drug-sensitive *P.berghei* screen. The value of this secondary screen has been discussed in several recent publications (WHO, 1973; Peters *et al.*, 1970b; Thompson, 1972; Kinnamon and Rothe, 1975).

What in fact has come out of these studies, what miraculous new drugs have emerged to justify the consumption of that kiloton of *Plasmodium*, those four million and more mice? The answer is, surprisingly little. However, this is in the very nature of drug screening. Kinnamon and Rothe (1975) have pointed out that, for each 3000 compounds that went in one end of the primary screen, only 54 came out at the other. All but 15 of these were rejected in the secondary screen, and those 15 then had to pass a rigorous series of tests to determine whether they would justify eventual clinical trial. On the average only 1 compound is examined in the pre-final simian screen before passing to pre-clinical toxicological evaluation. On this basis it is perhaps no wonder that a mere handful of potentially valuable drugs are at present in clinical trial, and not all of these have resulted directly from screening against rodent malaria.

Canfield and Rozman (1974) have reviewed the compounds (excluding several antibiotics) that have been selected for clinical studies. By 1974 from the quarter of a million compounds screened, a total of 26 compounds had been selected, 7 had received thorough clinical trials and were awaiting wider scale evaluation, 4 were actually in trial and a further 2 were being prepared for trial. The latter are now being studied. Some of these compounds are shown in Figure 1.

As evidence accumulated to underline the antimalarial value of combining a sulphonamide with a dihydrofolate reductase inhibitor ("antifol") (a fact long ago indicated by Goodwin (1952) and others), trials were organized with combinations of this type, but first more on empirical grounds than on the basis of experimental evidence. In spite of the accumulating evidence from studies in *P.berghei* that polytherapy should always be the aim in malaria prophylaxis and therapy (Peters, 1974b), there is still a great reluctance to test this principle in man.

Perhaps surprisingly, several of these "new" compounds have been known since the Second World War but were never adequately studied,

partly because of the loss of momentum in chemotherapy research after the end of the war, and partly because the activity data obtained in the old *P.gallinaceum* model were shown to be sometimes misleading when referred to primate, and especially human malaria. On retesting in the *P.berghei* systems, several compounds proved to be of far greater value than previously estimated, and they were therefore revived. For example, the 9-phenanthrenemethanol WR 33,063 was first described in 1946. When used as the standard comparison compound in the *P.berghei* screen it was found to stand up well to more recently synthesized compounds and, indeed, it has only been superseded in the 9-phenanthrenemethanol series by WR 122,455 (Figure 1, ix). From the old, phototoxic 4-quinolinemethanol SN 10,275 have now been developed, WR 30,090 and WR 142,490 (mefloquine) (Figure 1, x).

The choice of technique for the study of blood schizontocidal action depends upon the precise requirements of the individual investigator, and a selection is discussed by Peters (1970a, 1974a) and by WHO (1973). For drug screening a variety of procedures is available ranging

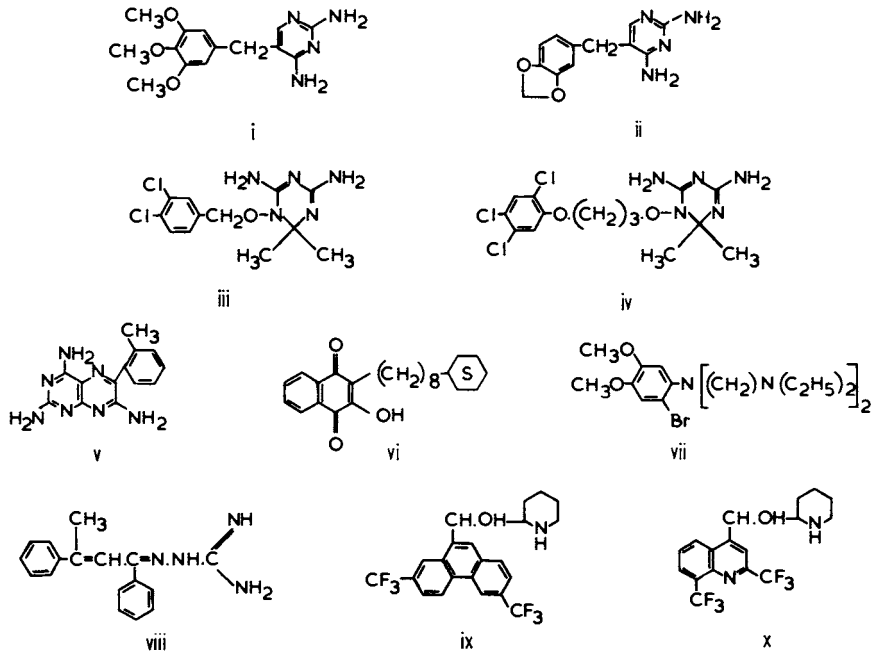

Figure 1. Structures of experimental antimalarials studied in man. (i) Trimethoprim; (ii) WR 40,070; (iii) clociguanil; (iv) WR 99,210; (v) WR 3090; (vi) menoctone; (vii) RC 12; (viii) WR 5677; (ix) WR 122,455; (x) mefloquine.

from the single-dose test of Rane in which survival time is the important parameter, to the "4-day test" of blood schizontocidal action used by Peters (1965). Mainly for historical reasons most studies have been made using one or other strain of *P.berghei* in random-bred albino mice. Fink and Kretschmar (1970) favoured *P.vinckei* and a single-dose regimen but, so far, few other workers have followed their example. The rare comparative reports available indicate few significant interspecies differences in drug responses, at least none greater than those known to exist between different strains. The exception is the different response between rodent plasmodia that reside in mature, and those that reside in immature red cells, e.g. *P.berghei* N strain and *P.yoelii* 17 X, the former being very sensitive to 4-aminoquinolines and the latter relatively resistant. *P.vinckei* (Fink and Kretschmar, 1970) and *P.chabaudi* (Peters, 1967) have a chloroquine sensitivity similar to *P.berghei* N strain. One or other systemic route of drug administration is favoured by most workers, but Thompson and his collaborators frequently employed oral dosing, often by the drug-diet method.

B. Causal Prophylaxis and Anti-relapse Drugs

Until the introduction of rodent malaria the only reliable model for the screening of chemicals for tissue schizontocidal activity against mammalian malaria parasites was *P.cynomolgi* in the rhesus monkey, hardly a suitable model for large-scale studies. The avian models developed in the 1930s in Germany, and in Britain and the USA during the Second World War yielded much information about compounds that exerted an action against the exoerythrocytic stages of avian parasites in various tissues of the reticuloendothelial system, but the data so obtained often proved impossible to correlate with subsequent evaluation in the simian model, or in man. For several years after the discovery of *P.berghei* attempts were made to elucidate the nature of its pre-erythrocytic cycle, but it was only through the combined field and laboratory investigations of Yoeli *et al.* (1964) that the road opened to the exploitation of rodent malaria for screening against tissue schizontocides. Even today some doubt remains as to whether there exists only a single, pre-erythrocytic cycle of rodent *Plasmodium* in liver parenchymal cells, or whether possibly, in Nature at least, dormant, chronic liver stages are formed (Landau, 1973). However, for all practical purposes, the short pre-erythrocytic liver cycle provides a relatively

simple target for drugs with activity against the tissue stages, and several techniques have been proposed for utilizing the rodent malarias in drug screening. None is as simple as screens for blood schizontocidal action since the infection of host mice must be by sporozoite inoculation, and the production of sporozoites carries inherent practical difficulties.

The relative merits of the different procedures have been discussed by Peters (1970a), Peters *et al.* (1975a), WHO (1973) and Fink (1974). The short (up to about 72 h) duration of the liver cycle introduces the complication that any compound that acts for a long time (e.g. slowly absorbed, poorly soluble substances or drugs that are strongly protein bound and slowly released) may act not only on the tissue stages but also upon the first (or even later) generation of erythrocytic stages that emerge from the maturing liver schizonts. Gregory and Peters (1970) attempted, not entirely successfully, to overcome this disadvantage by a mathematical analysis of their data, using *P.yoelii nigeriensis* as their test organism. In spite of the problems inherent in their technique we have successfully employed this procedure to examine over one hundred compounds for causal prophylactic activity (Peters *et al.*, 1975a). Fink (1974), using *P.yoelii* 17 X in NMRI mice with a slightly different technique, obtained essentially the same results in compounds that were studied by both procedures. He made the relevant comment that the rodent system may somewhat exaggerate the value of certain compounds that act on nucleic acid synthesis in view of the exceptionally high rate at which this proceeds in the forms in the rodent liver. This may indeed account for the higher level of sensitivity of the pre-erythrocytic stages even of some primate malaria species, for example to proguanil, as compared with the sensitivity of the asexual erythrocytic parasites (Fairley, 1946).

Unlike the tissue stages of avian malarias, it is impossible at the present time to cultivate the exoerythrocytic forms of any mammalian *Plasmodium*, including the rodent species, in tissue culture. One is therefore obliged to utilize a system in living rodents for the evaluation of causal prophylactic agents. With the foreknowledge that most compounds that possess activity against primary tissue schizonts of primates act also against secondary tissue stages, the evidence so far indicates that rodent malaria can be used with confidence to screen drugs for possible anti-relapse properties. There are, of course, always exceptions, the most notable in this case being proguanil. This compound is poorly active against rodent malaria when compared with its

excellent action against both tissue and blood schizonts in birds and primates, the reason being that it is only slowly or incompletely metabolized to its active triazine derivative by the rodent host. A second exception is the pyrocatechol compound RC 12 (Figure 1, vii) which was shown by Schmidt *et al.* (1966) to have a high level of tissue schizontocidal action against *P.cynomolgi* in rhesus monkeys. This compound is virtually inactive against *P.yoelii* 17 X and *P.y.nigeriensis* in the mouse.

C. Sporontocidal Agents

A certain parallel can be drawn between tissue schizontocidal action and the activity of a compound against the stages of *Plasmodium* in the anopheline mosquito, a phenomenon first explored by Terzian (1947) in *P.gallinaceum*-infected *Aedes aegypti*. While Terzian's procedures have been extended to include the direct study of drug action against *P.vivax* and even *P.falciparum* in *Anopheles stephensi* (Terzian, 1968; Gerberg *et al.*, 1968; Gerberg, 1971), remarkably little use has been made of rodent *Plasmodium* spp. for this purpose outside our own laboratory. Although many compounds have been examined for sporontocidal action in the course of the current US Army programme in a *P.gallinaceum* model, it would appear that the use of *P.berghei* or *P.yoelii* and its subspecies have presented too many practical difficulties for them ever to be fully exploited. This is unfortunate since it was reported that, at one stage, compounds were passing through the avian system at the rate of 1000 week^{-1}. This process would be more economical both in terms of manpower, and the quantities of drug used if a rodent malaria–*A.stephensi* system could be used.

For several years after the discovery of *P.berghei*, all attempts at achieving development of this parasite in *Anopheles* met with little success until Yoeli *et al.* (1964) pointed out the necessity for holding the mosquitoes during the period of the extrinsic cycle at a temperature (19 to 21°C) several degrees lower than that maintained in most insectaries. The discovery of *P.yoelii* and *P.chabaudi* in the Central African Republic by Landau (1965) was followed by the observation that sporogony in these species could proceed readily at 24 to 25°C (Landau and Killick-Kendrick, 1966) and this was soon confirmed for *P.chabaudi* in detailed studies by Wéry (1968). Bafort (1969) found that *P.vinckei* would develop in *A.stephensi* at temperatures ranging from 20

to 24°C. Unfortunately, even when using apparently standard conditions for the development of rodent plasmodia in *A.stephensi*, greatly varying results were obtained by most workers. Admittedly *A.stephensi* is far from being the natural vector species of any rodent *Plasmodium*. Nevertheless, it is common to obtain 100% oocyst infection rates with, for example, *P.berghei* NK 65 or *P.y.nigeriensis* in this anopheline in certain experiments, whereas, in others, few or no oocysts are seen.

We now recognize several factors that account, in part at least, for this great variability in the development of rodent plasmodial oocysts in *A.stephensi*. Almost at the same time Davies *et al.* (1971b) and Bird

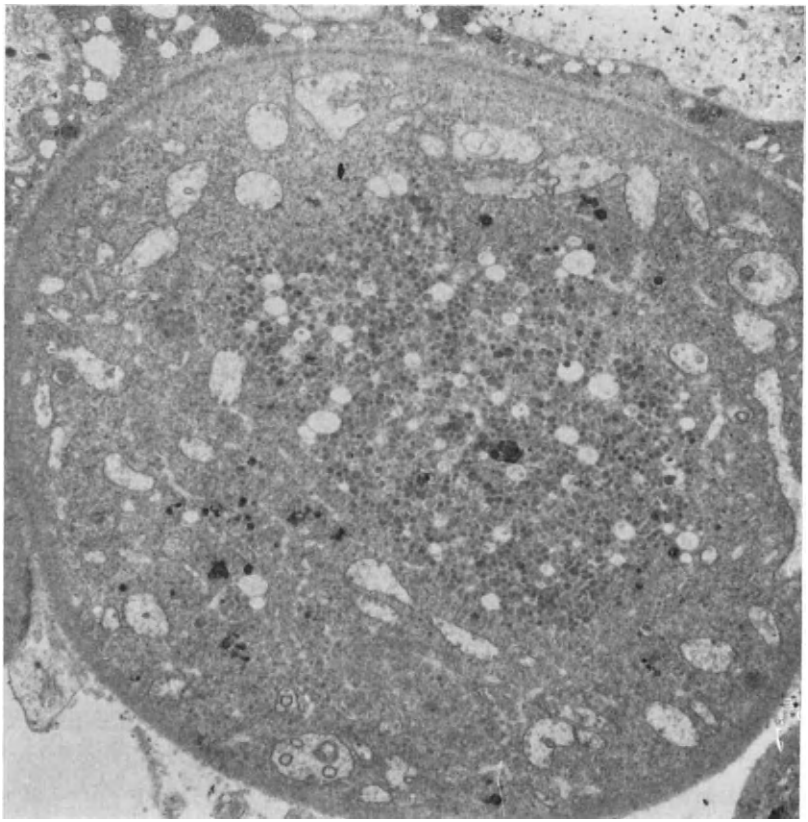

Figure 2. Oocyst of *P.y.nigeriensis* on the midgut wall of *A.stephensi*. Large numbers of virus-like particles are present within the oocyst cytoplasm and there is an apparent absence of nuclear material. Note the abnormally swollen nature of the parasite's mitochondria (×48 000).

et al. (1972) discovered virus-like particles within the oocysts of *P.y.nigeriensis* and *P.yoelii* 17 X respectively (Figure 2). In addition, both the Liverpool and London workers observed polyhedrosis-type viruses in the cells of the hosts' midguts (Davies *et al.*, 1971b; Bird *et al.*, 1972). There is little doubt that the organisms within the oocysts were cytotoxic. Furthermore, Hulls (1971) and Ward and Savage (1972) recognized that the development of *P.berghei* in *A.stephensi* was also adversely affected when the mosquito midgut was infected concomitantly by a species of microsporidian common in many laboratory colonies of this fly. To complicate the picture still further, Ramkaran (unpublished) found that the dietary content of para-aminobenzoic acid (PABA) of the gametocyte-carrying mouse upon which mosquitoes were fed, strongly influenced the subsequent development of *P.berghei* oocysts in those mosquitoes. (A more detailed account of this work is presented on p. 369.) In addition, the oocyst count could be influenced also by feeding the mosquitoes sugar solution containing certain levels of PABA after they had taken their infective blood meal. Thus nutritional factors in the mosquito hosts are also of importance in the development of the sporogonic stages of rodent malaria. Probably even the diet received by the anophelines during larval development is significant here since Beesley and Peters (1968) showed that the larvae of *A.stephensi* are susceptible to the action of several sulphonamides that are metabolic antagonists of PABA.

In spite of these difficulties the action of a number of antimalarials on the sporogonic stages of both *P.berghei* and *P.y.nigeriensis* has been examined. The results of these studies are discussed later (pp. 368–370).

D. The Relevance of Rodent Malaria to the Chemotherapy of Human Malaria

There is no doubt that it would not have been possible to launch the massive search for antimalarials that now is gradually being wound down if a *P.berghei*-mouse model had not been available. Although some doubts were cast about the technical value of the Rane screen when it first came into operation, it has unquestionably proved its value. Even if activity in some of the 250 000 compounds that passed through this screen has been missed, nevertheless the system has proved invaluable not only in detecting antimalarial activity in new chemical series, but also in providing a simple comparative picture of compounds within

individual chemical series both from the points of view of activity and toxicity. It is still too early to say how relevant the activity of blood schizontocidal action against a drug-sensitive *P.berghei* in the mouse is to either *P.falciparum* or *P.vivax* in man. However, of those new compounds that have emerged from the screen to pass all the hurdles of pre-clinical testing and have been examined in human volunteers infected with malaria, almost all have displayed at least some blood schizontocidal action. A number have subsequently had to be dropped because they were poorly tolerated. Several compounds were abandoned since they failed to control infection with chloroquine-resistant *P.falciparum*, even though they displayed activity against drug-sensitive strains, or because the activity was only apparent at doses approaching the toxic level. Only menoctone, a naphthoquinone (Figure 1, vi) has apparently been a complete failure, but this is possibly due to its lack of solubility and subsequent non-absorption from the human intestinal tract. RC 12 (Figure 1, vii) too failed to live up to its promise as a tissue schizontocide when tested in man. However, menoctone is very active in the rodent model, whereas RC 12 is inactive. Thus, as far as blood schizontocides are concerned, the drug-sensitive rodent malaria model seems, so far, to be a highly relevant one in relation to human malaria.

The use of a battery of drug-resistant strains of *P.berghei* gives clear indications of the limitations of a new drug. The triazine WR 33,839 (clociguanil) (Figure 1, iii) in our hands was only poorly active against a strain of *P.berghei* resistant to pyrimethamine, but active against a highly chloroquine-resistant line. In clinical trials against strains of *P.falciparum* sensitive to pyrimethamine, clociguanil proved very active (Laing, 1974; Rieckmann, 1971) but against a strain resistant to chloroquine and pyrimethamine it was ineffective (Rieckmann, 1971). This is what we would have forecast. Recently we have pointed out that the use of the moderately chloroquine-resistant NS line of *P.berghei* in secondary screening seems to be a good model for chloroquine-resistant *P.falciparum*; indeed the potent quinolinemethanols and phenanthrenemethanols mentioned earlier, which are very effective against *P.berghei* NS, are also very good blood schizontocides against chloroquine-resistant strains of *P.falciparum*. However, we believe that the highly resistant RC strain of *P.berghei* is valuable for its predictive value as to whether or not a parasite that is already chloroquine-resistant can be expected also to become resistant to another drug. Mefloquine and

WR 122,455 (Figure 1, x, ix), for example, are both effective against *P.berghei* NS and multiple-resistant *P.falciparum*. Both compounds have little or no activity against *P.berghei* RC, and it is proving relatively easy to develop strains resistant to them from *P.berghei* NS.

Causal prophylactic studies in rodent malaria have produced no startling results but, in general, compounds active against these parasites are in groups known also to display causal prophylactic or anti-relapse properties against human malaria (Peters *et al.*, 1975a). Thus 8-aminoquinolines are highly effective against *P.y.nigeriensis*. Amongst the members of this series that we have examined are several that are significantly more active on a mg kg^{-1} basis than primaquine which is the only 8-aminoquinoline now in common clinical use. It is not yet possible to say whether the order of activity of the 8-aminoquinolines that we observe in the rodent model will be paralleled in man, since so many other factors come into the picture, e.g. different pharmacokinetic and metabolic handling of the drugs in the mouse and man.

Several naphthoquinones and quinolones have shown good tissue schizontocidal action against *P.y.nigeriensis* but the only one so far tested in man (menoctone) was apparently not absorbed. There is good evidence to indicate that the dihydrofolate reductase inhibitors that have proved to be causally prophylactic in the mouse are also active in man (e.g. pyrimethamine, proguanil, cycloguanil, trimethoprim—Figure 1, i). The antibiotics tetracycline and clindamycin are also active causal prophylactics in both mouse and man.

There is still some doubt about the sulphonamides and sulphones which are active in the rodent system, but the causal prophylactic properties of these in man do not seem to have been sufficiently critically evaluated.

Negative correlations too are good, none of the 4-aminoquinolines, 4-quinolinemethanols, 9-phenanthrenemethanols or quinine, for example, showing tissue schizontocidal activity either in the mouse or in man.

The relevance of sporontocidal studies in the rodent malaria system to antimalarial activity in man is open to question. However, attention has already been drawn to the interesting parallel between the action of chloroquine upon the sporogony of chloroquine-resistant strains of *P.berghei* and *P.falciparum* (see p. 369), which may well carry important epidemiological implications.

III. ANTIMALARIAL DRUGS AS BIOLOGICAL PROBES

Antimalarial drugs may be used as biological probes if we have some prior knowledge of their mode of action against these or other organisms. As most compounds act only on certain stages of the parasite life cycle (Figure 3), we list some of them below in relation to these stages. Table II includes a selection of experimental substances as

Table II

Stages of life cycle in relation to site of drug action

Stage affected	Compounds
Asexual blood stages	Chloroquine and other 4-aminoquinolines, mepacrine, quinine and some quinine analogues 4-quinolinemethanols, 9-phenanthrenemethanols primaquine and other 8-aminoquinolines naphthoquinones, quinolones sulphonamides, sulphones pyrimethamine proguanil, cycloguanil and related triazines tetracycline and related analogues clindamycin and related analogues
Gametocytes (immature)	All the above
Gametocytes (mature)	?
Sporogonic stages	Naphthoquinones, quinolones sulphonamides, sulphones pyrimethamine proguanil, cycloguanil and related analogues ? antibiotics
Pre-erythrocytic stages	Primaquine and other 8-aminoquinolines napthoquinones, quinolones sulphonamides, sulphones pyrimethamine proguanil, cycloguanil and related triazines tetracycline, clindamycin and analogues

well as those currently in clinical use. The way in which some of these compounds act upon eukaryotic cells other than malaria parasites is well known, and indeed a compound such as chloroquine possesses a wide range of interesting pharmacological properties, apart from being an antimalarial. Much information was of course already available on the modes of action of older compounds such as proguanil from studies

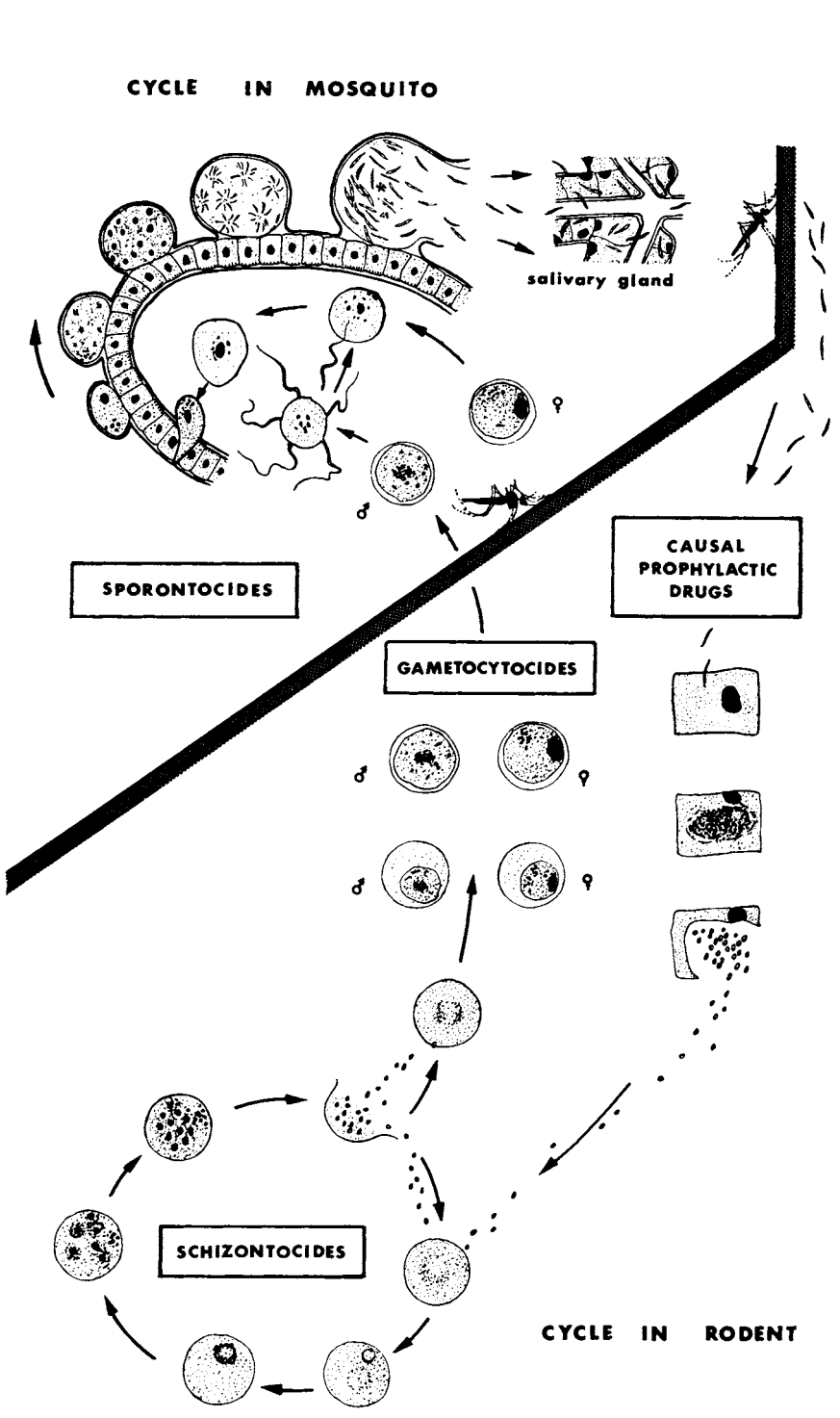

Figure 3. Diagrammatic representation of the life cycle of rodent malaria parasites showing the sites of action of antimalarial drugs (after Peters, 1970a).

carried out with other species of *Plasmodium*, and this information has been reviewed by, among others, Findlay (1951), Hill (1963) and Peters (1970a, 1974a). In more recent years, other reviewers have been able to take into account the work on rodent malaria, and the reviews of Thompson and Werbel (1972), Pinder (1973) and Steck (1971) are particularly valuable from the point of view of experimental medicinal chemistry.

Parasites of the genus *Plasmodium* have been shown by several workers to contain double-stranded DNA. Molecular biological techniques have underlined the ease with which several antimalarials interact relatively non-selectively with DNA, and this in turn has led a number of observers to conclude that this is the basis of their antimalarial action. Hahn (1974), for instance, and Olenick (1974) adopt this approach in their reviews on the mechanism of action of chloroquine and primaquine respectively.

However, the approach of Hahn and his colleagues, and of some other biological chemists, admirable as it is, ignores many basic features of the structure and physiology of both the parasites and the host cells within which they reside. Some of these features, as they are revealed through experimental chemotherapy, are discussed in the following pages. Because this will inevitably entail a certain amount of overlap with the review by Homewood (Chapter 4), the treatment of biochemical processes here will be relatively brief.

A. Chloroquine and Related Blood Schizontocides

1. Nature of the residual body

Macomber *et al.* (1967) and Warhurst and Hockley (1967) showed clearly that, in the presence of chloroquine, individual granules of haemozoin are aggregated into a single vesicle, together with other cytoplasmic constituents such as polysomes. The resultant mass (Figure 4B) resembles very closely the residual body that normally forms as the schizont matures, a process that has been carefully recorded by various investigators including Ladda (1968). Other blood schizontocides do not affect the haemozoin of *P.berghei* in this way. Thus primaquine and menoctone have virtually no action on the pigment (Howells *et al.*, 1970b). Quinine and the 9-phenanthrenemethanol WR 122,455 cause an apparent dissolution of haemozoin, but no

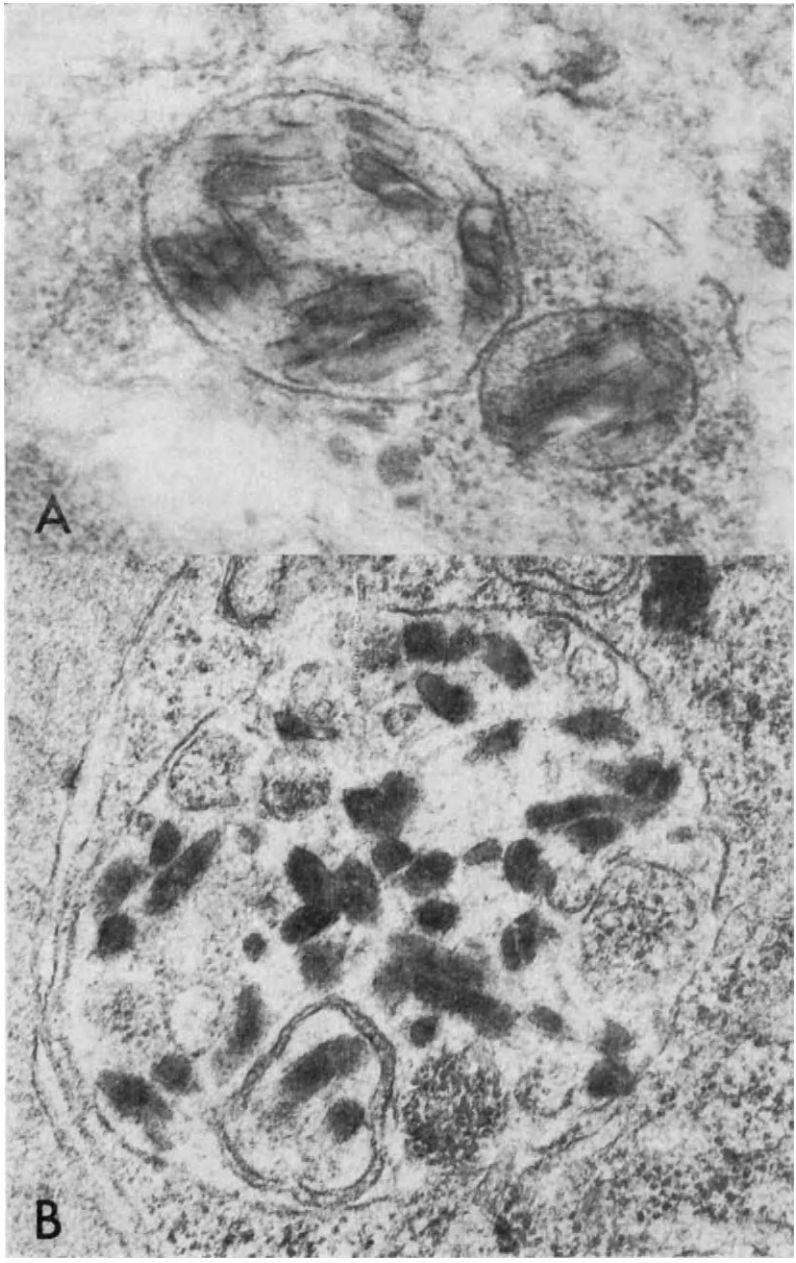

Figure 4A. Electron micrograph of a residual body from a schizont of normal untreated *P.berghei* (×60 000).

Figure 4B. Chloroquine-induced pigment clump in *P.berghei* (×64 000) (reproduced from Peters, 1970a).

clumping (Davies *et al.*, 1975). Dihydrofolate reductase inhibitors such as pyrimethamine cause no direct pigment changes (Figure 5).

This selective clumping action of chloroquine and certain other compounds stimulated several workers to exploit this property in an attempt to explore the nature of haemozoin, and its formation into a residual body in the natural process of schizogony. The ramifications of this research, and particularly the work of Warhurst and his associates are described in the following sections, and in Chapter 4.

2. *Chloroquine-induced haemozoin clumping as an investigative tool*

When *P.berghei* trophozoites are exposed to chloroquine *in vivo* and *in vitro*, within 10 to 30 min the dispersed haemozoin shows a tendency to granule formation. This is followed by secondary clumping, which is completed after 80 min (Warhurst and Robinson, 1971) (Figure 4B). At high concentrations of chloroquine, *in vitro* clumping is halted at the granulation phase (Warhurst *et al.*, 1974), but at 10^{-6}M chloroquine (the plasma concentration acquired during therapeutic use of this drug) secondary clumping normally occurs. The granulation phase was illustrated in electron micrographs by Ladda (1966) who exposed *P.berghei in vitro* to chloroquine at the high concentrations of 10^{-3} and 10^{-4}M, and found that the pigment vesicles became markedly enlarged, and contained large numbers of pigment bars. The ultrastructure of secondary pigment clumps illustrated by Macomber *et al.* (1967) for *P.berghei* and by Warhurst and Hockley (1967) for *P.knowlesi*, was found to resemble autophagic vacuoles (autophagosomes) containing the haemozoin grains released from their vacuoles, together with quantities of parasite cytoplasm (Figure 4A). Warhurst and Williamson (1970) found, moreover, that parasite RNA becomes degraded following chloroquine treatment and that this is detectable only after the autophagosome has formed.

Haemozoin clumping could not be induced by inhibitors of protein or nucleic acid synthesis, nor by the antimalarials quinine, primaquine, proguanil, cycloguanil or pyrimethamine (Warhurst and Robinson, 1971). The investigation of factors that could interfere with chloroquine-induced pigment clumping (CIPC) introduced a novel biochemical tool into these studies. Warhurst *et al.* (1971) found that protein synthesis inhibitors prevented CIPC and they later (Warhurst and

Baggaley, 1972; Warhurst *et al.*, 1972, 1974; Warhurst, 1973) confirmed that the chloroquine-induced autophagosome formation was a synthetic process sensitive to inhibitors of RNA synthesis and ribosomal protein synthesis and to temperature (clumping not occurring normally below 35°C nor above 38°C). CIPC was also substrate dependent, only 4% clumping occurring in the absence of glucose and only 18% if amino acids were omitted from the culture medium. Of the individual amino acids, only cysteine or methionine produced a marked influence on clumping (Homewood, quoted by Warhurst, 1973).

Homewood *et al.* (1972) also utilized the CIPC technique in conjunction with respirometry to study electron transport in *P.berghei*. The results of this study are discussed elsewhere (on p. 183).

Although the technique does not lend itself to drug screening (Warhurst, 1973), it did lead Robinson and Warhurst (1972) to discover the antimalarial activity of erythromycin. Tetracycline, however, also an effective antimalarial against *P.berghei*, was inactive on CIPC at 10^{-4}M. Cycloheximide and puromycin also inhibited CIPC (50% inhibition at 3×10^{-5}M and 3×10^{-6}M respectively), but were too toxic for use as antimalarials. The lack of effect of chloramphenicol and the marked effect of cycloheximide on CIPC indicate that ribosomes of eukaryotic type are involved (Warhurst *et al.*, 1974).

The CIPC phenomenon seemed to imply that the parasites possess structural sites to which the drug could bind, but the nature of these sites was quite unknown. Using ^{3}H-chloroquine, Fitch (1969) showed that at least three types of chloroquine binding occurred in parasitized erythrocytes with association constants of 10^{8}, 10^{5}, and 10^{3} mol^{-1}. Warhurst (1973) considered that high concentrations of chloroquine inhibit clumping by interrupting cellular synthesis, while low concentrations of the drug are associated with a "clumping site".

Further examination of the nature of the clumping site (Warhurst *et al.*, 1972; Warhurst, 1973; Warhurst and Thomas, 1975a) also involved the use of the CIPC technique in studies on the activity and mode of action of a variety of other antimalarials. Quinine was shown to inhibit CIPC competitively, with two molecules of the drug competing with each molecule of chloroquine for a clumping site. The clumping site was shown to have the characteristics of a classical "receptor" and structural similarities could be surmised between drugs that bind at the site. It was suggested that the clumping site was planar with co-planar electronegative and electropositive areas. The site was

very highly structure specific. Quinine displayed a marked competitive inhibition of CIPC, whilst the quinoline methanol WR 142,490 (mefloquine) and the phenanthrene methanol WR 122,455 (see Figure 1, x, ix) had affinities for the clumping site 100 and 40 times, respectively that of quinine. Because the affinities of amino-alcohol antimalarials for the clumping site and for the high affinity binding site differed, Warhurst and Thomas (1975a) concluded that these sites were separate.

They suggested that the clumping receptors are not lost from chloroquine-resistant malaria parasites, but are present in a modified form which is still accessible to suitable drugs. In moderate degrees of chloroquine resistance, where sensitivity to quinine is retained, such modifications could involve alteration of the electropositive area, perhaps by shielding with a lipophilic group, or by a slight increase in the distance between the charged areas of the receptor. At high levels of chloroquine resistance, when quinine is ineffective, it was suggested that the electronegative area might also be modified, so as to reduce its affinity for the charged part of the drug. The latter modification would have a marked action on the binding of cinchona alkaloids and derivatives, where the positively charged group is incapable of becoming co-planar with the aromatic ring. In drugs such as WR 122,455 and WR 142,490,* the receptor-fitting determinants are co-planar, and lipophilic electronegative groups are available on the ring; they would still be capable of binding to the modified receptor.

Warhurst *et al.* (1972) suggested that the clumping site may be localized on the digestive vacuole membrane. Compounds that bind to the site would, in fact, fall into the category of lysosomotropic drugs (De Duve *et al.*, 1974). Both the clumping site and high affinity site would appear to be necessary in order to achieve intracellular cytotoxic concentrations of a drug that is present only in low concentrations in the plasma. Whilst binding to these sites may be considered an essential prerequisite to the antimalarial activity of such compounds, it may not in itself be responsible for the antimalarial effects. The latter are probably brought about by interaction of the concentrated drug with certain metabolic pathways of the parasites. (*P.knowlesi* trophozoites

* In spite of having very high affinities both for the clumping site and the high affinity site, WR 142,490 and WR 122,455 differ markedly in another biological property, namely the ability to bind with DNA; WR 142,490 does not intercalate with DNA (Davidson *et al.*, 1975) whereas WR 122,455 does (Porter and Peters, 1976).

in vitro, for example, after several hours in the presence of 10^{-6}M chloroquine, show a great reduction of nucleic acid and protein synthesis and of glycolysis—Gutteridge and Trigg, 1972.)

3. Membrane transport systems

The fact that the intraerythrocytic malaria parasite is separated from the extracellular medium by not one, but three unit membranes, is sufficient to deter most would-be investigators of membrane transport systems in plasmodial infected cells.

Unlike that in several avian malarias, amino acid transport has not been investigated in *P.berghei*-infected erythrocytes. Homewood and Neame (1974) demonstrated that only parasitized mouse erythrocytes are permeable to L-glucose. Erythrocytes infected with *P.berghei* also have been shown to accumulate ^{3}H-chloroquine 100-fold from the plasma (Macomber *et al.*, 1966). That the drug was not washed out of the parasites by 0·14M NaCl, suggests that it was not bound to DNA. These workers further found that erythrocytes infected with chloroquine-resistant *P.berghei* accumulated this antimalarial to only one-half to one-third of the level attained by red cells containing chloroquine-sensitive parasites. More refined studies on *P.knowlesi* (Polet and Barr, 1969), chloroquine-sensitive *P.berghei* and *P.falciparum* (Fitch, 1969, 1970) later showed that the chloroquine concentration in parasitized erythrocytes was even greater than that shown by Macomber *et al.* (up to 600-fold).

These observations served as a basis for the sophisticated experiments carried out by Fitch and his co-workers (referred to on p. 366) to investigate the mode of action of chloroquine and pharmacologically related compounds on murine malaria parasites in relation to parasite membranes. Their data suggested that chloroquine uptake was attributable to a saturable process with a high affinity and specificity for chloroquine and processes that were not saturated by the highest concentrations of chloroquine employed in the experiments (Fitch, 1969). Significantly, the saturable high affinity sites were absent, or present only in very small numbers in chloroquine-resistant parasites. Kramer and Matusik (1971) later proposed that the high affinity binding sites were associated with the membrane of a "free" *P.berghei* lysate whilst the low affinity sites were cytoplasmic.

The high affinity binding site was found to be specific for derivatives

of 4-aminoquinolines and pharmacologically related drugs (Fitch, 1969, 1972; Fitch *et al.*, 1974b, c). Accumulation of chloroquine via this site was shown to be substrate dependent in chloroquine-sensitive *P.berghei* and *P.falciparum.* Chloroquine-resistant *P.berghei*-infected erythrocytes, however, required greater concentrations of glucose to stimulate drug uptake at chloroquine concentrations of 10^{-7}M or less, and drug accumulation by chloroquine-resistant *P.falciparum* was insensitive to glucose (Fitch *et al.*, 1974b, c). The blunted response of chloroquine-resistant *P.berghei* to glucose could be revived by higher concentrations of chloroquine (above 10^{-7}M) in the medium. With increasing concentrations of chloroquine the maximum accumulation of the drug by the resistant strain is at least as great as in erythrocytes infected with the chloroquine-sensitive *P.berghei* (Fitch *et al.*, 1975a). This effect was not observed with chloroquine-resistant *P.vinckei* or *P.y.yoelii* 17 X, nor with chloroquine-resistant *P.falciparum.*

Although the K_i values for amodiaquine and chloroquine accumulation were the same, erythrocytes infected with chloroquine-resistant *P.berghei* have a larger maximal capacity for accumulating amodiaquine which Fitch *et al.* (1975b) suggested occurred because chloroquine-resistant *P.berghei* obligatorily invaded immature erythrocytes. The immature erythrocytes possess a high affinity binding site for chloroquine and amodiaquine, which is independent of glucose supply and to which amodiaquine has greater access than chloroquine (Fitch *et al.*, 1975b). Mature erythrocytes treated with a non-specific protease from *Streptomyces griseus* also became able to accumulate chloroquine to a high degree (Fitch *et al.*, 1974a). Weidekamm *et al.* (1973) have shown that *P.berghei* selectively degrades certain proteins in the erythrocyte membrane. The "high affinity" site of Fitch may, therefore, be present in the erythrocyte membrane of *P.berghei*-infected cells and not simply in the parasites themselves.

The studies on chloroquine uptake by malaria parasites illustrate the difficulties inherent in investigations of membrane transport and the need to differentiate between the properties of the erythrocyte and those of the parasite membranes. The problem is complicated by different properties of the membranes of immature and mature erythrocytes, and the fact that alterations are induced in the erythrocyte membranes by the parasites themselves.

4. Feeding mechanisms and penetration of the red cell

Antimalarial attachment to receptor sites on membranes clearly may influence the mechanisms by which the plasmodia penetrate new host cells and feed on their contents once they have settled down to an intracellular life. Aikawa (1972) using electron microscope autoradiography, observed that ^{3}H-chloroquine was localized in pigment-containing digestive vacuoles of *P.berghei*. Homewood *et al.* (1972) also suggested that chloroquine and related compounds might be concentrated within the parasites' lysosomes and act by interfering with the feeding mechanism. In mouse erythrocytes infected with *P.berghei* (N strain) and incubated *in vitro* with 3×10^{-8} or 10^{-7}M mepacrine for five minutes, the drug appeared to be localized at the host–parasite interface and on the membranes of the parasites' digestive vacuoles (Warhurst and Thomas, 1975b). With longer times of incubation, however, the drug was distributed throughout the cytoplasm of the trophozoite but was not concentrated in the nucleus or within the digestive vacuoles. In developing and mature schizonts the drug was particularly concentrated in the conoid region of the merozoites, possibly within the paired organelles. Little fluorescence developed in mepacrine- and chloroquine-resistant (RC strain) *P.berghei* incubated with mepacrine. On the basis of these observations these workers suggested that the areas in which fluorescence was observed in the N strain parasites represent the high affinity binding sites for chloroquine and related compounds.

The recent important advances in our understanding of the mechanisms by which the merozoites attach to and penetrate the erythrocyte have been discussed by Sinden (Chapter 3). For the immunologist, particularly significant are the nature of the receptors on the surface of the host cell. Warhurst and Thomas' (1975b) study with mepacrine now suggests that "schizontocide" drugs may also block the penetration of new cells by *P.berghei* and *P.falciparum*, thus in a sense acting like antimalarial antibody. However, they could produce no evidence to support the suggestion of Fitch *et al.* (1974, 1975) that the high-affinity chloroquine-binding site is a property of the erythrocyte membrane and not of the parasite.

5. Dependence on host enzymes

The trophozoites of *P.berghei* in mature red cells obtain most of their energy through anaerobic glycolytic pathways that culminate in the production of lactate. During the investigation of the metabolic pathways of chloroquine-resistant *P.berghei*, Howells *et al.* (1970a) obtained evidence that seemed to indicate that, under chloroquine pressure, chloroquine-resistant trophozoites were able to open up an aerobic glycolytic Krebs cycle, a process that appears to occur once the parasites begin the sporogonic cycle in the anopheline vector. They postulated that, in these conditions, the genetic mechanisms for synthesis of enzymes of the Krebs cycle which are not normally functional in the vertebrate stages can be derepressed, thus producing a mechanism for survival of these parasites through the maximum utilization of glucose and, possibly, amino acid synthesis through transamination. Subsequent studies by Howells and Maxwell (1973) indicated that this hypothesis was in fact not valid, and that what was really happening was that the host cells were in some manner being induced to synthesize more than usual of their own Krebs cycle enzymes. (It should be recalled that this strain, *P.berghei* RC, preferentially invades immature erythrocytes.)

Several workers have noted how the presence of malaria parasites may induce an increase in enzyme production by host erythrocytes. Trager (1967), for example, showed that *P.lophurae* utilizes pyruvate kinase of its host, and Bennett and Trager (1967) demonstrated that the parasites require coenzyme A formed by the host cells. Following Howells' report, Peters (1973) suggested that the ability of *P.berghei* to induce increased enzyme production in its host cells may also account for the ease with which these parasites can develop multiple drug resistance, e.g. to both chloroquine and pyrimethamine, and suggested that this may also be happening in some strains of *P.falciparum*.

6. Transmission of chloroquine-resistant strains

Ramkaran and Peters (1969) observed that when mice infected with a cyclically transmissible, chloroquine-resistant line of *P.berghei* NK 65, were given chloroquine phosphate at doses ranging from 0·1 to 100 mg kg^{-1} and mosquitoes (*A.stephensi*) were fed on the mice 12 h later, more oocysts developed in the mosquitoes fed on mice dosed with 1·0

and 10 mg kg^{-1} chloroquine than in undosed controls or those dosed with 0·1 or 100 mg kg^{-1}. The maximal enhancement was observed following 1·0 mg kg^{-1}. No similar effect was observed with the chloroquine-sensitive L/9 line of *P.berghei* NK 65. Since the drug has no effect on the sporogonic development of either line when fed directly to the infected mosquitoes, it was concluded that chloroquine at an appropriate concentration can affect gametocytes of chloroquine-resistant parasites so as to enhance their infectivity to the vector. This phenomenon has subsequently been confirmed in other strains of murine malaria parasites, including *P.y.yoelii* 17 X (Peters *et al.*, 1970).

The mechanisms underlying this phenomenon remain uninvestigated and unexplained. Its potential significance to the spread of chloroquine-resistant *P.falciparum* was stressed in the original report. Wilkinson, Gould and Noeypatimond (R. N. Wilkinson, personal communication) have recently observed that the infectivity to *A.balabacencis* of the gametocytes of chloroquine-resistant *P.falciparum* is enhanced by chloroquine. The quinoline methanol WR 142,490 did not enhance the infectivity of gametocytes of chloroquine-resistant murine malaria parasites to *A.stephensi* (E. Daveds *et al.*, unpublished observation).

B. Diet, Sulphonamides and PABA Utilization

Tetrahydrofolic acid (FH_4) is a metabolically active co-factor or carrier for one-carbon units required for the synthesis of purine nucleotides and deoxythymidine monophosphate, for amino acid interconversion and ribosomal protein synthesis (Jaffe, 1972). In the mammalian host FH_4 is derived from dietary folic acid via dihydrofolate (FH_2) by reactions involving folate reductase and dihydrofolate reductase. Unlike mammals, however, plasmodia are unable to utilize pre-formed folic acid, lacking the enzyme folate reductase (Ferone and Hitchings, 1966) and must therefore synthesize folate containing co-factors *de novo* as do some pathogenic bacteria.

As early as 1940, Woods had related PABA to the action of sulphanilamide in bacterial systems. Thurston (1950a, b) showed that PABA antagonized the action of sulphadiazine against *P.berghei* in mice. A milk diet inhibited the development of *P.berghei* in mice (Maegraith *et al.*, 1952) and Hawking (1953) confirmed that this was attributable to a PABA deficiency.

The inhibition of malaria infections by sulphonamides is more readily antagonized by PABA than by folic acid (Thurston, 1954; Hill, 1963). Walter and Königk (1971) and Ferone (1973) ultimately demonstrated that various sulphonamides reduce the activity of dihydropteroate synthetase extracted from *P.chabaudi* and *P.berghei*, and showed that the drugs competitively inhibit PABA binding with this enzyme. Although the K_i values obtained by Ferone (1973) for sulphonamide–enzyme binding were difficult to relate to the ED_{50} values obtained against *P.berghei in vivo*, it was suggested that factors such as the different plasma binding, metabolism, and half-life, etc. of the drugs could be responsible for this discrepancy. Not all aspects of sulphonamide activity can easily be explained by the above generally accepted concept of the mode of action of these drugs.

Sulphones are also generally considered to exert their antimalarial activity by blocking PABA incorporation (reviewed by Peters, 1970a). Cenedella and Jarrell (1970), however, suggested that dapsone (DDS) may act by inhibiting glucose utilization by the parasites and a partial reversal of the *in vivo* activity of the sulphone was achieved by inducing hyperglycaemia in the host (Cenedella and Saxe, 1971).

Sulphonamides and sulphones have a well-known activity against the pre-erythrocytic stages of rodent malarias (Peters, 1970a; Thompson and Werbel, 1972). Sulphadiazine on the other hand, had no appreciable sporontocidal effect on *P.berghei* (Ramkaran and Peters, unpublished). PABA given to mosquitoes in a glucose solution, after they were infected with *P.berghei*, also enhanced sporogonic development, maximum enhancement being observed with an 0·01% PABA solution. When PABA was administered to the mosquitoes before the infective blood meal, an increase in numbers of oocysts was again observed, the increase being dose dependent up to a maximum (534% of control) with an 0·05% solution in 4% glucose. At 0·1% PABA no appreciable enhancement was observed. An increase in numbers of oocysts was also obtained by allowing the mosquitoes to feed on infected mice which had PABA in their drinking water. Enhancement was observed with PABA concentrations of 0·01 to 0·5%, with maximal effect at 0·05%.

More detailed work is needed on the mode of action of sulphonamides and sulphones, but a major gap in our understanding of the activity of these compounds is the total lack of information on the mechanism of sulphonamide resistance in *Plasmodium*. This is all the more surprising

when it is realized that interest in sulphonamides lapsed very largely as a result of the facility with which resistance to these compounds may be acquired by malaria parasites.

C. Antagonists of Dihydrofolate Metabolism

1. *Drug-induced morphological changes*

Valuable information on the morphological changes occurring during cell division of *Plasmodium* has been derived from light microscope and ultrastructural studies on parasites under the influence of agents that block various phases of mitosis. The morphological changes induced in malaria parasites for example by antagonists of dihydrofolate metabolism, such as pyrimethamine, proguanil and cycloguanil, are similar to those observed following exposure to sulphonamides. The parasites appear to be arrested in the process of cell division, with many bizarre forms of incomplete schizogony (Figure 5). Aikawa and Beaudoin (1968) previously showed that in *P.gallinaceum* pyrimethamine caused an arrest of nuclear division at metaphase, with no disruption of the mitotic apparatus.

Jacobs (1965) produced a pyrimethamine-resistant line of *P.berghei* which morphologically resembled the parent NYU 2 strain at the light microscope level. Rabinovitch (1968), on the other hand, described differences in the size of the trophozoites with marked pigment changes and alteration in the staining characteristics of a pyrimethamine-resistant strain. Peters (1970a) commented that the description given by Rabinovitch recalls that of gametocytes of a normal strain of *P.berghei*.

2. *The nature of parasite dihydrofolate reductase*

The pathway by which the malaria parasite synthesizes dihydrofolate from PABA and a pteridine is not utilized by the host and hence sulphonamides and sulphones, in competing with PABA, interfere specifically with the metabolism of the parasite. The conversion of dihydrofolate to tetrahydrofolate, on the other hand, is performed by both the parasite and its host and is mediated by the enzyme dihydrofolate reductase (DHFR). Ferone and Hitchings (1966) obtained a 95% inhibition of *P.berghei* DHFR *in vitro* with a concentration of $1{\cdot}5 \times 10^{-5}$M pyrimethamine, and found that the chemotherapeutic specificity of this

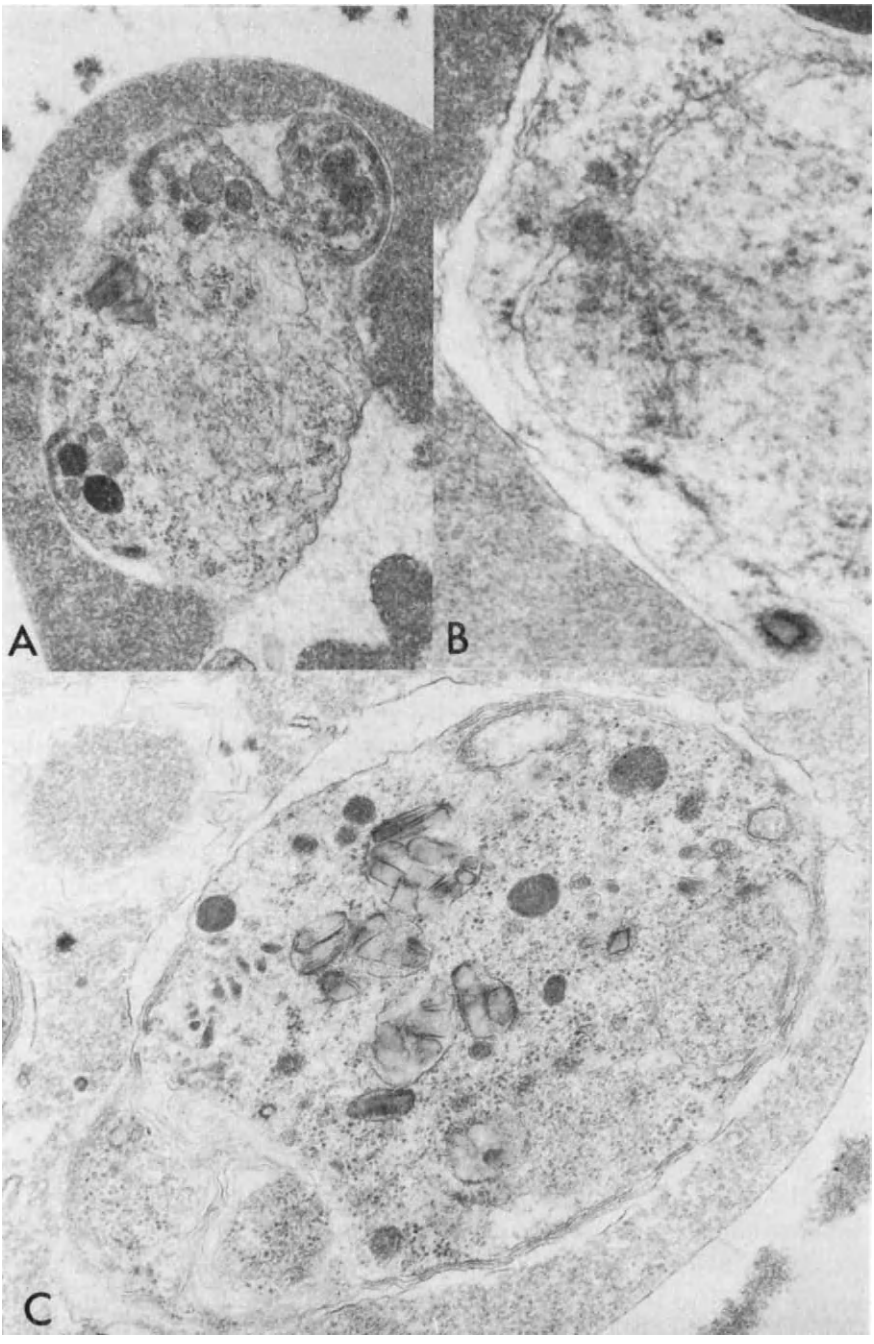

Figure 5. *P.y.nigeriensis* erythrocytic forms 24 h after treatment with pyrimethamine (5 mg kg^{-1} i.p).

(A) Arrested schizont showing some degree of merozoite differentiation ($\times 32\,000$).

(B) Arrested nuclear division in pre-schizont showing spindle figure ($\times 64\,000$).

(C) Pre-schizont showing a degree of clumping of the pigment. There is no obvious development of merozoites in this stage although the rhoptries and micronemes are differentiating ($\times 40\,000$).

drug was based on the different affinities for it of the host and parasite enzymes. Parasite DHFR was some 10 000 times more sensitive to pyrimethamine than was the reductase from erythrocytes (Ferone *et al.*, 1968, 1969). A similar difference was found in the affinity of host and parasite enzymes for cycloguanil.

Pyrimethamine binds stoichiometrically to isolated *P.berghei* DHFR and the degree of binding by pyrimethamine and three dihydrotriazine antimalarials *in vitro* correlated positively with their action *in vivo* (Ferone *et al.*, 1969). In pyrimethamine-resistant strains the reductase has a lower affinity both for pyrimethamine and dihydrofolate, though this is compensated for by higher enzyme levels in the parasite (Ferone, 1969; Diggens *et al.*, 1970). Mechanisms of resistance to dihydrofolate reductase inhibitors are discussed on p. 375.

Intraerythrocytic plasmodia synthesize pyrimidine nucleotides *de novo* (see Chapter 4), and for this the supply of deoxythymidine monophosphate (dTMP) is probably crucial (Jaffe, 1972). In *P.knowlesi* (Gutteridge and Trigg, 1972) and *P.berghei* (Jung *et al.*, 1975) DNA synthesis proved to be discontinuous, being greatest in the young ring and late trophozoite stages. Pyrimethamine, however, affected DNA synthesis only in the schizont stage and in both species the drug also inhibits growth primarily at the schizont stage. Gutteridge and Trigg (1972) suggested that if pyrimethamine does not cause an inhibition of DNA synthesis there may be some other metabolic process in the parasite requiring a fully functioning dihydrofolate reductase, which is only utilized during the process of schizogony. This idea is tenable only if dTMP for DNA synthesis is produced by a process which does not require the presence of a functioning dihydrofolate reductase, or if pyrimethamine penetrates to the malaria parasite only during schizogony. Gutteridge and Trigg (1972) considered that the latter was more probable. Pyrimidine uptake by malaria parasites is discussed in detail in Chapter 4.

3. Studies with drug combinations

Further light on sequential steps of parasite metabolic pathways has been shed by studies on antimalarial drug combinations. Richards (1966) demonstrated that pyrimethamine potentiated the schizontocidal action of sulphadoxine and of dapsone against *P.berghei* in the mouse, and this was confirmed and extended to combinations of

sulphadiazine and cycloguanil or proguanil by Peters (1968). The ED_{50} values for pyrimethamine and sulphadoxine in combination were reduced approximately seven-fold from the ED_{50} levels of the drugs when used individually (Richards, 1966). The obvious advantage of using synergistic combinations of drugs is the reduction in the dosage of the individual components required for antimalarial activity. It was also found that using sulphonamides and antifols in combination reduced the rate at which resistance to those compounds emerged. The ease with which resistance to both these classes of antimalarials can be induced has been commented on previously. Peters (1974b) additionally has demonstrated that, when such potentiating mixtures are given in combination with chloroquine, development of resistance to the latter is significantly delayed.

A further feature of the action of potentiating drug mixtures is that a synergistic effect can be observed even when the mixture is used on parasites which are resistant to one or both of the components. Richards (1966) showed that against a pyrimethamine-resistant strain of *P.berghei* with an ED_{50} (mg kg^{-1} daily $\times$ 7) for pyrimethamine of 10, and for sulphadoxine of 25, in combination the ED_{50}s of the two compounds were 0·02 and 5·0, respectively.

A similar drug mixture was effective against presumed pyrimethamine-resistant *P.falciparum* infections (Laing, 1968). A mixture of sulphalene and pyrimethamine was also active against strains of *P.berghei* that were highly resistant to the individual components (Peters, 1971).

Pyrimethamine–sulphadoxine combinations, and indeed pyrimethamine alone, were less effective against the NS cyclically transmissible strain of *P.berghei* when it was under chloroquine pressure than against parasites being passaged without chloroquine pressure (Peters *et al.*, 1973). This observation parallels that made on chloroquine-resistant *P.falciparum* infection in man in South-East Asia. These authors have suggested that, under chloroquine pressure, the parasites are situated within reticulocytes which are stimulated to produce metabolic intermediates that enable the parasite the better to resist the antimetabolic effects of the antimalarials. An interrelationship between resistance to the chloroquine and antifolate drugs was suggested by the observation of Carter (1972) that a chloroquine-resistant strain of *P.yoelii* reverted to chloroquine sensitivity when the mice were maintained on a low PABA diet.

A marked potentiation of the effect of sulphadoxine was also observed with pyrimethamine against the sporogonic stages of *P.berghei* (Ramkaran and Peters, 1969) and several examples of synergistic combinations active on the pre-erythrocytic stages of *P.berghei* were described by Vincke (1970).

4. Mechanisms of drug resistance

As indicated above, the mechanisms whereby resistance to sulphonamides is acquired by malaria parasites have not been investigated and remain obscure. Sulphonamide-resistant strains in general require less PABA than normal parasites, and a sulphonamide-resistant strain of *P.berghei* was produced by maintaining mice on a PABA-free diet (Ramakrishman *et al.*, 1956). It is widely accepted that sulphonamide resistance arises by the selection of pre-existing mutants from the "sensitive" population.

Resistance of malaria parasites to antifols has been shown to be associated with dihydrofolate reductases (DHFR) that differ from those of the parent strain. Thus Ferone (1969) and Diggens *et al.* (1970) have described DHFRs from pyrimethamine-resistant strains of *P.berghei* which show a reduced affinity both for pyrimethamine and dihydrofolate, although greater amounts of the enzyme were present in these parasites compared to the parent sensitive strains. The "resistant" enzyme described by Diggens *et al.* (1970), for example, required a 30-fold increase in the pyrimethamine concentration to achieve a 50% inhibition, and the affinity for dihydrofolate was reduced tenfold.

Pyrimethamine-resistant strains containing DHFR with a lowered affinity for dihydrofolate would need to produce more dihydrofolate. This is reflected in their increased sensitivity to sulphonamides and sulphones. An exception to this general rule was the partial resistance to dapsone observed in a cycloguanil-resistant strain of *P.berghei* (reviewed by Peters, 1970a). However, this sulphone may not act simply by competing with PABA in dihydrofolate synthesis (Cenedella and Jarrell, 1970; Cenedella and Saxe, 1971). Although resistance to antifols generally results in a hypersensitivity to sulphonamides, the converse is not true.

D. Electron Transport Mechanisms

1. Electron transfer chains in rodent malaria

Chloroquine-induced pigment clumping (CIPC) was used by Homewood *et al.* (1972) to investigate the nature of electron transport in *P.berghei*. CIPC, which is an energy-dependent process, occurred in the absence of oxygen and in the presence of 10^{-3}M cyanide. The process was inhibited by the electron transport inhibitors rotenone and antimycin A, both at 10^{-4}M. On the basis of their observations Homewood *et al.* concluded that two electron transfer chains were present in erythrocytes infected with *P.berghei*. So far the best evidence for electron transfer processes in *P.berghei* lies in the demonstration of a parasite CoQ_8 (Skelton *et al.*, 1970).

Interest in naphthoquinones as antimalarials appears to have been based on an assumption that vitamin K was intrinsic to the metabolism of the parasite—a not unreasonable assumption since the vitamin has been demonstrated in many other micro-organisms. Although a large number of naphthoquinones were active in avian malaria models, in 1968 Skelton and co-workers showed that vitamin K was not present in *P.lophurae* at a level compatible with a role in electron transfer mechanisms. However, co-enzymes Q_8 and Q_9 were found in the latter species, and later Skelton *et al.* (1970) found CoQ_8, but not vitamin K, in *P.berghei*. Skelton *et al.* (1968) suggested that the naphthoquinones may exert their antimalarial effect through inhibition of the biosynthesis or function of CoQ in the malaria parasite and tested the naphthoquinone menoctone in two sites in which CoQ participates, DPNH—oxidase and succinoxidase. They found that 10 to 25 nmol menoctone inhibited DPNH-oxidase by approximately 90% and 100 nmol of the drug reduced succinoxidase activity to only 7% of the control values. Naphthoquinones are also active on host CoQ systems but, for example, more than 100 times the concentration of drug was required to produce 50% inhibition of carbohydrate metabolism in normal duck erythrocytes compared with parasitized erythrocytes (Findlay, 1951).

Many of the naphthoquinones that showed high levels of activity against avian malaria had little activity against *P.berghei*. Mitochondrial swelling was observed in trophozoites of *P.berghei* following treatment *in vivo* with menoctone (Howells *et al.*, 1970b) suggesting that the drug

interferes with the mitochondrial-associated processes in this parasite. A similar morphological effect was observed in primaquine-treated trophozoites of *P.berghei* (Howells *et al.*, 1970) which also resembled the changes induced in the mitochondria of exoerythrocytic forms of *P.lophurae* and *P.fallax in vitro* by primaquine (Beaudoin and Aikawa, 1968; Aikawa and Beaudoin, 1969). However, another 8-aminoquinoline, pamaquine, has long been known to be inactive against the tissue forms of *P.gallinaceum* (Tonkin, 1946). The morphological changes induced by the naphthoquinone and 8-aminoquinoline, might be expected to be similar if the latter compounds, as generally accepted, exert their antimalarial effects via quinoline quinone metabolites (see review by Peters, 1970a). The attribution of activity to a metabolite of the 8-aminoquinoline might also explain the relative lack of effect *in vitro* of primaquine on DPNH-oxidase and succinoxidase activity in *P.lophurae* (Skelton *et al.*, 1969). These latter workers did, however, demonstrate that primaquine *in vitro* at a concentration of 10^{-9}M reduced the oxygen uptake of *P.lophurae*-infected duck erythrocytes by 50%. That 8-aminoquinolines and naphthoquinones act at different loci in the same metabolic pathway may perhaps be inferred from the synergistic effect observed with a mixture of these compounds on the exoerythrocytic stages of *P.cathemerium* (Walker and Richardson, 1948). Walker found that this drug mixture did not potentiate in its activity against the blood stages of *P.cathemerium*, and no potentiation of the schizogonic effect of menoctone and primaquine on *P.berghei* was observed when these compounds were administered together (Peters, 1970b). The effect of a menoctone–primaquine combination on the liver schizont of *P.berghei* does not, however, appear to have been investigated.

Primaquine administered in sugar solution to *P.berghei*-infected *A.stephensi* produced no observable changes in the ultrastructure of the oocyst (Davies *et al.*, 1971a). This might be expected if the drug acts in the vertebrate via a metabolite.

2. *Potentiation between cycloguanil and quinolinequinones*

Synergistic action between antimalarials may indicate that they act at different sites in the same metabolic pathway of the parasite, as appears to happen with sulphonamides and dihydrofolate reductase inhibitors. The demonstration of potentiation of the schizogonic action of

menoctone when it was administered with cycloguanil to *P.berghei*-infected mice (Peters, 1970b) was, however, difficult to explain in terms of this basic principle if the generally accepted, albeit poorly documented, concepts of the mode of action of these antimalarials are valid. However, this potentiation cannot be regarded simply in terms of synergism between antifols and antagonists of electron transport since no potentiation was observed with a mixture of pyrimethamine and menoctone (Porter and Peters, unpublished). The difference between these results is surprising and suggests that cycloguanil may exert an effect on the parasite other than by inhibiting DHFR (an effect not exerted by pyrimethamine).

Unlike menoctone and cycloguanil mixtures, primaquine and cycloguanil do not potentiate each other but have an additive effect against the blood stages of *P.berghei* (Peters, 1970b). A primaquine-resistant strain of *P.berghei* was, however, completely cross-resistant to cycloguanil, and a line primarily resistant to cycloguanil exhibited some degree of resistance to primaquine. Both strains were cross-resistant to menoctone.

There is thus an apparent interrelationship between the modes of action of cycloguanil, primaquine and menoctone. The additive effect observed with mixtures of primaquine and menoctone on *P.berghei* might suggest that the 8-aminoquinoline or its hypothetical quinone metabolites act at the same time as the naphthoquinone on the parasite electron transport system. This suggestion, however, is not supported by the different experimental results with primaquine–cycloguanil and menoctone–cycloguanil mixtures. Two quinolone esters were investigated for antimalarial activity by Ryley and Peters (1970). These esters were considered to have a primaquine-like effect on *P.berghei* and, significantly, their antimalarial effects were potentiated by sulphadiazine or chlorcycloguanil.

The physiological basis for the observed interactions between antifols and quinolinequinones remains obscure but provides one of the most intriguing areas of investigation in host–parasite–drug interactions.

E. Synthesis of Nucleic Acid

1. Chloroquine and parasite RNA

Tokuyasu *et al.* (1969) measured the sedimentation characteristics of ^{32}P-labelled RNA from *P.berghei* and obtained values of 4S, 15S and

25S, with the monomeric form of the malarial ribosome as 80S. Using ^{14}C-labelled orotic acid or ^{32}P as markers these workers obtained evidence only of a labelled 40S ribosomal subunit. The presence of an unlabelled 60S subunit was inferred and it was suggested that this 60S subunit could be of host origin. In a more recent study of *P.knowlesi* ribosomes, however, Sherman *et al.* (1975) dissociated 80S particles into 60 and 40S subunits, both of which had typically protozoan base compositions. It was concluded that the suggestion of Tokuyasu and his co-workers that the 60S ribosomal subunit of *P.berghei* is provided from host ribosomes seems most unlikely.

Warhurst and Williamson (1968) showed that following chloroquine treatment the 17·4S and 24·2S RNAs of *P.knowlesi* were degraded to 14–15S. This degradation occurred only after the formation of the autophagic vacuole (Warhurst and Williamson, 1970). At a therapeutically normal concentration of chloroquine ($\sim 10^{-6}$M) there is no inhibition of RNA synthesis in *P.berghei* (Homewood *et al.*, 1971; Warhurst *et al.*, 1974) or *P.knowlesi* (Warhurst, 1969). A chloroquine concentration of 2×10^{-2}M inhibited RNA synthesis in *P.knowlesi in vitro* by 50% (Warhurst, 1969). While this concentration is far higher than the normal range of serum concentration obtained *in vivo* ($\sim 10^{-6}$M), Gutteridge and Trigg (1972) found that 10^{-6}M chloroquine inhibited RNA synthesis after several hours *in vitro*. Presumably Warhurst (1969) examined the effect of chloroquine during the phase of accumulation into the parasite, cytotoxic effects being observable only some time after exposure to the drug. Primary exposure to higher concentrations, such as 2×10^{-3}M on the other hand results in cytotoxic effects being elicited more rapidly.

2. *Influence of drugs on nucleic acid synthesis*

The uptake of ^{32}P by intracellular *P.gallinaceum in vitro* was inhibited by quinine at $0{\cdot}5–3{\cdot}0 \times 10^{-5}$M but no inhibition of uptake was observed even with a 4×10^{-5}M concentration if the parasites were released from the host erythrocytes (Clarke, 1952). Schellenberg and Coatney (1961) later demonstrated that chloroquine, mepacrine and quinine inhibited uptake of ^{32}P into both RNA and DNA of *P.gallinaceum*. Chloroquine did not inhibit ^{32}P-uptake by *P.berghei* in these experiments and Peters (1970a) speculated that the strain of *P.berghei* utilized by Schellenberg and Coatney may have been chloroquine resistant. In *P.knowlesi* DNA

synthesis as measured by ^{3}H-labelled orotic acid uptake, was inhibited by only 13% by 10^{-6}M chloroquine, while 4×10^{-4}M was necessary to produce a 50% inhibition. As reported earlier, RNA synthesis was relatively less sensitive than DNA synthesis to chloroquine inhibition.

The evidence for the binding of chloroquine to double-stranded DNA and other polynucleotides has been reviewed extensively elsewhere (Peters, 1970a; Yielding *et al.*, 1971; Thompson and Werbel, 1972).

It has been proposed also that quinine forms an intercalation complex with DNA (see Hahn and Krey, 1971). The critical distance between the alicyclic amino group and the alcoholic hydroxyl of the quinine molecule was maintained in a quinolinemethanol selected by Hahn and Krey (1971), where a piperidine is substituted for the quinuclidine ring. This compound, α-piperidyl 6, 8 dichloro-2-phenyl-4-quinoline-methanol hydrochloride, did form a complex with DNA, though it was difficult to study owing to poor solubility and its property of precipitating DNA. In contrast, the highly active schizonticide WR 142,490, also a quinolinemethanol, did not bind to DNA (Davidson *et al.*, 1975). In other ways, however, the latter compound has many properties very close to those of quinine (Warhurst and Thomas, 1975a; Davies *et al.*, 1975). The 4-phenanthrenemethanol WR 122,455 does in fact intercalate with DNA (Porter and Peters, 1976).

The 8-aminoquinoline primaquine was also shown to interact with RNA and DNA and inhibit the activity of *E.coli* RNA polymerase. The interaction with DNA is not by intercalation (Holbrook *et al.*, 1971). It is difficult to evaluate results obtained *in vitro* on a non-malarial model with a drug that is considered to exert its *in vivo* effect through metabolites. Holbrook *et al.* (1971), however, considered that the interactions of primaquine with nucleic acids are significant aspects of its antimalarial activity.

3. *Synthetic deficiencies of the malaria parasite*

The review of malaria parasite biochemistry by Homewood (Chapter 4) adequately illustrates the fact that most aspects of parasite biochemistry and of host–parasite interactions remain obscure. This section is consequently restricted to a consideration of some examples where synthetic deficiencies of the parasite have been revealed by antimalarial drug action, or awareness of a deficiency has stimulated a search for agents which may serve as effective antimalarials.

One of the better documented aspects of the metabolism of the malaria parasite is its dependence on an exogenous source of preformed purines (see Chapter 4). The inability of the parasite to synthesize purines *de novo* stimulated Trigg *et al.* (1971) to investigate the antimalarial activity of the purine analogue cordycepin. Heischkeil (1974) considerably extended this study of antimetabolites based on purine analogues and observed a particularly good plasmodiostatic activity against *P.vinckei* infections with purine-6-sulphonic acid-3-N-oxide. In practice, however, these compounds have been unsatisfactory because of their toxicity to the host.

In contrast, the lack of a thymidylate salvage pathway in rodent malarias was suggested by the failure to demonstrate thymidine kinase in *P.chabaudi* (Walter *et al.*, 1970). Intra-erythrocytic murine malaria parasites are unable to utilize pre-formed pyrimidines, and the lack of incorporation of ^{3}H-pyrimidines into the oocysts and sporozoites of *P.berghei* (Davies and Howells, 1973; Jacobs *et al.*, 1974) indicates that this aspect of trophozoite metabolism is not controlled by permeability factors of the erythrocyte membrane.

Malaria parasites, lacking a folate reductase, apparently do not utilize folate derived from the host. Instead they synthesize dihydrofolate from a pteridine and PABA. The key reaction, catalysed by dihydropteroate synthetase, is competitively inhibited by sulphonamides.

The obligate parasitization of the immature, metabolically active erythrocytes by chloroquine-resistant strains of *P.berghei* suggests that chloroquine resistance is associated with an increased dependence by the parasite on host synthetic activities. This is further supported by the evidence, discussed earlier, of an enhancement of the metabolic activity of the reticulocyte when infected by chloroquine-resistant malaria parasites.

IV. CONCLUSIONS

In the final analysis, the value of rodent malaria in the search for new or improved antimalarials and in studying the mode of action of drugs and the mechanisms of resistance, rests entirely on the applicability of results obtained with this model to the malarias of man. The evidence presented in the first part of this chapter illustrates that, in general, the

rodent malarias can indeed serve as such a model and their practical value has been overwhelmingly demonstrated by their enabling over a quarter of a million compounds to be screened for antimalarial activity in little over a decade. That so little in the form of new drugs has emerged from this enormous expenditure of effort and resources is certainly no fault of the rodent malaria screen. Rather, as was discussed previously, it illustrates the essential limitation of empirical screening systems and emphasizes the necessity for a rational approach based on a more complete understanding of the biochemistry of the parasite and of the nature of its relationship to its host.

Whilst the major screening programmes for antimalarials are currently being run down, and are unlikely to be re-initiated in the foreseeable future, experimental and clinical studies have increasingly demonstrated the value of drug mixtures and combinations in the treatment of chloroquine-resistant malignant tertian malaria. In the absence of an armamentarium of new drugs it is probable that we shall have to rely increasingly on "polytherapy" in order to safeguard the effectiveness of those few effective compounds that we currently possess. The value of rodent malarias in investigating and selecting effective drug mixtures has been discussed. The availability of a wide range of drug-resistant strains of rodent malarias, against which drug mixtures can be tested, is of paramount importance.

In contrast to the amount of effort expended in producing and screening new compounds for potential antimalarial action, relatively few workers have studied the mode of action of the established antimalarials. These and other antiparasitic drugs can be considered as extremely valuable biological probes with which to explore little known areas of parasite biochemistry and physiology. Unfortunately many questions still remain unanswered regarding the mode of action even of chloroquine, the compound most investigated. One school of experimental chemotherapists favours the theory that antimalarial action is effected by intercalation of the drug with nucleic acids and a second school, including Fitch and Warhurst, believe that the drug may be generally cytotoxic but depends on a high affinity binding site to achieve the intracellular concentrations required for such an effect. The high affinity site studied by Warhurst and his colleagues, however, may be different from that studied by Fitch. Fitch, furthermore, suggests that the high affinity site may be present in the erythrocyte membrane and not even within the parasite. The fluorescence studies of Warhurst

and Thomas added a further facet to this problem by the observation of a concentration of mepacrine in the region of the paired organelles of the merozoites. Studies on the mechanism of resistance to chloroquine have similarly revealed the complexity of the problem rather than provide a definitive answer. With the exception of pyrimethamine, we can only speculate as to the method by which the malaria parasite becomes resistant to antimalarials.

Investigations on the mode of action of antimalarials and on the mechanisms of drug resistance must be linked with studies on the basic biochemistry and physiology of the parasites. It is abundantly clear that there is no aspect of the parasite metabolism which has been exhaustively studied or which is adequately understood (see Chapter 4). Large areas such as those of lipid synthesis and electron transport have been almost entirely neglected and others, such as the mechanism of haemoglobin digestion, remain unknown, apparently impregnable to the biochemical assaults which have been made to date.

The *cri de cœur* made in the introduction for an effective system for investigating malaria parasites and antimalarial drug action *in vitro* is now being increasingly echoed by workers in the field of malaria immunity for whom such cultivation techniques offer enormous prospects in terms of developing a malarial vaccine. It is anticipated that the rapidly increasing interest in the cultivation of *Plasmodium* will lead to significant improvements in these techniques in the near future.

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