THE DEVELOPMENT OF THE SPOROZOITE OF *PLASMODIUM GALLINACEUM* (APICOMPLEXA : HAEMOSPORINA)

by

David Peter Turner, B.Sc. (Lond.), A.R.C.S.

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of the University of London

Department of Zoology and Applied Entomology,
Imperial College Field Station,
Silwood Park,
Ascot,
Berkshire, SL5 7PY.

May 1980

TO MY MOTHER AND FATHER

WITH AFFECTION AND GRATITUDE

This day relenting God
Hath placed within my hand
A wondrous thing; and God
Be praised. At his command,

Seeking His secret deeds
With tears and toiling breath,
I find thy cunning seeds,
O million-murdering Death.

Sir Ronald Ross

Inspired by his discovery of the wonderful "pigmented cells" (oocysts) protruding from the stomach wall of a dapple-wing mosquito.

ABSTRACT

This thesis describes an investigation into the development of the P. gallinaceum sporozoite.

Observations by light microscopy failed to distinguish between sporozoites from mature oocysts and those from salivary glands. The only significant morphological change at the ultrastructural level occurred in the organisation of the rhoptry-microneme complex and resulted in a proliferation of the micronemes and a disappearance of the rhoptries in the salivary gland forms.

The cell surface properties of sporozoites were investigated by the techniques of free-flow electrophoresis and lectin-binding studies. The electrophoretic mobility of sporozoites was measured as a function of pH and data from these observations demonstrated that there was a significant reduction in cell surface charge of salivary gland sporozoites, compared to sporozoites from mature oocysts and qualitative differences between the two populations were shown to exist. The electrophoretic mobility of sporozoites was also examined after enzymatic and chemical modification of the cell surface groups. Trypsin treatment significantly reduced the electrophoretic mobility of both groups of sporozoites, although neuraminidase did not produce any change in cell surface charge.

Evidence for the presence of carbohydrates on the cell surface membrane of sporozoites was further sought by fluorescent lectin staining using a FITC-conjugated preparation of Concanavalin A. Results demonstrated that ligands similar to α-D-glucose and α-D-mannose are not present in an exposed or reactive form on the cell surface membrane of P. gallinaceum sporozoites.

The results of the free-flow electrophoresis and lectin-binding studies showed that the cell surface membrane of P. gallinaceum sporozoites lacks certain exposed carbohydrate and sialic acid residues which are common constituents of many cells, although it was shown to be rich in protein, possibly in association with amino, but not SH groups.

The biological significance of these findings was then examined by comparing the infectivity to chicks of sporozoites from mature oocysts and those from salivary glands. The latter group were shown to be 10,000 times more infective than sporozoites from mature oocysts. The infectivity of salivary gland sporozoites was further investigated after enzymatic modification of the cell surface membrane. Incubation with neuraminidase, which did not alter the electrophoretic mobility of sporozoites, did not have any effect on their infectivity, although trypsin treatment, which produced a significant decrease in the electrophoretic mobility, completely eliminated their infectivity to chicks.

Finally, following numerous attempts to culture primary exoerythrocytic schizonts by inoculation of infective salivary gland sporozoites in vitro, a suitable technique was eventually established.

The relevance of these findings is discussed.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. R. E. Sinden for his continued interest and encouragement during the period of this study.

Also, I am grateful to Professor P. C. C. Garnham, Dr. L. H. Bannister, Dr. R. S. Bray, Dr. N. Gregson and Dr. R. Killick-Kendrick for their help and discussion and to Mr. T. Ludlow and Dr. S. Young for their assistance with the statistics.

I would like to thank Professor T. R. E. Southwood for allowing me to make use of the Field Station facilities and the Science Research Council for financial support.

Special thanks are due to Mr. B. Balchin and Mr. S. Weir of Tetra Poultry Limited, who cheerfully supplied me with chicks and eggs for this work.

I would also like to thank Miss Patricia Hunt for patiently and accurately typing this thesis and Col. L. MacL. Young who proof read much of it. Finally, it is a joy to thank my wife, Christina, for the great deal of support and understanding she has always given me and who has made it all worthwhile.

TABLE OF CONTENTS

CHAPTER 1

1. GENERAL INTRODUCTION

The family Plasmodiidae Mesnil, 1903, contains the single genus Plasmodium Marchiafava and Celli, 1885 and includes nearly 100 species of true malaria parasites of mammals, birds and reptiles. Members of the genus are obligate intracellular parasites for almost all of their life cycle and have two hosts: a vertebrate in which asexual reproduction (schizogony) occurs and an invertebrate (mosquitoes) in which sexual reproduction occurs. Schizogony occurs in tissue cells, such as those of the liver parenchyma (mammalian malaria) or cells of the reticulo-endothelial system (avian malaria) which results in the production of a large number of merozoites. After entering other tissues, merozoites give rise to secondary asexual stages (the metacryptozoites and phanerozoites of avian malaria) and/or the sexual stages (gametocytes) in erythrocytes. The gametocytes, which are ingested by mosquitoes with a bloodmeal, develop in the midgut where fertilisation occurs, resulting in the formation of a motile ookinete, which penetrates the midgut wall and develops into an oocyst between the basement cell membrane and the basal lamina of the mosquito midgut wall. The oocyst then develops over a period of 4-15 days, depending on the species and the environmental conditions. On maturity, the oocyst ruptures, liberating sporozoites which progress towards the salivary glands, where they accumulate prior to being inoculated into another vertebrate when the mosquito takes its next blood meal.

Although malaria has been known from antiquity, it was not until the late nineteenth century that malaria parasites were first described, when Laveran, while working in North Africa in 1880, witnessed the dramatic nature of exflagellation and as Garnham (1966a) commented,

"and became at last convinced of the living nature of the various bodies that he had observed during the previous 2 years". Since Laveran's remarkable observations, 100 years of malaria research have elapsed and a great store of knowledge concerning this group of organisms has accumulated. But, however extensive our knowledge of the malaria parasites may at first seem, there are still fundamental questions which remain unanswered and many of these concern the sporozoite.

The migration of sporozoites from mature oocysts to the salivary glands and onto the final site of development in the vertebrate host is poorly understood, although the site of primary exoerythrocytic development for the avian malaria parasites is known. Sinden (1978) has voiced the feelings of many malariologists studying the rodent and simian parasites in stating that, "the greatest single omission is a total absence of knowledge as to the fate of the sporozoite following injection into the vertebrate host". The movements of sporozoites, ookinetes and merozoites remain little studied, as do their tactile responses to their target cells; for example, it is not known whether chemotaxis plays any role in the movement of sporozoites from mature oocysts to salivary glands. Also, receptor sites, which have been demonstrated for merozoites on the surface of erythrocytes (Miller et al., 1977), are not known for the other invasive stages. The function of many organelles is not well understood, "and will remain so until cytochemical techniques have been allied to electron microscopy of this group of organisms" (Garnham, 1966b). Finally, until very recently, the nature of malaria relapses has remained a mystery and although Krotoski et al. (1980) have demonstrated the occurrence of the resting stage, the "hypnozoite", the underlying mechanism of this phenomenon

remains unknown.

Thus the sporozoite poses many interesting problems and a thorough knowledge of this remarkable cell is fundamental to our greater understanding of the malaria parasites. In summary, it is able to tolerate a wide variety of habitats and crosses a number of cellular barriers; it initiates the malaria infection in the vertebrate; it is important in relapses, and it is regarded as of great potential for immunological studies (Nussenzweig, Cochrane and Lustig, 1978). Accordingly, studies were undertaken to examine further the development of sporozoites. Particular attention was paid to the cell surface properties, for it is known that the cell surface is important in a number of biological activities and the importance of the cell surface of merozoites is well documented (see for example, Bannister, 1977). Indeed the importance of the malaria parasite surface membrane was recently emphasised at a WHO workshop meeting (1979) which brought together experts in membrane biology, biochemistry, biophysics and immunology. They concluded that, "studies on the characterization and interaction of membranes from the parasite and their host cells are critical to the basic research on malaria which is required for the development of new and more subtle ways of controlling the disease. It is unlikely that the field of immunology will be the sole source of new methods for the control of the disease and membrane studies have a major application in the development of new methods of chemotherapeutic control. New drugs may also be developed on the basis of biological and biochemical characteristics of the parasite, for example, through the subtle modification of cellular receptivity to prevent invasion".

There were numerous reasons for choosing the avian malaria parasite, P. gallinaceum, for an investigation of this nature. When the study began in 1976, P. gallinaceum was the only species of Plasmodium to have been cultured from sporozoite inoculation, in vitro, although Strome, De Santis and Beaudoin (1979) and Sinden and Smith (1980) have now succeeded in culturing exoerythrocytic stages of P. berghei. However, P. gallinaceum still retains the advantage that the target cell of the sporozoite is known, being the cells of the reticulo-endothelial system near the site of bite, while culture of the rodent parasite has only been obtained in cultures of embryonic rat brain cells which are not selected in vivo. Also, there is a wealth of information on both parasite, P. gallinaceum and host, A. aegypti; both are easily maintained in the laboratory and A. aegypti, which is 100% susceptible to infection, produces salivary glands full of sporozoites. For these reasons, P. gallinaceum, with A. aegypti as host, is a very suitable model for studies on the biology of malarial sporozoites.

CHAPTER 2

2. HISTORICAL REVIEW

2.1. Introduction

An exhaustive account of the very many contributions to our knowledge of the malarial sporozoite and its immediate fate in the vertebrate host which have been made over the last 90 years, would be of such a length as to be out of place in this thesis. No such complete account has yet been written, but a number of excellent reviews are, however, available. The exhaustive work of Garnham (1966a) describes much of the early history and the life-cycles of the then known malaria parasites; Bray's (1957) monograph covers the exoerythrocytic cycle; up to date reviews are given in the work of Killick-Kendrick and Peters, "Rodent Malaria" (1978) which includes many of the recent studies on the cell biology and immunology of the rodent malaria parasites; and Gordon Harrisons book, "Mosquitoes, Malaria and Man" (1978) gives a very readable account of the "history of the hostilities since 1881", much attention being given to the early discoveries. However, in order to serve as a foundation for the experimental work described later in this thesis, a brief historical review of the relevant contributions which have led logically to our present state of knowledge is given below.

2.2. Biology of malarial sporozoites

2.2.1. Discovery and early history

The discovery of the sporogonic stages of malaria parasites in mosquitoes was made by Ross nearly twenty years after Laveran had described exflagellation of male gametocytes of the human parasites.

In 1897, Ronald Ross, driven by his own tenacity and inspired by the genius of his mentor, Patrick Mason, demonstrated that the grey mosquito (later, provisionally identified as Culex pipiens fatigans) was the invertebrate host of P. relictum and went on to describe the complete sporogonic cycle of this parasite (Ross, 1897, 1898). Ross was convinced that a similar cycle would exist for the human malaria parasites, although, due to his Government duties, he was unable to continue his research until 1899 in Sierra Leone, after the Italians had completed the work in such masterly detail.

It was in 1898, that Bignami succeeded in infecting a human volunteer with P. falciparum by the bite of naturally infected, wild-caught mosquitoes. Soon, the Italians had followed the complete sporogonic cycle of P. falciparum and P. vivax; P. malariae was to follow in 1899. The details of this cycle of development were published by Grassi (1900) in his classic monograph.

2.2.2. Fate of the sporozoite: discovery and early history of the exoerythrocytic forms

After the development of malaria parasites in their mosquito vectors and their subsequent transmission had been discovered, the gap remaining in the knowledge of the life cycles of malaria parasites concerned the events which occur between entry of the sporozoites into the vertebrate host and the infection of the erythrocytes. Since the time of the discovery of the stages in the mosquito vector, malariologists had held two theories of the development of malarial sporozoites once inoculated into a vertebrate. One was the obvious assumption that the sporozoites entered directly into the erythrocytes;

the other suggested that there was an occult phase of development which occurred between the injection of sporozoites and the blood parasitaemia. The latter view was supported by Grassi (1900) for, in his opinion, the nuclear differences between sporozoites and young erythrocytic trophozoites was too great to permit the sporozoite to transform into a blood parasite and that a prior cycle of development was needed. However, Schaudinn's (1903) allegedly lucid observations on the entry of sporozoites into erythrocytes which was later not only proved to be false, but for over 20 years hindered research into the development of the parasite during the pre-erythrocytic period, and all but removed Grassi's ideas from serious consideration.

After a period of 20 years much evidence, mostly of an indirect nature, was accumulating which questioned the accuracy of Schaudinn's reports. The evidence fell into several categories; the inability of chemoprophylaxis to prevent infection during the early stages of malaria infection (Sergent and Sergent, 1922; Yorke and MacFie, 1924; James, 1931); failure of attempts to repeat Schaudinn's observations (York and MacFie, 1924; Boyd and Stratman-Thomas, 1934); the existence of a period of noninfectivity of the blood and of the infectivity of certain organs following both mosquito bite and injection of sporozoites (Raffaele, 1936; 1937; Warren and Coggeshall, 1937; Kilkuth and Mudrow, 1938; Decourt and Schneider, 1938; Henry, 1939); and lastly, the existence of exoerythrocytic stages in the life cycles of certain species of malaria parasites (Ben-Harel, 1923; Huff, 1930; Raffaele, 1934; Huff and Bloom, 1935).

A milestone in the quest for the elusive occult stages of the malaria parasites was made by James and Tate (1937a; 1937b; 1938) who gave a detailed description of the exoerythrocytic stages of P. gallinaceum in the reticulo-endothelial system of infected chicks.

A little later, Kilkuth and Mudrow (1939) described the stages of P. cathemerium in the pectoral muscles of birds following sporozoite inoculation. The pre-erythrocytic cycle of P. gallinaceum was independently described by Mudrow (1940) in Germany and Shortt, Menon and Iyer (1940) in India. Reichenow and Mudrow (1943) then gave a detailed account of the pre-erythrocytic stages of P. relictum, following the inoculation of sporozoites into the tissues of canaries. Their work was closely followed by that of Huff and Coulston, who, in their classic paper of 1944, gave a complete account of "the development of P. gallinaceum, from sporozoite to erythrocytic trophozoite".

In 1947, Fairley and his colleagues in Australia reported that the blood of human volunteers was infective to other volunteers from 7 minutes to 30 minutes after the commencement of the act of biting of an infected mosquito. Thereafter, the blood remained negative until 6 days (P. falciparum) or 9 days (P. vivax) later. This work strongly suggested that there was some form of pre-erythrocytic development and provided great stimulus for research workers. Subsequently, there were many attempts in the 1940's to demonstrate the exoerythrocytic forms of the simian malaria parasites (see Bray, 1957). The first indication of the tissue stages was given by Garnham (1947) who demonstrated the development of Hepatocystis kochi in the liver parenchymal cells of African monkeys. Later, Shortt and his collaborators (1948; 1951) demonstrated the full cycle in a similar site, first with P. cynomolgi and then with P. vivax and P. falciparum.

2.2.3. Experimental research on malarial sporozoites

Since their discovery, the sporozoites of malaria parasites have intrigued parasitologists and much research concerning them has been conducted. Most interest has been shown in their morphology,

cultivation, and in the immunological responses which they evoke. It is not the purpose of this brief introductory review to reiterate all that has already been said, as a number of excellent reviews are available. The morphology of sporozoites has been dealt with by Aikawa (1971) and Sinden (1978); the cultivation of the sporogonic stages by Ball (1964, 1965, 1972) and Vanderberg, Weiss and Mack (1977); and the immunological responses to sporozoites by Nussenzweig (1977) and Nussenzweig, Cochrane and Lustig (1978).

Although the morphology of sporozoites as seen in Romanowsky stained preparations is well known (see Garnham, 1966a) it was not until 1942 that their ultrastructure was first investigated. Then, Emmel, Jakob and Golz examined the sporozoites of P. vivax and P. falciparum. Since then, improvements in microscope and preparative technology have led to a thorough understanding of sporozoite structure, although, as mentioned in Chapter 1, the functional interpretation of many organelles is not well understood. Although malarial sporozoites have been examined by transmission and scanning electron microscopy (see Aikawa, 1971; Sinden, 1978), and high voltage electron microscopy (Sinden, 1978), the histochemical techniques that have been used in the examination of merozoites, have not been employed to any great extent with the sporozoite. The morphology of the P. gallinaceum sporozoite is discussed fully in Chapter 4.

The continuous culture of the sporogonic stages of the malaria parasite have not, as yet, been achieved. However, culture systems able to support the transformation of gametocytes into ookinetes have been described (Alger, 1968; Weiss and Vanderberg, 1977), although subsequent stages of sporozoite formation, from the differentiating

oocyst to the formation of mature infective sporozoites, has only been accomplished by overlapping successive stages in different cultures (see Vanderberg, Weiss and Mack, 1977). In their review, the same authors consider that the different environments required at each phase of sporogony to be the major stumbling block for the successful continuous culture. Ookinete formation takes place in the lumen of the midgut, oocyst development in the haemocoele, and finally, maturation of the sporozoite occurs in the salivary glands.

It is clear that many problems remain to be solved before infective sporozoites can be regularly produced in vitro. Whether the production of sporozoites for use as a malaria vaccine depends on such a system, or improvements in the techniques for the recovery of infective, salivary gland sporozoites from whole mosquitoes, remains to be seen.

In 1978, Bray failed to detect circumsporozoite antibodies in areas of hyperendemic malaria and suggested that, "antibodies to sporozoites of human malaria parasites are not generated in nature, presumably because during the half an hour in which sporozoites circulate they are taken up in insufficient numbers to act as an antigenic stimulant". Recently, however, the converse has been reported; antibodies specific for P. falciparum have been detected by means of the circumsporozoite precipitation assay and indirect immunofluorescence in serum samples from Gambian (West African) adults (Nardin et al., 1979). It was therefore, suggested that, "antibodies to sporozoites are produced in areas of hyperendemic malaria in spite of the small number of parasites inoculated by a mosquito bite and the brief passage through the hosts blood stream" (Yoshida et al., 1980). The latter findings suggest that sporozoites have a high degree of

immunogenicity and interest for their use in the development of a malaria vaccine has increased.

The induction of immunity to sporozoites which have been inactivated by ultraviolet light, formalin fixation or mechanically disrupted was first demonstrated in P. gallinaceum (Mulligan, Russell and Mohan, 1941; Russell, Mulligan and Mohan, 1941; Richards, 1966). Recently, the studies of Nussenzweig and her collaborators in New York, have shown that total protection to rodent malaria can be achieved by intravenous injections of x-irradiated sporozoites (see Nussenzweig, 1977). The results of vaccination experiments with simian malarias however, have been less encouraging (Rieckmann et al., 1974, Clyde, 1975; Bray, 1976); the immune response being species specific, fairly short-lived (< 6 months) and occurs in only a limited number of individuals.

Immunity induced by sporozoite vaccination is strictly stage specific (Nardin and Nussenweig, 1978) and immune mice subsequently challenged with erythrocytic forms suffer fatal infections as observed in control animals (Nussenzweig et al., 1969). In addition, the protective antigen(s) are absent from oocyst sporozoites and are acquired at the time of their migration to the salivary glands (Vanderberg et al., 1972).

Recently a most interesting discovery has been made by Yoshida et al. (1980), who have been able to obtain monoclonal antibodies against sporozoites of P. berghei by the fusion of plasmacytoma cells with immune mouse spleen cells. The antibody is reported to bind to a protein with an approximate molecular weight of 44,000 (Pb44) which envelopes the surface membrane of the sporozoite. It is not known

whether Pb44 constitutes the major, or perhaps even the single antigen on the surface membrane of P. berghei sporozoites, but certainly, the availability of large amounts of this antibody should greatly enhance the purification and characterisation of this surface antigen and determine its role in protective immunity.

2.3. Biology of P. gallinaceum

2.3.1. Discovery and isolation

P. gallinaceum was probably first seen by Bronssais in 1910 in Indo-China. He isolated it from the domestic hen, Gallus domesticus transmitted it successfully by blood inoculation, and sent some blood films to Brumpt in Paris. Meanwhile, von Prowazek in 1912, had discovered the same organism in hens from Sumatra. Some years then passed, when in 1929, Brumpt, who had not lost interest in the parasite, tried without success to isolate it from the original locality and also from Cambodia. Then, in 1933, Crawford, who was working in Ceylon, reported on the presence of "relictum"-like parasites in the blood of dying imported fowls. Brumpt saw the report, re-examined Bronssaiss's original smears, and in 1935 described the organism as Plasmodium gallinaceum.

Brumpt, however, was not satisfied with the description of a parasite in blood films 25 years old. Encouraged by the importance of a malaria parasite which infected domestic hens, he set out on a tour of the Far East in attempts to isolate it, although the numerous chickens he examined were, at first, negative. On his way back to France, Brumpt collected a number of birds from Crawfords original outbreak in the hope of discovering a latent infection. In Paris he sub-inoculated into new birds which readily became infected, and the strain was eventually established.

2.3.2. Distribution and hosts

P. gallinaceum is widespread in South-East Asia, its distribution extending eastwards from Sri Lanka and southern India (Madras), to western Malaysia, Indo-China and the Philippines (Garnham, 1977). The reservoir hosts are different species of jungle fowl. Shortt, Menon and Iyer (1941) were the first to describe it from its natural host in Madras, Gallus sonneratii, and it has since been described from G. spudiceus in Malaysia and G. lafayetti in Sri Lanka (Fernando and Dissanaike, 1975).

Haiba (1948) claimed to have seen P. gallinaceum in chickens in southern Egypt and El-Kordy and Ahmed (1960) described its presence in Egypt and Syria where approximately one quarter of local and imported chickens showed infections. However, no isolations of the parasite or mosquito transmissions were made, consequently both these reports should be treated with some caution; furthermore, Garnham (1966a) commented that, his numerous investigations of suspected outbreaks of chicken malaria in Kenya, always proved to be aegyptianellosis. Also, Mettam (1943) reported a single unconfirmed diagnosis of P. gallinaceum in domestic fowls suffering from aegyptianellosis in Bauchi, West Africa. Malaria parasites in chickens from two localities in Wisconsin (U.S.A.) were reported by Krishnamurti et al. (1961), but it was later discovered that this strain had been derived from escaped laboratory infected birds. Seneviratna (1959) reported an outbreak in Colombo, Sri Lanka; Omar, Ismail and Lim (1962) noted the presence of the parasite in chicks in Malaysia, and succeeded in transmitting the infection to clean chicks with Aedes aegypti; and the parasite was also identified in chickens from Malaysia by Moorhouse and Wharton (1962).

Niles, Fernando and Dissanaike (1965) discovered the natural vector of P. gallinaceum in Sri Lanka to be Mansonia crassipes. In fact a large number of mosquitoes are able to transmit the parasite experimentally and P. gallinaceum appears to be more oligoxenous than any other malaria parasite. It has been shown to develop in at least forty different species of mosquitoes, belonging to six genera, viz., Aedes, Anopheles, Armigeres, Culex, Culiseta and Mansonia (Vargas, 1949; Brumpt, 1949). The domestic mosquito, Aedes aegypti, serves as an admirable experimental host since 100% readily become infected, producing salivary glands full of sporozoites.

P. gallinaceum provides a good example of ä "veterinary zoonosis" (Garnham, 1969). In the feral host, the infection is mild, the infection is capable of being transmitted to the local breeds of chickens, which occasionally show symptoms of the disease in a mild form, and when chickens of European or American affinities are introduced, the infection fulminates and many birds die of cerebral malaria.

Despite many records of its presence outside the Orient, P. gallinaceum seems to be largely confined to its habitat in Ceylon, in defiance of much trading in chickens and escape from research laboratories. This somewhat restricted zoogeography seems strange, especially as the chicken is such a ubiquitous bird and when mosquito species are abundant for its successful transmission. But as Garnham (1966a) has commented, "the direction of movement (of chickens) is usually from the uninfected to the infected regions of the world, and not the reverse, so the spread of the disease is rare.

2.3.3. Experimental research on P. gallinaceum

Not long after Brumpt had described P. gallinaceum, he generously distributed it to a number of scientific institutions and it became so well studied that at the time of publication of Garnham's monograph (1966a) P. gallinaceum had been the subject of over one thousand scientific papers. Since then, much of the early interest generated by this parasite has waned, for many of the advantages offered by P. gallinaceum, particularly its ready availability and the ease of its transmission by A. aegypti, have been overshadowed by the discovery of the malaria parasites of rodents (see Killick-Kendrick and Peters, 1978). Rodent malaria parasites, by infecting mammals, are more comparable to the human parasites and therefore more acceptable as laboratory models. Interestingly though, the ancient lineage of P. falciparum, by far the most important species infecting man, has recently been discussed (Sinden et al., 1978) and it is possible that this parasite is more closely related to the malaria parasites infecting birds, rather than mammals.

Between the time of Hewitt's (1940) monograph and Huff's review (1963), studies on the chemotherapy of P. gallinaceum had attracted a great deal of attention, due largely to the strategic importance of human malaria during the second World War. In 1939, at the onset of hostilities, P. gallinaceum had been available for 4 years and was already known to be of some use in chemotherapeutic studies. Subsequently, the parasite proved to be a great boon for the screening of antimalarial drugs, carried out mainly by the Americans, during the 1940's (see Peters, 1970). In fact, P. gallinaceum was so widely studied during the 1940's that it would be superfluous to mention all

the contributions that this species has made to our understanding of the malaria parasites. Instead, the reader is refered to the work of Garnham (1966a) and the reviews of Huff (1963, 1968). However, an attempt is made to mention some of the more important and unusual findings concerning P. gallinaceum.

In addition to the numerous studies on the structure and function of the parasite during the various stages of its life cycle, P. gallinaceum has also contributed much to our knowledge concerning the susceptibility of mosquitoes to infection by malaria parasites. Ward (1963) was able, by careful genetic selection, to obtain a highly resistant strain of A. aegypti. Over a period of 26 generations, the susceptibility of A. aegypti to infection by P. gallinaceum, was reduced by 98% and, in the absence of selection did not return to normal during 13 subsequent generations.

Weathersby (1952) demonstrated that sporogonic development of P. gallinaceum could occur in the haemocoelomic cavity, as well as on the gut wall of susceptible A. aegypti. He introduced gametocytes and oocysts into the haemocoelomic cavity and observed normal sporogony, resulting in the production of sporozoites which invaded the salivary glands and were infective to chicks. Later (1960) Weathersby extended his observations on the development of P. gallinaceum by introducing phanerozoites from the brain of an infected chick embryo into the haemocoele of A. aegypti. He maintained the mosquitoes for 3 days, fed them on clean birds and after a short prepatent period, a mild parasitaemia was observed in 29% of the chicks. This experiment demonstrated the ability of exoerythrocytic merozoites to mimic sporozoites by invading the salivary glands and establishing an infection in the vertebrate host.

P. gallinaceum has contributed greatly to our understanding of the exoerythrocytic cycle in avian malaria (see Bray, 1957) both by observations in vivo and in vitro. Some of the history concerning the exoerythrocytic forms is covered earlier in this chapter and the tissue culture of P. gallinaceum is discussed in Chapter 8. One of the interesting controversies which existed (and some may argue still exists) was the belief that dimorphism of the exoerythrocytic schizonts occurred. The German workers, Reichenow and Mudrow-Reichenow, in a series of publications in the 1940's, believed that the merozoites of metacryptozoites and the subsequent phanerozoites were of two distinct sizes; the macromerozoites measuring from 1·5-2·5 μm in length and being the precursors of the macroschizonts, the merozoites of which invade tissue cells; and the micromerozoites, roughly spherical in shape, measuring from 1·0-1·5 μm in diameter, destined to form the microschizonts, the merozoites of which invade erythrocytes. Huff, however, in 1952 carefully measured a number of merozoites from different schizonts, found a normal distribution and concluded that dimorphism did not occur. Huff's curiosity in the relationship between avian malaria parasites and their host cells continued and his review of 1963, considered that one area of future research was a study of the factors which determine the choice of host cell which is made by the parasite and why this choice changes during the course of infection. P. gallinaceum would be an excellent parasite for studying these interactions with its different patterns of exoerythrocytic schizogony (Haas et al., 1948).

The fine structure of P. gallinaceum has been examined at all stages of its life cycle and the reader is refered to the authorative review of Aikawa (1971); the fine structure of the sporozoite is

discussed in Chapter 4.

Recent interest in P. gallinaceum has concerned the factors which stimulate exflagellation (Nijhout, 1979; Nijhout and Carter, 1978); immunological studies with the sexual stages (Gwadz, 1976; Carter and Chen, 1976); and the influence of digestive enzymes on the development of ookinetes (Gass, 1979).

CHAPTER 3

3. GENERAL MATERIALS AND METHODS

3.1. Parasite

The strain of P. gallinaceum used in these experiments was strain 8A obtained from the Wellcome Laboratories of Tropical Medicine, Beckenham, by courtesy of Dr. W. H. G. Richards. This is the strain originally isolated by Brumpt in 1935 from domestic hens in Sri Lanka. It has been transmitted for many years in the Wellcome Laboratories, and in this laboratory has been maintained since October, 1976.

3.2. Avian hosts

The chicks used during this investigation were Ranger Cockerels (Ross 1), supplied by Ross Poultry Limited, Andover; or Tetra Cockerels (Tetra 1), supplied by Tetra Poultry Limited, Dunsfold, Surrey. Chicks from 1 day to 3 weeks old were used, not only for the ease with which the smaller birds could be handled but also for the rather higher proportion of sexual parasites that are reported to be produced in younger birds (Garnham, 1966a). They were kept at an ambient temperature of 25°C and were fed on chick crumbs (coccidiostat-free) obtained from Lilico and Son, Betchworth, Surrey; water was provided ad libitum.

The chick embryos used both for the preparation of tissue cultures and for strain maintenance were also obtained from Tetra Poultry Limited and were kept in a chick incubator at 39°C until the appropriate day of development (see Chapter 8).

3.3. Mosquito hosts

Aedes aegypti was used exclusively for this study. The mosquitoes were derived from a stock that had been bred in the laboratory of COPR (Centre for Overseas Pest Research) Porton Down, for many years and the eggs used to start the colony were kindly provided by Dr. A. B. Hadaway.

The methods used for rearing the mosquitoes were similar to those outlined by Shute and Maryon (1966) and Garnham (1966a) and the method is given only briefly here. The mosquitoes were kept under constant conditions of 12 hours daylight and 12 hours darkness at a temperature of 28°C and relative humidity of 70-90%. Larvae were raised in plastic washing-up bowls and fed on coarse whole meal breadcrumbs. The pupae were filtered from the larval bowls and allowed to emerge in mosquito cages from small crystallising dishes. A 4% solution of glucose was provided and regular blood meals were offered. Eggs were laid on inverted moist filter paper cones and stored either damp for up to 3 weeks or dessicated for several months.

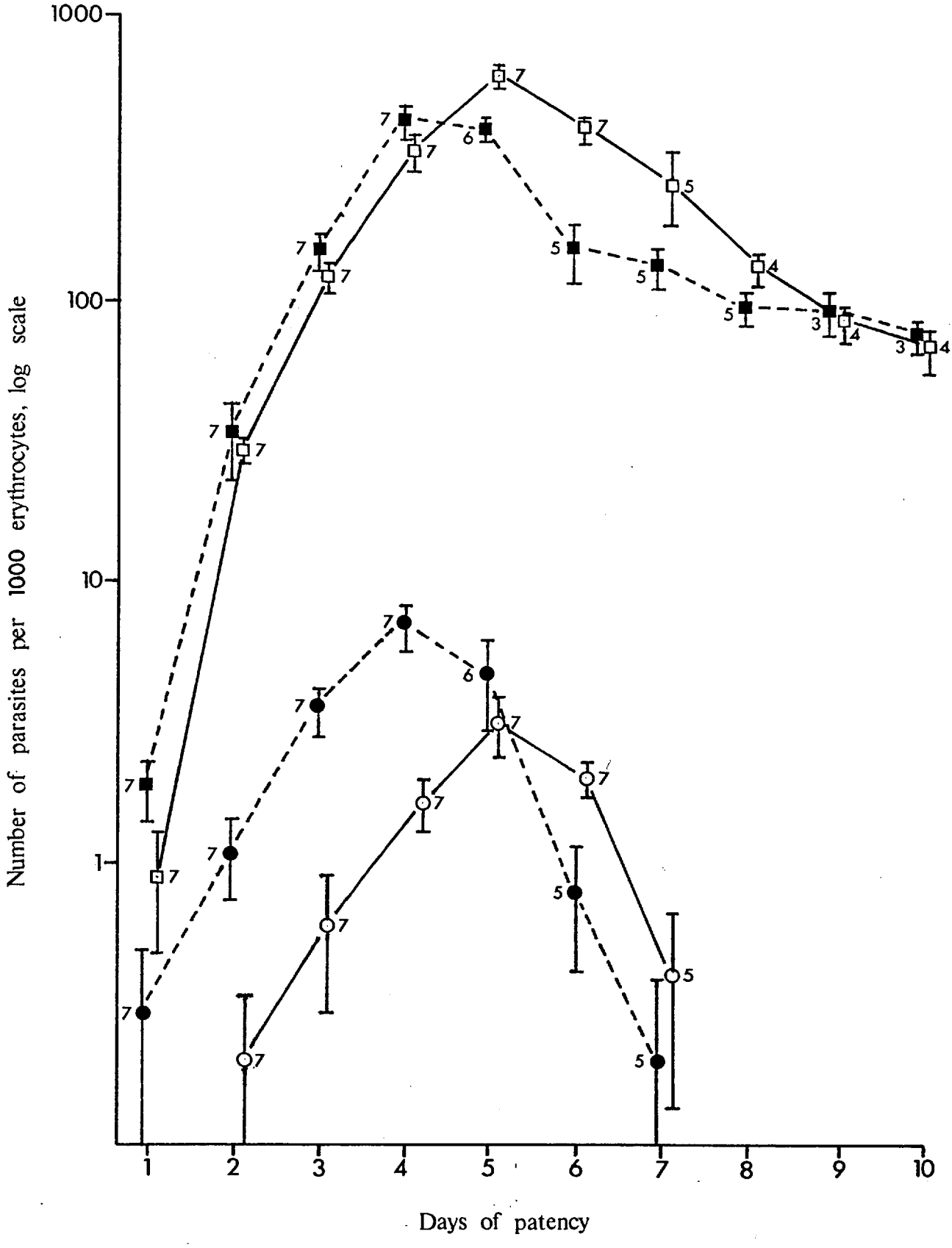
3.4. Parasite transmission

The parasite was transmitted in chicks by sporozoite inoculation or blood passage. Initially 2 or 3 blood passages were alternated with a sporozoite induced infection, but latterly, sporozoite induced infections were used exclusively because of the marked reduction in gametocyte numbers (approximately 60% at the peak day of gametocytaemia) that was observed following blood passage (Fig. 3.1.).

For the production of sporozoites, mosquitoes, 3-4 days old, were fed on infected birds showing gametocytes in Giemsa stained thin blood smears (see p. 42). The blood was taken by pricking the vein

Fig. 3.1. Course of P. gallinaceum infection in chicks inoculated with sporozoites or parasitized blood. Figures beside each point (mean ± S.E.M.) indicate the number of chicks examined. ●- - - - -● sporozoite induced gametocytaemia; O———O blood induced gametocytaemia; ■- - - - -■ sporozoite induced asexual parasitaemia; □———□ blood induced asexual parasitaemia.

For blood induced infections 7 chicks were inoculated intravenously, each with 0.1 ml of blood containing approximately 5.4×10^5 parasites. For sporozoite induced infections 7 chicks were inoculated intravenously, each with approximately 100,000 sporozoites. Daily blood films were taken and 1,000 erythrocytes were examined for the occurence of gametocytes or asexual forms.


1000
100
10
1
Number of parasites per 1000 erythrocytes, log scale
1
2
3
4
5
6
7
8
9
10
Days of patency

on the surface of the leg. Feeds were usually offered 1-2 days before the maximum gametocytaemia, for although Eyles (1951) has shown that for P. gallinaceum the highest oocyst numbers in A. aegypti corresponds to the peak gametocytaemia, the majority of workers, using the same experimental system, consider that the highest average oocyst count tends to preceed the highest gametocyte numbers by 1-2 days (Lumsden and Bertram, 1940; Cantrell and Jordan, 1946; Huff and Marchbank, 1955; Huff, Marchbank and Shiroishi, 1958). Mosquitoes fed at this time usually had between 50-100 oocysts on the midgut and assured a plentiful supply of sporozoites in the salivary glands (25,000-50,000).

After 5 or 6 days a few mosquitoes from each batch were dissected and the presence of oocysts on the midguts observed; after another 5-6 days (10-12 days after the infective feed) a few mosquitoes from the same group were dissected and checked for the presence of sporozoites in the salivary glands. These routine midgut and salivary gland dissections were carried out by the well established techniques of Shute and Maryon (1966) and Garnham (1966a) and were performed in tissue culture medium 199 (M199, Gibco-Biocult). A 'Wild' M20 microscope fitted with a phase contrast condenser was used throughout for the examination of mid-guts and salivary glands.

For sporozoite-induced infections, 100-200 mosquitoes with heavy salivary gland infections, were lightly ground in 2 mls of M199 in a loose-fitting teflon/glass homogenizer. The resulting suspension was spun at 25g for 5 minutes to sediment the larger pieces of mosquito debris and the supernatant was injected intramuscularly, normally 0.4 mls containing approximately 200,000 sporozoites, into each of 4 chicks. This method was considered suitable for the routine

passage of the strain, although as Garnham (1966a) has commented, there is a loss of parasites which adhere to the chitinous debris; also, the final suspension contains a large ammount of fat, muscle and bacterial contamination. Thus when a quantitative infection was required, the salivary glands were dissected in the normal way, pooled in M199 and stored on ice. A suspension of sporozoites was then prepared by gentle homogenisation in a teflon/glass homogenizer and the number of sporozoites counted using an improved Neubeubeur haemocytometer. The counting chamber was loaded with a sample of the suspension and allowed to stand in a damp chamber for 10 minutes to allow the sporozoites to settle. Portions of this suspension containing the appropriate number of sporozoites were then inoculated into the jugular vein of the chicks.

For each blood passage, 0.2 mls of heavily parasitized blood (60-90% erythrocytes infected) was obtained in a syringe (wetted with heparin, 5,000 units/ml) from the jugular vein of the donor bird and injected intravenously into the recipient.

As a safeguard during this study the parasite was cryopreserved (Appendix 1). Twice during the course of this study it was necessary to initiate infections with cryopreserved material.

3.5. Harvesting of sporozoites

Dissections for oocyst sporozoites were carried out on days 8-10 when distinct sporozoites could be seen by phase microscopy within the oocysts and dissections for salivary gland sporozoites were performed on days 12-16; sporozoites older than 16 days were either discarded or used for routine passage.

For many experiments, notably in the determination of electrophoretic mobilities (see Chapter 5) it was necessary to produce a large number of sporozoites relatively free of mosquito and microbial contamination. Recent attempts to separate and purify sporozoites (some of which have been briefly reviewed by Krier, 1977) have included density gradient centrifugation (Chen and Schneider, 1969; Schneider and Chen, 1969; Krettli, Chen and Nussenzweig, 1973; Beaudoin et al, 1977; Vermeulen, Munster and Meuwissen, 1979) and column separation (Moser et al, 1978; Mack, Vanderberg and Nawrot, 1978). However none of these methods has gained universal acceptance, as for example, has the technique of separating bloodstream forms of pathogenic trypanosomes on a DEAE-cellulose column (Lanham, 1968; Lanham and Godfrey, 1970) because all suffer from the same disadvantages, including poor separation from microbial contaminants and low recovery rates. Also, if whole mosquitoes are used a heterogenous population of sporozoites is produced, consisting of individuals from the oocysts, haemocoele and salivary glands, irrespective of the age of infection. Therefore it was decided to use the technique of mass mosquito dissection which was successfully employed by Shortt and Garnham in London and Huff and his colleagues in America in their pioneering work on the exoerythrocytic cycle in mammalian and avian malaria respectively. This method produces a relatively clean suspension containing a large number of sporozoites and many mosquitoes can be rapidly processed if experienced dissecters are used.

It was however necessary to decide on a centrifugation regime to enable the suspension to be washed, after for example, enzyme treatment (see Chapters 5 and 7). A small bench centrifuge (Microfuge,

Burkard Scientific, Rickmansworth) with a fixed relative centrifugal force (r.c.f.) of 14,000·g was found suitable and experiments were performed to determine the length of time it was necessary to spin a sporozoite suspension to recover maximum numbers of sporozoites.

The salivary glands of approximately 100 mosquitoes were dissected and a suspension produced in 1.0 ml of M199 by gentle homogenisation in a loose fitting teflon/glass grinder[1]. This was spun at 25 g to remove the large pieces of mosquito tissue and the number of sporozoites in the supernatant was adjusted to 10^6 and 0.5 mls of M199 were added to produce a final volume of 1.5 mls. This was then spun on the Microfuge for 2 seconds[2], the supernatant removed and the numbers of sporozoites in the residue counted using an improved Neubaeur haemocytometer. This procedure was repeated for times of 4, 8, 15, 30 and 60 seconds and 2, 4 and 5 minutes; each time the numbers of sporozoites in the residue was counted.

The experimental design is shown in Fig. 3.2. and the recovery of sporozoites in the residue in Fig. 3.3. All the sporozoites were deposited after 2 minutes centrifugation, but as 86% were sedimented after 1 minute the latter was adopted as the standard time for recovery and washing. Therefore the method selected to produce large numbers of relatively pure sporozoites was as follows. Dissection of

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1. Mature oocyst sporozoites were liberated by very light homogenisation of whole guts. If performed carefully, ripe oocysts are ruptured and the guts which are left intact, are deposited after the initial spin at 25 g.

2. The Microfuge took approximately 20 seconds to run down and this obviously has a considerable effect on the very short spins.

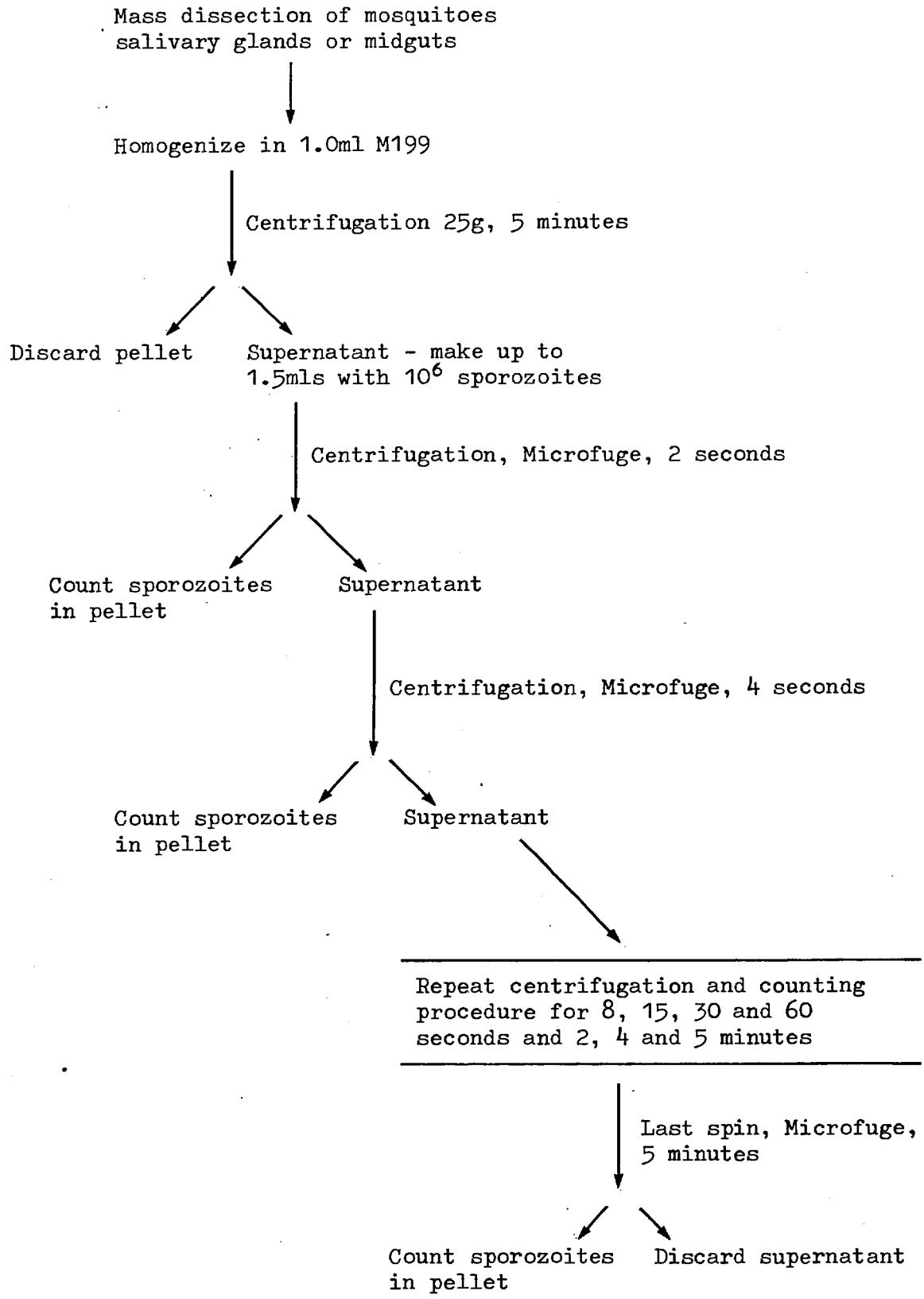


Fig. 3.2. Flow diagram illustrating the experimental technique used to determine the optimum time of centrifugation for the recovery of P gallinaceum sporozoites from the microfuge.

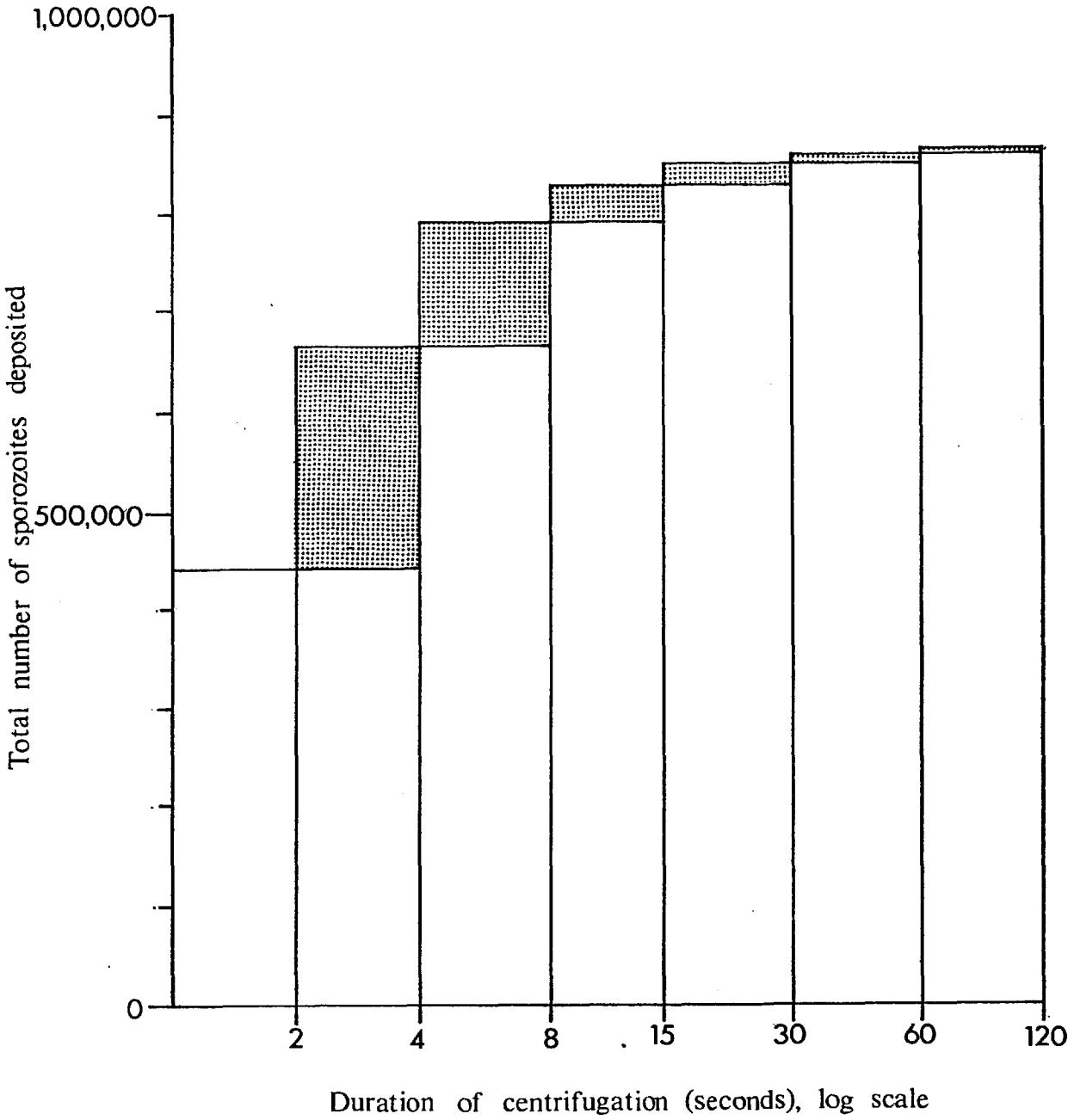


Fig. 3.3. Effect of the duration of centrifugation on the recovery of P. gallinaceum sporozoites on the Microfuge. Stippling represents the number of sporozoites deposited in sequential residues.

salivary glands (or midguts) into 1.5 mls of M199 and a preliminary spin at 25 g followed by centrifugation of the supernatant on the Microfuge for 1 minute and resuspension of the sporozoite pellet into the desired volume of medium to yield between 1 to 3 x 10^6 sporozoites/ml.

When applied to mature oocyst sporozoites the Microfuge technique gave equally good recovery rates.

As earlier separation techniques (see for example Chen and Schneider, 1969) had shown a significant loss of sporozoite infectivity, it was essential that the infectivity of Microfuge recovered sporozoites was investigated. This was performed in the following manner. A suspension of sporozoites was produced from salivary gland dissections of a batch of mosquitoes from the same group as used for the centrifuge routine and chicks were injected intravenously, 5 with 10,000 sporozoites each and 5 with 1,000 sporozoites each. The sporozoites remaining in this suspension were spun for 5 minutes on the Microfuge and again chicks were infected with the same doses as before. The prepatent period of the infections were then recorded by the observation of daily blood films. At least 10,000 erythrocytes were examined before a chick was considered negative. As can be seen in Table 3.1. the Microfuge recovered sporozoites exhibited no loss of infectivity to chicks at these high doses and subsequent experiments have confirmed these observations with much lower doses (see Chapter 7). Thus the centrifugation procedure described above was considered suitable for the recovery of sporozoites.

Table 3.1. Infectivity of Microfuge recovered sporozoites (MS) compared with control sporozoites (CS) from the salivary glands of P. gallinaceum infected mosquitoes.

Expt. No.	No. of sporozoites inoculated	No. of chicks infected/no. inoculated		Mean prepatent period (days)	
		CS	MS	CS	MS
1	10,000	5/5	5/5	6·2	5·8
	1,000	5/5	5/5	6·4	6·8
2	10,000	5/5	5/5	5·8	5·2
	1,000	5/5	5/5	6·8	6·2
3	10,000	5/5	5/5	6·0	6·2
	1,000	5/5	5/5	6·4	6·4

CHAPTER 4

4. MORPHOLOGICAL OBSERVATIONS

4.1. Introduction

The morphology of many species of malarial sporozoites is well documented by light microscopy (see Garnham, 1966a) and since the first description of the ultrastructure of the sporozoites of P. falciparum and P. vivax (Emmel, Jacob and Goltz, 1942), sporozoites have been the subject of a number of morphological investigations (see Aikawa, 1971 and Sinden, 1978). Despite these studies, there is no clear morphological explanation to account for the major changes that occur in the physiological organization of malarial sporozoites as they leave the mature oocyst and migrate to the salivary glands of the mosquito host. These physiological differences include changes in motility (Vanderberg, 1974), infectivity (Vanderberg, 1975) and antigenicity (Vanderberg et al., 1972) and it is likely that these changes are related to the different invasive capacities that are exhibited by sporozoites. On leaving the mature oocyst, the sporozoite must be adapted to locate and penetrate the cells of the mosquito's salivary gland; subsequently it must be adapted for survival in the vertebrate and for location and penetration of a hepatocyte, in mammalian malaria, or the cells of the reticulo-endothelial system, in avian malaria.

This chapter compares the morphology of sporozoites from mature oocysts and salivary glands and the results are discussed in relation to the differences that exist between the two populations. It is not the purpose of this chapter to provide a detailed description of the morphology of the P. gallinaceum sporozoite as this has been published elsewhere (Terzakis, 1968; Terzakis, Sprinz and Ward, 1976; Sinden and Garnham, 1973).

4.2. Materials and Methods

4.2.1. Preparation and examination of material for light microscopy

Infected salivary glands and midguts were dissected into a very small amount of M199 as described previously (p.33). The tissues were gently teased apart with fine syringe needles and smeared with the edge of a square coverslip. Smears were allowed to air dry and then fixed momentarily (~3 seconds) in methanol before staining in 10% Giemsa stain (Revector), in phosphate buffer at pH 7·2 (Appendix 2) for 1 hour. Smears were washed briefly in tap water and allowed to air dry before examination.

Sporozoites in stained smears of infected salivary glands and midguts were measured by first drawing midlines at a magnification of x 1,500 with the aid of a drawing tube attached to a 'Wild' M20 microscope. The lengths of the sporozoites were then obtained by plotting their lengths on a computerised drawing board (X-Y digitiser, Feranti Cetec) which was considered to be more accurate and less time-consuming than using calibrated dividers (see Killick-Kendrick, 1973).

Outline drawings of sporozoites were also made and after the position of the nucleus and micropore had been recorded the Giemsa stain was removed and the smears processed for the Feulgen reaction. The smears were washed in xylene and taken down through graded alcohols to water, washed in cold IN HCl, then hydrolysed in IN HCl at 60° for 10-15 minutes. After a rinse in cold IN HCl, the smears were washed in distilled water. The smears were then dehydrated through a series of graded alcohols, rinsed in xylene and mounted in "Euparal Vert". Slides of chick erythrocytes were processed with the test slides to serve as controls.

4.2.2. Preparation and examination of material for electron microscopy

The infected mosquitoes were dissected and examined as described previously (p.33). The infected midguts were prepared using the method of Sinden and Garnham (1973). The midguts were fixed in a small drop of 3·0% glutaraldehyde in 0·1M phosphate buffer pH 7·4 and were then transferred to 2·0 mls of fixative and fixation was continued overnight at 4°C. Following fixation the material was washed in 0·15M phosphate buffer pH 7·4 for 3 hours. The glands were post-fixed in 1% osmium tetroxide in veronal acetate buffer pH 6·6 containing 0·005M calcium chloride. After dehydration in graded alcohols the specimens were transferred to propylene oxide and subsequently embedded in Araldite CY212.

The infected salivary glands were prepared using a modified Karnovsky fixative (see Sinden, Canning and Spain, 1976). The material was fixed for 10 minutes at room temperature in Karnovsky's fixative containing 4-5mM calcium chloride and then for a further 1 hour at 4°C. Specimens were then washed twice in 0·12M cacodylate buffer pH 7·4. Following the first wash, the glands were embedded in 2% agar in the same buffer. The agar was allowed to solidify then cut into cubes having sides of approximately 2mm and then processed in the usual manner and post-fixed for 1 hour in 0·1M cacodylate buffer containing 2·5g/100ml osmium tetroxide at 4°C. The material was then washed in 0·1M sodium acetate and then immersed in 0·25g/100ml aqeous uranyl acetate for 1 hour. After two 10 minute washes in 0·1M sodium acetate and one each in 35% and 50% (by vol.) acetone, preparations were stained en bloc overnight in 70% acetone containing 1g/100ml uranyl acetate and 1g/100ml phosphotungstic acid at 4°C. Following final dehydration in acetone the material was embedded in Araldite CY212.

Thick sections, cut at 0·5μm with diamond and glass knives on a LKB Ultramicrotome, were transferred to glass slides and stained with toluidine blue. The sections were examined for the presence of parasites. Thin sections were placed on grids coated with formvar, stained in 2% (w/v) aqeous uranyl acetate for 10 minutes, rinsed in distilled water and then dried. They were then stained in Reynold's lead citrate for 10 minutes, washed in 2 changes of 0·02M NaOH, rinsed in distilled water and dried on filter paper. Sections were examined in a Phillips E.M. 300 operated at accelerating voltages of 60 and 80 kV.

4.3. Results

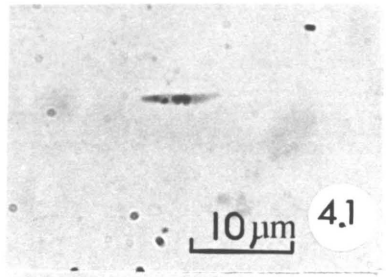
4.3.1. Morphology with the light microscope

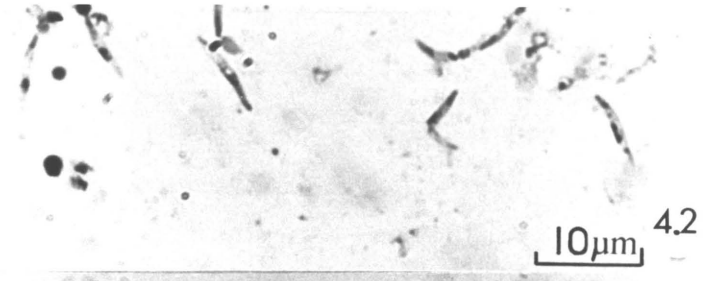
The P. gallinaceum sporozoite has the typical morphological characteristics of the avian species of malaria parasites. Thus it is rather short and thick, sickle or bow-shaped and with tapering ends. The anterior end is occupied by a deeply staining mass which represents the organelles comprising the apical complex. The nucleus, stains deeply with Giemsa, has an irregular contour, is always single but often appears fragmented (Figs. 8.1 to 8.4). In fresh preparations of crushed salivary glands small numbers of sporozoites are often seen in close apposition (Fig. 8.6), although after a few minutes they usually become dissociated (Fig. 8.5).

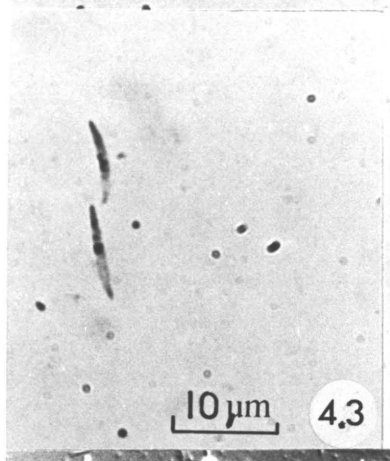
The lengths of sporozoites in dry-fixed Giemsa stained preparations are given in Table 4.1. There was no significant difference between the lengths of sporozoites in mature oocysts and sporozoites in salivary

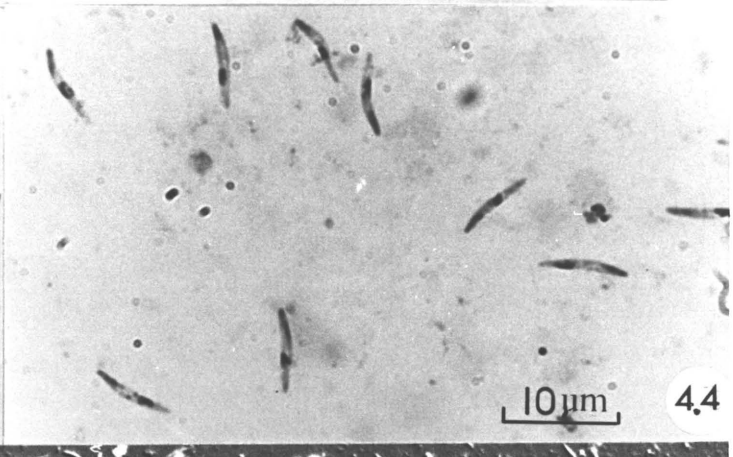
Figs. 4.1 to 4.4. Sporozoites of P. gallinaceum in a Giemsa stained smear of the salivary glands of A. aegypti.

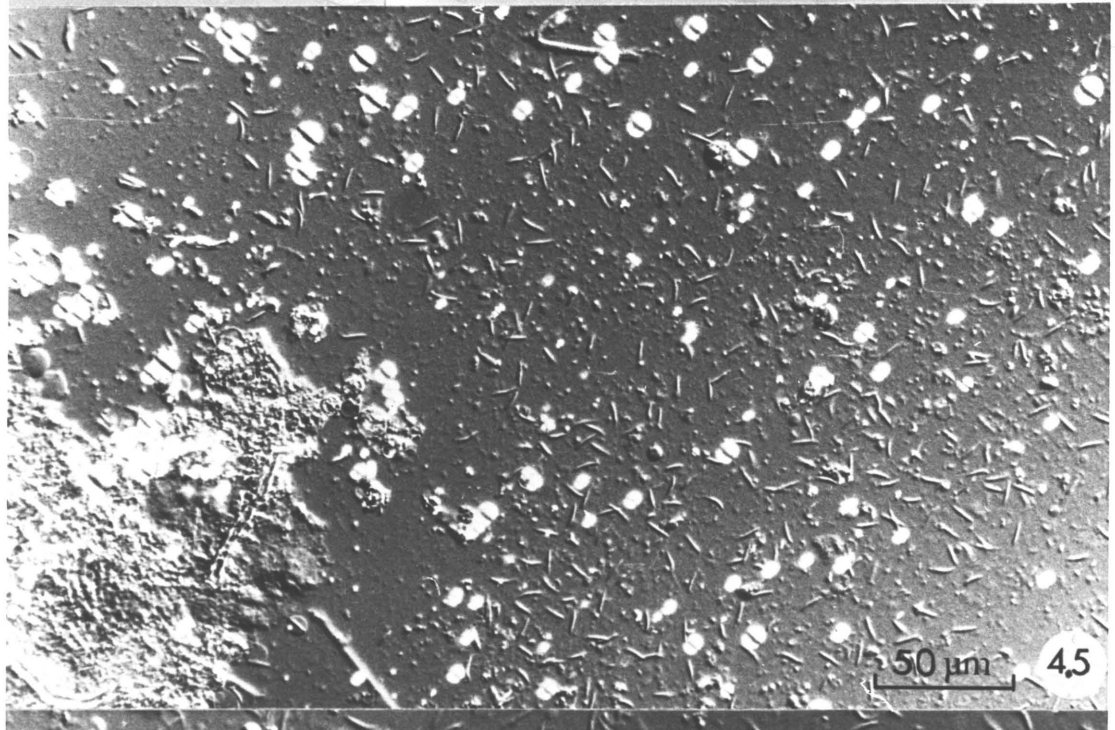
Figs. 4.5 and 4.6. Sporozoites of P. gallinaceum liberated from a squashed salivary gland of A. aegypti. Photographed with Leitz Nomarski interference contrast microscopy.


10 µm
4.1


10µm
4.2


10 µm
4.3


10 µm
4.4


50 µm
4.5

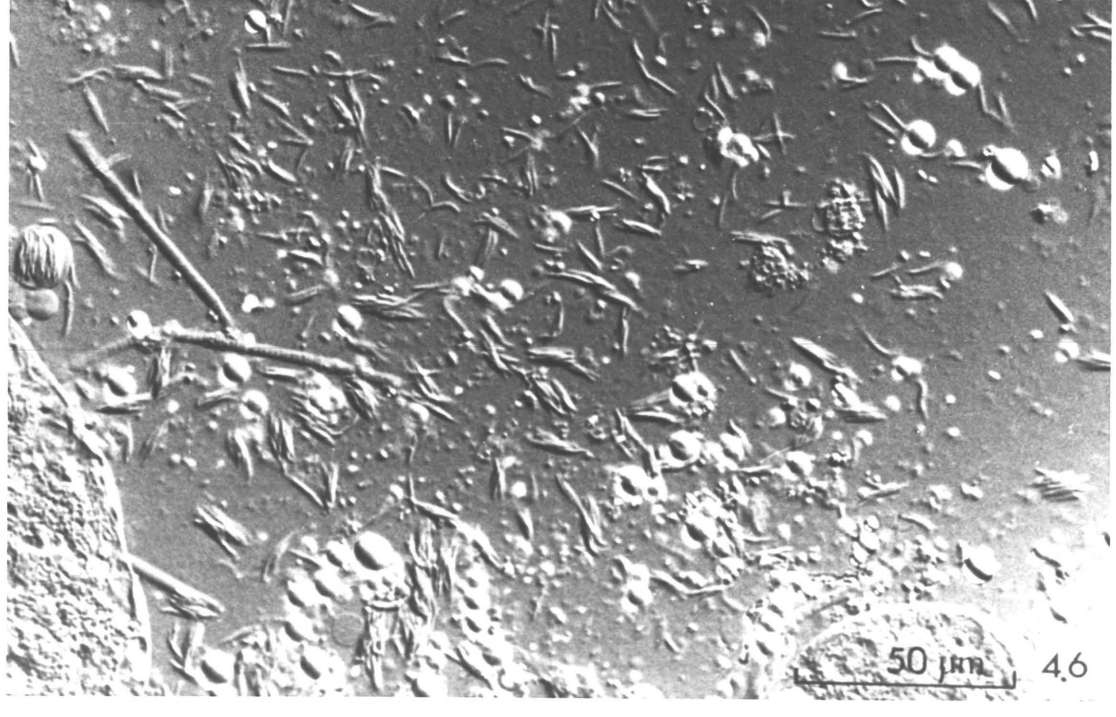

50 µm
4.6

Table 4.1. Mensural data of the lengths of P. gallinaceum sporozoites from mature oocysts and salivary glands [1].

Source of sporozoites	Length (μm)	Range
Mature oocysts	8·16 ± 0·14	10·85 - 5·74
14 day old[2] salivary gland forms	8·20 ± 0·15	10·72 - 5·64
35 day old salivary gland forms	7·64 ± 0·17	10·57 - 5·16

Table 4.2. A comparison of the demonstrability of the micropore in P. gallinaceum sporozoites from mature oocysts and salivary glands [3].

Source of sporozoites	Sporozoites with a demonstrable micropore (%)
Mature oocysts	80·8
14 day old salivary gland forms	82·4
35 day old salivary gland forms	43·4

1. Methanol fixed, Giemsa stained preparations; 100 observations for each sample.

2. "Day old" refers to days after the infective blood meal. At 28°C the salivary glands become invaded between the ninth and tenth after the infective blood meal.

3. Methanol fixed, Giemsa stained preparations; 500 observations for each sample.

glands ($p>0{\cdot}2$, Student's t-test). There was however, a significant reduction in the lengths of sporozoites with extended residence in the salivary glands ($p<0{\cdot}001$, Student's t-test).

Sporozoites processed for the Feulgen reaction confirmed the results of Dasgupta (1959) in that they displayed a strongly staining and prominent nucleus; moreover, the presumed micropore did not stain, thus demonstrating that the Giemsa stained material was in fact a micropore and had not been mistaken for a piece of fragmented nuclear material. There was no significant difference between the occurrence of the micropore in mature oocyst sporozoites and in salivary gland sporozoites ($p>0{\cdot}02$, Chi-square test), although in the older sporozoites, the incidence of the micropore decreased significantly ($p>0{\cdot}001$, Chi-square test). The presence of the micropore in sporozoites is shown in Table 4.2. The nuclei of chick erythrocytes processed with the test slides served as controls to show that the reaction was unaffected by pre-staining with Giemsa, as was previously shown by Canning and Sinden (1975).

4.3.2. Morphology with the electron microscope

Sporozoites from mature and nearly mature oocysts (Figs. 4.7 to 4.12) are relatively straight with a circular or eliptical cross section. The surface of the sporozoite consists of a plasmalemma (the surface membrane) and beneath this, two pellicular membranes (the cytoplasmic membranes) and the asymmetrically arranged subpellicular microtubules (Fig. 4.12) which possibly function as a cytoskeleton, imparting rigidity to the parasite (Aikawa *et al.*, 1966). The plasmalemma of the sporozoites appears naked (Figs. 4.7 and 4.8) but on close examination may be sparsely covered with fibrillar material (Figs. 4.9 to 4.12).

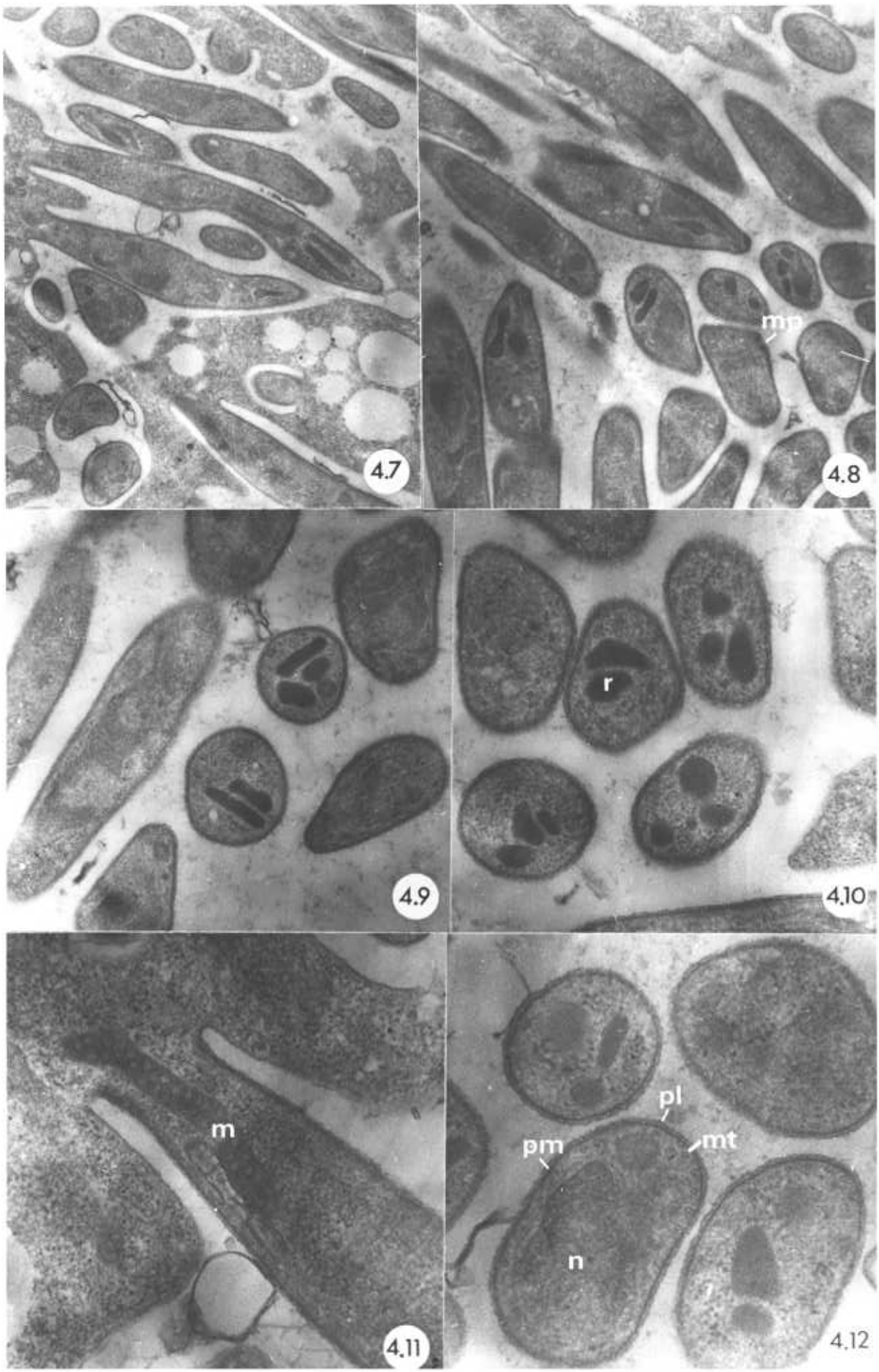
Figs. 4.7 to 4.12. Sections through P. gallinaceum sporozoites in mature and nearly mature oocysts.

Fig. 4.7 (x 22,818) and Fig. 4.8 (x 24,160). Transverse and obliquely sectioned sporozoites in nearly mature oocysts. Note the micropore (mp) in Fig. 4.8.

Fig. 4.9 (x 30,672) and Fig. 4.10 (x 40,849). Transverse and obliquely sectioned sporozoites in mature oocyst, showing numerous electron-dense rhoptries (r).

Fig. 4.11. L.S. of budding sporozoite. Note that the newly formed inner pellicular membranes are confined to the sporozoite bud and the transfer of the mitochondrion (m) into the sporozoite bud x 38,885.

Fig. 4.12. T.S. of sporozoites in mature oocyst. Note that the sporozoites are invested by a pellicle consisting of an outer plasmalemma (pl) and a pair of inner pellicular membranes (pm). Beneath these lie the asymmetrically distributed subpellicular microtubules (mt). The nucleus (n) is large x 52,495.


4.7
mp
4.8
4.9
r
4.10
m
4.11
pl
pm
mt
n
4.12

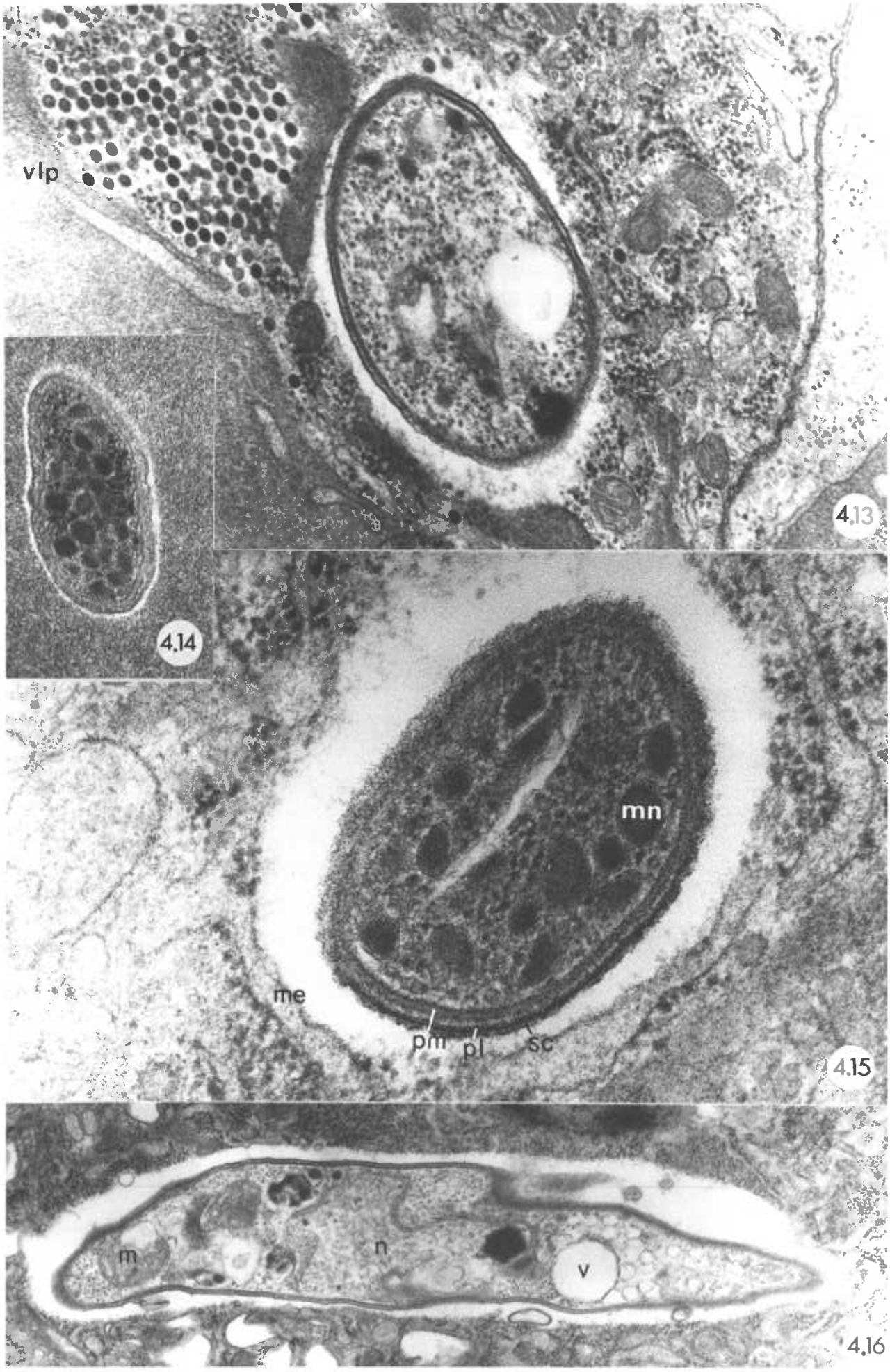
Figs. 4.13 to 4.16. Sections through P. gallinaceum sporozoites in the salivary glands of A. aegypti.

Fig. 4.13. T.S. of sporozoite lying "naked" within cytoplasm of salivary gland cell. Note the virus-like particles (vlp) in the salivary gland, but not in the sporozoite x 59,254.

Fig. 4.14. T.S. of sporozoite (anterior end) within secretion of salivary glands x 67,627.

Fig. 4.15. T.S. of sporozoite (anterior end) within cytoplasm of salivary gland cell. Note vacuole is partially membrane bound (me) and the electron-dense, membrane bound micronemes (mn). Also, as in the mature oocyst sporozoites, the outer plasmalemma (pl) and the pellicular membranes (pm). Note the fibrillar surface coat (sc) x 113,642.

Fig. 4.16. L.S. of degenerate sporozoite within cytoplasm of salivary gland cell. Note the distorted nucleus (n) and vacuolated cytoplasm (v). Mitochondria (m) are present x 24,181.


vlp
4.13
4.14
mn
me
pm
pl
sc
4.15
m
n
v
4.16

Sporozoites in mature oocysts possess membrane limited rhoptries which have an electron-dense matrix (Figs. 4.10 and 4.12). The nucleus is large, elongate and centrally placed.

The morphology of sporozoites from the salivary glands differs little from that seen in mature oocyst sporozoites, except in the organization of the rhoptry-microneme complex; rhoptries are not seen in salivary gland sporozoites, and are instead replaced by an extensive system of micronemes (Figs. 4.14 and 4.15). A prominent surface coat was observed on one sporozoite (Fig. 4.15).

Sporozoites from salivary glands were observed intracellularly, either lying in a membrane bound parasitophorous vacuole (Fig. 4.15), or naked in the cytoplasm (Fig. 4.13 and 4.16), or extracellularly within the matrix of the salivary gland secretion (Fig. 4.14).

4.4. Discussion

The morphological observations reported in this chapter are in close accord with those of earlier workers (Huff and Coulston, 1944; Garnham, 1966a; Terzakis, 1968; Terzakis, Sprinz and Ward, 1976; Sinden and Garnham, 1973). However, particular attention in this investigation was given to a comparison between sporozoites from mature oocysts and salivary glands, since any changes in their morphology may be related to their different invasive behaviour.

Studies by the Sergent brothers (Sergent and Sergent, 1918), who infected mosquitoes with P. relictum and kept them at a temperature of 8-25°C for 5 months, not only demonstrated that after this time their length's were reduced from about 14 µm to 8-10 µm, but also that they were no longer infective. In the present investigation, the lengths

of sporozoites were also found to decrease with prolonged residence in the salivary glands and earlier, Porter, Laird and Dusseau (1954) had found that the infectivity of P. gallinaceum sporozoites was reduced to one fourtieth after 4 weeks in the salivary glands of susceptible A. aegypti. A similar decrease in infectivity of sporozoites with extended residence in the salivary glands has been reported for P. vivax (Boyd and Stratman-Thomas, 1934) and P. falciparum (Garnham, 1966a). The latter author reporting that after 40 days sporozoites were non-infective, although no morphological differences were apparent.

In the search for differences between sporozoites from mature oocysts and salivary glands, the occurrence of the micropore was considered a suitable candidate for investigation. The micropore, first described by Garnham et al. (1961) and at first thought by these authors to represent the point of exit of an emergent sporoplasm, has subsequently been described as a characteristic organelle of the Sporozoa (Scholtyseck and Melhorn, 1970) and is now considered to be a cytostome for the ingestion of food material (Aikawa et al., 1966). Although the micropore is likely to be dormant in sporozoites its presence has attracted some recent attention. Sinden and Garnham (1973) have commented on the extreme rarity or absence of the micropore in the sporozoites of the rodent plasmodia which contrasts strongly with the avian and primate species. These authors compared their morphological findings of the incidence of the micropore to the available data on the infectivity of sporozoites and suggested that the absence of the micropore in sporozoites of rodent malarial parasites was an indication of their incomplete morphogenesis. In the present investigation the micropore was demonstrated by routine Giemsa

staining in approximately the same proportion of mature oocyst and salivary gland sporozoites (80·8% and 82·4%); although its incidence decreased in older sporozoites (43·4%). However, as is demonstrated later (Chapter 7) the infectivity of salivary gland sporozoites is 10,000 times greater than that of mature oocyst sporozoites, so obviously the presence of the micropore cannot be the sole indicator of sporozoite infectivity and other changes must be occuring as well.

At the ultrastructural level, the only significant morphological difference observed between sporozoites from mature oocysts and salivary glands was in the organization of the rhoptry-microneme complex. In the sporozoites from salivary glands the rhoptries, which were present in sporozoites from mature oocysts, were not seen and were replaced by an extensive system of micronemes. A similar transition in the morphology of the rhoptry-microneme complex between oocyst and salivary gland sporozoites has been noted in other species of malarial parasites, including, P. vivax, P. cynomolgi, P. yoelii nigeriensis, P. vinckei chabaudi, and P. gallinaceum (Sinden and Garnham, 1973); P. falciparum (Sinden and Strong, 1978) and Leucocytozoon dubreuili (Wong and Desser, 1976).

Although the rhoptry-microneme complex is one of the most striking and characteristic features of the invasive stages of the Sporozoa its true function remains enigmatic, although it is assumed by many authors (for references see Chapter 9) that it aids in the penetration of the cellular barriers that are crossed. Garnham, Bird and Baker (1960) and Garnham et al. (1961) in some earlier studies on the ultrastructure of malarial sporozoites, suggested that these organelles secreted proteolytic enzymes which dissociated the cell

surface membrane of the host cell and thus facilitated invasion. However, it is now apparent, from studies with particularly Eimeria (see Long and Speer, 1977) and malarial merozoites (see Bannister, 1977), that the host cell membrane is not dissociated, at least not during the invasive process, but invaginated at the site of entry of the invading organism and therefore it seems likely that proteolytic digestion of the cell surface membrane is not the mechanism of entry. Instead, some recent investigations suggest the involvement of cationic proteins with the invasion of host cells. Kilejian (1976) isolated a protein, unusually rich in histidine (73%), which was associated with the rhoptries and micronemes of P. lophurae merozoites. This protein caused invagination and osmotic fragility to the surface membrane of erythrocytes and was therefore incriminated to assist in their penetration. Furthermore, a protein with similar properties and effects was isolated from the trophozoites of Toxoplasma gondii (Lycke, Carlberg and Norry, 1975). Therefore, by analogy with these closely related species, it is likely that the rhoptries and micronemes of malarial sporozoites secrete polycationic surfactant substances which aid in the invagination of host cell membranes, whether it be salivary gland cells or macrophages, and their subsequent intracellular localisation. However, ultrastructural evidence to support the suggestion that these organelles secrete material is not convincing, although as Bannister (1977) has pointed out, these organelles are so large and numerous that a partial emptying (rhoptries) or loss of a few of them (micronemes) would be hard to detect.

From the observations reported in this chapter, together with those of other workers (see Aikawa, 1971 and Sinden, 1978) it is impossible to distinguish between the contents of the rhoptries and

micronemes of malarial sporozoites. Although further work is clearly needed to isolate the constituents of these organelles and to determine their functional independence, it is likely that the rhoptries of mature oocyst sporozoites assist in their exit from the mature oocyst and/or in their entry into the salivary glands, and the micronemes of salivary gland sporozoites assist in their entry into cells of the reticulo-endothelial system (see Chapter 9).

Another interesting and fundamentally important aspect of sporozoite morphology is the appearance of the surface membrane, particularly as a need has recently been expressed for a closer examination of the cell surface membrane of malarial sporozoites (Bannister, 1977). Although malarial sporozoites have been reported to possess a very thin surface coat of fibrillar material loosely surrounding the outer sporozoite membrane (Cochrane et al., 1976), Sinden (1978) has commented that the surface membrane of the sporozoite is covered by a disperse fibrous material which is probably derived from the cystic fluid. Indeed, my own observations are in agreement with those of Sinden, in that the surface membrane of both mature oocyst and salivary gland sporozoites appears naked, except for some scanty fibrillar material, and are devoid of a true surface coat as seen in the bloodstream forms of pathogenic trypanosomes (Vickerman, 1969). However, a prominent surface coat was observed on one intracellular salivary gland sporozoite and although little is known about the surface membrane of malarial sporozoites (see Chapters 5 and 6) it is interesting to note that a similar coat has been observed on intracellular sporozoites of L. dubreuili (Wong, 1979).

Ookinetes also appear to lack a surface coat (Garnham, Bird and

Baker, 1962; Canning and Sinden, 1973) although recently Gass (1979) has used ruthenium red to detect the presence of acidic mucopolysaccharides on the surface of P. gallinaceum ookinetes and concluded that this stage has a prominent surface coat. However, Luft (1971) has commented that considerably more work is required to establish the specificity of ruthenium red for acidic mucopolysaccharides and also, there is an added difficulty of interpreting reactions of ookinetes taken from bloodmeals, for it is possible that the dense layer seen with ruthenium red at the cell surface could, at least in part, be due to adsorbed serum glycoproteins.

In summary, this chapter has discussed the morphology of P. gallinaceum sporozoites from mature oocysts and salivary glands. With the light microscope, sporozoites from mature oocysts and recently invaded salivary glands were indistinguishable in length, gross morphology and in the incidence of the micropore. In old salivary gland infections, sporozoites became shorter and the micropore was seen less often; these changes were correlated with a gradual loss of infectivity with prolonged residence in the salivary glands. With the electron microscope, the changes observed in the organization of the rhoptry-microneme complex were suggested to be related to the different invasive capacities that are exhibited by sporozoites, although a precise functional interpretation of the role of the rhoptries and micronemes remains unclear.

CHAPTER 5

5. CELL SURFACE CHARGE: MEASUREMENT OF CELLULAR ELECTROPHORETIC MOBILITY

5.1. Introduction

In the previous chapter, it has been demonstrated that although sporozoites from mature oocysts and salivary glands have the same dimensions, ultrastructural changes occur in their internal organisation which result in a proliferation of the micronemes and a disappearance of the rhoptries in the salivary gland forms (Sinden and Garnham, 1973; Sterling, Aikawa and Vanderberg, 1973). It is considered that the function of these organelles is to aid in penetration of some of the cell barriers that are crossed by sporozoites (Sinden, 1978), although it would seem unlikely that this change in the rhoptry-microneme complex would account for all the physiological changes, such as those that occur in infectivity (Vanderberg, 1975) motility (Vanderberg, 1974) and antigenicity (Vanderberg et al., 1972) that are associated with the progression of malarial sporozoites from the mature oocyst to residence in the salivary glands. Presumably, the cell surface, which is known to be of importance in the recognition, attachment and penetration of the malarial merozoite to erythrocytes (Ladda, Aikawa and Sprinz, 1969; Bannister et al. 1975; Bannister, 1977) is intimately involved with at least some of these changes. Although the cell surface is known to be of such importance to a number of biological phenomena such as adhesion and antigenicity, little is known about the function of the surface membrane of malaria parasites; this is

particularly true of the sporozoite. The nature of the cell surface of P. gallinaceum sporozoites was therefore investigated by the technique of free-flow electrophoresis. The investigation of a cell surface by this technique can, when coupled with the appropriate chemical and enzymatic manipulation of the surface groups, provide information of both a general and specific nature (Ambrose, 1966; Shaw, 1969; Mehrishi, 1972). This information should be helpful in understanding more completely the structure of sporozoites and their interaction with a variety of cells.

This chapter reports studies on the free-flow electrophoretic behaviour of P. gallinaceum sporozoites from mature oocysts and salivary glands and discusses these results in terms of some of the surface features of sporozoites.

5.2. Materials and Methods

5.2.1. Preparation of sporozoites and electrophoretic measurements

Sporozoites from salivary glands and mature oocysts were prepared as described previously (p. 33). Suspensions containing 1-3 x 10^6 sporozoites/ml were produced in M199.

The microelectrophoretic mobilities of sporozoites were measured in the apparatus of Bangham et al. (1958) and Seaman and Heard (1961) as supplied by Rank Bros., Bottisham, Cambridge. It was fitted with reversible blacked platinum electrodes. The microscope was supplied with a X40 water immersion objective lens. Mobilities were measured at 24°C, 15-25 observations being made for each sample, with reversal of current to minimize the effects of polarization. A squared eye-piece graticule was used to divide the observed field into squares with sides

30μm. The potential gradient was adjusted so that the to and fro transit time lay between 10 and 15 seconds. To ensure that all readings were taken at the stationary phase, the microscope was set so that only the sporozoites at the stationary phase were in focus. After each run the electrophoresis cell was drained and washed out several times with distilled water before the next solution was added.

Electrophoretic mobilities, EPM, expressed as μm/s/V/cm ± standard error of the mean (S.E.M.), were determined by the equation,

$$EPM = \frac{L}{T} \times \frac{l}{V}$$

where, L is the distance travelled by the sporozoite (μm)

T is the time taken (seconds)

V is the voltage

and l is the effective length of the electrophoresis tube (cm) calibrated from the resistance of the cell when filled with a buffer of known conductance.

5.2.2. Calibration of electrophoresis cell: determination of the stationary phase

In a narrow closed electrophoresis cell containing a suspension of particles in an electric field, two electrokinetic phenomena occur, namely, the electro-osmotic and electrophoretic effects. The electro-osmotic effect is produced by the electrical charges assumed by the capillary walls which results in the movement of liquid towards the cathode and a return flow of liquid through the centre of the cell. Between these regions of electro-osmotic movements there are two

regions where no net movement of liquid occurs, which form the stationary phase.

The electrophoretic effect refers to the motion of the particles with respect to the medium, and therefore for measuring the true electrophoretic mobility of cells, the position of the stationary phase must be determined. This was calculated experimentally using fresh human erythrocytes suspended in M/15 phosphate buffer, pH 7.4 (Appendix 2).

Human erythrocytes were collected in a heparinised syringe (see p. 34), washed twice in phosphate buffered saline (Appendix 2) then twice in the M/15 phosphate buffer. For each washing the erythrocytes were centrifuged at 2,500 rev./min. for 5 minutes at 4°C (M.S.E. 1800, 8 x 50 ml angle head). Their mobilities were then examined at 50 μm intervals from the wall of the capillary and a graph was constructed of erythrocyte mobility against distance from the capillary wall (Fig. 5.1). As the mobility of human erythrocytes is known to be 1.3 μm/s/V/cm (see references in Sherbet, 1978) the position of the stationary level was found to be at 260 μm from the wall of the capillary.

5.2.3. Chemical and enzymatic modification of sporozoite surface groups

Trypsin treatment:

1-2 x 10^6 sporozoites were suspended in 1.0 ml of 0.01% trypsin (Sigma, Type 3) in M199 at pH 7.4 and incubated at 37°C in a shaking water bath. Trypsin, inactivated by heating to 60°C for 30 minutes, was incubated with sporozoites (5 x 10^5/ml) to serve as one control,

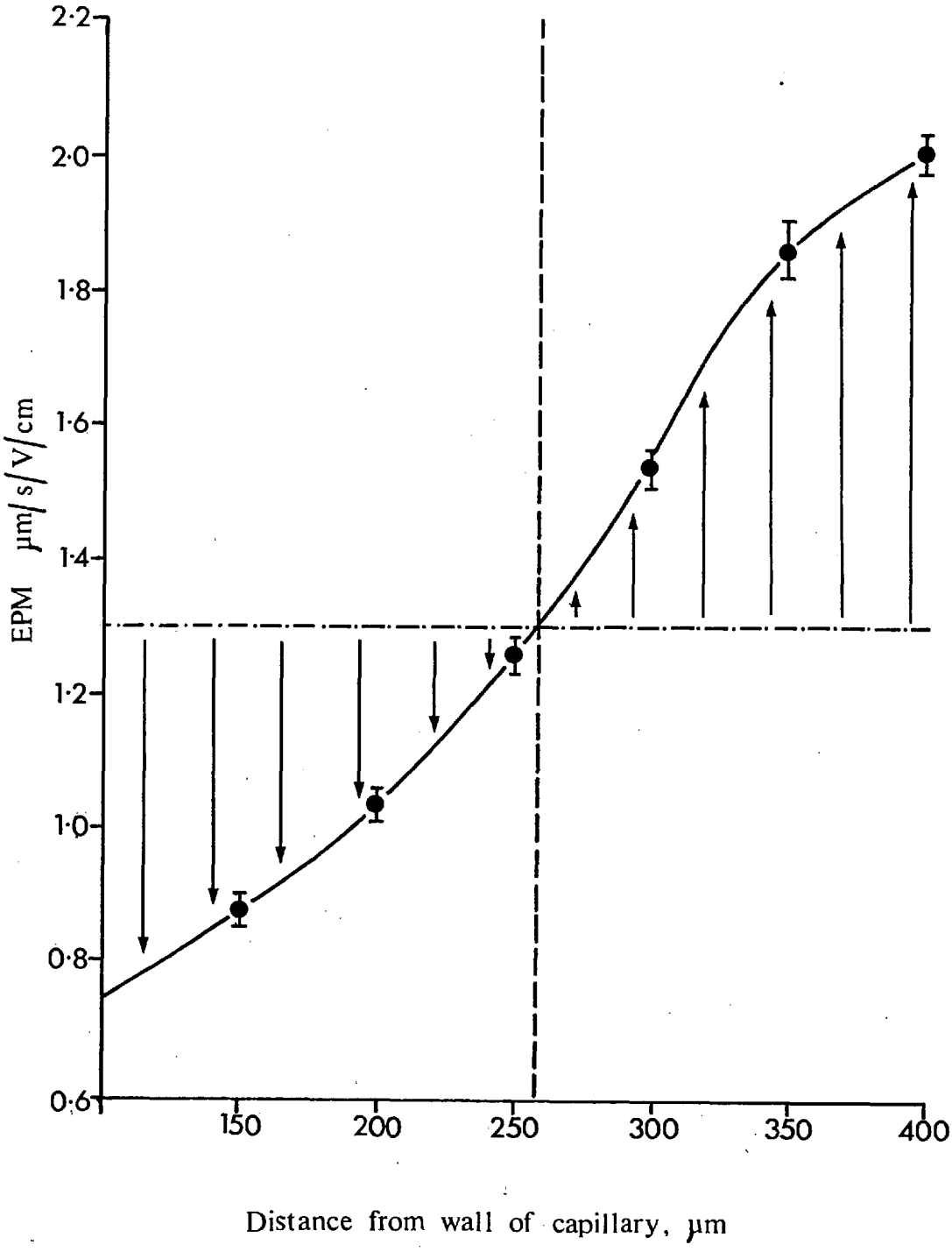


Fig. 5.1. Depth-mobility curve for human erythrocytes in M/15 phosphate buffer, pH 7·4.——————— stationary level;·—·—·—·— known erythrocyte mobility. Each point represents the mean of 25-40 measurements, with standard error. Arrows represent velocity of liquid due to electro-osmotic effect.

the other being an incubation of 5×10^5 sporozoites in 1.0 ml M199, without enzyme, for 30 minutes at 37°C. Sporozoites were washed twice in M199 after treatment and resuspended in M199 for electrophoretic measurements. The activity of the trypsin was checked by its effect on the mobility of fresh human erythrocytes.

Neuraminidase treatment

Neuraminidase from Clostridium perfrigens (Sigma) was used in an attempt to detect sialic acid on the sporozoite surface. The sporozoites ($1\text{-}2 \times 10^6$) were suspended in 1.0 ml M199 and neuraminidase was added to give a concentration of 1 unit/ml and the cells were incubated at 37°C in a shaking water bath. Controls were set up as for the trypsin treatment and again, fresh human erythrocytes demonstrated the activity of the neuraminidase. Cells were washed twice in M199 after treatment and resuspended in M199 for electrophoretic measurements.

Modification of amino groups by citraconic anhydride (CA)

Citraconic anhydride was dissolved in acetone and added to $1\text{-}2 \times 10^6$ sporozoites in PBS* (Appendix 2) to give final concentrations of 0.01 and 0.0001M CA and so that the concentration of acetone did not exceed 1% (v/v). Acetone was also added to the control. The cell suspension was incubated for 30 minutes at 23°C and washed twice in M199 before resuspension in the same medium for electrophoretic measurements.

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*Incubation was carried out in PBS to avoid any reaction of the citraconic anhydride with the amino groups present in M199.

Modification of thiol groups by 5,5'-dithio-bis (2-nitrobenzoic) acid (DTNB)

Incubation was carried out according to the conditions of Lee (1972). Sporozoites were suspended in 40mM Tris-HCl (pH 8.2) at a density of 1-2 x 10^6 sporozoites/ml and a freshly made solution of DTNB (Sigma), in the same buffer, was added to give concentrations of 0.001M and 0.005M. The sporozoite suspensions were incubated and washed after treatment as described in the preceeding paragraph.

5.3. Results

5.3.1. Variation of electrophoretic mobility with development of sporozoite

The mean mobility of sporozoites from salivary glands was found to be lower (0.92 $\pm$ 0.01 µm/s/V/cm) than that of sporozoites from mature oocysts (1.19 $\pm$ 0.02 µm/s/V/cm) and the difference was statistically significant ($p<0.001$, Student's t-test). This represents a decrease in mobility of 22.7%. In both cases the sporozoites were randomly orientated in the electrophoresis chamber indicating that the surface charge is distributed evenly over the surface so there can be no gross localization of charge on any part of the sporozoites, such as is found with spermatozoa (Bangham, 1961).

The distribution of sporozoite mobilities are shown in Fig. 5.2 and that of both sporozoites from salivary glands and midguts is normal.

As sporozoites from mature oocysts and salivary glands are the same size (see Chapter 4) and the electrophoretic mobility is a measurement of surface charge density, this decrease in mobility must

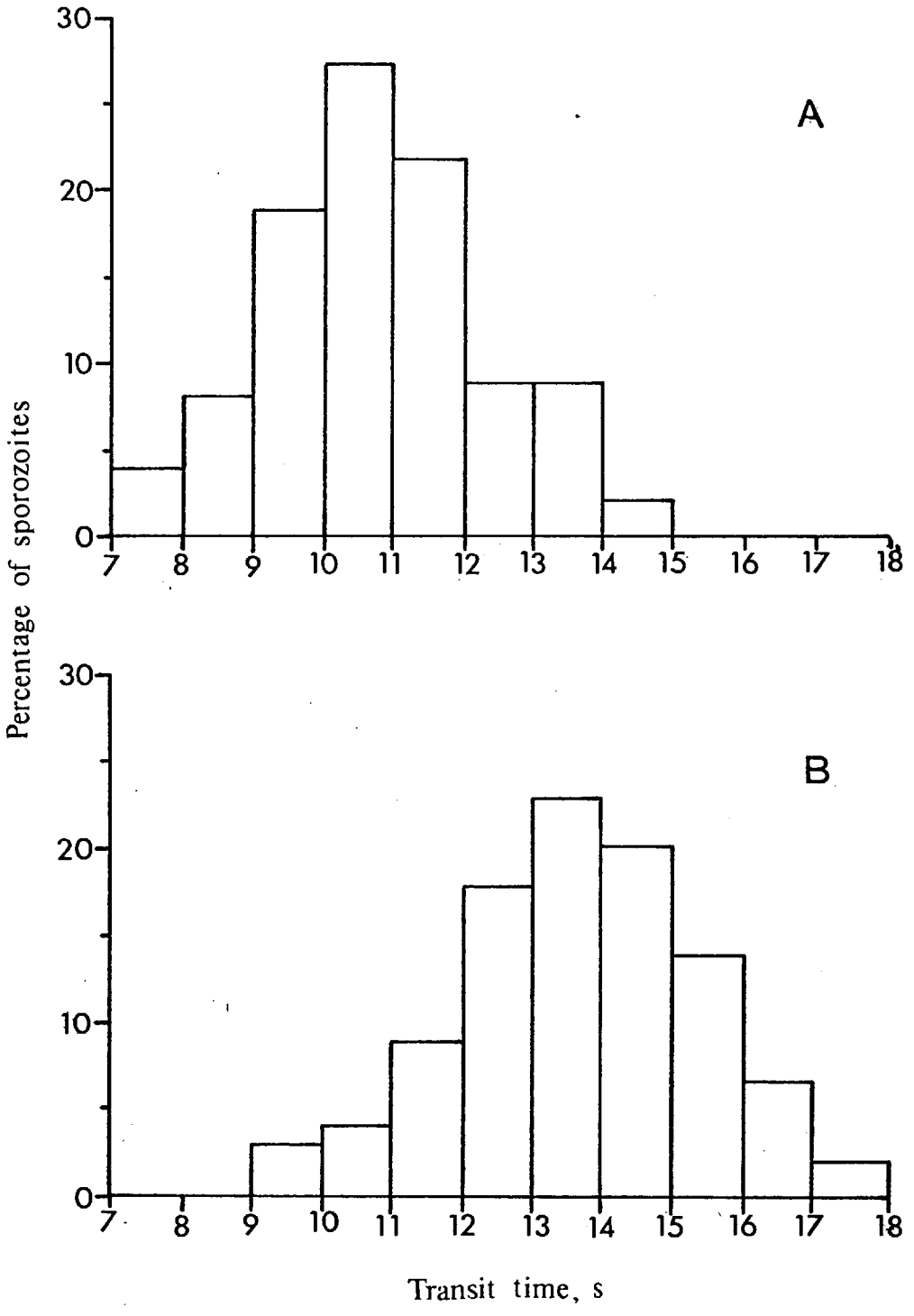

Fig. 5.2. Distribution of electrophoretic mobilities of P. gallinaceum sporozoites. The distribution of mobility is measured in terms of transit time, in seconds, for 60 μm. Electrophoretic mobilities were determined in M199 at pH 7·4. A. mature oocyst sporozoites; B. salivary gland sporozoites. 100 observations for each sample.

represent a decrease in the negatively charged groups at the surface. There are a number of possible explanations for the decrease in electrophoretic mobility as the sporozoite leaves the rupturing oocyst and moves to the salivary gland and include, changes in membrane composition, conformation and structure, and the appearance (or disappearance) of surface components.

The remainder of this chapter describes experiments to identify the ionogenic groups involved.

5.3.2. Mobility of sporozoites as a function of pH

The effect of changes in the pH of the suspending medium on the mobility of sporozoites was investigated within the narrow, but physiologically important, range of pH 6.0-8.0. Sporozoites maintained a net negative charge over this pH range, but the way in which the mobility of salivary gland forms and of oocyst forms altered with respect to changes in pH was different, suggesting that qualitative differences exist between the cell surface ionogenic groups of the two populations (Fig. 5.3). At pH 7.0, the surface groups of sporozoites from salivary glands appear to be fully ionised, indicating that the groups responsible for the surface charge are ionised at a lower pH than 7.0 and suggesting the presence of carboxyl groups (pKa* 4.5), such as those of glutamic and aspartic acids. However the surface charge of oocyst sporozoites increased above pH 6.5 suggesting the presence of phosphate groups, possibly linked to sugars or proteins; whilst the most likely group to ionise at this pH range would be the imidazolyl grouping of histidine (pKa 5.6-7.0).

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*pKa of a group may be defined as that pH at which ionization of the group is one-half.

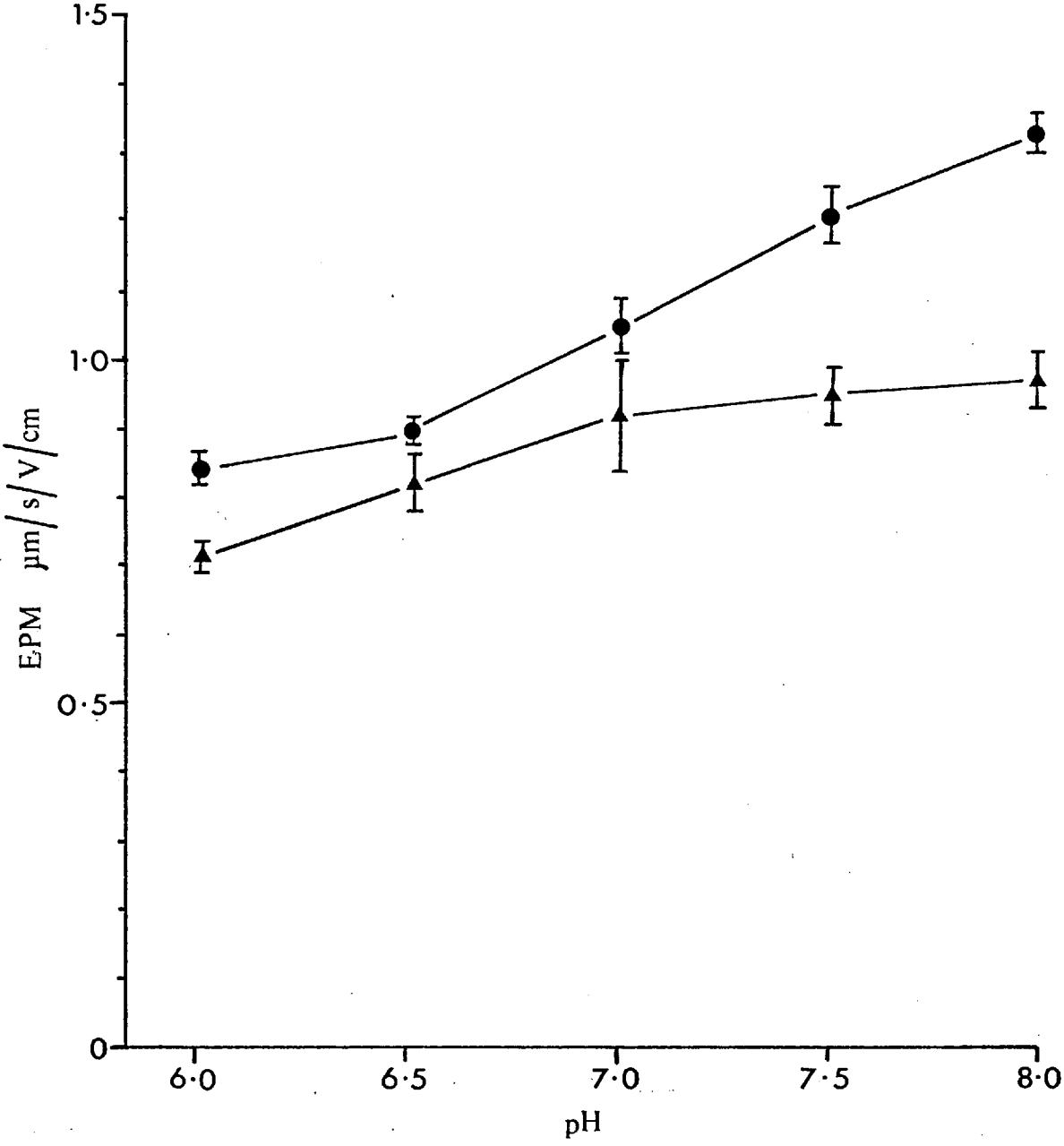


Fig. 5.3. Effect of pH of the suspending medium on the electrophoretic mobility of P. gallinaceum sporozoites. Sporozoites were harvested, washed and resuspended in M199, pH 7·4 The pH value of the suspension was varied by additions of 0·05M-HCl or NaOH, and was measured before and after electrophoretic measurements. Each point represents the mean of 15-25 measurements, with standard error. ●———● mature oocyst sporozoites, ▲———▲ salivary gland sporozoites.

5.3.3. The action of enzymes on sporozoite mobility

The action of two enzymes, trypsin and neuraminidase, was studied.

Incubation of sporozoites with trypsin considerably reduced their cell surface charge (Table 5.1). The mobility of oocyst forms was reduced by 28.3% and that of salivary gland forms by 23.6%; in both cases the difference was statistically significant ($p<0.001$). The effect was apparent after 30 minutes and incubation for a further 30 minutes produced no significant decrease in mobility (Fig. 5.4). Heat-inactivated trypsin had no effect on the mobility of sporozoites, indicating that the proteolytic action of this enzyme was responsible for the decrease in surface charge. The control incubation of erythrocytes with trypsin reduced their mobility by 32.3% (Table 5.1) and is consistent with established data (Ponder, 1951; Seaman and Heard, 1960). These results indicate that a significant proportion of the surface charge of sporozoites is associated with protein and is compatible with the pH-mobility data.

Neuraminidase however, had no effect on the mobility of sporozoites (Table 5.2) even with a long incubation of 60 minutes (Fig. 5.5). These results indicate that sialic acid residues, which are responsible for much of the negative charge associated with mammalian cells (see Sherbet, 1978) are not present in an exposed or reactive form on the cell surface of sporozoites. Under the same conditions, the mobility of human erythrocytes was reduced by 71%, which, as for the trypsin treatment is consistent with the numerous reports in the literature (Eylar et al., 1962; Seaman and Cook, 1965; Mayhew and Weiss, 1968; Zalik, Sanders and Tilley, 1972).

Table 5.1. Effect of trypsin treatment on the electrophoretic mobility of P. gallinaceum sporozoites

Cells and treatment	Electrophoretic mobility (μm/s/V/cm)	Statistical probability, P*
A. Salivary gland sporozoites		
None	0·93 ± 0·03+	
Control incubation, 60 min.	0·92 ± 0·04	P>0·2
Heat-inactivated trypsin, 60 min.	0·90 ± 0·02	P>0·2
Trypsin, 60 min.	0·71 ± 0·01	P<0·001
B. Mature oocyst sporozoites		
None	1·20 ± 0·06	
Control incubation, 60 min.	1·11 ± 0·02	P>0·05
Heat-inactivated trypsin, 60 min.	1·16 ± 0·06	P>0·2
Trypsin, 60 min.	0·86 ± 0·04	P<0·001
C. Human erythrocytes		
None	1·36 ± 0·03	
Trypsin, 60 min.	0·92 ± 0·02	P<0·001

Electrophoretic mobilities were measured in M199 at pH 7·4.
* Probability from Student's t-test, control versus treated.
+ Mean ± standard error, 15-25 measurements for each value.

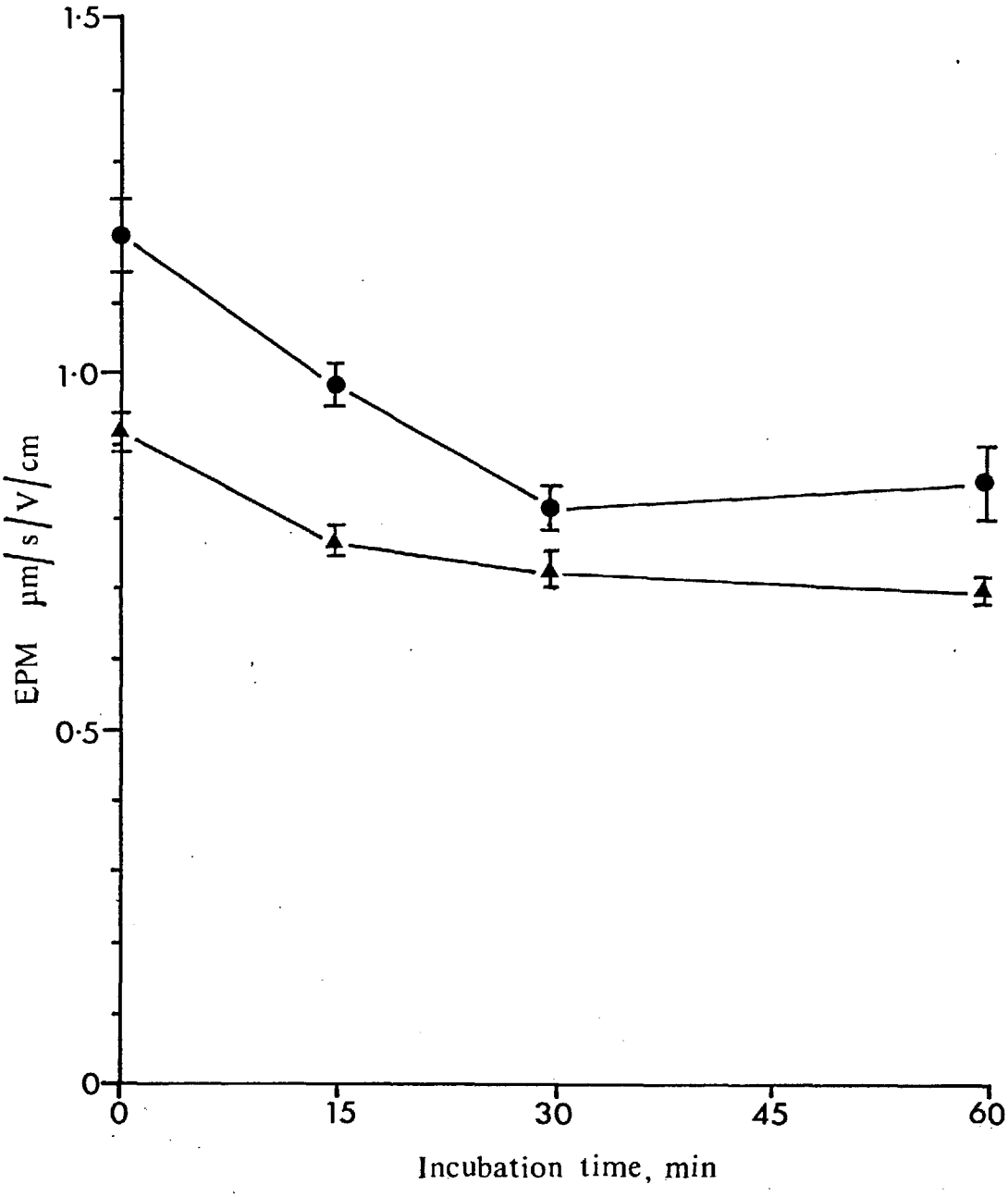


Fig. 5.4. Effect of trypsin on the electrophoretic mobility of P. gallinaceum sporozoites. Sporozoites were incubated for varying lengths of time with 0·01% trypsin in M199, pH 7·4, at 37°C. Each point represents the mean of 15-25 measurements, with standard error. ●———● mature oocyst sporozoites, ▲———▲ salivary gland sporozoites.

Table 5.2. Effect of neuraminidase treatment on the electrophoretic mobility of P. gallinaceum sporozoites

Cells and treatment	Electrophoretic mobility (μm/s/V/cm)	Statistical probability, P*
A. Salivary gland sporozoites		
None	0·91 ± 0·04+	
Control incubation, 60 min.	0·90 ± 0·02	P>0·2
Heat-inactivated neuraminidase, 60 min.	0·92 ± 0·02	P>0·2
Neuraminidase, 60 min.	0·92 ± 0·02	P>0·2
B. Mature oocyst sporozoites		
None	1·19 ± 0·05	
Control incubation, 60 min.	1·19 ± 0·06	P>0·2
Heat-inactivated neuraminidase, 60 min.	1·12 ± 0·06	P>0·2
Neuraminidase, 60 min.	1·18 ± 0·06	P>0·2
C. Human erythrocytes		
None	1·33 ± 0·01	
Neuraminidase, 60 min.	0·38 ± 0·01	P<0.001

Electrophoretic mobilities were measured in M199 at pH 7·4.
* Probability from Student's t-test, control versus treated.
+ Mean ± standard error, 15-25 measurements for each value.

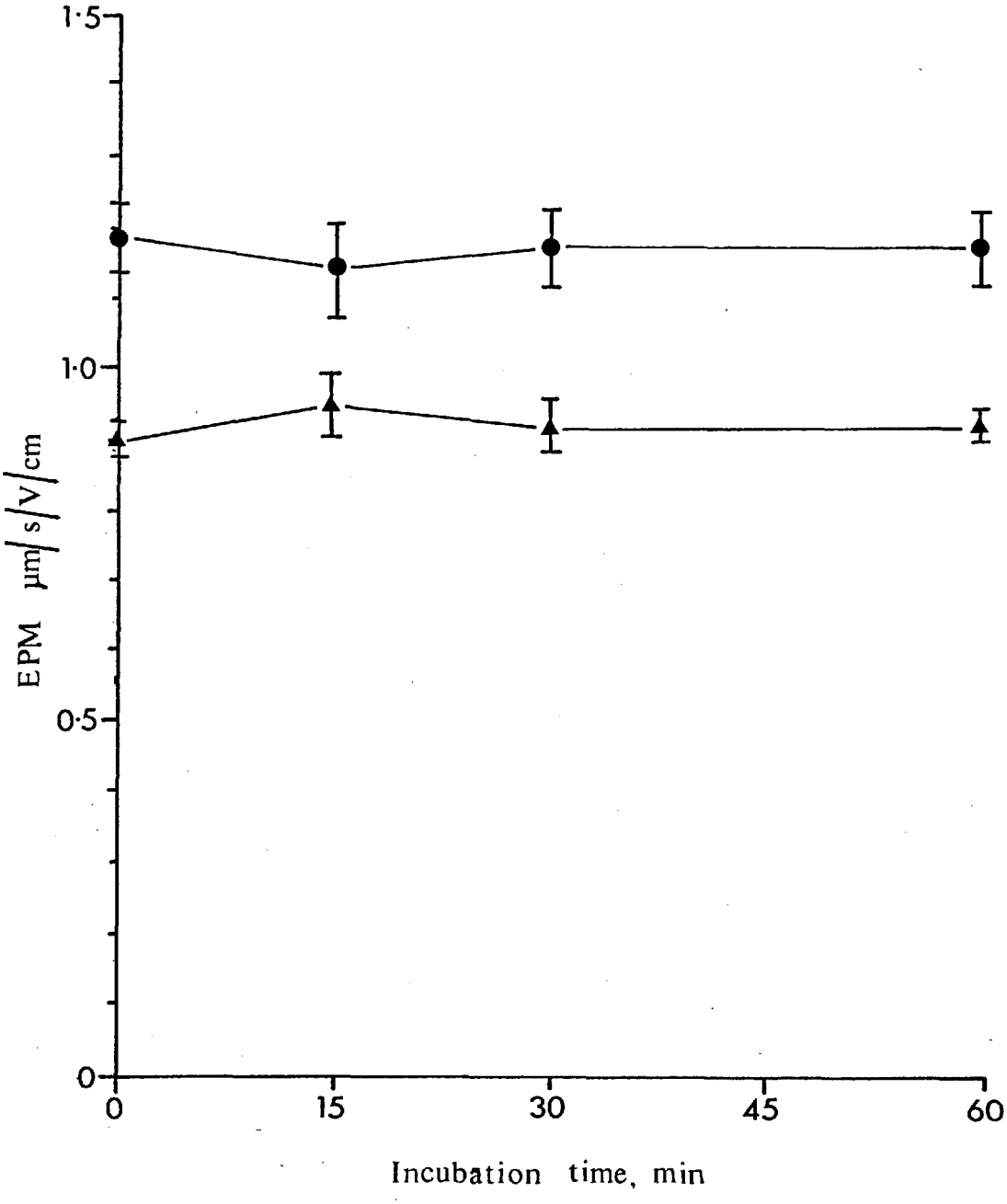
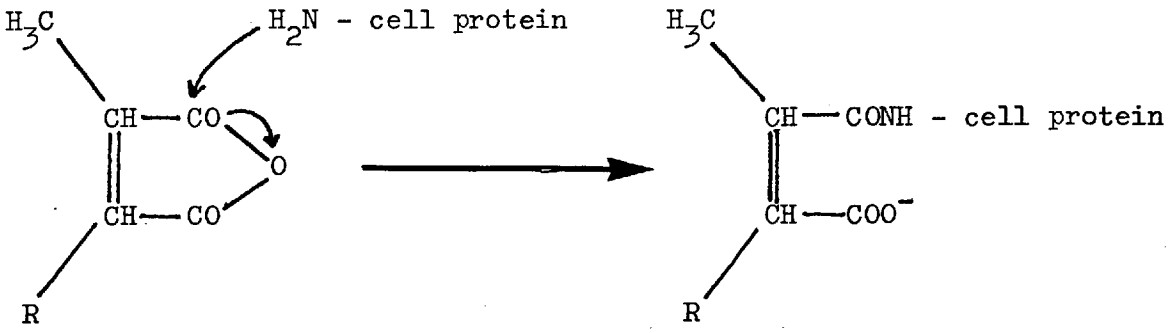


Fig. 5.5. Effect of neuraminidase on the electrophoretic mobility of P. gallinaceum sporozoites. Sporozoites were incubated for varying lengths of time in 1 unit/ml neuraminidase in M199 at 37°C. Each point represents the mean of 15-25 measurements, with standard error. ●——● mature oocyst sporozoites, ▲——▲ salivary gland sporozoites.

5.3.4. The effect of an amino blocking agent on sporozoite mobility

Citraconic anhydride, which reacts with amino groups and converts them into residues bearing negatively charged groups, as shown in Fig. 5.6. (Dixon and Perham, 1968; Mehrishi, 1970) produced a small but significant increase in the mobility of both groups of sporozoites (Table 5.3), thereby demonstrating the occurrence of amino groups at the cell surface which could be associated with either protein or sugar residues.



R = citraconic anhydride

Fig. 5.6. Reaction of citraconic anhydride with cell surface amino groups

The reaction of citraconic anhydride is not specific for amino groups and it will also modify available thiol (SH) groups (Gibbons and Perham, 1970). Therefore sporozoites were incubated with 5,5'-dithio-bis-(2-nitrobenzoic) acid (DTNB) which is a specific reagent for SH groups (Ellman, 1959; Holbrook and Stinson (1970). Any SH groups present at the surface react with DTNB to produce compounds with negatively charged 2-nitrobenzoate residues. Treatment of sporozoites, both from mature oocysts and salivary glands, with DTNB failed to alter their mobility significantly (Table 5.3) thus it appears that they do not possess accessible SH groups at the cell

Table 5.3. Effect of citraconic anhydride and 5,5'-dithio-bis-(2-nitrobenzoic)acid on the electrophoretic mobility of *P. gallinaceum* sporozoites

Cells and treatment	Electrophoretic mobility (μm/s/V/cm)	Statistical probability, P*
A. Salivary gland sporozoites		
1. Citraconic anhydride (CA)		
Control ‡	0·91 ± 0·03+	
CA 0·0001M	0·96 ± 0·02	P<0·1
CA 0·01M	1·03 ± 0·04	P<0·02
2. 5,5'-Dithio-bis-(2-nitrobenzoic) acid (DTNB)		
Incubated control	0·91 ± 0·02	
DTNB 0·001M	0·90 ± 0·03	P>0·2
DTNB 0·005M	0·92 ± 0·03	P>0·2
B. Mature oocyst sporozoites		
1. Citraconic anhydride (CA)		
Control ‡	1·18 ± 0·04	
CA 0·0001M	1·22 ± 0·04	P>0·2
CA 0·01M	1·37 ± 0·03	P<0·01
2. 5,5'-Dithio-bis-(2-nitrobenzoic) acid (DTNB)		
Incubated control	1·19 ± 0·04	
DTNB 0·001M	1·20 ± 0·05	P>0·2
DTNB 0·005M	1·21 ± 0·03	P>0·2

Electrophoretic mobilities were measured in M199 at pH 7·4.
* Probability from Student's t test, control versus treated.
\+ Mean ± standard error, 15-25 measurements for each value.
‡ Control sporozoites incubated with acetone, acetone being the solvent for CA.

surface and that the increase in mobility recorded after reaction with citraconic anhydride was due solely to the reactivity of accessible amino groups.

5.4. Discussion

The electrophoretic properties of cells are due mainly to the presence of ionogenic groups at their peripheries and all cells carry a net charge which is generally net negative. Thus, the mobility of sporozoites in an electric field is not surprising, nor is the fact that they carry a net negative charge. What is surprising perhaps, is that the cell surface charge decreases with development in the mosquito vector; salivary gland sporozoites are significantly less negatively charged than sporozoites from mature oocysts.

The distribution of anionic residues at the cell surface is believed to play an important role in the interaction of cells with their environment. The charge differences recorded in the present study could play an important role in the association of sporozoites with their host cells. It is possible that a relatively low surface charge on the surface of sporozoites would assist in their close apposition to target cells, and thus might facilitate invasion.

From the distribution of sporozoite mobilities shown in Fig. 5.2 it can be seen that only one population of sporozoites appear to be present in either location in the mosquito, as their distribution, from a plot on probability paper, appears to be normal. However, the numbers examined (100 for each group) are relatively small for a statistical analysis of this nature; perhaps the technique of continuous free-flow electrophoresis (described later in this chapter with regard to sporozoite separation) would permit a more detailed examination of this question.

Both groups of sporozoites appear to possess significant quantities of accessible protein at the surface which contribute to the net negative charge, since trypsin treatment caused a marked reduction in their electrophoretic mobilities. It is considered unlikely that the enzyme binds to the cell surface to any extent because heat-inactivated trypsin did not alter the electrophoretic mobility of control sporozoites and so the decrease in mobility associated with trypsin treatment cannot be explained by simple adsorption to the surface. This would cause an almost instantaneous change in mobility after the addition of the enzyme to the sporozoite suspension; as can be seen from Fig. 5.4, the reduction in mobility was not complete until after about 30 minutes incubation.

It is probable that at least some of the cell surface protein is associated with amino groups, as reaction with citraconic anhydride produced a small but significant decrease in electrophoretic mobility. Thiol groups however, which are associated solely with proteins are not detectable.

In mammalian cells a large proportion of the negative charge at the cell surface is due to carboxyl groups associated with N-acetyl-neuraminic acid residues (NANA) which are sensitive to neuraminidase (Cook, Heard and Seaman, 1961; Cook and Jacobson, 1968; Cook, 1968; Vassar, Kendall and Seaman, 1969; Lightman and Weed, 1970; Patinkin, Schlessinger and Doljanski, 1970; Kojima and Maekawa, 1970). In the protozoa however, sialic acid appears to be rare or absent and is considered to be something of an "evolutionary substance" (Warren, 1963). Indeed, incubation of sporozoites with neuraminidase had no effect on the mobility of either groups and so it appears that sialic acid residues are not exposed at the surface. This does

not mean that sporozoites lack sialic acid, it may be that sialic acid residues are inaccessible at the surface. This has been shown to be the case for myelin sialic acid which is in the form of the mono-sialoganglioside GM1, and is resistant to the action of neuraminidase (Gregson, 1977). However, it is likely that sialic acid residues are scarce in the malaria parasites. Miller et al. (1973) were unable to detect the binding of ferric oxide colloid to the surface of asexual parasites of P. knowlesi and to the sporozoites of P. berghei and suggested that the surface of malaria parasites is qualitatively different from the surface of mammalian erythrocytes in the absence of NANA. Seed, Aikawa and Sterling (1973) and Seed et al. (1974) have provided further cytochemical evidence to suggest that sialic acid residues are rare on the surface membranes of erythrocytic forms. Recently, Sherman and Jones (1979) have compared quantitatively the ammount of sialic acid in the membrane of erythrocytic forms of P. lophurae to the amount present in the erythrocyte surface membrane and have found that the parasite surface membrane contains little sialic acid compared to the red cell membrane (8 nmoles sialic acid/mg membrane protein versus 79 nmoles sialic acid/mg membrane protein).

Limited as our knowledge is concerning the nature of the sporozoite surface membrane, much attention has recently been focussed on the membrane properties of malaria infected erythrocytes and freed erythrocytic parasites and it is clear that compositional, structural and functional differences exist between the limiting membrane of erythrocytic forms of plasmodia and their host cells. These include differences in phospholipids (Rock et al., 1971; De Zeeuw et al., 1972), differences in membrane structure as revealed by freeze-fracture microscopy (Seed et al., 1971; Mezoely, Steere and Bahr, 1972;

Seed et al., 1973; Mazzen, Gull and Gutteridge, 1975; McLaren et al., 1977; McLaren et al., 1979), differences in the staining affinity for glycoproteins (Langreth, 1975), differences in staining with positively charged iron colloids (Seed, Aikawa and Sterling, 1973; Miller et al., 1973; Seed et al., 1974; Takahashi and Sherman, 1978) and differences in membrane proteins (Weidenkamm et al., 1973; Wallach and Conley, 1977; Konigk and Mirtsch, 1977; Shakespeare, Trigg and Tappenden, 1979; Sherman and Jones, 1979). The latter authors suggest that parasite mediated modifications of existing membrane polypeptides, rather than insertion of plasmodial membrane protein into the erythrocyte plasma membrane, are responsible for these differences. It seems then that the erythrocytic parasites surface is rich in phospholipids while sialic acid residues and mucopolysaccharides appear to be scarce.

Although knowledge concerning the surface charge of malaria parasites remains limited, numerous attempts have been made to demonstrate the surface charge of bloodstream trypanosomes (for review of early literature, see Broom, Brown and Hoare, 1936). The net negative charge of erythrocytes was used in a simple adhesion test by Broom and Brown (1937; 1939) to investigate the surface charge of T. gambiense. They demonstrated that trypanosomes of the first blood parasitaemia were positively charged, and those of subsequent relapse populations were either weakly positive or negatively charged. Following inoculation into culture medium, or entering the tsetse fly, the trypanosomes all assumed a net negative charge. In the salivary glands of the fly, reversion to net positive took place. Later Hollingshead, Pethica and Ryley (1963) quantitatively studied the surface charge of bloodstream and culture forms of T. rhodesiense and bloodstream forms of some other species. They found that the isoelectric

point of bloodstream forms of T. rhodesiense was about pH 7.0, indicating that circulating trypanosomes probably carry little, or no net surface charge. The isoelectric point of culture (fly midgut) forms was about pH 3.0 and the electrophoretic mobility at pH 7.0 was found to be 0.91 μm/s/V/cm. However, electrophoretic measurements showed no differences between relapse populations of the same strain. Vickerman (1969) has commented on these observations and suggests that the cyclical loss and aquisition of the surface coat is likely to account for the changes in the electrophoretic behaviour of trypanosomes at different stages in their life-cycles. Unfortunately, no such statement can be made with reference to the P. gallinaceum sporozoite because there appears to be no surface coat associated with either salivary gland or mature oocyst sporozoites (see Chapter 4).

Observations on the electrophoretic mobility of trypanosomes have recently been extended by De Souza et al. (1977) who have examined the surface charge of T. cruzi by direct measurement of cellular electrophoretic mobility and by the binding of cationised ferritin and demonstrated that the surface charge increased during development from epimastigote to trypomastigote. Indeed, the relatively high mobility of bloodstream trypomastigotes of T. cruzi may explain why it is difficult to separate these forms on a DEAE-cellulose column.

The present investigation represents the first quantitative data on the cell surface charge of malaria sporozoites, although it has previously been suggested by Miller et al. (1973), who used a cytochemical technique to demonstrate n-acetyl neuraminic acid (NANA), that sporozoites are less negatively charged than their host cells. Further evidence concerning the variability in net negative surface charge of sporozoites has come from the work of Moser et al. (1978), who, during

their studies on the separation of sporozoites from mosquito contaminants on a DEAE-cellulose column, have commented on differences in the elution behaviour among sporozoites of different species of Plasmodium, as well as among sporozoites of the same species derived from different organs of the mosquito. They suggested that this may be due to intrinsic differences in surface charge, or variation in the ionic strength of the sample, which was not excluded because the conductivity of the samples were not standardised. In the light of the present work, it is probable that differences in cell surface charge are responsible and that both intraspecific as well as inter-specific variations are likely to exist.

Although this study represents the first direct measurement of the surface charge of malarial sporozoites, the mobility of erythrocytic forms of P. berghei, prepared by mechanical means, have been examined; they too have a net negative charge which is similar to that of erythrocytes (Seed and Krier, 1976). However, Bannister (pers. comm.) has found that merozoites of P. knowlesi, obtained by the sieve technique of Dennis et al. (1975) have a negative surface charge which is significantly lower than that of erythrocytes and is thought to be caused by the anionic glycoproteins of the cell coat (Bannister, Butcher and Mitchell, 1977).

Recently the technique of continuous free-flow electrophoresis which has proved to be highly successful in separating homologous cell populations from hetergenous cell groups (Zeiller, Liebich and Hannig, 1971; Zeiller, Hannig and Pascher, 1971; Zeiller et al., 1972) and also for the purification and isolation of subcellular organelles (Stahn, Maier and Hannig, 1970; Heidrich, Stahn and Hannig 1970) has been applied to the separation of the erythrocytic stages of two rodent

malaria parasites, P. vinckei and P. berghei (Heidrich et al., 1979; Suzuki et al., 1979) from a mixture of free parasites and uninfected and infected erythrocytes. It is likely that this technique will be extended to obtain merozoite rich fractions for studies on merozoite vaccination and also in the examination of membrane changes that occur in parasitized cells. The isolated parasites maintain their viability and both groups of workers have reported that infected erythrocytes and free parasites are less negatively charged than uninfected erythrocytes. Indeed, differences in the cell surface charge between oocyst and salivary gland sporozoites, and their probable difference from the mobility of mosquito tissue, offers exciting new prospects for their isolation in quantities large enough to permit detailed biochemical and immunological investigations.

In summary, these results have shown that there is a significant reduction in cell surface charge of the salivary gland sporozoite compared to the mature oocyst sporozoite; qualitative differences appear to exist; much of the cell surface charge appears to be attributable to protein possibly in association with amino, but not SH groups; and sialic acid residues appear to be absent.

Finally, Dubremetz et al. (1979) have noticed a significant increase in the number of intramembranous protein particles of salivary gland sporozoites, compared to the oocyst forms, in P. yoelii nigeriensis. Their observations combined with the present electrophoretic differences, suggest that there is a significant reorganization of the cell surface membrane which is likely to be responsible for other important aspects of sporozoite maturation. For example, the dramatic increase in infectivity between sporozoites in

mature oocysts and sporozoites in salivary glands that have been reported for P. berghei, an observation confirmed in the present study on P. gallinaceum (see Chapter 7). Whether these changes occur during passage through the haemocoele, or after invasion of the salivary glands, is, as yet, unknown.

CHAPTER 6

6. CELL SURFACE SACCHARIDES: LECTIN-BINDING PROPERTIES

6.1. Introduction

In the preceding chapter the net negative charge of the surface membrane of the P. gallinaceum sporozoite was shown to decrease as the sporozoite moves from the mature oocyst to residence in the salivary gland and the possible ionogenic groups involved were discussed. This chapter describes experiments which were undertaken to characterize further the cell surface of the sporozoite. The basis for these studies was provided by findings that saccharides are present on the cell surface of a large number of animal cells and that these carbohydrates contain negatively charged radicals which have an important role in various membrane functions (see review by Cook and Stoddart, 1973). Any reagent which is specific for sugars is therefore an invaluable aid for an investigation of the cell surface.

Lectins are proteins which bind to specific carbohydrate residues and are therefore useful probes for assessing some of the properties of the cell surface components, such as the relative mobility of intramembranous macromolecules and the localization of carbohydrate containing receptor sites (Lis and Sharon, 1973; Nicolson, 1974). Lectins react with specific carbohydrate residues and are able to agglutinate erythrocytes and a wide variety of other cells (Sharon and Lis, 1972; Lis and Sharon, 1973; Nicolson, 1974). Some lectins also stimulate blast transformation in lymphocytes (Andersson et al., 1972; Greaves and Janossy, 1972; Pearson et al., 1979). These properties of lectins have led to them being used to study membrane structure and lymphocyte transformation (Loor, 1974).

Dwyer (1974) first reported on the presence of terminal saccharide residues on the surface of a parasitic protozoan (Leishmania donovani promastigotes). Since then, lectins have been employed in the investigation of receptor sites on the surface of a variety of these organisms (Table 6.1.) although analysis of the data presented in these observations shows that the lectin-binding sites present on or within the cell surface membranes are dependant on the species, strain, developmental stage and in the case of some trypanosomes, the antigenic variant.

In contrast to the increasing amount of literature on the ability of trypanosomatids to bind lectins, information concerning the ability of malaria parasites to do so, is limited. However, Seed and Krier (1976) and Bannister et al. (1979) have commented on the scarcity of lectin-binding sites on the surface of erythrocytic stages (maturing intra-erythrocytic forms and free merozoites). Subsequently, David et al., (1978) and David and Hommel (1979) have described a method for isolating merozoites of P. chabaudi and P. knowlesi, which is based on the ability of the lectin Concanavalin A (Con A) to bind to erythrocytes, but not merozoites.

Among the various lectins that have been examined for their ability to bind to the surface of parasitic protozoa, Con A appears to be the most potent inducer of cell agglutination. Consequently, Con A, isolated from the jack bean (Canavalia enisformis) and specific for terminal residues similar to α-D-glucose and α-D-mannose (see review by Goldstein, 1976) was used in this study. This chapter describes the investigation of the reactivity of Con A with sporozoites from mature oocysts and salivary glands.

Table 6.1. Lectin-binding studies on a number of parasitic protozoa

Species	Reference
Entamoeba histolytica	Martinez-Palomo, Gonzales-Robles and De La Torre, 1973 Pinto Da Silva and Martinez-Palomo, 1974
L. braziliensis	Dawidowicz, Hernandez and Infante, 1975 De Souza and Brasil, 1976 Infante *et al.*, 1980
L. donovani	Dwyer, 1974 Dwyer, 1977
L. enrietti	Pardoe *et al.*, 1975
L. mexicana	Infante *et al.*, 1980
P. berghei	Seed and Krier, 1976
P. knowlesi	Bannister *et. al.*, 1979
T. gondii	Sethi *et al.*, 1977
T. brucei	Steiger, 1975 Cross and Johnson, 1976 Seed, Seed and Brindley, 1976
T. congolense	Jackson, Honiberg and Holt, 1978
T. cruzi	Alves and Coli, 1974 Schottelius, 1976 Chiari *et al.*, 1978
T. duttoni	Dwyer and D'Alesandro, 1974;
T. equiperdum	Jackson and Fisher, 1975 Baltz, Baltz and Pautrizel, 1976 Jackson, 1977
T. lewisi	Dwyer, 1976
T. musculi	Dwyer and D'Alesandro, 1976

6.2. Materials and Methods

6.2.1. Preparation of sporozoites and treatment for lectin examination

Sporozoites from mature oocysts or salivary glands were prepared as described previously (p. 33) and suspensions containing 1-3 x 10^6 sporozoites/ml were produced in PBS (Appendix 2). Sporozoites were either used fresh, or fixed in 0.2% glutaraldehyde in PBS for 10 minutes at 4°C, immediately before incubation with FITC - conjugated Con A. Trypsin treated sporozoites were incubated for 30 minutes in 0.01% trypsin at 37°C as described before (p. 59).

6.2.2. Lectin and saccharide inhibitor

Fluorescein isothiocyanate-Concanavalin A (FITC - Con A) prepared by affinity chromatography was purchased from Miles-Yeda (Israel). The competitive saccharide inhibitor, alpha-methyl-D-mannoside (α-MM) was obtained from Sigma (Poole, Dorset). The desired concentrations of the lectin and saccharide inhibitor were prepared in PBS. The saccharide inhibitor, α-MM, was used at a final concentration of 100 mM.

6.2.3. Labelling of sporozoites with FITC - Con A

Suspensions of washed sporozoites, both glutaraldehyde fixed and unfixed (approximately 10^5 sporozoites in 0.2 mls of PBS) were added to 0.2 mls of the appropriate dilution of test solution of FITC - Con A so that the final lectin concentration ranged from 10-100 µg/ml. The incubation was carried out at 37°C for 30 minutes. After incubation, the sporozoites were recovered by centrifugation on the Microfuge (p. 35) and washed three times with PBS at room temperature before resuspension in 0.1 ml of PBS. The diluted lectins were held on ice before use.

The stained preparations were examined under phase contrast and for fluorescence using a Leitz Orthoplan incidence fluorescence-phase contrast microscope. The intensity of fluorescence was scored on a arbitary scale of 0 to ++, where 0 indicated no apparent fluorescence and ++ indicated bright to brilliant fluorescence. At least 20 cells were examined for each test carried out.

6.3. Results

6.3.1. Reactivity of FITC - Con A with sporozoites

Both unfixed and glutaraldehyde fixed sporozoites, when incubated at 37°C with FITC - Con A at concentrations of 10 and 100 µg/ml, showed no sign of fluorescence. Similarly, sporozoite preparations that had been pretreated with 0.01% trypsin prior to incubation with FITC - Con A also revealed no evidence of fluorescence. In contrast, fresh unfixed and glutaraldehyde fixed rabbit erythrocytes displayed strong uniform fluorescence of the surface membrane. Incubation of rabbit erythrocytes with 100 µg/ml of Con A in the presence of 100 mM solution of competing sugar, α-MM, effectively prevented any staining. These observations suggest that specific lectin-binding sites are absent on the cell surface of P. gallinaceum sporozoites.

6.4. Discussion

The presence of lectin-binding sites on the cell surface of many mammalian cells is well documented (see for example, Sharon and Lis, 1972). However, on the basis of the observations presented here, it appears that sporozoites of P. gallinaceum from mature oocysts and salivary glands lack residues similar to α-D-mannose and α-D-glucose as they were unable to bind detectable amounts of FITC - Con A to their

Table 6.2. Summary of attempts to demonstrate binding of FITC - Con A to P. gallinaceum sporozoites [1].

Inhibitor	Inhibitor concentration	FITC - Con A (µg/ml) 0	10	100
		Salivary gland forms		
None	0	-	-	-
α-Methyl-D-mannoside	100 mM	-	-	-
		Mature oocyst forms		
None	0	-	-	-
α-Methyl-D-mannoside	100 mM	-	-	-
		Rabbit erythrocytes		
None	0	-	+	++
α-Methyl-D-mannoside	100 mM	-	-	-

1. Identical results were obtained in all tests with living, glutaraldehyde-fixed and trypsin treated sporozoites.

surfaces. Indeed, surface fluorescence was not apparent after glutaraldehyde fixation of sporozoites, or after incubation of sporozoites with trypsin.

Several hypotheses can be put forward to explain the inability to demonstrate binding of FITC - Con A to the surface of sporozoites. While the lectin failed to react with sporozoites, it does not follow that the appropriate carbohydrate residues are absent from their surfaces. It may be that the specific lectin-binding sites are present at the cell surface, but are in such small quantities, as to fall below the sensitivity for detection by fluorescence microscopy. Alternatively, it is possible that FITC - Con A does bind to the sporozoite surface which results in rapid capping, followed by shedding of the lectin receptor cap during the handling and incubation procedure. Such a capping process is known to occur with great rapidity (a few seconds at 37°C) when the anionic sites at the surface of E. nieschulzi and Sarcocystis tenella sporozoites are labelled with fluorescein conjugated cationised ferritin (Dubremetz and Ferreira, 1978). This hypothesis is invalid in the present investigations because experiments were also performed with glutaraldehyde fixed sporozoites and capping is known to be dependant on the lateral mobility of membrane components (Unanue and Karnovsky, 1974; Zucker-Franklin, Liebes and Silber, 1979). Aldehyde fixation of some mammalian cells is reported to produce changes in membrane rigidity which effects the reactivity of such cells with lectins (Gibson, Marquardt and Gordon, 1975; Nicholson, 1974; Temmink et al., 1975). For example, Con A induced agglutination of Ehrlich carcinoma cells is unaffected after fixation (Inoue, 1974) and that of erythrocytes is enhanced (Marquardt and Gordan, 1975), although during investigations on the nature of the cell surface

saccharides on a number of parasitic protozoa, aldehyde fixation does not appear to effect lectin-binding (Dwyer, 1976; Dwyer, 1977; Dwyer and D'Alesandro, 1976).

It is possible that the lectin-binding sites are masked at the surface and not available in an exposed form since it is known that nontransformed mammalian cells do not agglutinate unless exposed to trypsin (Burger, 1969; Inbar, 1969; Inbar, Rabinowitz and Sachs, 1969). Similarly, it is known that lectin-binding sites on the cell surface of bloodstream T. equiperdum (Jackson, 1977) and bloodstream T. brucei (Cross and Johnson, 1976) are exposed after a brief incubation with trypsin. However trypsin treatment of P. gallinaceum sporozoites failed to reveal lectin-binding sites.

The higher concentration of lectin used in the present investigations (100 µg/ml) is the norm for lectin studies (Lis and Sharon, 1973) and higher concentrations of lectin may not necessarily increase agglutination or, in this case, fluorescence, as the binding sites become saturated (Nicholson, 1974). Indeed, lectin studies are best investigated at low concentrations (Reisner, Lis and Sharon, 1976) and the technical data supplied by Miles Laboratories with the Con A claims maximum fluorescence with mouse splenocytes between 15-25 µg/ml.

On the evidence of the observations reported in this chapter, it seems that residues similar to α-D-mannose and α-D-glucose, common to the surface of erythrocytes and many other cells, are absent or in very low concentrations at the surface of P. gallinaceum sporozoites. These results extend those of previous workers (Seed and Krier, 1976; David et al., 1978; David and Hommel, 1979; Bannister et. al., 1979) who have suggested that lectin-binding sites are rare or absent at the cell surface of the erythrocytic stages of the malaria parasites.

It would be interesting to compare the results obtained here with the sporozoites of other species of Plasmodium and determine whether this apparent lack of lectin-binding sites is a consistent feature of the genus because there seems to be some species differences with Leishmania. Cloned promastigotes of L. donovani are able to bind Con A, phytohaemagglutinin-P and -M, fucose binding protein, soybean agglutinin and wheat germ agglutinin (Dwyer, 1977) and although L. braziliensis promastigotes are able to agglutinate with Con A they do not bind to phytohaemagglutinin-P and with extended culture lose the ability to agglutinate with Ricinus communis agglutinin (Dawidowicz et al., 1975).

CHAPTER 7

7. INFECTIVITY OF P. GALLINACEUM SPOROZOITES

7.1. Introduction

It has been demonstrated, as described in Chapter 5, that changes take place in the cell surface charge of the sporozoite from the time that it leaves the rupturing oocyst up to the time that it is found in the salivary gland. It is probable that these changes are associated with the different invasive potentials that are required of the sporozoite. On leaving the oocyst, the sporozoite must be adapted to locate and penetrate the salivary gland of the mosquito host; subsequently, it must be adapted for survival in the vertebrate and for location and penetration of the cells of the reticulo-endothelial system.

Accordingly, observations were extended on the development of the sporozoite to a consideration of the ontogeny and nature of sporozoite infectivity. Thus, this chapter describes the investigation of the infectivity of the sporozoite, with two main aims:

(a) To discover if salivary gland sporozoites of P. gallinaceum are more infective than sporozoites from mature oocysts, as has been demonstrated to be the case with P. berghei (Vanderberg, 1975).

(b) To investigate the effect of two enzymes, trypsin and neuraminidase, on the infectivity of salivary gland sporozoites. In the free-flow electrophoretic studies that were described in Chapter 5, trypsin has been shown to produce a significant reduction in the cell surface charge of the sporozoite, while neuraminidase does not affect the net negative charge of the sporozoite.

The only evidence to suggest the importance of trypsin-sensitive components on the surface of sporozoites has come from the investigations of Cochrane et al (1976) who noted the non-infectiousness of sporozoites of P. berghei after incubation with trypsin or pronase; both enzymes were used at a concentration of 1 mg/ml and incubation was for 30 minutes at 37°C. Unfortunately, however, the consequence of these observations were not discussed.

7.2. Materials and Methods

7.2.1. Preparation and inoculation of sporozoites

The salivary glands and midguts of the infected mosquitoes were dissected and a suspension produced in M199 as previously described (p. 33). The volume of the medium was then measured and a sample of the suspension was examined in a Neubauer haemocytometer to determine the concentration of sporozoites. Tenfold dilutions were then prepared so that the required doses (100,000, 10,000, 1,000, 100 and 10 sporozoites) were obtained in volumes of 0.2 mls (Killick-Kendrick, 1973; Shute et al., 1976). The lowest dilutions were given first and the highest last and all inoculations were performed intravenously. The time taken from the beginning of the dissections until the last inoculation was just over 2 hours and throughout the procedure sporozoite suspensions were kept cool on crushed ice.

Giemsa stained thin blood films from the chicks were examined at 4 days and thereafter every day for 20 days to determine the proportion which developed a patent parasitaemia and the first day of patency in each case. Approximately 100,000 erythrocytes were examined before a chick was considered negative.

7.2.2. Enzyme treatment of sporozoites

The salivary glands of the infected mosquitoes were dissected in a small volume of M199 and their concentration estimated as described above. This suspension was then divided into 6 portions of approximately the same volume and the sporozoites in each deposited using the Microfuge. The sporozoites in the pellet were then re-suspended in 1.0 ml of the appropriate solution and incubated at 37°C for 30 minutes in a shaking water bath. The enzyme solutions were prepared in exactly the same way as for the electrophoretic mobility studies (p. 59). After incubation the sporozoites in each suspension were washed twice and resuspended in M199. The number of sporozoites in each sample was counted and dilutions of 1,000, 500, 100 and 10 sporozoites were prepared. The inoculation procedure and subsequent examination of the birds was as before, except that this procedure took slightly longer, just over 3 hours.

7.3. Results

7.3.1. Infectivity of oocyst versus salivary gland sporozoites

The results, as shown in Table 7.1., demonstrate that a dose of 10,000 oocyst sporozoites was the minimum number required to produce a patent infection in chicks. At a dose of 100,000, sporozoites from mature oocysts produced infections in only 3/5 chicks; in both cases the prepatent period was considerably extended, with means of 10.0 and 9.3 days respectively.

In contrast, the infectivity of salivary gland sporozoites was much greater. Even a dose of 10 sporozoites was sufficient to produce a patent parasitaemia in 3/5 chicks and inoculums of 100 and above

Table 7.1. Infectivity of *P. gallinaceum* sporozoites from mature oocysts and salivary glands

Source of sporozoites	No. of sporozoites inoculated	No. of chicks infected/no. inoculated	Mean prepatent period in days	Range
	10	0/5	-	-
	100	0/5	-	-
Oocyst	1,000	0/5	-	-
	10,000	1/5	10.0	-
	100,000	3/5	9.3	9-10
	10	3/5	9.0	8-10
	100	5/5	7.6	7-9
Salivary gland	1,000	5/5	7.0	6-8
	10,000	5/5	6.4	6-7
	100,000	5/5	4.6	4-6

invariably produced infections. The prepatent period was reduced from 9 days to a mean of 4.6 days as the dose increased from 10 to 100,000 sporozoites.

Although the technique used to assess sporozoite infectivity (incidence of patency and length of prepatent period) does not allow a statistical analysis to distinguish between small differences in infectivity among different doses of sporozoites, it was sufficient to demonstrate (a) that sporozoites from salivary glands are approximately 10,000 times more infective than sporozoites from mature oocysts and (b) there is a reduction in the length of the prepatent period as the number of sporozoites inoculated is increased.

7.3.2. Effect of enzymes on sporozoite infectivity

Having shown that trypsin treatment (0.01%) of P. gallinaceum sporozoites completely eliminated their infectivity to chicks (Table 7.2) the possibility remained that infection had simply been blocked by adsorption of trypsin to the sporozoite surface. Accordingly, sporozoites were incubated with heat-inactivated trypsin as a control. Such treatment did not change their degree of infectivity (Table 7.2) hence it has been clearly demonstrated that the proteolytic activity of trypsin was responsible for the total inhibition of infectivity.

Treatment of sporozoites with neuraminidase (1 unit/ml) did not have any effect on their infectivity (Table 7.2).

Control incubation at 4°C gave similar results to those obtained in the preceeding section and demonstrated that sporozoites of approximately the same age, from different batches of mosquitoes, at least for

Table 7.2. Effect of enzyme treatment on the infectivity of *P. gallinaceum* sporozoites (salivary gland forms)

Treatment	No. of sporozoites inoculated	No. of chicks infected/no. inoculated	Mean prepatent period in days	Range
None, control incubation 4°C for 30 min.	10	3/5	8·5	8-9
	100	5/5	8·0	7-9
	500	5/5	7·2	7-8
	1,000	5/5	6·8	6-7
None, control incubation 37°C for 30 min.	10	1/5	9·0	-
	100	4/5	9·0	8-10
	500	5/5	7·2	7-8
	1,000	5/5	7·0	6-8
Trypsin, 0·01% 37°C for 30 min.	10	0/5	-	-
	100	0/5	-	-
	500	0/5	-	-
	1,000	0/5	-	-
Trypsin 0·01% (heat-inactivated) 37°C for 30 min.	10	1/5	8·0	-
	100	5/5	8·0	7-9
	500	5/5	7·6	7-9
	1,000	5/5	6·8	6-8
Neuraminidase 1 unit/ml 37°C for 30 min.	10	2/5	8·0	7-9
	100	5/5	7·2	6-8
	500	5/5	6·8	6-7
	1,000	5/5	6·6	6-7
Neuraminidase 1 unit/ml (heat-inactivated) 37°C for 30 min.	10	2/5	8·5	8-9
	100	5/5	8·2	8-9
	500	5/5	7·4	7-8
	1,000	5/5	7·0	6-8

P. gallinaceum in A. aegypti, show similar levels of infectivity. Also, there appears to be no loss in infectivity after 30 minutes on ice. In contrast, incubation of sporozoites at 37°C for 30 minutes produced a slight decrease in their infectivity and the prepatent periods were also slightly longer than those for the control incubation at 4°C (Table 7.2). These results are consistent with those of Fink and Schica (1969) who studied the survival of sporozoites of P. berghei in M199. They found that all of 5 mice became infected when inoculated with 2,500 sporozoites, even when they had been kept for 3 hours at 4°C, but the prepatent periods were slightly prolonged. Also, sporozoites clearly showed a loss of infectivity at temperatures higher than 4°C.

7.4. Discussion

Information concerning the infectiousness of sporozoites that had not yet invaded the mosquito's salivary gland has recently been reviewed by Vanderberg (1975) who commented on, "a lively controversy in the malaria literature of the late 1930's and early 1940's". By that time, well over a quarter of a century had elapsed since Ross's discovery of the "germinal rods" (sporozoites) of P. relictum in the salivary gland of the grey mosquito (later, provisonally identified as Culex pipiens fatigans) and it was well established that inoculation of sporozoites from the salivary gland of an infected mosquito during the feeding process was the natural route of malaria transmission. However, the fascination with the infectiousness of oocyst sporozoites has remained since Schaudinn's (1903) observation of the penetration of human erythrocytes by sporozoites of P. vivax; for which he used sporozoites from mature oocysts and not those dissected from salivary glands.

In 1937, both Missiroli (1937a, 1937b) and Neri (1937) recorded infections in canaries that had been inoculated with emulsions of crushed oocysts containing sporozoites of P. relictum. Working with same parasite, Sandicchi (1937; 1938), Jerace (1937) and Brooke (1942) were unable to produce infections in their avian hosts by the inoculation of oocyst sporozoites and unsuccessful attempts to infect birds with P. cathemerium were performed by Gambrell (1937) and Brooke (1942), which prompted Sandicchi (1938) to question the validity of Missiroli and Neri's results, suggesting that their inocula were contaminated with sporozoites from salivary glands that had ruptured during dissection. Shute (1943) however, in a series of experiments with the human malaria parasites P. vivax, P. ovale and P. falciparum succeeded in obtaining infections in 5/12 patients that had been inoculated with crushed oocysts from A. malculipennis but suggested that the failure of some inoculations to produce an infection was due to faulty technique and not that the sporozoites were immature. Shute also noted that infections from oocyst sporozoites were followed by the normal incubation period, the production of viable gametocytes, and where applicable, the occurrence of relapses. A little later, Trembley, Greenberg and Coatney (1951) were able to obtain infections in 4/12 chicks that had been inoculated with single intact oocysts of P. gallinaceum.

By this time most workers were in agreement that sporozoites from mature oocysts were able to produce patent infections in the appropriate vertebrate host, but many justifiably considered that contamination with even a few salivary gland sporozoites could greatly affect the results. This could so easily have been the case in the work of Yoeli and Boné (1967) when they were isolating strains of rodent malaria parasites from naturally infected A. dureni millecampsi. They found that 14/14

rats given single pairs of infected salivary glands became infected with P. b. berghei; but infections arose in only 3/14 rats inoculated with infected midguts. However, in 1960, Yoeli and Most had obtained infections of P. berghei in rats under conditions in which no salivary gland infections were observed. This suggested that oocyst sporozoites have some degree of infectivity and this was conclusively demonstrated to be the case by Ball and Chao (1961) who showed that sporozoites from oocysts of P. relictum, formed in vitro in the absence of salivary gland, were infective to canaries. Similar observations were recorded by Schneider (1968) who succeeded in infecting chicks with P. gallinaceum sporozoites, from oocysts grown in vitro and later by Walliker and Robertson (1970) with P. berghei grown under similar conditions.

These later reports confirm unequivocally the inherent infectivity of oocyst sporozoites, but the question of the degree of infectivity remains. The only quantitative work has come from Vanderberg (1975) who has reported a 10,000 fold increase in the infectivity of P. berghei sporozoites as they progress from oocyst to salivary gland. My own observations have illustrated that a similar 10,000 fold increase in infectivity occurs with P. gallinaceum.

The significance of the number of infective sporozoites of P. gallinaceum has not been investigated in a satisfactory manner. The early work of Coatney, Cooper and Trembley (1945a; 1945b) reported on the incidence and course of infection in young chicks following the subcutaneous or intramuscular injection of pooled sporozoites and intact salivary glands and although this method is adequate for

inducing fairly reproducable infections for the use of mass screening of antimalarial drugs it tells us little about the infectivity of sporozoites. More recently Porter, Laird and Dusseau (1954) attempted to assess the relationship between the numbers of sporozoites inoculated and the intensity of the resulting infections and tried accurately to determine the number of sporozoites in a mixture of homogenized salivary gland and chick blood by recording the ratio of erythrocytes to sporozoites in dried Giemsa stained films. The inherent errors in Porter's techniques and the difficulty he must have experienced in estimating accurately doses of sporozoites, probably explains the small differences between his results and those presented here. In essence, Porter and his colleagues always produced infections after the intravenous inoculation of 500 sporozoites, in 3/6 chicks after inoculation of 50 sporozoites, and never produced infections after inoculation with 5 sporozoites. However, the present experimental work always produced infections with 100 or more sporozoites and in these two experiments, infections were produced in 6/10 chicks inoculated with 10 sporozoites.

It is interesting to compare the infectivity of P. gallinaceum sporozoites with other species and in particular the very low infectivity rates exhibited by sporozoites of the rodent malaria parasites (see Wéry, 1968). This has been suggested by Sinden and Garnham (1973) to be correlated to the absence or extreme rarity of the micropore and is discussed in more detail in Chapter 4. Recently, Sinden (pers. comm.) has found it necessary to inoculate mice with at least 1,000 sporozoites from dissected salivary glands to produce infections with P. berghei and P. yoelii nigeriensis and although

Pacheco et al. (1979) have obtained similar results with sporozoites procured from whole mosquitoes and recovered by density gradient centrifugation, the earlier work of Killick-Kendrick (1973) indicated a much higher degree of infectivity by obtaining infections of P. berghei nigeriensis (now, P. y. nigeriensis, Killick-Kendrick, 1974) in 6/6 mice given only 80 sporozoites by intravenous inoculation. This apparent difference in recorded infectivity of P. y. nigeriensis sporozoites has probably been caused by a combination of two factors; continued passage in laboratory hosts and the choice of an unnatural route for inoculation. It is known that continued passage in laboratory hosts causes changes in the virulence of erythrocytic forms and in the viability of gametocytes (Wery, 1968; Killick-Kendrick, 1971) and it would not be unnatural to assume that similar changes occur in the infectivity of sporozoites. Also Wery and Killick-Kendrick (1967) and Wery (1968) have reported on the greater number of tissue schizonts that develop following intravenous, rather than intraperitoneal,inoculation of sporozoites. Both Sinden (1979) and Pacheco et al. (1979) used the intraperitoneal route.

In conclusion, it seems likely that this change in infectivity is related to other aspects of sporozoite maturation and would support the suggestion that the reduction in cell surface charge (as discussed in Chapter 5) is caused by a reorganisation of the surface membrane in preparation for survival in the vertebrate and successful penetration of the cells of the reticulo-endothelial system. Therefore, in order to characterize this development further it was decided to investigate the action of the two enzymes, trypsin and neuraminidase, used in the electrophoretic studies, on the infectivity of salivary gland forms.

Nothing is known of the attachment and penetration stages in the primary exoerythrocytic phase of the malaria infection. However, the interaction between malarial sporozoites and their host cells of the reticulo-endothelial system is highly specific in that malaria parasites have a restricted host range. This specificity suggests that receptors on the red cell surface are involved in the attachment and invasion by merozoites and this is thought to be determined, at least in part, by the surface of the erythrocyte and it would seem that an erythrocyte membrane receptor triggers the invasion process (McGhee, 1953; Butcher, Mitchell and Cohen, 1973; Miller et al., 1973). It must be seriously considered whether a similar system could be operating for the sporozoite-macrophage interaction and further contribute to this host specificity. Brumpt (1949) was the first to test the behaviour of P. gallinaceum in various birds and found that geese, pheasants, partridges and peacocks were susceptible, whereas canaries and passerine birds were totally resistant. The nature of this host specificity was further examined (Huff and Coulston, 1944; Huff, 1951, 1957) and it was shown to be a complex process, affecting one stage in a certain species of bird and another in a different bird (Table 7.3). Although some species of birds are entirely insusceptible to infection with P. gallinaceum, sporozoites can survive in their blood for quite a long time. Pigeons for example, although totally resistant to infection, can support sporozoites in their blood stream for up to 4 minutes (Singh et al., 1954) and canaries for half an hour (Raffaele, 1955). This prompted Garnham (1966a) to suggest that, "the immune barrier is not so much in the blood, as in the non-receptive lymphoid-macrophage cells". Perhaps these cells lack the specific receptor necessary for sporozoite invasion.

Table 7.3. Susceptibility of abnormal hosts to P. gallinaceum *

Avian host	Cryptozoite	Erythrocytic stage	Phanerozoite
Canary	Low	Nil	Nil
Duck	Abundant	Slight	Rare
Guinea-fowl	Nil	Slight	Nil
Goose	Abundant	Slight	?
Turkey	Present	Light	Present
Pheasant	Present	Moderate	Rare
Partridge	Present	Slight	Absent

* from Garnham (1966a).

The existence of cellular receptors for bacteria (Clawson , Roa and White, 1975; Gibbons and Van Houte, 1975; Ofek et al., 1975) and viruses (Springer, 1970; Howe and Lee, 1972) are well established, although it has not been until comparatively recently that some attention has been given to their possible occurrence on the cell surface of a number of parasitic protozoa. Banks (1979) has suggested that the cell surface of bloodstream T. congolense contains a protein-associated site which is responsible for binding to sialic acid of some host cells; Chang (1977) has observed that the attachment of L. donovani promastigotes to macrophages appears to follow saturation kinetics, suggesting that there may be a ligand-receptor binding effect; and in the same year Miller, McAuliffe and Mason (1977) reviewed the available evidence for the presence of receptors on the cell surface of erythrocytes for a variety of primate malarias.

McGhee (1953) was the first to propose that invasion of erythrocytes by malaria merozoites was initiated by their interaction with specific receptors on the erythrocyte surface. This assumption was inspired by the observation on the preference of P. lophurae merozoites to invade duck erythrocytes when exposed to both duck and chick erythrocytes in the chick embryo. Later, Sherman (1966) concluded that malaria parasites lack receptor sites similar to those established for bacteria and viruses after failing to inhibit the invasion of duck erythrocytes by merozoites of P. lophurae after trypsin, chymotrypsin and neuraminidase digestion of the erythrocyte surface. Ladda, Aikawa and Sprinz (1969) also suggested that there were no specific receptor sites for the penetration of erythrocytes by avian and mammalian merozoites. However, recent work, by particularly Miller and his associates in America, on human and primate malarias,

have indicated the presence of specific determinants on the erythrocyte surface which interact with receptors on the merozoites and which are necessary for successful invasion (Butcher, Mitchell and Cohen, 1973; Miller et al., 1973; Miller et al., 1975; Dvorak et al., 1975; Miller et al., 1976; Mason et al., 1977; Miller, 1977).

In this section, data is presented which illustrates that trypsin, by its proteolytic activity, blocks the infectivity of P. gallinaceum sporozoites. First, the concentration of trypsin which eliminated sporozoite infectivity (0.01%) corresponded to that concentration which altered their surface membrane proteins as shown by free-flow electrophoretic measurements (p. 66). Second, heat-inactivated trypsin had no effect on sporozoite infectivity so any non-specific inhibition of infectivity by adsorption of the enzyme on the sporozoite surface can be ruled out. Finally, trypsin treated sporozoites still retained their motility, a property Vanderberg (1974) has correlated with sporozoite infectivity. However, in this instance trypsin treated sporozoites were not infective. Therefore it is likely that the proteolytic activity of the trypsin removed a presumed receptor for cells of the reticulo-endothelial system from the surface of the sporozoite and that motility per se is not directly responsible for infectivity.

CHAPTER 8

8. EXOERYTHROCYTIC SCHIZOGONY IN VITRO

8.1. Introduction

In the previous chapters, some aspects of the developmental biology of the P. gallinaceum sporozoite have been examined and particular interest has been shown in the development and nature of sporozoite infectivity. Accordingly, observations were extended on the development of P. gallinaceum in vitro and attempts were made to establish cultures of cryptozoites (primary exoerythrocytic schizonts), by the inoculation of sporozoites into tissue cultures.

Routine procedures for growing the continuing exoerythrocytic stages (phanerozoites) of avian malaria parasites in continuous culture are well established and have been available for many years (Hawking, 1945; Huff, 1963; 1968). Cultures have been initiated from embryos infected in ovum (Pipkin and Jensen, 1956) or from the introduction of exoerythrocytic merozoites into established cell cultures (Davis, Huff and Palmer, 1966).

There have been many reports in the literature describing unsuccessful attempts to grow cryptozoites from sporozoites in tissue culture (Rhodhain, Gavrilov and Cowez, 1940; Paraense, Meyer and Menezes, 1942, Huff and Coulston, 1944; Hawking, 1945; Porter, 1948). Appreciating the importance of a successful procedure for the growth of cryptozoites, further attempts were made by Dublin and his colleagues in America, who reported on the successful development of sporozoites of P. gallinaceum into cryptozoites in cultures of normal chicken macrophages (Dublin, Laird and Drinnon, 1949; 1950; Laird, Dublin and Drinnon, 1950.

Success with P. gallinaceum was also achieved by Jensen, Huff and Shiroishi (1964) and these four publications, have, until recently, represented the only published data on the successful development of the primary exoerythrocytic stages of malaria parasites from sporozoites in vitro. However, during the course of this study, Strome, De Santis and Beaudoin (1979) and Sinden and Smith (1980) have reported the development and partial maturation of the exoerythrocytic schizonts of P. berghei in cultures of embryonic rat brain.

Considering the paucity of knowledge concerning the early development of the malarial sporozoite in the vertebrate host and the potential of an in vitro system for its investigation, it was decided to try to delineate the conditions necessary for the invasion and development of sporozoites, in vitro. The results of a number of investigations are reported in this chapter.

8.2. Materials and Methods

8.2.1. Sterilisation procedure

In general, the aseptic techniques described by Paul (1966) were used throughout. Glassware and instruments were cleaned in 2% Decon 90 (Decon Laboratories Ltd.), washed thoroughly in running tap water, then in 3 changes of distilled water and autoclaved by moist heat at 15 lbs/inch^2 for 20 minutes. Coverslips were washed twice in absolute alcohol, air dried, then sterilised by dry heat at 160°C for 2 hours. A laminar flow cabinet was used for all tissue culture work.

8.2.2. Preparation of tissue cultures: general methods

Cultures of chick brain and liver cells were set up from 12-13 day old embryos; macrophage cultures were established from the spleens

of 17-19 day old embryos and cultures of chick kidney cells were obtained from 1-3 week old chicks. The general method for the preparation of cell cultures was,however, the same for all cell types and is given below.

The appropriate tissue was removed and cut into cubes having sides of approximately 1-2 mm. The tissue was then added to an Erlenmeyer flask containing 10 mls of 0.25% trypsin and 0.02% EDTA (ethylene diamine tetra-acetic acid, disodium salt) in PBS without calcium and magnesium and incubated at 37°C for 7 minutes. The suspension was drawn up a pasteur pipette a few times and the tissue allowed to settle. The supernatant was removed and centrifuged at 250g for 10 minutes. The supernatant of this spin was then removed and the pellet containing the cells of the first trypsinisation, re-suspended in 5 mls of medium. This procedure was repeated for the tissue remaining after the first trypsinisation. For most experiments, cells were dispensed onto 16 mm circular glass coverslips in the wells of multiwell petri dishes (Sterilin Ltd.) at concentrations of between 2-4 x 10^5 cells/well. Each well contained 2 mls of medium. Tissue cultures that were established for centrifugation experiments were grown in Falcon flasks prior to their detachment and use in 5 ml flat bottomed plastic tubes.

Cultures were incubated at either 37°C or 41°C and the medium was replaced after 24 hours and thereafter every 48 hours. Sporozoites were inoculated 3-4 days after the establishment of macrophage cultures and from 3-15 days after the establishment of the kidney or brain cultures.

The medium used in most experiments was tissue culture medium 199 (M199) with Hank's Salts, 10% foetal calf serum, 2% l-glutamine with penicillin (100 units/ml) and streptomycin (100 µg/ml). The medium, which contained 0.35g of sodium bicarbonate/litre, was gassed with a mixture of 5% CO_2 and 95% air to pH 7.2. All tissue culture products were obtained from Gibco-Biocult Ltd., Paisley, Scotland.

8.2.3. Preparation of tissue cultures: other methods

Chick spleen macrophages

Some of the macrophage cultures used in the initial experiments were set up as follows. The spleens of normal 17-19 day old embryos were removed, transferred to a petri dish and cut into cubes having sides of approximately 1 mm. Six to eight of these pieces were then placed in each well of a multiwell petri dish, to which a 16 mm diameter glass coverslip and 2 mls of medium had previously been added. After 24 hours the tissue pieces were dislodged and the medium changed. Subsequent medium changes were made at 48 hour intervals.

Mouse peritoneal macrophages

A mouse was inoculated intraperitoneally with 2 mls of a 2% starch solution in M199 and after 3 days was sacrificed. The peritoneal cavity was washed out with 2 mls of M199 and the suspension spun at 250g for 5 minutes. The supernatant was discarded and the pellet resuspended in M199. The cells were then washed twice in medium before being plated out at approximately 1 x 10^6 cells/16 mm coverslip. The medium was changed after 12 hours and thereafter every 48 hours. Sporozoites were inoculated 1-3 days later.

8.2.4. Sporozoite inoculation

A. aegypti were immersed momentarily (~3 seconds) in 70% ethanol, dried on sterile filter paper and washed twice in M199 containing the standard quantities of antibiotics (penicillin 100 units/ml and streptomycin 100 µg/ml). Each mosquito was dissected in a drop of medium and the salivary glands from all the mosquitoes were pooled and stored on ice. Glands were gently teased apart, the sporozoite density calculated and known numbers of sporozoites applied to tissue monolayers. Normally, 50,000-100,000 sporozoites were added to each well. Parallel control cultures were set up with salivary glands from non-infected mosquitoes. After incubation, the coverslips were removed, fixed in methanol and stained in 10% Giemsa as described previously (p. 42) and the preparations examined for erythrocytic parasites.

During the course of this study, it was considered necessary to check that the sterile dissection of mosquitoes was not adversely effecting the infectivity of sporozoites. Accordingly, experiments were conducted to examine this possibility. Mosquitoes were dissected by the sterile technique described above and a suspension of sporozoites produced in M199. Doses of 1,000 and 10,000 sporozoites were then prepared, inoculated intravenously into each of 5 chicks and the incidence of patency and the length of the prepatent period recorded using methods as described previously (p. 91). Control infections were set up from mosquitoes that had not been subjected to alcohol sterilisation.

8.2.5. Centrifugation procedure

The method was essentially that of Strome, De Santis and Beaudoin (1979). When the brain, spleen or kidney cells in the Falcon flasks became confluent, the medium was poured off and the cells rinsed briefly with the trypsinising solution. A further 5 mls of trypsinising solution was added and the cultures were incubated for 7 minutes at 37°C. The supernatant was removed and centrifuged at 250g for 10 minutes to sediment the detached cells and the supernatant was discarded. The pellet was resuspended in medium and 1.0 ml of this suspension, containing approximately 25,000 cells, was inoculated into each of 6 small plastic centrifuge tubes, to which a 13 mm circular glass coverslip had previously been added. Cultures were incubated for 24 hours prior to sporozoite inoculation. These preparations were centifuged with sporozoite suspensions at 1,600g for 30 minutes and then incubated at 37°C.

8.2.6. Production of phanerozoites in vivo and in vitro

During the course of the tissue culture investigations P. gallinaceum was propagated in chick embryos by the weekly passage of brain tissue from infected embryos onto the chorioallantoic membrane of normal 7 day old embryos, by methods similar to those used by Huff et al. (1960) and many earlier workers (see review by Pipkin and Jensen, 1956).

The embryonic culture of P. gallinaceum was initiated by transferring portions of the brain of a chick that had been inoculated intravenously 15 days previously with erythrocytic parasites and whose brain,

after examination of a Giemsa stained smear, exhibited numerous exoerythrocytic parasites, onto the chorioallantoic membrane of chick embryos.

Normal, 7 day old chick eggs were candled, the position of the air sac in each marked, a small hole cut in the shell and the shell membrane removed. Several pieces of infected brain were placed upon the exposed membrane in the area having the greatest concentration of blood vessels. The hole in the shell was sealed with sellotape. After 7 days incubation at 37°C the embryos were dissected. The brain and spleens were removed, stained with Giemsa, and examined for exoerythrocytic parasites. The most heavily infected brains were used to propagate the strain. The brain from a 14-15 day old embryo was cut into 15-20 pieces and provided sufficient inoculum for about 8 embryos. The most heavily infected spleens were used to prepare monolayer tissue cultures, although brains were also used, using the method described on p. 106.

Tissue cultures of infected spleens (and brains) were maintained at 37°C and the medium, M199 with Hanks Salts supplemented with 10% foetal calf serum, 2% l-glutamine and penicillin/streptomycin solution to 100 units/ml and 100 µg/ml, respectively, was changed every 48 hours. After 4-7 days culture, the cells were dislodged by light trypsinisation and used to infect other cultures of brain and spleen cells that had been set up 2-4 days prior to inoculation from uninfected embryos. On most occasions the infectivity of the parasites in the cultures were examined by inoculating chicks with a suspension of cells that were used to infect clean cultures and observing the subsequent infections.

8.3. Results

8.3.1. Phanerozoites in vivo and in vitro

Following preliminary experiments which failed to establish the growth of cryptozoites in vitro, control cultures of phanerozoites were set up to establish the conditions which would support the growth of exoerythrocytic forms of P. gallinaceum (see Fig. 8.1).

The brains and spleens of chicken embryos, infected in ovum 7 days previously, showed, on examination of Giemsa stained tissue smears, developing phanerozoites (Fig. 8.2). Development of phanerozoites was also demonstrable in tissue cultures set up from infected embryonic material and maintained for 7 days. Uninfected cultures of brain and spleen cells were successfully infected following the inoculation of parasitised cells from existing cultures. These cultures were observed for 5-10 days and phanerozoites, were observed (Figs. 8.3 and 8.4). These phanerozoites retained their infectivity when inoculated into young chicks.

8.3.2. Infectivity of sporozoites prepared by sterile procedure

Following the sterile dissection of mosquitoes, sporozoites showed no loss of infectivity to chicks (Table 8.1). The results are consistent with those obtained in the previous chapter.

8.3.3. Development of cells in vitro

As is usually the case in tissue culture systems where a gas overlays the free surface of the liquid medium, most of the cells in culture became somewhat undifferentiated, although several types, namely,

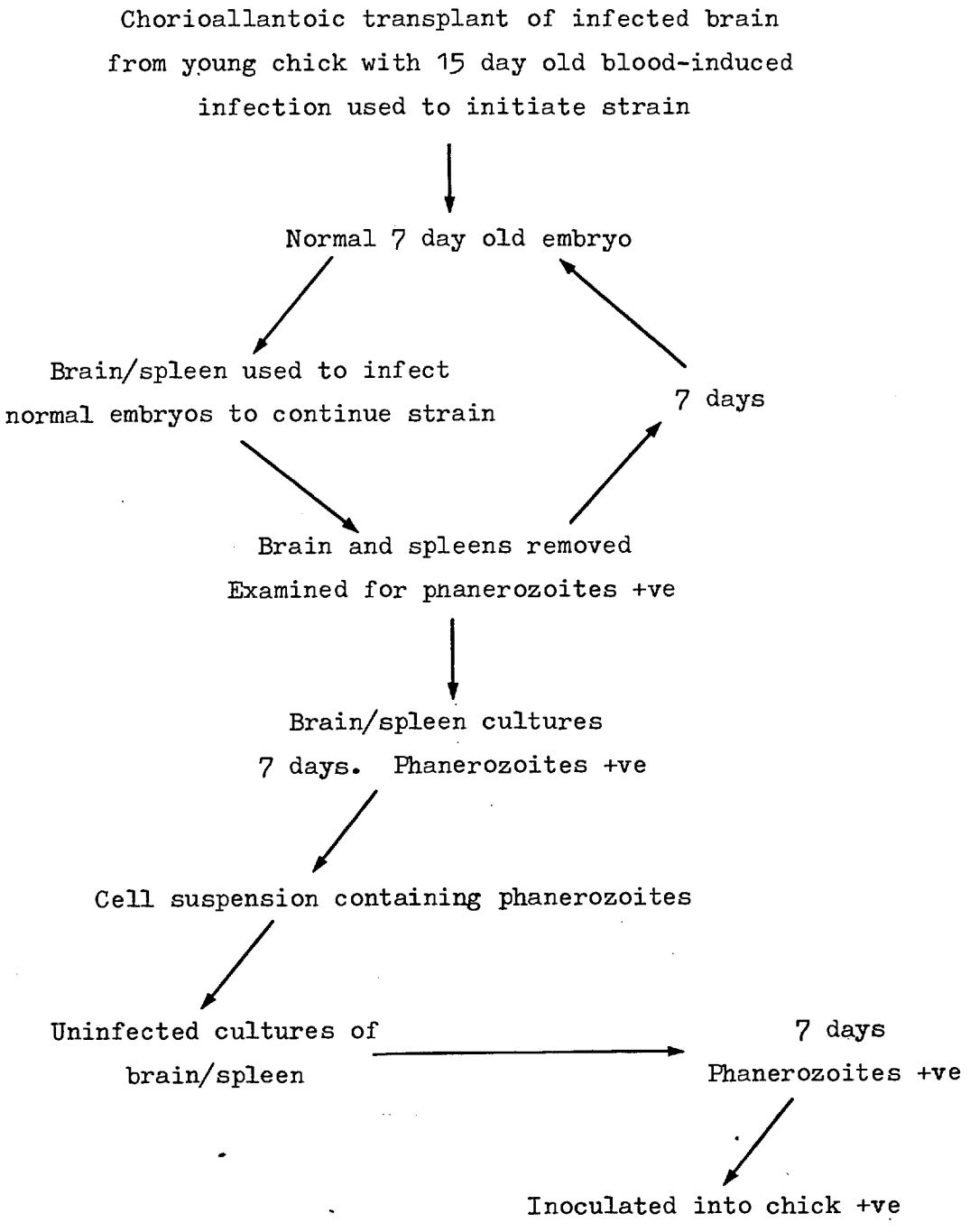


Fig. 8.1. Flow diagram illustrating the experimental procedure for the production of phanerozoites *in vivo* and *in vitro*.

Figs. 8.2 to 8.9. Aspects of the exoerythrocytic growth of P. gallinaceum in tissue culture; Giemsa stained preparations.

Fig. 8.2. Exoerythrocytic schizonts (phanerozoites) of P. gallinaceum in the brain of a 14 day old chick embryo; 7 days after in ovum infection.

Fig. 8.3. Mature exoerythrocytic schizont (phanerozoite) of P. gallinaceum in a culture of embryonic chick brain cells.

Fig. 8.4. Nearly mature exoerythrocytic schizont (phanerozoite) of P. gallinaceum in a culture of embryonic chick spleen cells.

Fig. 8.5. Appearance of embryonic chick spleen cells after 3 days in culture.

Fig. 8.6. Appearance of embryonic chick brain cells after 10 days in culture.

Fig. 8.7. Appearance of adult chick kidney cells, established from 1 week old chicks, after 5 days in culture.

Fig. 8.8. Appearance of mouse peritoneal macrophages after 24 hours in culture.

Fig. 8.9. Appearance of embryonic chick liver cells after 4 days in culture.

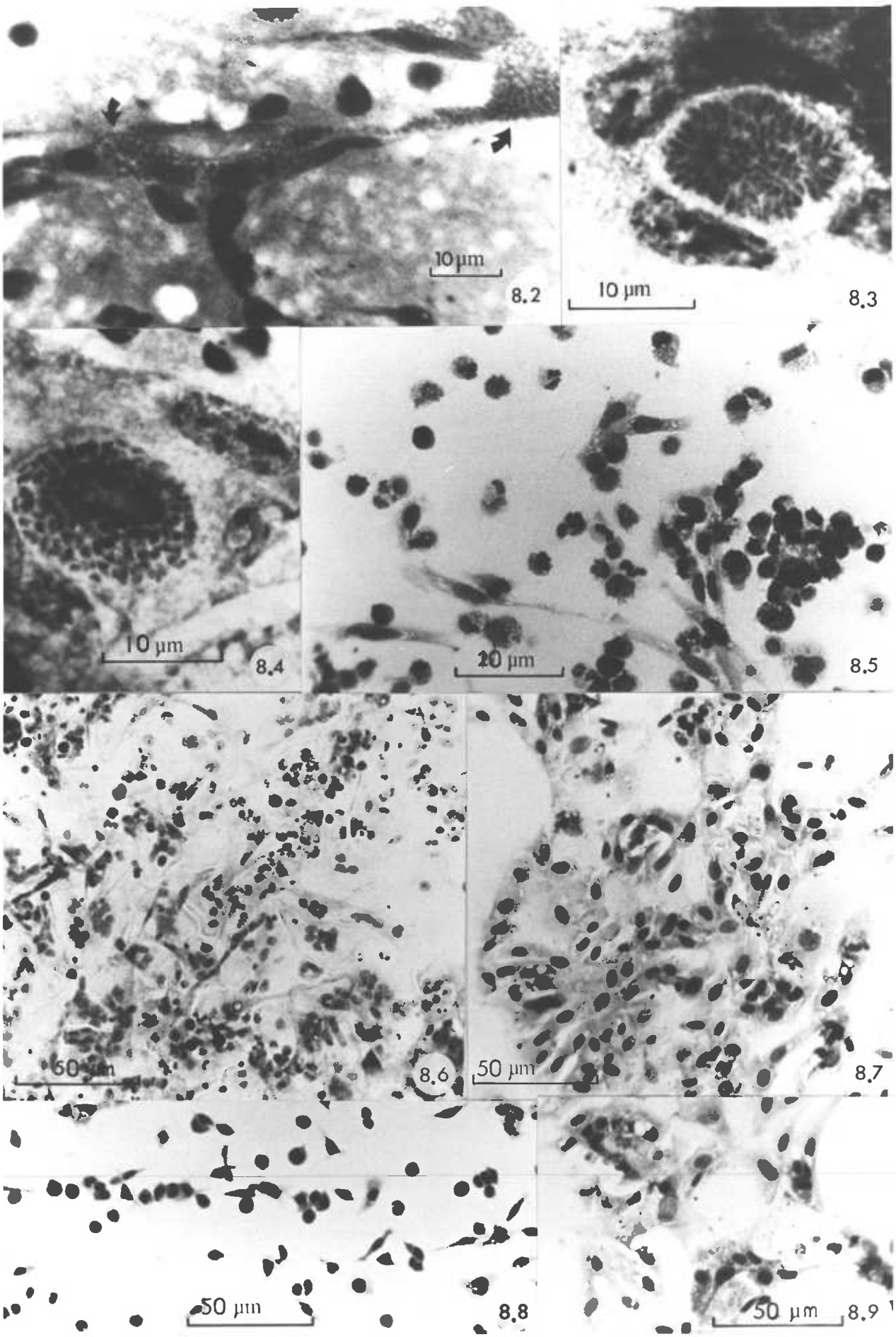

10 µm
8.2
10 µm
8.3
10 µm
8.4
20 µm
8.5
50 µm
8.6
50 µm
8.7
50 µm
8.8
50 µm
8.9

Table 8.1. Infectivity of P. gallinaceum sporozoites from salivary glands following sterile dissection of mosquitoes

Preparation of sporozoites	No. of sporozoites inoculated	No. of chicks infected/no. inoculated	Mean prepatent period in days	Range
Following sterile dissection of mosquitoes	1,000	5/5	7·0	6-8
	10,000	5/5	6·8	6-7
Control	1,000	5/5	7·4	6-9
	10,000	5/5	7·0	6-8

macrophage, fibroblast-like cells and epithelial-like cells, could be distinguished.

Excellent cultures of macrophages were obtained from spleen explants or trypsinised tissue. Macrophages became firmly attached to the coverslip as a single cell layer. There was also good growth of fibroblast-like cells which were narrow and spindle shaped with an elongate nucleus. Fibroblast-like cells became more prominent with extended culture (>5 days). Macrophages assumed a vacuolated appearance to their cytoplasm after about 48 hours in culture, although fibroblast-like cells did not show such obvious vacuolation. A few, small, epithelial-like cells were also observed (Fig. 8.5).

Cells of a similar morphology to those observed in spleen cultures were seen in cultures of chick brain cells (Fig. 8.6) their relative proportions were different. Macrophages were less abundant and the growth of fibroblast-like cells and epithelial-like cells more vigorous.

Chick kidney cells grew to form a monolayer of epithelial-like cells (Fig. 8.7). Fibroblast-like cells were also conspicuous, although macrophages were rare.

In contrast to the few macrophages seen in cultures of chick kidney cells, cultures of nearly pure macrophages were obtained from the peritoneal cavity of mice. Mouse peritoneal macrophages became firmly attached to the glass coverslip to form a single cell layer (Fig. 8.8).

Growth of chick liver was comparatively slow, but macrophages, fibroblast-like cells and epithelial-like cells, could all be distinguished (Fig. 8.9).

8.3.4. Attempts at establishing cryptozoites

Following the successful establishment, maintenance and growth of phanerozoites in tissue culture, attempts were made, using similar culture conditions, to establish cryptozoites from sporozoites *in vitro*.

Despite the success obtained with the culture of the phanerozoites, numerous attempts to establish cryptozoites using a number of cell types and different combinations of media, serum and other supplements, continually failed (Tables 8.2 and 8.3). However, during the duration of this work, Strome, De Santis and Beaudoin (1979) reported the development of exoerythrocytic schizonts of *P. berghei* in tissue culture, by using a centrifugation technique to bring sporozoites in close contact with cell monolayers. In this study, centrifugation was therefore also applied to the cultivation of *P. gallinaceum* cryptozoites, although with very limited success. Suspensions of sporozoites were centrifuged onto a variety of cell types (chick embryonic spleen; chick embryonic liver; chick kidney; rat embryonic brain) and for each cell type, two different media were used. The first, M199 with the standard additives that had been used to maintain phanerozoites (p.111), the other, a very complex medium (Appendix 4) that has been used to culture the phanerozoites of *P. fallax* and *P. lophurae* (Davis, Huff and Palmer, 1966; Beaudoin, Strome and Clutter, 1969). The latter medium was also used by Strome, De Santis and Beaudoin (1979) for the exoerythrocytic growth of *P. berghei*. For each cell type and media combination three experiments were performed and 12 coverslips examined for each. After many unsuccessful experiments, one cryptozoite was seen in a culture of embryonic chick brain, 30 hours after sporo-

Table 8.2. Different cell systems used in attempts to cultivate *P. gallinaceum* cryptozoites *in vitro**

Cell type	No. of experiments	No. of coverslips examined	No. of positive cultures
Chick Embryonic Spleen	35	117	0
Chick Embryonic Liver	3	12	0
Chick Embryonic Brain	3	12	1
Chick Kidney	3	12	0
Rat Embryonic Brain	3	12	0
Mouse Peritoneal Macrophages	2	10	0

* Medium used was, M199 with Hank's Salts, 10% foetal calf serum, 2% l-glutamine with penicillin (100 units/ml) and streptomycin (100 µg/ml).

Table 8.3. Different media systems used in attempts to cultivate P. gallinaceum cryptozoites in cultures of embryonic chick spleen macrophages*

Medium	No. of experiments	No. of coverslips examined
M199 + 10% CS + P/S	14	33
M199 + 20% CS + P/S	3	12
M199 + 10% CS (heat-inactivated) + P/S	3	12
M199 + 10% FCS + P/S	3	12
M199 + 10% CS	3	12
HBSS + 10% CS + P/S	3	12
BME + 10% CS + P/S	3	12
Tyrode's + 20% CS + P/S	3	12

* All experiments were unsuccessful.
M199 - Medium 199 (with Hank's Salts and 2% l-glutamine).
CS - Chick serum.
PS - Penicillin (100 units/ml) and streptomycin (100 µg/ml).
FCS - Foetal calf serum.
HBSS - Hank's Balanced Salt Solution.
BME - Basal Medium Eagle's (with Hank's Salts).

zoite inoculation. The growth had been achieved by using the centrifugation procedure, but without the complex medium of the American workers. The parasite had 10 distinct nuclei, was in an apparently healthy condition and was situated in a fibroblast-like cell (Fig. 8.10). It was prolate spheroidal in shape and measured 9.6 by 7.2 μm. Despite extensive microscopical examination of subsequent preparations, this remained the only cryptozoite observed.

8.4. Discussion

The early attempts to grow the exoerythrocytic forms of avian malarial parasites began at about the time of their discovery and have progressed steadily during the last four decades. Much of the early work has been reviewed by Porter (1948) and Huff (1951); later reviews were published by Pipkin and Jensen (1956) and Huff (1964). The phanerozoites of a number of avian species have now been successfully grown *in vitro*. Phanerozoites have been shown to be capable of developing in a variety of different tissues and culture media and maintain their infectivity with extended cultivation (Huff, 1964). Using a method involving the alternate subculturing of parasites in roller tube and hanging drop cultures, Musacchio and Meyer (1955) have maintained *P. gallinaceum* in continuous culture for several years with little loss of virulence and infectivity. Thus, the procedures reported in this chapter for the maintenance and growth of *P. gallinaceum* phanerozoites are not surprising, although success in cultivating the exoerythrocytic forms of *P. gallinaceum* is considerably more difficult than with the two other species of avian parasites that are commonly used, *P. fallax* and *P. lophurae* (Beaudoin, 1977). Therefore, the routine tissue culture methods which have been used for

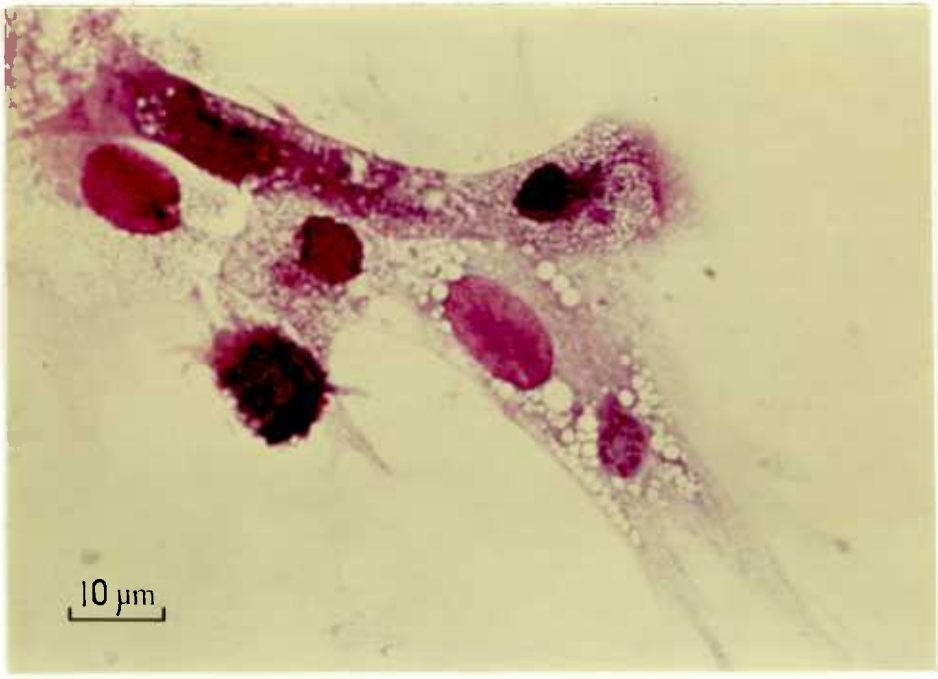


Fig. 8.10. Photomicrograph of exoerythrocytic schizont (cryptozoite) of P. gallinaceum cultured for 30 hours in vitro in embryonic chick brain cell.

the maintenance of phanerozoites, are capable of supporting plasmodial growth, and permit the invasion of uninfected cells by exoerythrocytic merozoites.

Although methods for the cultivation of the phanerozoites of the avian malarial parasites are well established, the culture of cryptozoites has always presented problems. Hawking (1945) failed in attempts to grow cryptozoites in spleen cultures by inoculating sporozoites. However, he was able to demonstrate the growth of cryptozoites in tissue cultures of spleen cells by inoculating chicks with large numbers of sporozoites and then growing explants from their spleen, one hour later. It seems then, that a simple, rather crude tissue culture medium (tyrodes, chick serum and embryo extract) was capable of supporting development of cryptozoites, and supports the results obtained in the present study, in suggesting that the entry of the sporozoite into cultured cells, not their development thereafter, remains the difficult problem in the culture of cryptozoites.

Numerous early workers also failed in their attempts to transform sporozoites into cryptozoites in tissue culture (Rhodhain, Gavrilov and Cowez, 1940; Paraense, Meyer and Menezes, 1942; Huff and Coulston, 1944; Porter, 1948). The first success was reported by Dublin, Laird and Drinnon (1949) who succeeded in growing cryptozoites from sporozoites inoculated into cultures of spleen macrophages, set up 3 to 4 days earlier from 17-19 day old chick embryos. Dublin and his colleagues reported the presence of parasites in about 0.2% of cells after 48 hours in culture. Subsequent development of metacryptozoites occurred (Dublin, Laird and Drinnon, 1950) and although their numbers were reduced to something like one-tenth of observed cryptozoites,

they retained the ability to infect chicks (Laird, Dublin and Drinnon, 1950). Some years later, Jensen, Huff and Shiroishi (1964) grew cryptozoites in cultures of spleen macrophages in M199 with 20% foetal calf serum by using Rose multipurpose chambers and cellophane dialysis membranes. Since then, there have been no reports of the development of cryptozoites of the avian malarial parasites in tissue culture. Such a dearth of reports is the more surprising, since the avian system must surely be considered a promising approach for the study of the basic biology of this phase of the malaria infection and should help in the development of new chemotherapeutic and immunological agents for its destruction.

As outlined above, success in the development and maturation of cryptozoites of avian malaria has only been rarely achieved and attempts to extend these techniques to mammalian exoerythrocytic forms have not, as yet, been successful, although recent work has given some encouragement. In some earlier studies, Dublin (1947) made cultures of bone marrow from humans infected with sporozoites of P. vivax and P. faciparum and reported "bodies suggesting erythrocytic forms", although the first published attempt at infecting cells with sporozoites in vitro was not made until 1976. Then, Doby and Barker (1976) inoculated sporozoites of P. vivax into cultures of embryonic hepatic cells and reported intracytoplasmic forms. They were, however, cautious not to state with any certainty whether these inclusions represented exoerythrocytic forms. Indeed, my own observations and those of Sinden and Smith (1980) have demonstrated that artefacts and cellular inclusions are very common in cells after the inoculation of sporozoite suspensions.

Foley, Kennard and Vanderberg (1978a; 1978b) have used

suspensions of rat liver cells, containing exoerythrocytic forms of P. berghei, infected in vivo, to initiate primary monolayer cultures of rat hepatic cells. These cultures remain infective after 18-48 hours in vitro, but their ability to continue maturation cannot be assured. They may have been simply maintained as there was a progressive loss of infectivity with time in culture. More recently, Strome, De Santis and Beaudoin (1979) have reported the growth and partial maturation of exoerythrocytic schizonts of P. berghei in cultures of embryonic rat brain. These preliminary observations have been extended by Sinden and Smith (1980) who, by using the same technique, studied quantitively the invasion and subsequent development of sporozoites in vitro. As yet, complete maturation of schizonts has to be reported; the rate of development reported in vitro is about a half that observed in vivo; very few parasites are seen and their numbers decline with time. This technique is reported to be dependant upon centrifugation so it is likely that the entry process is totally artificial.

In examining the conclusions that may be drawn from the experiments described in this chapter concerning the conditions necessary for invasion and growth of P. gallinaceum sporozoites in cultured cells, the three principle components of the tissue culture systems - the sporozoite, the host cell, and the medium - are considered separately.

It is possible that the sporozoites were rendered noninfective after the mosquitoes had been subjected to the sterile dissection procedure. However, the infectivity to chicks of sporozoites prepared in this way was not affected (Table 8.1) and therefore the recurring

inability of sporozoites to transform into cyptozoites in tissue cultures could not have been due to their noninfectiousness.

It is also possible that the cell preparations were unsuitable for the growth of cryptozoites, although this is considered unlikely, as they were able to support the development of phanerozoites and also, the same cells (prepared by the author) supported the intracellular growth of Eimeria tenella sporozoites (Urquhart, 1980). The suitability of the cell cultures was also supported by observations on the cryptozoite that was found; it was of a healthy appearance and showed no signs of vacuolation or degeneration; also its rate of development was consistent with cryptozoites observed in vivo (Huff and Coulston, 1944).

A variety of tissue culture media and supplements were tested for their ability to promote the invasion and development of sporozoites into cultured cells - all with limited success. Indeed, the earlier reports had demonstrated that a relatively simple medium was able to support the growth of phanerozoites (Hawking, 1945) and cryptozoites (Dublin, Laird and Drinnon, 1949, 1950; Laird, Dublin and Drinnon, 1950) and a variety of media have subsequently been used for the growth of the exoerythrocytic stages of the avian malaria parasites (Davis, Huff and Palmer, 1966).

In summary, the major obstacle to the cultivation of cryptozoites seems to be the invasion of sporozoites into cultured cells, and not their subsequent development. This suggestion is supported by the fact that the only parasitised culture obtained in these experiments had been subjected to centrifugation. It is possible therefore, that this process enhanced the close apposition of sporozoite to host cell, allowing the association with a specific surface receptor responsible

for the attachment of sporozoites. Although, unlike the exoerythrocytic development of P. berghei in vitro, successful growth of P. gallinaceum is not apparently dependant upon centrifugation.

The experiments reported in this chapter show that the successful transformation of P. gallinaceum sporozoites into cryptozoites in culture is certainly more difficult to achieve than the few reports in the literature would suggest. Furthermore, it is the opinion of the author that several unsuccessful and unpublished attempts to repeat Dublin's work have been made (e.g. Bray, pers. comm.).

CHAPTER 9

9. GENERAL DISCUSSION

9.1. Introduction

The P. gallinaceum sporozoite surmounts many difficult problems and adverse conditions in its progression from the mature oocyst to its final site of development in the reticulo-endothelial cells of its avian host. During this journey, the sporozoite encounters a number of different metabolic and ionic conditions; it must resist attention from the defence systems of its mosquito and vertebrate hosts; it must cross a number of very different cellular barriers; and finally, it must avoid destruction by the macrophage's lysosomal enzymes. The various adaptations which enable sporozoites to meet and successfully overcome each of these difficulties are discussed in relation to the results obtained in this thesis, together with those of other workers.

9.2. Escape from the oocyst

The fibrous wall of the mature oocyst is the first barrier to be crossed in the sporozoite's migration to the cells of the vertebrate's reticulo-endothelial system. Although it is generally assumed that the mature oocyst bursts and liberates sporozoites into the haemocoele (Garnham, 1966a). Sinden (1974, 1975) has suggested that in some instances sporozoites may leave via holes formed by ruptures in the oocyst wall. Sinden further postulates that the sporozoites may penetrate the oocyst wall actively, perhaps orientating themselves to some biochemical gradient between the surrounding haemocoelomic fluid and the midgut epithelium of the mosquito. These openings have

also been reported by Strome and Beaudoin (1974) in the mature oocyst of P. berghei. As was demonstrated by the ultrastructural study (see Chapter 4) the sporozoites in mature oocysts have well developed rhoptries and it is possible that these organelles could secrete a substance(s) which would aid in the breakdown of the oocyst wall. Rosales-Ronquillo and Silverman (1974) have commented on the fact that ookinetes in vitro are capable of invaginating the membrane of erythrocytes, presumably by a secretion from the rhoptries. Thus, active penetration of the oocyst wall, as suggested by Sinden (1974) seems a likely possibility. To support this hypothesis, Beaudoin, Strome and Tubergen (1974) have, while studying the ectopic development of P. berghei, noted infective sporozoites in the lumen of the gut and observed that, within the epithelial cells, the sporozoites, like the intraerythrocytic merozoite, lies within a well defined membrane limited parasitophorous vacuole. This is unlike the ookinete where no membrane is seen and the parasite is strictly intracytoplasmic (Garnham, Bird and Baker, 1962; Canning and Sinden, 1973).

8.3. Passage through the haemocoele

Sporozoites leaving the mature oocyst and entering the haemocoele have to avoid the mosquito's phagocytes and humoral responses if they are to progress unhindered to the salivary glands. Recently, Foley (1978) has demonstrated that A. stephensi possess haemocytes which are capable of interacting with the sporogonic stages of malaria parasites in vitro, although suggesting that in vivo, the mosquito's cellular defence mechanism is numerically overwhelmed by the parasite during its sojourn in the haemocoele. Foley points out that adult mosquitoes have only about 10,000 haemocytes which is about

the same as the number of sporozoites in a single oocyst (Pringle, 1965). It is important however, to consider that, unlike laboratory transmissions where susceptible mosquitoes may become infected with hundreds of oocysts, in areas of hyperendemic P. falciparum malaria, the oocyst rate rarely exceeds five per mosquito (Bray, pers. comm.). Thus, haemocytes may be of some importance in regulating natural transmission. There is also some evidence to suggest that haemocytes can interact with sporozoites even before the sporozoites have reached the haemocoele. Sinden (1978) has observed a number of P. vivax sporozoites inside a cell, tentatively identified as a haemocyte, within the midgut epithelium of an infected mosquito.

The immune response of insects is poorly understood and in the past has been little investigated (see Weathersby, 1975 and Lackie, 1980). It is thought however, that no immunity to repeated infection with malaria occurs in mosquitoes as Sergent (1940) showed that C. pipiens which had been infected up to four months earlier were completely susceptible to new infections of P. relictum and observed that oocysts of three different ages were capable of developing normally at the same time. Similar experimental results were obtained by Garnham (1955) with P. gallinaceum and A. aegypti var. queenlandensis.

Recently, much interest has been shown in invertebrate immunity and the humoral factors which have attracted most attention are the haemagglutinins, so-called because of their ability to agglutinate vertebrate erythrocytes by having receptors specific for certain carbohydrate residues (Sharon and Lis, 1972; Lis and Sharon, 1973; Nicolson, 1974). Lackie (1980) has suggested that molecules

containing such specific binding sites could become involved with the organisms system of defence and come to be incorporated as "recognition molecules" in the immune system. Amirante and Mazzali (1978) have been able to demonstrate a direct interaction between haemocytes of the cockroach Leucophaea maderae and agglutinins for rabbit erythrocytes. These authors have found that agglutinins are present on the cell surface and in the cytoplasm of granulocytes and spherule cells. They further suggest that, as well as being membrane bound, agglutinins are released into the haemolymph. Further evidence for the involvement of agglutinins in the response of invertebrates to foreign material has come from the work of Hardy, Fletcher and Olafsen (1977) who have found that exposure of oysters to bacteria stimulated an increased titre of agglutinins and subsequently demonstrated the opsonic effect of purified agglutinins on the phagocytosis of bacteria. In Chapter 6, it was demonstrated that P. gallinaceum sporozoites do not bind concanavalin A and carbohydrate residues, similar or identical to α-D-mannose and α-D-glucose are absent or in very low concentrations at the cell surface. Although concanavalin A is of plant origin, it establishes a molecular link between vertebrate erythrocytes and haemocytes of the snail Helix pomatia (Renwrantz and Cheng, 1977) thus illustrating that non-native agglutinins may enhance adhesion between haemocytes and foreign biotic material. It is suggested, therefore, that the absence or inaccessibility of lectin-binding sites at the cell surface of malarial sporozoites would be advantageous for their survival in the mosquito host. By failing to react with agglutinins, the presumed mediators of the immune response, sporozoites would avoid surveillance from the phagocytes.

It is not known whether it is by chance that the haemocoelomic sporozoites reach the salivary glands or if a directional migration or chemotaxis is involved. If sporozoites do actively migrate through the haemocoele, they must be metabolising and using an energy source. Mack and Vanderberg (1978) used sporozoite motility as an indicator of energy production/consumption and found that salivary gland forms of P. berghei utilised glycolysis, the Kreb's cycle and convential electron transport through the cytochrome chain. These findings are consistent with previous histochemical work (Howells, Peters and Homewood, 1972) and so it seems likely that sporozoites in the haemocoele use a similar pathway for their energy production as sporozoites leaving the mature oocyst are well equiped with cristate mitochondria (see Chapter 4). Probably nutrients are absorbed by the sporozoites from the haemocoele in the same way that oocysts are believed to take up amino acids and sugars from the haemolymph of infected mosquitoes (Mack, Samuels and Vanderberg, 1979a, 1979b). Whether this alteration in the composition of the haemolymph affects the functioning of haemocytes is unknown.

Sporozoites that have left the oocyst are not only found in the salivary glands but also in the aorta, between the muscle fibres and even in the palps or legs (Garnham, 1966a) and perhaps as few as 2% reach the salivary glands (Shute, 1945). This evidence suggests that there is no chemotactic response involved in the migration of sporozoites to the salivary glands. Thus it is probably the random movement of the haemolymph which carries haemocoelomic sporozoites into the vicinity of the salivary glands. However, there may well be some electrostatic attraction between sporozoites and the salivary glands

and probably a specific cell surface receptor is involved in the invasive process. Sporozoites that do not reach the salivary glands usually quickly disappear (Huff, 1949), but small numbers may be found in organs other than the salivary glands for as long as 31 days after infection (Oelerich, 1967).

8.4. Sporozoites in the salivary glands

The entry and localisation of malarial sporozoites in the salivary glands of their mosquito hosts has recently been discussed (Sterling, Aikawa and Vanderberg, 1973; Sinden, 1978). Two important points are particularly relevant to the work described in this thesis. Sporozoites must invade the salivary gland cells very quickly, for this process has never been observed in numerous studies (e.g. Garnham et al., 1961; Sinden and Garnham, 1973; Sterling, Aikawa and Vanderberg, 1973). Also, sporozoites in the salivary gland show differences in the organisation of the rhoptry-microneme complex when compared with sporozoites from mature oocysts (see Chapter 4).

In recent years it has become clear that sporozoites in salivary glands are very different from those in mature oocysts. Sporozoites of rodent and simian parasites are reported to have protective antigen(s) which are absent from oocyst forms (Vanderberg et al., 1972), and although sporozoites do possess antigens common to blood stages these are not exposed on viable sporozoites and can only be detected when sporozoites have been air-dried and frozen prior to their interaction with immune sera (Nussenzweig, 1977). Vanderberg (1974) has described qualitative differences between the motility of oocyst and salivary gland sporozoites of P. berghei, but as Sinden (1978) has commented, "Yoeli's original description (1964) of movement of salivary gland sporozoites is very similar to that of Vanderberg's

oocyst form, suggesting that the motility of sporozoites may differ quantitatively rather than qualitatively between the two forms". In addition to these reported changes in antigenicity and motility, earlier in this thesis differences in the electrophoretic mobility (Chapter 5) and infectivity (Chapter 7) were demonstrated. The changes in surface properties that were demonstrated by the free-flow electrophoretic investigations would account for the differences in antigenicity that are likely to exist for P. gallinaceum as well as for the differences in infectivity that do exist. A change in membrane structure may be related to the different receptors that are required for these two distinct phases of the life-cycle. Sporozoites leaving the mature oocyst must presumably have receptors for salivary gland cells. These appear to be different from those required for invasion of the macrophage as sporozoites from mature oocysts are 10,000 times less infective to the vertebrate than those from salivary glands (see Chapter 7). In the salivary glands the structure of the membrane changes and new receptors are exposed. It seems clear then, from the results presented here together with those of other workers, that sporozoites in salivary glands undergo a structural and physiological re-organisation: presumably the sporozoites are becoming adapted for survival in the vertebrate, although when these changes begin to take place is unknown. Although it may be suggested that penetration of the salivary gland cell is the trigger for this re-organisation there exists some circumstantial evidence to show that this may not be the case. Vanderberg (1975) has suggested that the development of infectivity by the P. berghei sporozoite may be time, rather than site, dependant and it is possible that as soon as the sporozoites leave the mature oocyst they begin to change. Although in his paper Vanderberg

stressed that stringent precautions were taken to prevent contamination of haemocoelomic sporozoites with those from accidentally disrupted oocysts and salivary glands, this possibility cannot be dismissed. So whether these changes in sporozoite biology occur in the haemocoele or after penetration of the salivary glands remains unresolved. One way to investigate this would be to examine the electrophoretic behaviour of sporozoites that had recently invaded the salivary glands to determine if they possess surface properties characteristic of oocyst or salivary gland populations. However, in the present study this was not possible as very large numbers of sporozoites were required for each experiment and when salivary glands are first infected, sporozoites are present only in small numbers.

9.5. Inoculation into the vertebrate host

Vanderberg (1977) has shown that very few sporozoites about 1% of the total number in the salivary glands, are injected into the vertebrate when a mosquito takes a blood meal. In understanding the malarial infection it is crucial to know what happens to these sporozoites once they have been inoculated. A. aegypti is a pool or capillary feeder (see Christophers, 1960) and Griffiths and Gordon (1952) have observed the feeding process of A. aegypti in tissues of live rodents and noticed that when the fascicle was searching for blood, discharges of a clear fluid, evidently salivary gland secretion, took place into the tissue. Presumably sporozoites would be discharged with this and these would be the ones to initiate primary exoerythrocytic development in the tissue macrophages. Other sporozoites presumably enter the blood vessils which remains infective for no longer than 30 minutes even after the inoculation of massive doses of

sporozoites (Coulston, Cantrell and Huff, 1945). Under natural conditions, sporozoites inoculated into the blood are found developing only in the macrophages of the spleen, although following the intravenous inoculation of large numbers of sporozoites the macrophages of the lung, liver and muscle may become infected (Coulston, Cantrell and Huff, 1945).

An immediate problem the sporozoite has to encounter is the defence system of the host. In blood flukes there is evidence that host serum proteins can become absorbed onto the surface so that the parasite is able to mimic its host antigenically and hence avoid rejection as foreign tissue (Smithers, Terry and Hockley, 1968). Although there is no evidence of this phenomenom occuring with malarial sporozoites the possibility must be considered. Probably the best way for the sporozoite to avoid undue attention from the defence systems of the vertebrate would be if it were to gain intracellular entry very quickly and this is known to take no longer than 6 hours with P. gallinaceum (Huff and Coulston, 1944). However, sporozoites of Leucocytozoon dubreuli have been found in an impression smear of a robins liver 11 days after receiving sporozoites (Khan, Desser and Fallis, 1969), although it is not clear whether these were intracellular or extracellular.

Despite previous unsuccessful attempts (Bray, 1978) antibodies to sporozoites in areas of endemic malaria have now been demonstrated (Nardin et al., 1979) although their role in protective immunity is not known. Immune sera is known to agglutinate P. knowlesi merozoites in vitro, the antibody apparently binding to the surface coat and this agglutination is thought to reduce erythrocyte invasion in vitro

(Miller, Aikawa and Dvorak, 1975). Butcher and Cohen (1972) however, hold a different view in that they believe that receptor sites are neutralised *in vitro* and this is the cause of reduced invasion *in vivo*. Probably both mechanisms occur and although agglutination of malarial sporozoites has been described (Russell, Mulligan and Mohan, 1941), and antibody is known to bind to the surface membrane of sporozoites *in vitro*, it is not known whether these events have any importance *in vivo*.

Recently, Strome, De Santis and Beaudoin (1979) in their discussion of the culture of the exoerythrocytic forms of *P. berghei*, consider that, "the ability of sporozoites to invade and develop in cells derived from tissues as widely divergent as embryonic rat brain and embryonic turkey brain demonstrates that the malarial parasite does not have the rigid requirements for a particular host cell". Although this may be the case *in vitro*, *in vivo* it most certainly is not: avian malarial sporozoites and young cryptozoites have never been observed in any other cells except macrophages and the exoerythrocytic schizonts of mammalian malaria have only been observed in liver parenchymal cells. Also, it should be considered that the complete development of the exoerythrocytic stages of *P. berghei* may not have been successful because the choice of cell, and not the parasites growth requirements, were wrong. Moreover, Sinden and Smith (1980) have suggested that the low infectivity rates of sporozoites *in vitro* may in part be due to the use of the wrong cell which may lack specific surface ligands responsible for initial binding of the sporozoite.

9.6. Entry and survival of intracellular sporozoites

The entry of Plasmodium merozoites into erythrocytes is well documented (Ladda, Aikawa and Sprinz, 1969; Bannister et al., 1975; Bannister, 1977; Dvorak et al., 1975; Miller, McAuliffe and Mason, 1977; Miller et al., 1977, 1979; Aikawa et al., 1978) and in recent years there has been much interest in the mechanism by which a number of parasitic protozoa enter their host cells. Thus, Eimeria (see Long and Speer, 1977), Toxoplasma (Jones, Yeh and Hirsch, 1972; Lycke, Carlberg and Norry, 1975), Babesia (Rudzinska et al., 1975; Rudzinska, 1976) and Leucocytozoon (Wong, 1979) all appear to use much the same method. The invading organism attaches to the host cell, presumably by a specific receptor at the cell surface, and enters, apical end first, within an invagination of the host cell membrane. In some cases the parasitophorous vacuole membrane breaks down soon after entry and the membrane forming the vacuole is newly formed e.g. Toxoplasma (Lycke, Carlberg and Norry, 1975).

It has been suggested by many authors that the rhoptries and micronemes consist of a complex sequence of interconnected organelles with secretory activities which are related to cell penetration (see Aikawa, 1971; Scholtyseck and Melhorn, 1970 and Sinden, 1978). Although the mechanism is unclear, it would seem that the rhoptry-microneme complex secretes a surfactant substance(s) which alters the physico-chemical properties of the host cell membrane, thus allowing a stretching which is necessary if the membrane is to invaginate and surround the parasite (Bannister, 1977). However, McLaren et al. (1979)

in a freeze-fracture study on the interaction between P. knowlesi merozoites and erythrocytes have suggested that the increase in surface area of the invaginated host cell membrane is beyond the limits of simple membrane stretching and may involve the incorporation of material released from the merozoite.

Recently, Aikawa et al. (1978) have examined the invasion of erythrocytes by malarial merozoites and have reported a moving junction between the erythrocyte and parasite. The area of the erythrocyte membrane to which the merozoite becomes attached thickens and forms a junction with the plasma membrane of the merozoite; as the merozoite enters, the junction moves to maintain its position at the orifice of the invagination. When entry is complete the junction becomes part of the parasitophorous vacuole. Although this appears to be a suitable model for the entry of merozoites into erythrocytes, it is possible that the entry of sporozoites into actively phagocytic macrophages, is different.

Lyke, Carlberg and Norry (1975) have isolated a protein with membrane-active properties from the trophozoites of T. gondii. It appears that this protein aids the invasive process by enhancing phagocytosis and it must be considered if a similar process enhances the invasion of macrophages by sporozoites. Griffin et al. (1975) and Griffin, Griffin and Silverstein (1976) have proposed that endocytosis of particles by macrophages involves specific attachment, which triggers uptake, followed by a process called "zippering" which results from the sequential and circumferential interaction of receptors on the macrophage's surface with ligands on the particle's (parasite?) surface. These particle bound ligands form a template which guides the macrophage's plasma membrane over the particles

surface. Whether "zippering" is really a different process from invagination via a moving junction requires some clarification and which mechanism the malarial sporozoite uses remains unknown. However, it is evident from the results presented in Chapter 7, that trypsin sensitive components of the cell surface membrane of P. gallinaceum sporozoites are necessary for the invasion process.

Once inside in a macrophage, presumably in a parasitophorous vacuole, the developing sporozoite must be able to avoid destruction by the lysosomal enzymes of the host cell. Mechanisms which enable parasites of macrophages to survive and multiply in their host cells include an absence of lysosomal fusion e.g. T. gondii (Jones and Hirsch, 1972) and Mycobacterium tuberculosis (Armstrong and Hart, 1971); resistance to lysosomal enzymes e.g. Leishmania (Alexander and Vickerman, 1975; Chang and Dwyer, 1976, 1978) and Mycobacterium lepraemurium (Draper and Rees, 1970); and escape from the parasitophorous vacuole into the cytoplasm of the host cell e.g. T. cruzi (Dvorak and Hyde, 1973; Milder and Kloetzel, 1980). However, speculation as to which mechanism the developing cryptozoites of P. gallinaceum employs must await the development of a satisfactory technique for its in vitro culture.

9.7. Summary

This thesis has described an investigation into the development of the P. gallinaceum sporozoite.

Although with the light microscope, sporozoites from mature oocysts and salivary glands were indistinguishable, changes were observed in their ultrastructure which resulted in a proliferation of the micronemes and a loss of the rhoptries in the salivary gland forms.

Results of the free-flow electrophoresis studies demonstrated qualitative differences in the cell surface ionogenic groups between the two populations and lectin-binding studies showed that carbohydrate ligands similar to α-D-mannose and α-D-glucose, which are common components of many cells, were absent from the cell surface of *P.gallinaceum* sporozoites.

In addition to the changes in morphology and cell surface properties, sporozoites in salivary glands were found to be 10,000 times more infective than those in mature oocysts and their infectivity dependant upon trypsin-sensitive components at the cell surface.

Finally, the difficulties of culturing cryptozoites, from sporozoites *in vitro*, were discussed.

Although these results have demonstrated that changes take place in the physiological and structural organisation of sporozoites as they progress from the mature oocyst to the salivary glands, further work is needed, not only to discover where and when these changes take place, and what triggers them, but also to determine how the sporozoite reaches and penetrates the cells of the salivary glands. Also, there is clearly a need for new and efficient methods of isolating infective salivary gland sporozoites from infected mosquitoes, to allow more detailed biochemical and immunological investigations of the cell surface. Finally, the mechanism of the entry of malarial sporozoites into cells of the vertebrate, the initiation of exoerythrocytic schizogony and, in avian malaria, the avoidance of the digestive activities of the host cell, are unknown. They seem set to remain so until such time that a suitable tissue culture system is established that will allow the elucidation of the early stages of the malarial infection.

REFERENCES

AIKAWA, M. (1967). Ultrastructure of the pellicular complex of Plasmodium fallax. Journal of Cell Biology, 35, 103-113.

AIKAWA, M. (1971). Plasmodium: The fine structure of malarial parasites. Experimental Parasitology, 30, 284-320.

AIKAWA, M. (1977). Variations in structure and function during the life cycle of malarial parasites. Bulletin of the World Health Organization, 55, 139-156.

AIKAWA, M., HEPLER, P.K., HUFF, C.G. and SPRINZ, H. (1966). The feeding mechanism of avian malarial parasites. Journal of Cell Biology, 28, 355-373.

AIKAWA, M., MILLER, L.H., JOHNSON, J. and RABBEGE, J. (1978). Erythrocyte entry by malaria parasites. A moving junction between erythrocyte and parasite Journal of Cell Biology, 77, 72-82.

ALEXANDER, J. and VICKERMAN, K. (1975). Fusion of host cell secondary lysosomes with the parasitophorous vacuoles of Leishmania mexicana-infected macrophages. Journal of Protozoology, 22, 502-508.

ALGER, N.E. (1968). In vitro development of Plasmodium berghei ookinetes. Nature, London, 218, 774.

ALVES, M.J.M. and COLI, W. (1974). Agglutination of Trypanosoma cruzi by concanavalin A. Journal of Protozoology, 21, 575-578.

AMBROSE, E.J. (1966). Electrophoretic behaviour of cells. Progress in Biophysics and Molecular Biology, 16, 243-265.

AMIRANTE, G.A. and MAZZALI, F.G. (1978). Synthesis and localisation of hemoagglutinins in hemocytes of cockroach Leucophaea maderae L. Developmental and Comparative Immunology, 2, 435-440.

ANDERSSON, J., EDELMAN, G.M., MÖLLER, G. and SJÖBERG, O. (1972). Activation of B lymphocytes by locally concentrated concanavalin A. European Journal of Immunology, 2, 233-235.

ARMSTRONG, J.A. and HART, P.D. (1971). Response of cultured macrophages to Mycobacterium tuberculosis with observations on fusion of lysosomes with phagosomes. Journal of Experimental Medicine, 134, 713-740.

BALL, G.H. (1964). In vitro culture of the mosquito phase of avian malaria. Journal of Parasitology, 50, 3-10.

BALL, G.H. (1965). Adaptation of the malarial parasite, particularly to its insect host. In "Host-parasite relationships". (A.J. McCauley, ed.). Corvallis, Oregon State University Press, pp. 73-96.

BALL, G.H. (1972). Use of invertebrate tissue culture for the study of Plasmodia. In "Invertebrate tissue culture" Vol. II. (C. Vago, ed.). Academic Press, New York. pp. 321-343.

BALL, G.H. and CHAO, J. (1961). Infectivity to canaries of sporozoites of Plamodium relictum developing in vitro. Journal of Parasitology, 47, 787-790.

BALTZ, T., BALTZ, D. and PAUTRIZEL, R. (1976). Affinité de la concanavaline A pour Trypanosoma equiperdum applications á l'isolement de la fraction glycoprotéique spécifique du type antigénique. Annales d'Immunologie (Institute Pasteur) 127C, 761-774.

BANGHAM, A.D. (1961). Electrophoretic characteristics of ram and rabbit spermatozoa. Proceedings of the Royal Society of London, B, 155, 292-305.

BANGHAM, A.D., FLEMANS, R., HEARD, D.H. & SEAMAN, G.V.F. (1958). An apparatus for microelectrophoresis of small particles. Nature, London, 182, 642-644.

BANKS, K.L. (1979). In vitro binding of Trypanosoma congolense to erythrocytes. Journal of Protozoology, 26, 103-108.

BANNISTER, L.H. (1977). The invasion of red cells by Plasmodium. In "Symposium of the British Society of Parasitology", No. 15 (A. Taylor and R. Muller, eds.). Blackwell Scientific Publications, Oxford. pp. 27-55.

BANNISTER, L.H. (1980). Personal communication.

BANNISTER, L.H., BUTCHER, G.A. and MITCHELL, G.H. (1977). Recent advances in understanding the invasion of erythrocytes by merozoites of Plasmodium knowlesi. Bulletin of the World Health Organization, 55, 163-169.

BANNISTER, L.H., BUTCHER, G.A., DENNIS, E.D. and MITCHELL, G.H. (1975). Structure and invasive behaviour of Plasmodium knowlesi merozoites in vitro. Parasitology. 71, 483-491.

BANNISTER, L.H., BUTCHER, G.A., McLAREN, D.J. and MITCHELL, G.H. (1979). Ultrastructural observations on changes in merozoites of Plasmodium knowlesi maintained in the absence of red cells. Journal of Protozoology, 26, 77A.

BEAUDOIN, R.L. (1977). Should cultivated exoerythrocytic parasites be considered as a source of antigen for a malaria vaccine. Bulletin of the World Health Organization, 55, 373-376.

BEAUDOIN, R.L., STROME, C.P.A. and CLUTTER, W.G. (1969). A tissue culture system for the study of drug action against the tissue phase of malaria. Military Medicine, 134, 979-985.

BEAUDOIN, R.L., STROME, C.P.A. and TUBERGEN, T.A. (1974). Plasmodium berghei berghei: ectopic development of the ANKA strain in Anopheles stephensi. Experimental Parasitology, 36, 189-201.

BEAUDOIN, R.L., STROME, C.P.A., MITCHELL, F. and TUBERGEN, T.A. (1977). Plasmodium berghei: immunization of mice against the ANKA strain using the unaltered sporozoite as an antigen. Experimental Parasitology, 42, 1-5.

BEN-HAREL, S. (1923). Studies on bird malaria in relation to the mechanism of relapse. American Journal of Hygiene, 6, 652-685.

BIGNAMI, A. (1898). The inoculation theory of malarial infection: account of a successful experiment with mosquitoes. Lancet, 1461-1463, 1541-1544.

BOYD, M.F. and STRATMAN-THOMAS, W.K. (1934). Studies in benign tertian malaria. 7. Some observations on inoculation and onset. American Journal of Hygiene, 20, 288-295.

BRAY, R.S. (1957). "Studies on the exo-erythrocytic cycle in the genus Plasmodium". H.K. Lewis and Co., London.

BRAY, R.S. (1976). Vaccination against Plasmodium falciparum; a negative result. Transactions of the Royal Society of Tropical Medicine and Hygiene, 70, 258.

BRAY, R.S. (1978). Absence of circumsporozoite antibodies in areas of hyperendemic malaria. Journal of Parasitology, 64, 410.

BRAY, R.S. (1980). Personal communication.

BROOKE, M.M. (1942). Inoculation of canaries with sporozoites from isolated malarial oocysts, American Journal of Hygiene, 35, 134-137.

BROOM, J.C. and BROWN, H.C. (1937). Studies in trypanosomiasis. I. The electric charge of trypanosomes in tsetse flies. Transactions of the Royal Society of Tropical Medicine and Hygiene, 31, 81-86.

BROOM, J.C. and BROWN, H.C. (1939). Studies in trypanosomiasis. III. The electric charge of trypanosomes in the salivary gland of tsetse flies. Transactions of the Royal Society of Tropical Medicine and Hygiene, 32, 545-548.

BROOM, J.C., BROWN, H.C. and HOARE, C.A. (1936). Studies in microcataphoresis. II. The electric charge of haemoflagellates. Transactions of the Royal Society of Tropical Medicine and Hygiene, 30, 87-100.

BRUMPT, E. (1935). Paludisme aviare: Plasmodium gallinaceum n.sp. de la poule domestique. Compte rendu hebdomadaire des séances de l' Académie des sciences, 200, 783-786.

BRUMPT, E. (1949). "Précis de Parasitologie", 6th edition, Masson et Cie, Paris.

BURGER, M.M. (1969). A difference in the architecture of the surface membrane of normal and virally transformed cells. Proceedings of the National Academy of Sciences of the United States of America, 62, 994-1001.

BUTCHER, G.A. and COHEN, S. (1972). Antigenic variation and protective immunity to Plasmodium knowlesi malaria. Immunology, 23, 503.

BUTCHER, G.A., MITCHELL, G.H. and COHEN, S. (1973). Mechanism of host specificity in malarial infection. Nature, London, 244, 40-42.

CANNING, E.U. and SINDEN, R.E. (1973). The organization of the ookinete and observations on nuclear division in oocysts of Plasmodium berghei. Parasitology, 67, 29-40.

CANNING, E.U. and SINDEN, R.E. (1975). Nuclear organization in gametocytes of Plasmodium and Hepatocystis: a cytochemical study. Zeitschrift für Parasitenkunde, 46, 297-299.

CANTRELL, W. & JORDAN, H.B. (1946). Changes in the infectiousness of gametocytes during the course of Plasmodium gallinaceum infections. Journal of Infectious Diseases, 78, 153-159.

CARTER, R. and CHEN, D.H. (1976). Malaria transmission blocked by immunisation with gametes of the malaria parasite. Nature, London, 263, 57-60.

CHANG, K.P. (1977). Leishmania donovani-hamster macrophage surface interactions in vitro: Quantitation and characterization of host-parasite attachment using tritium-labelled promastigotes. Proceeding of International Congress of Protozoology, 5, 436.

CHANG, K.P. and DWYER, D.M. (1976). Multiplication of a human parasite (Leishmania donovani) in phagolysosomes of hamster macrophages in vitro. Science, 193, 678-680.

CHANG, K.P. and DWYER, D.M. (1978). Leishmania donovani - hampster macrophage interactions in vitro: cell entry, intracellular survival and multiplication of amastigotes. Journal of Experimental Medicine, 147, 515-530.

CHEN, D. & SCHNEIDER, I. (1969). Mass isolation of malaria sporozoites from mosquitoes by density gradient centrifugation. Proceedings of the Society for Experimental Biology and Medicine, 130, 1318-1321.

CHIARI, E., DE SOUZA, W., ROMANHA, A.J., CHIARI, C.A. and BRENER, Z. (1978). Concanavalin A receptors on the cell membrane of Trypanosoma cruzi. Acta tropica, 35, 113-121.

CHRISTOPHERS, S.R. (1960). "Aedes aegypti (L.) the yellow fever mosquito". Cambridge University Press.

CLAWSON, C.C., RAO, G.H.R. and WHITE, J.G. (1975). Platelet interaction with bacteria IV. Stimulation of the release action. American Journal of Pathology, 81, 411-419.

CLYDE, D.F. (1975). Immunisation of man against falciparum and vivax malaria by use of attenuated sporozoites. American Journal of Tropical Medicine and Hygiene, 24, 397-401.

COATNEY, G.R., COOPER, W.C. and TREMBLEY, H.L. (1945a). Studies on Plasmodium gallinaceum Brumpt II. The incidence and course of infection in young chicks following the inoculation of infected salivary glands. American Journal of Hygiene, 41, 119-122.

COATNEY, G.R., COOPER, W.C. and TREMBLEY, H.L. (1945b). Studies on Plasmodium gallinaceum Brumpt III. The incidence and course of infection in young chicks following the subcutaneous inoculation of pooled sporozoites. American Journal of Hygiene, 42, 323-329.

COCHRANE, A.H., AIKAWA, M., JENG, M. and NUSSENZWEIG, R.S. (1976). Antibody-induced ultrastructural changes of malarial sporozoites. Journal of Immunology, 116, 859-867.

COOK, G.M.W. (1968). Glycoproteins in membranes. Biological Reviews. 43, 363-369.

COOK, G.M.W. & JACOBSON, W. (1968). The electrophoretic mobility of normal and leukaemic cells. Biochemical Journal, 107, 549-557.

COOK, G.M.W. and STODDART, R.W. (1973). "Surface carbohydrates of the eukaryotic cell". Academic Press, London.

COOK, G.M.W., HEARD, D.H. & SEAMAN, G.V.F. (1961). Sialic acids and the electrokinetic charge of the human erythrocyte. Nature, London, 191, 44-47.

COULSTON, F.W., CANTRELL, W. and HUFF, C.G. (1945). The distribution and localization of sporozoites and pre-erythrocytic stages in infections with Plasmodium gallinaceum. Journal of Infectious Diseases, 76, 226-238.

CRAWFORD, M. (1945). Plasmodium gallinaceum, a malaria parasite of of the domestic fowl. Veterinary Record, 57, 395-396.

CROSS, G.A.M. and JOHNSON, J.G. (1976). Structure and organization of the variant-specific surface antigens of Trypanosoma brucei. In "Biochemistry of parasites and host-parasite relationships," (H. Van den Bossche, ed.). North-Holland Publishing Co., Amsterdam, pp. 413-420.

DASGUPTA, B. (1959). The Feulgen reaction at different stages of the life cycle of certain sporozoa. Quarterly Journal of Microscopical Science, 100, 241-255.

DAVID, P.H. and HOMMEL, M. (1979). Purification of plasmodial merozoites by differential elution from a Sepharose-Concanavalin A column. Transactions of the Royal Society of Tropical Medicine and Hygiene, 73, 326.

DAVID, P.H., HOMMEL, M., BENICHOU, J.C., EISEN, H.A. and PEREIRA DA SILVA, L.H. (1978). Isolation of malaria merozoites: release of Plasmodium chabaudi merozoites from schizonts bound to immobilized concanavalin A. Proceedings of the National Academy of Sciences of the United States of America, 75, 5081-5084.

DAVIS, A.G., HUFF, C.G. and PALMER, T.T. (1966). Procedures for maximum production of exoerythrocytic stages of Plasmodium fallax in tissue cultures. Experimental Parasitology, 19, 1-8.

DAWIDOWICZ, K., HERNANDEZ, A.G. and INFANTE, R.B. (1975). The surface membrane of Leishmania. 1. The effects of lectins on different stages of Leishmania braziliensis. Journal of Parasitology, 61, 950-953.

DECOURT, P. and SCHNEIDER, J. (1938). Note preliminaire sur la recherche de la localisation et de la morphologie des plasmodes pendent les périodes d'intestation latente. Bulletin de la Société de pathologie exotique, 31, 609-614.

DENNIS, E.D., MITCHELL, G.H., BUTCHER, G.A. and COHEN, S. (1975). In vitro isolation of Plasmodium knowlesi merozoites using polycarbonate sieves. Parasitology, 71, 475-481.

DE SOUZA, W. and BRASIL, R.P. (1976). An electron microscopic and cytochemical detection of concanavalin A receptors on the cell membrane of Leishmania braziliensis guyanensis. Zeitschrift für Parasitenkunde, 50, 1-9.

DE SOUZA, W., ARGUELLO, C., MARTINEZ-PALOMO, A., TRISSL, D., GONZÁLES-ROBLES, A. and CHIARI, E. (1977). Surface charge of Trypanosoma cruzi. Binding of cationised ferritin and measurement of cellular electrophoretic mobility. Journal of Protozoology, 24, 411-415.

DE ZEEUW, R.A., WIJSBEEK, J., ROCK, R.C. and McCORMICK, G.J. (1972). Composition of phospholipids in Plasmodium knowlesi membranes and in host rhesus erythrocyte membranes. Proceedings of the Helminthological Society of Washington, 39 (special issue) 412-418.

DIXON, H.B.F. & PERHAM, R.N. (1968). Reversible blocking of amino groups with citraconic anhydride. Biochemical Journal, 109, 312-314.

DOBY, J.M. and BARKER, R. (1976). Essais d'obtention in vitro des formes pré-erythrocytaires de Plasmodium vivax en cultures de cellules hepatiques humaines inoculées par sporozoites. Comptes rendus des séances de la Société de biologie, 170, 661-665.

DRAPER, P. and REES, R.J.W. (1970). Electron transparent zone of mycobacterium may be a defence mechanism. Nature, London, 228, 860-861.

DUBLIN, I.N. (1947). Bodies suggesting exoerythrocytic forms of Plasmodium vivax in tissue culture. Proceedings of the Society for Experimental Biology and Medicine, 65, 154-156.

DUBLIN, I.N., LAIRD, R.L. and DRINNON, V.P. (1949). The development of sporozoites of Plasmodium gallinaceum into cryptozoites in tissue culture. Journal of the National Malaria Society, 8, 175-180.

DUBLIN, I.N., LAIRD, R.L. and DRINNON, V.P. (1950). Further observations on the development of sporozoites of Plasmodium gallinaceum into cryptozoites in tissue culture. Journal of the National Malaria Society, 9, 119-127.

DUBREMETZ, J.F. and FERREIRA, E. (1978). Capping of cationised ferritin by coccidian zoites. Journal of Protozoology, 25, 9B.

DUBREMETZ, J.F., TORPIER, G. MAUROIS P., PRENSIER, G. and SINDEN, R.E. (1979). Structure de la pellicule du sporozoite de Plasmodium yoelii. Compte Rendu Hebdomadaire des séances de l'Academie des Sciences. 288, 623-626.

DVORAK, J.A. and HYDE, T.D. (1973). Trypanosoma cruzi: intreaction with vertebrate cells in vitro. 1. Individual interactions at the cellular and subcellular levels. Experimental Parasitology, 34, 268-283.

DVORAK, J.A., MILLER, L.H., WHITEHOUSE, W.C. and SHIROISHI, T. (1975). Invasion of erythrocytes by malaria merozoites. Science, 187, 748-750.

DWYER, D.M. (1974). Lectin-binding saccharides on a parasitic protozoan. Science, 184, 471-473.

DWYER, D.M. (1976). Cell surface saccharides of Trypanosoma lewisi. II. Lectin-mediated cell agglutination and fine structure cytochemical detection of lectin binding sites. Journal of Cell Science, 22, 1-19.

DWYER, D.M. (1977). Leishmania donovani: surface membrane carbohydrates of promastigotes. Experimental Parasitology, 41, 341-358.

DWYER, D.M. and D'ALESANDRO, P.A. (1974). Cell surface carbohydrates in Trypanosoma duttoni. Journal of Protozoology, 21, 430.

DWYER, D.M. and D'ALESANDRO, P.A. (1976). The cell surface of Trypanosoma musculi bloodstream forms. II. Lectins and immunologic studies. Journal of Protozoology, 23, 262-271.

DYKSTRA, M.J. and ALDRICH, H.C. (1978). Successful demonstration of an elusive cell coat in amebae. Journal of Protozoology, 25, 38-41.

EL-KORDY, M.A. and AHMED, H.N. (1960). Incidence of malaria infection in the domesticated fowl in the United Arab Republic. Journal of the Egyptian Veterinary Medical Association, 20, 119-125.

ELLMAN, G.L. (1959). Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics, 82, 70-77.

EMMEL, L., JAKOB, A. and GOLZ, H. (1942). Electronenoptische Untersuchungen an Malaria-Sporozoiten. Deutsche Tropenmedizinische Zeitschrift, 46, 254-258.

EYLAR, E.H., MADOFF, M.A., BRODY, O.V. and ONCLEY, J.L. (1962). The contribution of sialic acid to the surface charge of the erythrocyte. Journal of Biological Chemistry, 237, 1992-2000.

EYLES, D.E. (1951). Studies on Plasmodium gallinaceum I. Characteristics of the infection in the mosquito, Aedes aegypti. American Journal of Hygiene, 54, 101-112.

FAIRLEY, N.H. (1947). Sidelights on malaria in man obtained by sub-inoculation experiments. Transactions of the Royal Society of Tropical Medicine and Hygiene, 40, 621-676.

FERNANDO, M.A. and DISSANAIKE, A.S. (1975). Studies on Plasmodium gallinaceum and Plasmodium juxtanucleare from the Malayan jungle fowl Gallus gallus spadiceus. Southeast Asian Journal of Tropical Medicine and Public Health, 6, 25-32.

FINK, E. and SCHICA, E. (1969). Influence of synthetic insect T.C. medium on the survival of malaria sporozoites in vitro (Plasmodium berghei yoeli and P. cathemerium). Zeitschrift für Parasitenkunde, 32, 93-101.

FOLEY, D.A. (1978). Innate cellular defence by mosquito haemocytes. In "Comparative pathobiology", Vol. 4. (L.A. Bulla and T.C. Cheng, eds.). Plenum Press, New York. pp. 113-144.

FOLEY, D.A., KENNARD, J. and VANDERBERG, J.P. (1978a). Plasmodium berghei: infective exoerythrocytic schizonts in primary monolayer cultures of rat liver cells. Experimental Parasitology, 46, 166-178.

FOLEY, D.A., KENNARD, J. and VANDERBERG, J.P. (1978b). Plasmodium berghei: preparation of rat hepatic cell suspensions that include infective exoerythrocytic schizonts. Experimental Parasitology, 46, 179-188.

GAMBRELL, W.E. (1937). Variation in gametocyte production in avian malaria. American Journal of Tropical Medicine, 17, 689-727.

GARNHAM, P.C.C. (1947). Exoerythrocytic schizogony in Plasmodium kochi Laveran. A preliminary note. Transactions of the Royal Society of Tropical Medicine and Hygiene, 40, 719-722.

GARNHAM, P.C.C. (1955). The comparative pathogenicity of protozoa in their vertebrate and invertebrate hosts. In "Mechanisms of microbial pathogenicity", Cambridge University Press, pp. 191-206.

GARNHAM, P.C.C. (1966a). "Malaria parasites and other Haemosporidia". Blackwell, Oxford.

GARNHAM, P.C.C. (1966b). Locomotion in the parasitic protozoa. Biological Reviews, 41, 561-586.

GARNHAM, P.C.C. (1969). Malaria parasites as medical and veterinary zoonoses. Bulletin de la Société de pathologie exotique, 62, 325-332.

GARNHAM, P.C.C. (1977). The origins, evolutionary significance and dispersal of haemosporidian parasites of domestic birds. In "Origins of pest, parasite, disease and weed problems" (J.M. Cherrett and G.R. Sagar, eds.). Blackwell Scientific Publications, Oxford.

GARNHAM, P.C.C., BIRD, R.G. and BAKER, J.R. (1960). The fine structure of the sporozoites of Haemoeba (= Plasmodium) gallinaceum, Transactions of the Royal Society of Tropical Medicine and Hygiene, 54, 274-278.

GARNHAM, P.C.C., BIRD, R.G. and BAKER, J.R. (1962). Electron microscope studies on the motile stages of malaria parasites. III. The ookinetes of Haemamoeba and Plasmodium. Transactions of the Royal Society of Tropical Medicine and Hygiene, 56, 116-120.

GARNHAM, P.C.C., BIRD, R.G., BAKER, J.R. and BRAY, R.S. (1961). Electron microscope studies of motile stages of malaria parasites II. The fine structure of the sporozoites of Laverania (Plasmodium) falciparum. Transactions of the Royal Society of Tropical Medicine and Hygiene, 55, 93-102.

GASS, R.F. (1979). The ultrastructure of cultured Plasmodium gallinaceum ookinetes: a comparison of intact stages with forms damaged by extracts from blood fed, susceptible Aedes aegypti. Acta tropica, 36, 323-334.

GIBBONS, I. and PERHAM, R.N. (1970). The reaction of aldolase with 2-methylmaleic anhydride. Biochemical Journal, 116, 843-849.

GIBBONS, R.J. and VAN HOUTE, J. (1975). Bacterial adherence in oral microbial ecology. Annual Review of Microbiology, 29, 19-44.

GIBSON, D.A., MARQUARDT, M.D. and GORDON, J.A. (1975). Cell rigidity: effect of concanavalin A-mediated agglutinability of fibroblasts after fixation. Science, 189, 45-46.

GOLDSTEIN, I.J. (1976). Carbohydrate binding specificity of concanavalin A. In "Concanvalin A as a tool" (H. Bittinger and H.P. Schnebli eds.). Wiley, London. pp. 55-65.

GRASSI, B. (1900). "Studi di un zoologo sulla malaria". Roma, R. Accad. Lincei. (from Garnham, 1966a).

GREAVES, M. and JANOSSY, G. (1972). Elicitation of selective T and B lymphocyte responses by cell surface ligands. Transplantation Reviews, 11, 87-130.

GREGSON, N.A. (1977). The surface properties of isolated rat brain myelin: A microelectrophoretic study. Journal of Neurochemistry, 29, 895-903.

GRIFFIN, F.M., GRIFFIN, J.A. and SILVERSTEIN, S.C. (1976). Studies on the mechanism of phagocytosis II. The interaction of macrophages with anti-immunoglobulin. Ig G coated bone marrow derived lymphocytes. Journal of Experimental Medicine, 144, 788-809.

GRIFFIN, F.M., GRIFFIN, J.A., LEIDER, J.E. and SILVERSTEIN, S.C. (1975). Studies on the mechanism of phagocytosis I. Requirements for circumferential attachment of particle bound ligands to specific receptors on the macrophage plasma membrane. Journal of Experimental Medicine, 142, 1263-1282.

GRIFFITHS, R.B. and GORDON, R.M. (1952). An apparatus which enables the process of feeding by mosquitoes to be observed in the tissue of a live rodent together with an account of the ejection of saliva and its significance in malaria. Annals of Tropical Medicine and Parasitology, 46, 311-319.

GWATZ, R.W. (1976). Malaria: successful immunisation against sexual stages of P. gallinaceum. Science, 193, 1150-1151.

HAAS, B.H., WILCOX, A., LAIRD, R.L., EWING, F.M. and COLEMAN, N. (1948). Response of exoerythrocytic forms to alterations in the life cycle of Plasmodium gallinaceum. Journal of Parasitology, 34, 306-320.

HAIBA, M.H. (1948). Plasmodia of common Egyptian birds. Journal of Comparative Pathology, 58, 81-93.

HARDY, S.W., FLETCHER, T.C. and OLAFSEN, J.A. (1977). Aspects of cellular and humoral defence mechanisms in the Pacific oyster, Crassostrea gigas. In "Developmental Immunobiology" (J.B. Solomon and J.D. Horton, eds). Amsterdam, Elsevier/North-Holland.

HARRISON, G. (1978). "Mosquitoes, malaria and man: a history of the hostilities since 1880". John Murray, London.

HAWKING, F. (1945). Growth of protozoa in tissue culture. 1. Plasmodium gallinaceum, exoerythrocytic forms. Transactions of the Royal Society of Tropical Medicine and Hygiene, 39, 245-263.

HEIDRICH, H.G., STAHN, R. and HANNIG, K. (1970). The surface charge of rat liver mitochondria and of their membranes. Clarification of some controversies concerning the mitochondrial structure. Journal of Cell Biology, 46, 137-150.

HEIDRICH, H.G., RUSSMANN, L., BAYER, B. and JUNG, A. (1979). Free-flow electrophoresis for the separation of malaria infected and uninfected mouse erythrocytes and for the isolation of free parasites (Plasmodium vinckei): a new rapid technique for the liberation of malaria parasites from their host cells. Zeitschrift für Parasitenkunde, 58, 151-159.

HENRY, C. (1939). Pouvoir infestant du sang cours de l'incubation du paludisme de la poule (P. gallinaceum) inoculé par moustiques. Bulletin de la Société de pathologie exotique, 32, 30-34.

HEWITT, R. (1940). "Bird malaria". John Hopkins Press, Baltimore.

HOLBROOK, J.J. & STINSON, R.A. (1970). Reactivity of the essential thiol group of lactate dehydrogenase and substrate binding. Biochemical Journal, 120, 289-297.

HOLLINGSHEAD, S., PETHICA, B.A. and RYLEY, J.F. (1963). The electrophoretic behaviour of some trypanosomes. Biochemical Journal, 89, 123-127.

HOWE, C. and LEE, L.T. (1972). Virus-erythrocyte interactions. Advances in Virus Research, 17, 1-49.

HOWELLS, R.E., PETERS, W. and HOMEWOOD, C.A. (1972). Physiological adaptability of malaria parasites. In "Comparative biochemistry of parasites" (H. Van den Bossche, ed.). Academic Press, New York, pp. 235-258.

HUFF, C.G. (1930). Plasmodium elongatum n.sp., an avian malarial organism with an elongate gametocyte. American Journal of Hygiene, 11, 385-391.

HUFF, C.G. (1949). Life cycles of malaria parasites with special reference to the newer knowledge of pre-erythrocytic forms. In "Malariology" (M.F. Boyd, ed.). W.B. Saunders, pp. 54-64.

HUFF, C.G. (1951). The significance of new findings in the life cycle of malarial parasites. In "Parasitic infections in man" (H. Most ed.). Columbia University Press, New York. pp. 9-18.

HUFF, C.G. (1952). Studies on the exoerythrocytic stage of Plasmodium gallinaceum during the "transitional phase". Experimental Parasitology, 1, 392-405.

HUFF, C.G. (1957). Organ and tissue distribution of the exoerythrocytic stages of various avian malaria parasites. Experimental Parasitology, 6, 143-162.

HUFF, C.G. (1963). Experimental research on avian malaria. Advances in Parasitology, 1, 1-65.

HUFF, C.G. (1964). Cultivation of the exoerythrocytic stages of malarial parasites. American Journal of Tropical Medicine and Hygiene, 13, 171-177.

HUFF, C.G. (1968). Recent experimental research on avian malaria. Advances in Parasitology, 6, 293-311.

HUFF, C.G. and BLOOM, W. (1935). A malarial parasite infecting all blood and blood forming cells of birds. Journal of Infectious Diseases, 57, 231-249.

HUFF, C.G. and COULSTON, F. (1944). The development of Plasmodium gallinaceum from sporozoite to erythrocytic trophozoite. Journal of Infectious Diseases, 75, 231-249.

HUFF, C.G. & MARCHBANK, D.F. (1955). Changes in infectiousness of malarial gametocytes. I. Patterns of oocyst production in seven host-parasite combinations. Experimental Parasitology, 4, 256-270.

HUFF, C.G., MARCHBANK, D.F. & SHIROISHI, T. (1958). Changes in infectiousness of malarial gametocytes. II. Analysis of the possible causative factors. Experimental Parasitology, 7, 399-417.

HUFF, C.G., PIPKIN, A.C., WEATHERSBY, A.B. and JENSEN, D.V. (1960). The morphology and behaviour of living exoerythrocytic stages of Plasmodium gallinaceum and P. fallax and their host cells. Journal of Biophysical and Biochemical Cytology, 7, 93-102.

INBAR, M. (1969). Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells. Proceedings of the National Academy of Sciences of the United States of America, 63, 1418-1425

INBAR, M., RABINOWITZ, Z. and SACHS, L. (1969). The formation of variants with a reversion of properties of transformed cells. III. Reversion of the structure of the cell surface membrane. International Journal of Cancer, 4, 690-696.

INFANTE, R.B., HERNANDEZ, A.G., RIGGIONE, F. and DAWIDOWICZ, K. (1980). A new method for the partial purification of Leishmania amastigotes from cutaneous lesions. Parasitology, 80, 105-112.

INOUE, M. (1974). Cell agglutination mediated by Concanavalin A and the dynamic state of the cell surface. Journal of Cell Science, 14, 197-202.

JACKSON, P.R. (1977). Lectin binding by Trypanosoma equiperdum. Journal of Parasitology, 63, 8-14.

JACKSON, P.R. and FISHER, F.M. (1975). Lectin binding of Trypanosoma equiperdum. Journal of Parasitology, 61, 85.

JACKSON, P.R., HONIGBERG, B.M. and HOLT, S.C. (1978). Lectin analysis of Trypanosoma congolense bloodstream trypomastigote and culture procyclic surface saccharides by agglutination and electron microscope techniques. Journal of Protozoology, 25, 471-481.

JAMES, S.P. (1931). Some general results of a study of induced malaria in England. Transactions of the Royal Society of Tropical Medicine and Hygiene, 24, 477-525.

JAMES, S.P. and TATE, P. (1937a). New knowledge of the life cycle of malaria parasites. Nature, London, 139, 545- 546.

JAMES, S.P. and TATE, P. (1937b). Preparations illustrating the recently discovered cycle of avian malaria parasites in reticulo-endothelial cells. Transactions of the Royal Society of Tropical Medicine and Hygiene, 31, 4-5.

JAMES, S.P. and TATE, P. (1938). Exoerythrocytic schizogony in P. gallinaceum Brumpt, 1935. Parasitology, 30, 128-139.

JENSEN, D.V., HUFF, C.G. and SHIROISHI, T. (1964). The use of Rose multipurpose chambers and dialysis membranes in the cultivation of exoerythrocytic stages of avian malarial parasites. American Journal of Tropical Medicine and Hygiene, 13, 653-658.

JERACE, F. (1937). Sul potere infettante delgi sporozoiti di Plasmodium relictum. Rivista di Malariologia, 16, 398-403.

JONES, T.C. and HIRSCH, J.G. (1972). The interaction between Toxoplasma gondii and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. Journal of Experimental Medicine, 136, 1173-1194.

JONES, T.C., YEH, S. and HIRSCH, J.G. (1972). The interactions between Toxoplasma gondii and mammalian cells. 1. Mechanisms of entry and intracellular fate of the parasite. Journal of Experimental Medicine, 136, 1157-1172.

KHAN, R.A., DESSER, S.S. and FALLIS, A.M. (1969). Survival of sporozoites of Leucocytozoon in birds for 11 days. Canadian Journal of Zoology, 47, 347-350.

KILEJIAN, A. (1976). Does a histidine-rich protein from Plasmodium lophurae have a function in merozoite penetration? Journal of Protozoology, 23, 272-277.

KILKUTH, W. and MUDROW, L. (1938). Malariaübertragungsversuche mit Blut and Organen Sporozozoiteninfizierter Kanarienvogel, Rivista di Malariologie, 17, 1-14 (from Huff and Coulston, 1944).

KILKUTH, W. and MUDROW, L. (1939). Die Entwicklung der sporozoiten von P. cathemerium im Kanarienvogel. Zentralblatt für Bakteriologie, Parasitenkunde, Infecktionskrankheiten und Hygiene (Abteilung 1), 145, 81-88 (from Huff and Coulston, 1944).

KILLICK-KENDRICK, R. (1971). The collection of strains of murine malaria parasites in the field, and their maintenance in the laboratory by cyclical passage. In "Symposium of the British Society of Parasitology", No. 9. (A. Taylor and R. Muller, eds.). Blackwell Scientific Publications, Oxford, pp. 39-64.

KILLICK-KENDRICK, R. (1973). Parasitic protozoa of the blood of rodents. 1. The life cycle and zoogeography of Plasmodium berghei nigeriensis subsp. nov. Annals of Tropical Medicine and Parasitology, 67, 261-277.

KILLICK-KENDRICK, R. (1974). Parasitic protozoa of the blood of rodents: a revision of P. berghei. Parasitology, 69, 225-237.

KILLICK-KENDRICK, R. and PETERS, W. (1978). "Rodent malaria". Academic Press, London.

KOJIMA, K. & MAEKAWA, A. (1970). Difference in electrokinetic charge of cells between two cell types of ascites hepatoma after removal of sialic acid. Cancer Research, 30, 2858-2862.

KONIGK, E. and MIRTSCH, S. (1977). Plasmodium chabandi infection of mice: specific activities of erythrocyte membrane - associated enzymes and patterns of proteins and glycoproteins of erythrocyte membrane preparations. Zeitschrift für Tropenmedizin und Parasitologie, 28, 17-22.

KRETTLI, A., CHEN, D.H. & NUSSENZWEIG, R.S. (1973). Immunogenicity and infectivity of sporozoites of mammalian malaria isolated by density - gradient centrifugation. Journal of Protozoology, 20, 662-665.

KRIER, J.P. (1977). The isolation and fractionation of malaria-infected cells. Bulletin of the World Health Organization, 55, 317-333.

KRISHNAMURTI, P.V., PEARDON, D.L., TODD, A.C. and McGIBBON, W.H. (1961). Natural occurrence of a Plasmodium in chickens in Wisconsin. Technical Abstracts of the University of Wisconsin. (from Garnham, 1966a).

KROTOSKI, W.A., KROTOSKI, D.M., GARNHAM, P.C.C., BRAY, R.S., KILLICK-KENDRICK, R., DRAPER, C.C., TARGETT, G.A.T. and GUY, M.W. (1980). Relapses in primate malaria: discovery of two populations of exoerythrocytic stages. Preliminary note. British Medical Journal, 153-155.

LACKIE, A.M. (1980). Invertebrate immunity. Parasitology, 80, 393-412.

LADDA, R.L., AIKAWA, M. and SPRINZ, H. (1969). Penetration of erythrocytes by merozoites of mammalian and avian malarial parasites. Journal of Parasitology, 55, 633-644.

LAIRD, R.L., DUBLIN, I.N. and DRINNON, V.P. (1950). The infection of chicks with pre-erythrocytic stages of Plasmodium gallinaceum grown in tissue culture. Journal of the National Malaria Society, 9, 128-131.

LANGRETH, S.G. (1975). Trophozoite growth in an intraerythrocytic malaria parasite, Plasmodium lophurae. Journal of Cell Biology, 67, 234A.

LANHAM, S.M. (1968). Separation of trypanosomes from the blood of infected rats and mice by anion exchangers. Nature, London, 218, 1273-1274.

LANHAM, S.M. & GODFREY, D.G. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Experimental Parasitology, 28, 521-534.

LAVERAN, A. (1880). Note sur un nouveau parasite trouvé dans le sang de plusieurs malades atteints de fiévere palustre. Bulletin Academie de Medicine, Paris, 9, 1235-1236. (from Garnham, 1966a).

LEE, K.C. (1972). Cell electrophoresis of the cellular slime mould Dictyostelium discoideum I. Characterization of some of the cell surface ionogenic groups. Journal of Cell Science, 10, 229-248.

LIGHTMAN, M.A. & WEED, R.I. (1970). Electrophoretic mobility and N-acetyl-neuraminic acid content of normal human and leukemic lymphocytes and granulocytes. Blood, 35, 12-22.

LIS, H. and SHARON, N. (1973). The biochemistry of plant lectins (phytohaemagglutins). Annual Review of Biochemistry, 43, 541-574.

LONG, P.L. and SPEER, C.A. (1977). Invasion of host cells by coccidia. In "Symposium of the British Society of Parasitology", No. 15 (A. Taylor and R. Muller, eds.). Blackwell Scientific Publications, Oxford, pp. 1-26.

LOOR, F. (1974). Binding and redistribution of lectins on lymphocyte membranes. European Journal of Immunology, 4, 210-220.

LUFF, J.H. (1971). Ruthenium red and violet. II. Fine structural localization in animal tissues. Anatomical Record, 171, 369-416.

LUMSDEN, W.H.R. & BERTRAM, D.S. (1940). Observations on the biology of Plasmodium gallinaceum, Brumpt, 1935, in the domestic fowl, with special reference to the production of gametocytes and their development in Aedes aegypti (L). Annals of Tropical Medicine and Parasitology, 34, 135-160.

LYCKE, E., CARLBERG, K. and NORRBY, R. (1975). Interactions between Toxoplasma gondii and its host cells: function of the penetration - enhancing factor of Toxoplasma. Infection and Immunity, 11, 853-861.

MACK, S.R. and VANDERBERG, J.P. (1978). Plasmodium berghei: energy metabolism of sporozoites. Experimental Parasitology, 46, 317-322.

MACK, S.R., SAMUELS, S. and VANDERBERG, J.P. (1979a). Hemolymph of Anopheles stephensi from non-infected and Plasmodium berghei infected mosquitoes. 2. Free amino acids. Journal of Parasitology, 65, 130-136.

MACK, S.R., SAMUELS, S. and VANDERBERG, J.P. (1979b). Hemolymph of Anopheles stephensi from non-infected and Plasmodium berghei - infected mosquitoes. 3. Carbohydrates. Journal of Parasitology, 65, 217-221.

MACK, S.R., VANDERBERG, J.P. and NAWROT, R. (1978). Column separation of Plasmodium berghei sporozoites. Journal of Parasitology, 64, 166-168.

MARQUARDT, M.D. and GORDON, J.A. (1975). Glutaraldehyde fixation and the mechanism of erythrocyte agglutination by concanavalin A and soybean agglutinin. Experimental Cell Research, 91, 310-316.

MARTINEZ-PALOMA, A., GONZALES-ROBLES, A. and DE LA TORRE, M. (1973). Selective agglutination of pathogenic strains of Entamoeba histolytica induced by Con A. Nature, London, 245, 186-187.

MASON, S.J., MILLER, L.H., SHIROISHI, T. DVORAK, J.A. and McGINNISS, M.H. (1977). The Duffy blood group determinants: Their role in the susceptibility of human and erythrocytes to Plasmodium knowlesi malaria. British Journal of Haematology, 36, 327-335.

MAYHEW, E. and WEISS, L. (1968). Ribonucleic acid at the periphery of different cell types, and effect of growth rate on ionogenic groups in the periphery of cultured cells. Experimental Cell Research, 50, 441-453.

MAZZEN, L., GULL, K. and GUTTERIDGE, W.E. (1975). A freeze-fracture study of the host/parasite interface of the malaria parasite Plasmodium chabaudi. Journal of Protozoology, 22, 54A.

MEHRISHI, J.N. (1970). Positively charged amino groups on the surface of normal and cancer cells. European Journal of Cancer, 6, 127-137.

MEHRISHI, J.N. (1972). Molecular aspects of the mammalian cell surface. Progress in Biophysics and Molecular Biology, 25, 3-68.

METTAM, R.W.M. (1943). Report of the Veterinary Department of Nigeria, 1941. Appendix 9. (from Garnham, 1966a).

MEZOELY, C.A.M., STEERE, R.L. and BAHR, G.F. (1972). Morphologic studies on the freeze-etched avian malaria parasite Plasmodium gallinaceum. Proceedings of the Helminthological Society of Washington, 39, 149-173.

MILDER, R. and KLOETZEL, J. (1980). The development of Trypanosoma cruzi in macrophages in vitro. Interaction with lysosomes and host cell fate. Parasitology, 80, 139-145.

MILLER, L.H. (1977). Hypothesis on the mechanism of invasion of erythrocytes by malaria merozoites. Bulletin of the World Health Organization, 55, 157-162.

MILLER, L.H., AIKAWA, M. and DVORAK, J.A. (1975). Malaria (Plasmodium knowlesi) merozoites: immunity and the surface coat. Journal of Immunology, 114, 1237-1242.

MILLER, L.H., McAULIFFE, F.M. and MASON, S.J. (1977). Erythrocyte receptors for malaria merozoites. American Journal of Tropical Medicine and Hygiene, 26, 204-208.

MILLER, L.H., AIKAWA, M., JOHNSON, J.G. and SHIROISHI, T. (1979). Interaction between cytochalasin B-treated malarial parasites and erythrocytes. Attachment and junction formation. Journal of Experimental Medicine, 149, 172-184.

MILLER, L.H., DVORAK, J.A. SHIROISHI, T. and DUROCHER, J.R. (1973). Influence of erythrocyte membrane components on malaria merozoite invasion. Journal of Experimental Medicine, 138, 1597-1601.

MILLER, L.H., MASON, S.J., CLYDE, D.F. and McGINNISS, M.H. (1976). The resistance factor to Plasmodium vivax in blacks. The Duffy blood-group genotype, Fy Fy. New England Journal of Medicine, 295, 302-304.

MILLER, L.H., POWERS, K.G., FINNERTY, J. and VANDERBERG, J.P. (1973). Difference in surface charge between host cells and malarial parasites. Journal of Parasitology, 59, 925-927.

MILLER, L.H., MASON, S.J., DVORAK, J.A., McGINNIS, M.H. and ROTHMAN, I.K. (1975). Erythrocyte receptors for (Plasmodium knowlesi) malaria: Duffy blood group determinants. Science, 189, 561-563.

MILLER, L.H., HAYNES, J.D., McAULIFFE, F.M., SHIROISHI, T., DUROCHER, J.R. and McGINNIS, M.H. (1977) Evidence for differences in erythrocyte surface receptors for the malarial parasites, Plasmodium falciparum and Plasmodium knowlesi. Journal of Experimental Medicine, 146, 277-281.

MISSIROLI, A. (1937a). Sullo sviluppo degli parassiti malarici. III. nota. Rivista di Malariologia, 16, 99.

MISSIROLI, A. (1937b). Sullo sviluppo delgi sporozoiti di Plasmodium praecox. Rivista di Malariologia, 16, 181-184.

MOORHOUSE, D.E. and WHARTON, R.H. (1962). Haemamoeba (= Plasmodium) gallinaceum in Malaya. Medical Journal of Malaya, 17, 93.

MOSER, G., BROHN, F.H., DANFORTH, H.D. & NUSSENZWEIG, R.S. (1978). Sporozoites of rodent and simian malaria, purified by anion exchangers, retain their immunogenicity and infectivity. Journal of Protozoology, 25, 119-124.

MUDROW, L. (1940). Klinische und Parasitologische befunde und Chemotherapeutische ergebnisse bei der Hühner-malaria. Archiv für Schiffbau und Tropenhygiene, 44, 257-275. (from Bray, 1957).

MULLIGAN, H.W., RUSSELL, P.F. and MOHAN, B.N. (1941). Active immunisation of fowls against Plasmodium gallinaceum by injections of killed homologous sporozoites. Journal of the National Malaria Institute of India, 4, 25-34.

MUSACCHIO, O. and MEYER, H. (1955). Plasmodium gallinaceum in tissue culture. Observations after one year of cultivation. Parasitology, 45, 1-4.

McGHEE, R.B. (1953). The infection by Plasmodium lophurae of duck erythrocytes in the chick embryo. Journal of Experimental Medicine, 97, 773-782.

McLAREN, D.J., BANNISTER, L.H., TRIGG, P.L. and BUTCHER, G.A. (1977). A freeze-fracture study on the parasite/erythrocyte interrelationship in Plasmodium knowlesi infections. Bulletin of the World Health Organization, 55, 199-203.

McLAREN, D.J., BANNISTER, L.H., TRIGG, P.I. and BUTCHER, G.A. (1979). Freeze fracture studies on the interaction between the malaria parasite and the host erythrocyte in Plasmodium knowlesi infections. Parasitology, 79, 125-139.

NARDIN, E.H. and NUSSENZWEIG, R.S. (1978). Stage - specific antigens on the surface membrane of sporozoites of malaria parasites. Nature, London, 274, 55-57.

NARDIN, E.H., NUSSENZWEIG, R.S., McGREGOR, I.A. and BRYAN, J.H. (1979). Antibodies to sporozoites: their frequent occurrence in individuals living in an area of hyperendemic malaria. Science, 206, 597-599.

NERI, P. (1937). Il potere infettante delgi sporozoiti di Plasmodium relictum prima del loro ingresso nelle ghiandole salivare. Rivista di Malariologia, 16, 461-464.

NICOLSON, G.L. (1974). The interactions of lectins with animal cell surfaces. International Review of Cytology, 39, 89-190.

NIJHOUT, M.M. (1979). Plasmodium gallinaceum: exflagellation stimulated by a mosquito factor. Experimental Parasitology, 48, 75-80.

NIJHOUT, M.M. and CARTER, R. (1978). Gamete development in malaria parasites: bicarbonate dependant stimulation by pH in vitro. Parasitology, 76, 39-55.

NILES, W.J., FERNANDO, M.A. and DISSANAIKE, A.S. (1965). Mansonia crassipes as the natural vector of filaroids, Plasmodium gallinaceum and other plasmodia of fowls in Ceylon. Nature, London, 205, 411-412.

NUSSENZWEIG, R.S. (1977). Immunoprophylaxis of malaria: sporozoite-induced immunity. In "Immunity to blood parasites of animals and man" (L.H. Miller, J.A. Pino and J.J. McKelvey, eds.). Plenum Publishing Corporation, New York. pp. 75-87.

NUSSENZWEIG, R.S., COCHRANE, A.H. and LUSTIG, H.J. (1978). Immunological responses. In "Rodent Malaria" (R. Killick-Kendrick and W. Peters, eds.). Academic Press, London. pp. 247-307.

NUSSENZWEIG, R.S., VANDERBERG, J.P., MOST, H. and ORTON, C. (1969). Specificity of protective immunity induced by X-irradiated P. berghei sporozoites. Nature, London, 222, 488-489.

OELERICH, S. (1967). Comparative studies on the occurrence of malaria sporozoites in the salivary glands and other organs of mosquitoes. Zeitschrift für Tropenmedizin und Parasitologie, 285-291.

OFEK, I., BEACHEY, E.H. JEFFERSON, W. and CAMPBELL, G.L. (1975). Cell membrane-binding properties of group A streptococcal lipoteichoic acid. Journal of Experimental Medicine, 141, 990-1003.

OMAR, A.R., ISMAIL, Y. and LIM, S.Y. (1962). Plasmodium gallinaceum infections among chickens in Malaya. Journal of the Malayan Veterinary and Medical Associations, 3, 75-80, 81-104.

PACHERO, N.D., STROME, C.P., MITCHELL, F., BAWDEN, M.P. and BEAUDOIN, R.L. (1979). Rapid, large-scale isolation of Plasmodium berghei sporozoites from infected mosquitoes. Journal of Parasitology, 65, 414-417.

PARAENSE, L., MEYER, H. and MENEZES, V. (1942). Estudos sobre Plasmodium gallinaceum. Comportamento dos esporozoites em cultura de baco embrionario. Revista Brasileira de Biologia 2, 89-94.

PARDOE, G.J., JAQUET, H., HAHN, R. and WAJNAROWSKI, W. (1975). The immunochemistry of surface antigens of Leishmania enrietti. Behring Institute Mitteilungen, 58, 30-39.

PATINKIN, D., SCHLESSINGER, M. & DOLJANSKI, F. (1970). A study of surface ionogenic groups of different types of normal and leukemic cells. Cancer Research, 30, 489-497.

PAUL, J. (1966). "Cell and tissue culture". 4th edition. Churchill Livingstone, Edinburgh.

PEARSON, T.W., ROELANTS, G.E., LUNDIN, L.B. and MAYOR-WITHEY, K.S. (1979). The bovine lymphoid system: binding and stimulation of peripheral blood lymphocytes by lectins. Journal of Immunological Methods, 26, 271-282.

PETERS, W. (1970). "Chemotherapy and drug resistance in malaria". Academic Press, London and New York.

PINTO DA SILVA, P. and MARTINEZ-PALOMO, A. (1974). Induced redistribution of membrane particles, anionic sites and con A receptors in Entamoeba histolytica. Nature, London, 249, 170-171.

PIPKIN, A.C. & JENSEN, D.V. (1956). Avian embryos and tissue culture in the study of parasitic protozoa. I. Malaria parasites. Experimental Parasitology, 7, 491-530.

PONDER, E. (1951). Effects produced by trypsin on certain properties of the human red cell. Blood, 6, 350-356.

PORTER, R.J. (1948). Studies in tissue culture of exoerythrocytic schizogony in avian malarial parasites. Journal of Parasitology, 34, 300-305.

PORTER, R.J., LAIRD, R.L. and DUSSEAU, E.M. (1954). Studies on malarial sporozoites. II Effect of age and dosage of sporozoites on their infectiousness. Experimental Parasitology, 3, 267-274.

PRINGLE, G. (1965). A count of the sporozoites in an oocyst of Plasmodium falciparum. Transactions of the Royal Society of Tropical Medicine and Hygiene, 59, 289-290.

RAFFAELE, G. (1934). Sul comportamento delgi sporozoiti nel sangue dell' ospite. Rivista di Malariologia (from Huff and Coulston, 1944).

RAFFAELE, G. (1936). Potere infettante del sangue durante l'incubazione della malaria aviaria. Rivista di Malariologia, 15, 77-87. (from Huff and Coulston, 1944).

RAFFAELE, G. (1937). Sullo sviluppo iniziale dei parassiti malarici nell 'ospite vertebrato. Rivista di Malariologia, 16, 185-198. (from Garnham, 1966a).

RAFFAELE, G. (1955). Alcune osservazioni sulla vitalitá delgi sporozoiti. Rivista di Malariologia, 34, 215-226.

REICHENOW, E. and MUDROW, L. (1943). Der Entwicklungsgang von Plasmodium praecox im Volgelkörper. Deutshe Tropenmedizinische Zeitschrift, 47, 289-299.

REISNER, Y., LIS, H. and SHARON, N. (1976). On the importance of the binding of lectins to cell surface receptors at low lectin concentrations. Experimental Cell Research, 97, 445-448.

RENWRANTZ, L.R. and CHENG, T.C. (1977). Agglutinin-mediated attachment of erythrocytes to hemocytes of Helix pomatia. Journal of Invertebrate Pathology, 29, 97-100.

RHODHAIN, J., GAVRILOV, W. and COWEZ, S. (1940). Essais d'infection de culture de tissues d'embryon de poulet par les sporozoites de Plasmodium gallinaceum. Comptes rendus des Seances de la Société de Biologie, 134, 261-264.

RICHARDS, W.H.G. (1966). Active immunisation of chicks against P. gallinaceum by activated homologous sporozoites and erythrocytic parasites, Nature, London, 212, 257-259.

RIECKMAN, K.H., CARSON, P.E., BEAUDOIN, R.L., CASSELLS, J.S. and SELL, K.W. (1974). Sporozoite induced immunity in man against an Ethiopian strain of Plasmodium falciparam. Transactions of the Royal Society of Tropical Medicine and Hygiene, 68, 258-259.

ROCK, R.C., STRANDEFER, J.C., COOK, R.T., LITTLE, W. and SPRINZ, H. (1971). Lipid composition of Plasmodium knowlesi membranes: comparison of parasites and microsomal subfractions with host rhesus erythrocyte membranes. Comparative Biochemistry and Physiology, 38, 425-437.

ROSALES-RONQUILLO, M.C. and SILVERMAN, P.H. (1974). In vitro development of the rodent malarial parasites, Plasmodium berghei. Journal of Parasitology, 60, 819-824.

ROSS, R. (1897). On some peculiar pigmented cells found in two mosquitoes fed on malarial blood. British Medical Journal, 2, 1786-1788.

ROSS, R. (1898). Report on the cultivation of Proteosoma, Labbé, in grey mosquitoes. Indian Medical Gazette, 33, 401-408, 448-451. (from Garnham, 1966a).

RUDZINSKA, M.A. (1976). Ultrastructure of intraerythrocytic Babesia microti with emphasis on the feeding mechanism. Journal of Protozoology, 23, 224-233.

RUDZINSKA, M.A., TRAGER, W., LEWENGRUB, S. and GUBERT, E. (1975). Invasion of Babesia microti into erythrocytes. Journal of Protozoology, 22, 28A.

RUSSELL, P.F., MULLIGAN, H.W. and MOHAN, B.N. (1941). Specific agglutinogenic properties of inactivated sporozoites of P. gallinaceum. Journal of the National Malaria Institute of India, 4, 15-24.

SANDICCHI, G. (1937). Contributo alla conoscenza della biologia delgi sporozoiti degli ematozoi malarici. Rivista di Malariologia, 16, 111-117.

SANDICCHI, G. (1938). Sul potere infettante delgi sporozoiti immaturi. Rivista di Malariologia, 17, 78-82.

SCHAUDINN, F. (1903). Studien über Krankheitserregende Protozoen. Arbeiten aus dem K. Gesundheitsamte, 19, 169-250.

SCHNEIDER, I. (1968). Cultivation in vitro of Plasmodium gallinaceum oocysts. Experimental Parasitology, 22, 178-186.

SCHNEIDER, I. & CHEN, D.H. (1969). Isolation of malaria sporozoites on Renografin serum albumin gradients. American Zoologist, 9, 501.

SCHOLTYSECK, E. and MEHLHORN, H. (1970). Ultrastructural study of characteristic organelles (paired organelles, micronemes, micropores) of sporozoa and related organisms. Zeitschrift für Parasitenkunde, 34, 97-129.

SCHOTTELIUS, J. (1976). Die wirkung von lektinen, proteklinen und blutgruppentestseren auf verschiedene Trypanosomenarten. Zeitschrift für Parasitenkunde, 50, 182-183.

SEAMAN, G.V.F. and COOK, G.M.W. (1965). Modification of the electrophoretic behaviour of the erythrocyte by chemical and enzymatic methods. In "Cell electrophoresis" (E.J. Ambrose, ed.). Churchill, London. pp. 48-65.

SEAMAN, G.V.F. and HEARD, D.H. (1960). The surface of the washed human erythrocyte as a polyanion. Journal of General Physiology, 44, 251-268.

SEAMAN, G.V.F. & HEARD, D.H. (1961). A microelectrophoresis chamber of small volume for use with biological systems. Blood, 18, 599-604.

SEED, T.M. and KRIER, J.P. (1976). Surface properties of extracellular malaria parasites: Electrophoretic and lectin-binding characteristics. Infection and Immunity, 14, 1339-1347.

SEED, T.M., AIKAWA, M. and STERLING, C.R. (1973). An electron-microscope cytochemical method for differentiating membranes of host red cells and malaria parasites. Journal of Protozoology, 20, 603-605.

SEED, T.M., SEED, J.R. and BRINDLEY, D. (1976). Surface properties of bloodstream trypanosomes (Trypanosoma brucei brucei). Zeitschrift für Tropenmedizin und Parasitologie, 27, 202-212.

SEED, T.M., AIKAWA, M., STERLING, C. and RABBEGE, J. (1974). Surface properties of extracellular malaria parasites: morphological and cytochemical study. Infection and Immunity, 9, 750-761.

SEED, T., PFISTER, R., KRIER, J. and JOHNSON, A. (1971). Plasmodium gallinaceum: fine structure by freeze-etch technique. Experimental Parasitology, 30, 73-81.

SEED, T.M., AIKAWA, M., PRIOR, R.B., KRIER, J.P. and PFISTER, R.M. (1973). Plasmodium sp: topography of intra- and extracellular parasites. Zeitschrift für Tropenmedizin und Parasitologie, 24, 525-535.

SENEVIRATNA, P. (1959). An outbreak of bird malaria in a flock of domestic fowls. Ceylon Veterinary Journal, 6, 51-52.

SERGENT, ET. (1940). A la différence de l'hôte vertébré, la Culex infecté de Plasmodium relictum n'accquiert pas la prémunition contre une réinfection par le même Plasmodium. Archives des Instituts Pasteur de Algéria, 18, 295-298.

SERGENT, ED. and SERGENT, ET. (1918). Sur le paludisme des oiseaux du au Plasmodium relictum (vel Proteosoma). Annales de l'Institut Pasteur, Paris, 32, 382-388.

SERGENT, ED. and SERGENT, ET. (1922). Étude expérimentale du paludisme des oiseaux. (Plasmodium relictum). Suite des recherches sur l'action de la quinne XX11e note. Archives des Instituts Pasteur de l'Afrique du Nord, 2, 320-329.

SETHI, K.K., RAHMAN, A., PELSTER, B. and BRANDIS, H. (1977). Search for the presence of lectin-binding sites on Toxoplasma gondii. Journal of Parasitology, 63, 1076-1080

SHAKESPEARE, P.G., TRIGG, P.I. and TAPPENDEN, L. (1979). Some properties of membranes in the simian malaria parasite Plasmodium knowlesi. Annals of Tropical Medicine and Parasitology, 73, 333-343.

SHARON, N. and LIS, H. (1972). Cell agglutinating and sugar specific proteins. Science, 177, 949-959.

SHAW, D.J. (1969). "Electrophoresis". Academic Press, New York.

SHERBET, G.V. (1978). "The biophysical characterisation of the cell surface". Academic Press, London.

SHERMAN, I.W. (1966). In vitro studies of factors affecting penetration of duck erythrocytes by avian malaria (Plasmodium lophurae). Journal of Parasitology, 52, 17-22.

SHERMAN, I.W. and JONES, L.A. (1979). Plasmodium lophurae: membrane proteins of erythrocyte-free plasmodia and malaria-infected red cells. Journal of Protozoology, 26, 489-501.

SHORTT, H.E. and GARNHAM, P.C.C. (1948). The pre-erythrocytic development of P. cynomolgi and P. vivax. Transactions of the Royal Society of Tropical Medicine and Hygiene, 41, 485-495.

SHORTT, H.E., MENON, K.P. and IYER, P.V.S. (1940). The form of P. gallinaceum present in the incubation period of infection. Indian Journal of Medical Research, 28, 273-276.

SHORTT, H.E., MENON, K.P. and IYER, P.V.S. (1941). The natural host of P. gallinaceum (Brumpt, 1935). Journal of the Malaria Institute of India, 4, 175-178.

SHORTT, H.E., FAIRLEY, N.H., COVELL, G., SHUTE, P.G. and GARNHAM, P.C.C. (1951). The pre-erythrocytic stage of P. falciparum. Transactions of the Royal Society of Tropical Medicine and Hygiene, 44, 405-419.

SHUTE, P.G. (1943). Successful transmission of human malaria with sporozoites which have not come into contact with the salivary glands of the insect host, Journal of Tropical Medicine and Hygiene, 46, 57-58.

SHUTE, P.G. (1945). An investigation into the number of sporozoites in the salivary glands of Anopheles mosquitoes. Transactions of the Royal Society of Tropical Medicine and Hygiene, 38, 493-498.

SHUTE, P.G. & MARYON, M. (1966). "Laboratory techniques for the study of malaria". 2nd edition, Churchill, London.

SHUTE, P.G., LUPASCU, G.H., BRANZEI, P., MARYON, M., CONSTANTINESCU, P., BRUCE-CHWATT, L.J., DRAPER, C.C., KILLICK-KENDRICK, R. and GARNHAM, P.C.C. (1976). A strain of Plasmodium vivax characterized by prolonged incubation: the effect of numbers of sporozoites on the length of the prepatent period. Transactions of the Royal Society of Tropical Medicine and Hygiene, 70, 474-481.

SINDEN, R.E. (1974). Excystment by sporozoites of malaria parasites. Nature, London, 252, 314.

SINDEN, R.E. (1975). The sporogonic cycle of Plasmodium yoelii nigeriensis: A scanning electron microscope study. Protistologica, 11, 31-39.

SINDEN, R.E. (1978). Cell biology. In "Rodent Malaria" (R. Killick-Kendrick and W. Peters, eds.). Academic Press, London. pp. 85-168.

SINDEN, R.E. (1980). Personal communication.

SINDEN, R.E. and GARNHAM, P.C.C. (1973). A comparative study on the ultrastructure of Plasmodium sporozoites within the oocyst and salivary glands, with particular reference to the incidence of the micropore. Transactions of the Royal Society of Tropical Medicine and Hygiene, 67, 631-637.

SINDEN, R.E. and SMITH, J. (1980). Culture of the liver stages (exoerythrocytic schizonts) of rodent malaria parasites from sporozoites in vitro. Transactions of the Royal Society of Tropical Medicine and Hygiene, 74, 134-136.

SINDEN, R.E. and STRONG, K. (1978). An ultrastructural study of the sporogenic development of Plasmodium falciparum in Anopheles gambiae. Transactions of the Royal Society of Tropical Medicine and Hygiene, 72, 477-791.

SINDEN, R.E., CANNING E.U. and SPAIN, B.J. (1976). Gametocytogenisis and fertilization in Plasmodium yoelii nigeriensis: a transmission electron microscope study. Proceedings of the Royal Society, London, B, 193, 55-76.

SINDEN, R.E., CANNING, E.U., BRAY, R.S. and SMALLEY, M.E. (1978). Gametocyte and gamete development in Plasmodium falciparum. Proceedings of the Royal Society of London, B, 201, 375-399.

SINGH, J., NAIR, C.P., DAVID, A. and KRISHNAN, K.S. (1954). Natural immunity of domestic pigeons (Columba livia Gmelin) to experimental infection with P. gallinaceum. Indian Journal of Malariology, 7, 117-126.

SMITHERS, S.R., TERRY, R.J. and HOCKLEY, D.J. (1968). Do adult schistosomes masquerade as their hosts? Transactions of the Royal Society of Tropical Medicine and Hygiene, 62, 466-467.

SPRINGER, G.P. (1970). Importance of red cell surface structures in reactions between man and microbes. In "Blood and tissue antigens" (D. Aminoff, ed.). Academic Press, New York, pp. 265-291.

STAHN, R., MAIER, K.P. and HANNIG, K. (1970). A new method for the preparation of rat liver lysosomes. Journal of Cell Biology, 46, 576-591.

STEIGER, R.F. (1975). Ultracytochemistry of the surface coat/pellicle complex in Trypanosoma brucei. Acta tropica, 32, 152-158.

STERLING, C.R., AIKAWA, M. and VANDERBERG, J.P. (1973). The passage of Plasmodium berghei sporozoites through the salivary glands of Anopheles stephensi: An electron microscope study. Journal of Parasitology, 59, 593-605.

STROME, C.P.A. and BEAUDOIN, R.L. (1974). The surface of the malaria parasite. I. Scanning electron microscopy of the oocyst. Experimental Parasitology, 36, 131-142.

STROME, C.P.A., DE SANTIS, P.L. and BEAUDOIN, R.L. (1979). The cultivation of the exoerythrocytic stages of Plasmodium berghei from sporozoites in vitro. In Vitro, 15, 531-536.

SUZUKI, M., SAWASAKI, Y., WAKI, S., IOKAWA, H., ASADA, T. and NAKAJIMA, H. (1979). Separation of Plasmodium berghei-parasitized rat erythrocytes by means of carrier-free electrophoresis. Bulletin of the World Health Organization, 57, 129-132.

TAKAHASHI, Y. and SHERMAN, I.W. (1978). Plasmodium lophurae: cationised ferritin staining, an electron microscope cytochemical method for differentiating malarial parasite and host cell membranes. Experimental Parasitology, 44, 145-154.

TEMMINK, J.H.M., COLLARD, J.G., SPITS, H. and ROOS, E. (1975). A comparative study of four cytochemical detection methods of concanavalin A binding sites on the cell membrane. Experimental Cell Research, 92, 307-322.

TERZAKIS, J.A. (1968). Uranyl acetate, a stain and a fixative. Journal of Ultructure Research, 22, 168-184.

TERZAKIS, J.A. (1972). Virus-like particles and sporozoite budding. Proceedings of the Helminthological Society of Washington, 39, 129-137.

TERZAKIS, J.A., SPRINZ, H. and WARD, R.A. (1967). The transformation of the Plasmodium gallinaceum oocyst in Aedes aegypti mosquitoes. Journal of Cell Biology, 34, 311-326.

TREMBLEY, H.L., GREENBERG, J. and COATNEY, G.R. (1951). Strain differences in Plasmodium gallinaceum. II. Experiences with the sporozoite and single oocyst passage of the B1 strain, Journal of the National Malaria Society, 10, 68-75.

UNANUE, E.R. and KARNOVSKY, M.J. (1974). Ligand-induced movement of lymphocyte membrane macromolecules: capping, cell movement and microtubular function in normal and lectin-treated lymphocytes. Journal of Experimental Medicine, 140, 1207-1220.

URQUHART, C.M. (1980). Tissue culture of coccidia. Ph.D. thesis, University of London.

VANDERBERG, J.P. (1974). Studies on the motility of Plasmodium sporozoites. Journal of Protozoology, 21, 527-537.

VANDERBERG, J.P. (1975). Development of infectivity by the Plasmodium berghei sporozoite. Journal of Parasitology, 61, 43-50.

VANDERBERG, J.P. (1977). Plasmodium berghei: quantitation of sporozoites injected by mosquitoes feeding on a rodent host. Experimental Parasitology, 42, 169-181.

VANDERBERG, J.P., WEISS, M.M. and MACK, S.R. (1977). In vitro cultivation of the sporogonic stages of Plasmodium: a review. Bulletin of the World Health Organization, 55, 375-390.

VANDERBERG, J.P., NUSSENZWEIG, R.S., SANABRIA, Y., NAWROT, R. and MOST, H. (1972). Stage specificity of anti-sporozoite antibodies in rodent malaria and its relationship to protective immunity. Proceedings of the Helminthological Society of Washington, 39, 514-525.

VARGAS, L. (1949). Culicine and aedine mosquitoes and the malaria infections of lower animals. In "Boyds Malariology", Vol. 1.

VASSAR, P.S., KENDALL, M.J. & SEAMAN, G.V.F. (1969). Electrophoresis of human leukocytes. Archives of Biochemistry and Biophysics, 135, 350-355.

VERMEULEN, A., MUNSTER, J.V. and MEUWISSEN, J.H.E. (in press). Purification and partial characterization of P. berghei sporozoites.

VICKERMAN, K. (1969). On the surface coat and flagellar adhesion in trypanosomes. Journal of Cell Science, 5, 163-193.

VON PROWAZEK (1912). Beiträge zur Kenntnis der Protozoen und verwandter Organismen von Sumatra. Archiv für Protistenkunde, 26, 250-274.

WALLACH, D.F.H. and CONLEY, M. (1977). Altered membrane proteins of monkey erythrocytes infected with simian malaria. Journal of Molecular Medicine, 2, 119-136.

WALLIKER, D. and ROBERTSON, E. (1970). Cultivation in vitro of sporozoites of Plasmodium berghei. Transactions of the Royal Society of Tropical Medicine and Hygiene, 64, 5.

WARD, R. (1963). Genetic aspects of the susceptibility of mosquitoes to malarial infections. Experimental Parasitology, 13, 328-341.

WARREN, L. (1963). The distribution of sialic acids in nature. Comparative Biochemistry and Physiology, 10, 153-171.

WARREN, A.J. and COGGLESHALL, L.T. (1937). Infectivity of blood and organs of canaries after inoculation of sporozoites. American Journal of Hygiene, 26, 1-10.

WEATHERSBY, A.B. (1952). The role of the stomach wall in the exogenous development of Plasmodium gallinaceum as studied by means of haemocoele injections of susceptible and refractory mosquitoes. Journal of Infectious Diseases, 91, 198-205.

WEATHERSBY, A.B. (1960). Experimental infection of Aedes with exoerythrocytic stages of Plasmodium gallinaceum. Experimental Parasitology, 9, 334-337.

WEATHERSBY, A.B. (1975). The haemocoel as barrier to parasitic infection in insects. In "Invertebrate Immunity" (K. Moramorosch and R.E. Shope eds.). Academic Press, New York and London. pp. 273-288.

WEIDENKAMM, E., WALLACH, D.F.H., LIN, P.S. and HENDRICKS, J. (1973). Erythrocyte membrane alterations due to infection with Plasmodium berghei. Biochimica et biophysica acta, 323, 539-546.

WEISS, M.M. and VANDERBERG, J.P. (1977). Studies on Plasmodium ookinetes II. In vitro formation of Plasmodium berghei ookinetes. Journal of Parasitology, 77, 932-934.

WERY, M. (1968). Studies on the sporogony of rodent malaria parasites. Annale de la Societé Belge de Médecine Tropicale, 48, 1-137.

WERY, M. and KILLICK-KENDRICK, R. (1967). Differences in the number of tissue schizonts of Plasmodium berghei yoelii in the livers of individual mice, and a comparison of two routes of inoculation of sporozoites. Transactions of the Royal Society of Tropical Medicine and Hygiene, 61, 447.

WONG, T.C. (1979). A ultrastructural study on the development of Leucocytozoon dubreuli Mathis and Leger, 1911 (Apicomplexa: Haemosporina) in its avian host, the American robin (Turdus migratorius). Ph.D. thesis, University of Toronto.

WONG, T.C. and DESSER, S.S. (1976). Fine structure of oocyst transformation and the sporozoites of Leucocytozoon dubreuili. Journal of Protozoology, 23, 115-126.

WORLD HEALTH ORGANISATION (1979). "The membrane pathobiology of tropical diseases". Schwabe and Co., Basel.

YOELI, M. (1964). Movement of the sporozoites of Plasmodium berghei. Nature, London. 201, 1344-1345.

YOELI, M. and BONÉ, G. (1967). Studies on Anopheles dureni, Edwards. Rivista di Malariologia, 43, 3-17.

YOELI, M. and MOST, H. (1960). The biology of a newly isolated strain of Plasmodium berghei in a rodent host and in experimental mosquito vectors. Transactions of the Royal Society of Tropical Medicine and Hygiene, 54, 549-555.

YORKE, W. and MacFIE, J.W.S. (1924). Observations on malaria made during treatment of general paralysis. Transactions of the Royal Society of Tropical Medicine and Hygiene, 18, 13-33.

YOSHIDA, N., NUSSENZWEIG, R.S., POTOCNJAK, P., NUSSENZWEIG, V. and AIKAWA, M. (1980). Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. Science, 207, 71-73.

ZALIK, S.E., SANDERS, E.J. and TILLEY, C. (1972). Studies on the surface of chick blastoderm cells. 1. Electrophoretic mobility and pH-mobility relationships. Journal of Cellular Physiology, 79, 225-234.

ZEILLER, K., HANNIG, K. and PASCHER, G. (1971). Free-flow electrophoretic separation of lymphocytes. Separation of graft versus host reactive lymphotcytes of rat spleens. Zeitschrift für Physiologische Chemie, 352, 1168-1173.

ZEILLER, K., LIEBICH, H.G. and HANNIG, K. (1971). Free-flow electrophoretic separation of lymphocytes. Two thoracic duct lymphocyte subpopulations studied after prolonged cannulation and immunization. European Journal of Immunology, 1, 315-321.

ZEILLER, K., HOLZBERG, E., PASCHER, G. and HANNIG, K. (1972). Free-flow electrophoretic separation of T and B lymphocytes. Evidence for various sub-populations of B cells. Zeitschrift für Physiologische Chemie, 353, 105-112.

ZUCKER-FRANKLIN, D., LIEBES, L.F. and SILBER, R. (1979). Differences in the behaviour of the membrane and membrane-associated filamentous structures in normal and chronic lymphocyte leukemia (CLL) lymphocytes. Journal of Immunology, 122, 97-107.

APPENDIX 1

1. Cryopreservation of erythrocytic parasites

1.1. Cryoprotectant

20% glycerol in PBS (Appendix 2).

1.2. Procedure

Blood from a rising parasitaemia of a sporozoite-induced infection was obtained in a syringe (wetted with heparin, 5,000 units/ml) from the jugular vein of the infected bird, mixed 1:1 with the cryoprotectant and left for 30 minutes at room temperature to equilibriate. The mixture was then divided into 1·0 ml portions, dispensed into small plastic ampoules and held in liquid nitrogen vapour for 1 hour before storage in cans submerged in liquid nitrogen.

For subsequent inoculations, ampoules were removed from the liquid nitrogen and held under running tap water until liquifaction was complete.

APPENDIX 2

2. Phosphate buffers

2.1. Phosphate buffer for Giemsa staining

Na_2HPO_4 (anhydrous)	3·0g
KH_2PO_4 (anhydrous)	0·6g
Distilled water	up to 1 litre

2.2. M/15 phosphate buffer (Sorrenson's) pH 7·4

M/15 KH_2PO_4	19·6ml
M/15 Na_2HPO_4	80·4ml

2.3. Phosphate buffer (Dulbecco's) pH 7·4

$CaCl_2$ (anhydrous)	0·1g
KCl	0·2g
KH_2PO_4	0·2g
$MgCl_2 \cdot 6H_2O$	0·1g
NaCl	8·0g
$Na_2HPO_4 \cdot 2H_2O$	1·15g

APPENDIX 3

3. Solutions for the Feulgen reaction

3.1. Schiff's reagent

2·5g of basic fuchsin was dissolved in 500 ml of boiling, distilled water. The mixture was allowed to cool to 52°C, filtered, and 50 ml of IN HCl was added. When cooled to 25°C, 2·5g of $Na_2S_2O_5$ was added and the solution put in a refrigerator over-night. The next day the solution was shaken with 5g of activated charcoal before being filtered, stored in the dark at 4°C, and kept not longer than 2 weeks.

3.2. Bisulphite rinse

Always to be made fresh

10% $Na_2S_2O_5$	10 ml
IN HCl	10 ml
Distilled water	200 ml

APPENDIX 4

Tissue culture medium used for the growth of exoerythrocytic schizonts of *P. berghei**

	ml
1. Diploid growth medium	
Medium 199 with L-glutamine + Hank's salts	50
Basal Medium Eagle (BME) without L-glutamine with 1% sodium bicarbonate (7·5%)	50
L-glutamine, 200 mM solution	0·5
Foetal calf serum	10
Nonessential amino acids 100X	1·0
Penicillin (5,000 units/ml)-streptomycin (5,000 µg/ml/solution	1·0
Folinic acid 10^{-5} M	1·0
2. Mouse liver medium	
Diploid growth medium (as above)	100
Basal medium Eagle vitamins 100X	1·0
Nonessential amino acids 100X	2·0
Minimum essential medium amino acids 100X	2·0
Sodium pyruvate 100 mM solution	1·0
Insulin 400 units/ml	0·25
Penicillin (5,000 units/ml)-streptomycin (5,000 µg/ml) solution	1·0
Sodium bicarbonate 7·5% wt/vol aqueous solution	3·0

* from Strome, De Santis and Beaudoin (1979).