The biology of *Plasmodium* in the mosquito

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Introduction

Development of *Plasmodium* wihin the mosquito encompasses some of the most divergent stages of parasite growth and is no less compex than the entire cycle in the vertebrate host. It is however far less intensely studied and will remain so until techniques for the in vitro culture of the sporogonic stages have been mastered.

The female mosquito draws blood infected with malarial gametocytes from the capillaries of the vertebrate host into her midgut, where within 8–15 min these sexual parasites have escaped the enveloping erythrocyte. The male gametocyte then produces 8 microgametes (sperm) and the female a single macrogamete (egg). The fertilized macrogamete (zygote) differentiates into a single motile ookinete over the next 10–25 h by a process comparable to both merozoite and sporozoite formation. The ookinete migrates from the bloodmeal through the midgut wall but fails to penetrate the enveloping basal lamina. The ensuing replicative oocysts thus develop between the basement membrane and the basal lamina of the midgut. Each oocyst produces many thousands of invasive sporozoites over a period of 7–12 days. The sporozoites escape from the oocyst and then invade the salivary glands, here they stay for possibly very long periods[72] until injected into another vertebrate host when the next bloodmeal is taken.

Parasite development

Gametocytes (figures 1 and 2)

The biological functions of the gametocytes are 2-fold: ecologically they are responsible for the transmission of the parasite from the vertebrate to the mosquito vector, biologically they are responsible for the sexual processes of the malarial parasite. Gametocyte organisation obviously reflects both these functions.

The gametocytes of *Plasmodium* are morphologically separable into 2 classes; those of mammalian origin and those of reptilian or avian origin. The exceptions are *P. reichenovi* and *P. falciparum* which despite their primate origin are of 'avian' morphology. Coincidentally these parasites are equally unusual in having a prolonged period of development and persistence in the peripheral circulation of their host. For most species mature gametocytes persist in the capillary blood only for a brief period, e.g., 24 h and are infective for only a part of this time[62] such that the 'infectivity' of the gametocytes coincides with the time of biting of the mosquito*. Whilst models proposing this transient infectivity suggest it is an innate property of the parasite other data must be considered. It has been shown that changes in the circadian rhythm of the host and/or surgical operations such as pinealectomy will change predictably both the patterns of infectivity and of parasite distribution in the host[58, 59, 119]. Thus the control of infectivity in these instances *may* be determined by an interaction of the host and parasite. The concept of directed infectivity[62] cannot hold for *P. falciparum*, the gametocyte in vivo has a long half life (2.4 days) and a prolonged period of infectivity[127], properties particularly evident when mosquitoes are infected from cultured parasites. Thus we must conclude that 2 strategies of infectivity have coevolved the 'directed' or 'active' strategy whereby the parasites muster finite resources for a brief period when the vector may be expected to bite; and the 'passive' approach where to the parasite following its maturation (capacitation) persists with minimal expenditure of energy for prolonged periods such that vectors will be infected irrespective of the times at which they take a bloodmeal.

Irrespective of their 'avian' or 'mammalian' sub-grouping, male and female gametocytes are each of highly conserved morphology[3, 120]. The female (macrogametocyte) has a small (and presumably haploid) nucleus within which lies a prominent nucleolus. Her cytoplasm contains extensive endoplasmic reticulum and numerous mitochondra. Additionally numerous small membrane bound electron dense osmiophilic bodies are

Figure 1. Transmission electron micrograph (TEM) of mature intraerythrocytic microgametocyte of *Plasmodium falciparum*. Adjacent to the small central nucleus (n) lie numerous large grains of electron dense pigment. Note also extensive endoplasmic reticulum, mitochondria and dense small osmiophilic bodies. × 18,000.

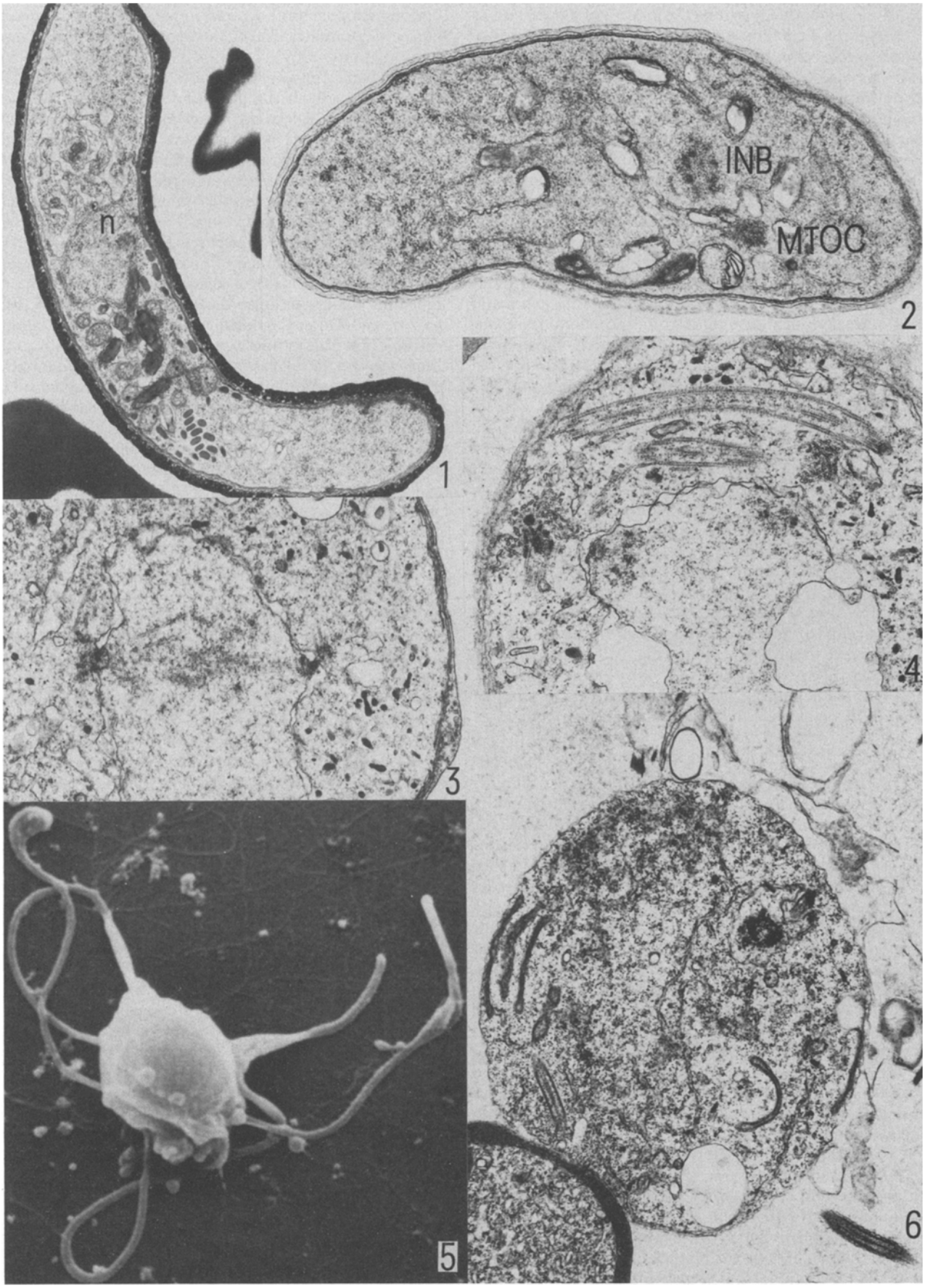
Figure 2. TEM of mature microgametocyte of *P. falciparum* in parasitophorous vacuole which almost totally occupies the host erythrocyte. The large digitate nucleus is surrounded by pigment crystals (leached vacuoles). Within the nucleus lies the intranuclear body (INB) which is located close to the cytoplasmic microtubule organizing center (MTOC). Note the paucity of mitochondria, endoplasmic reticulum and osmiophilic bodies. × 22,300.

Figure 3. First gametogenic nuclear division in activated microgametocyte of *P. yoelii*. The single large mitotic spindle traverses the nucleus and at each pole the spindle plaque is situated in a nuclear pore. Kinetosomes are present on the cytoplasmic face of these two nuclear pores. × 26,300.

Figure 4. Third gametic division in microgametocyte of *P. yoelii*. The 8 haploid genomes are connected via the small hemispindles to the cytoplasmic kinetosomes. The kinetosome has developed a terminal electron dense granule, and is surrounded by a basket of membranous tubules. Each of the 8 kinetosomes has developed a 22-μm long intracytoplasmic axoneme. × 32,800.

Figure 5. A scanning electron micrograph of an exflagellating microgametocyte of *P. yoelii* showing the microgametes emerging from the parental cell. × 8000.

Figure 6. Fertilized zygote of *P. berghei*. The condensed male nucleus is visible (top right) and the naked intracytoplasmic microgamete axoneme (bottom left). An oblique section of the axoneme of a free microgamete is seen (bottom right). × 16,000.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INB
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found, these organelles mediate the escape of the non-motile parasite from the erythrocyte – and may be anticipated to bear strong resemblances to the rhoptry and micronemes of the invasive stages.

The male (microgametocyte) contrasts with the female in having a large octoploid digitate nucleus within which lies an electron dense intranuclear body (INB) attached to a pore in the nuclear envelope. All the chromosomes are attached by their kinetochores to the INB. The INB extends through the nuclear pore and is continuous with an electron dense microtubule organizing center (MTOC) on the cytoplasmic face of the nuclear envelope. Within the cytoplasm there are relatively few ribosomes, a poorly developed endoplasmic reticulum – suggesting a limited potential for de novo protein synthesis; and few osmiophilic bodies.

Gametogenesis (figures 3–5)

Microgametogenesis in vitro is, optimally, dependent upon a number of environmental factors, e.g., a pH of 7.5–8.4 (with optima at pH 8.0 for avian and pH 7.8 for rodent parasites); a bicarbonate concentration of at least 30 mM[93] and a fall in temperature of at least 5 °C from that of the vertebrate host[101, 122]. Other influential factors include the addition of cyclic AMP analogues, or inhibitors of phosphodiesterase[85]; and mosquito exflagellation factor (MEF) – a small heat stable molecule found in the vector's head and gut[92]. Many of these properties can be correlated to the physical and chemical changes in the ingested bloodmeal, even MEF may be a neurotransmitter controlling bloodmeal digestion. The duration of microgametogenesis is both temperature and species dependent, e.g. at 20–22 °C it may take 7–15 min for *P. falciparum* in vitro[119], although exflagellation may be detected after shorter periods in the fluid excreted by feeding *Anopheles*.

Gametogenesis involves 2 simultaneous but independent events, escape from the host cell and the maturation and production of the gametes. Escape from the erythrocyte is mediated by the secretion of the contents of the osmiophilic bodies into the parasitophorous vacuole (PV), resulting in the disruption of host (but not parasite) membranes. Aikawa et al.[1] have suggested the female rapidly dissolves the PV membrane and plasmalemma, the male only disrupts the PVM. This observation must correlate with the abundance of osmiophilic bodies in the former, and with the fact that the vigorous lashing of the liberated microgametes breaks down the erythrocyte plasmalemma in the latter. Escape is also enhanced by a significant increase (× 3) in parasite volume which is recognizable cytologically as a decrease in density of the cytoplasm.

Macrogametogenesis at the morphological level involves little more than escape from the host cell. It is not apparently inhibited by actinomycin D[139] but is blocked by inhibitors of protein synthesis indicating that de novo protein synthesis occurs during macrogamete and zygote formation[78, 150], although whether these are *new* gene products remains unknown. Monoclonal antibodies can be made which are specific for the surface of the macrogamete and these have a transmission blocking (TB) potential, which suggests that proteins are newly, or more dominantly, expressed on the surface of the macrogamete as compared to the macrogametocyte. Briefly summarized microgametogenesis involves 3 incredibly rapid mitotic divisions[121] (in the absence of DNA replication) resulting in the segregation of 8 haploid genomes from the polyploid microgamtocyte. Simultaneously 8 22-μm-long axonemes are assembled on the cytoplasmic MTOC (which it will be recalled in physically connected to the intranuclear genome). This physical linkage ensures the incorporation of both the motile apparatus and the genome into the gamete as it shoots out of the cell surface (in 15–45 sec!).

The component events in microgametocytes are: – within seconds of activation the cytoplasmic MTOC produces 8 kinetosomes arranged as planer orthogonal tetrads; the axonemes immediately initiate assembly on the kinetosomes in the conventional manner, this polymerisation is susceptible to antimicrotubule agents e.g. colchicine or vinblastine[124]; within 3 min the INB splits and the 2 daughter structures (spindle poles) migrate within the persistent nuclear envelope to opposite sides of the nucleus, this automatically segregates the kinetosomes such that 4 lie at each spindle pole. Spindle pole migration is susceptible to the antimicrofilament agent cytochalasin B[124]. The next 2 divisions sequentially halve the kinetosome and genome number to produce 8 haploid hemispindles each associated with a single cytoplasmic kinetosome and fully assembled axoneme. At the final division the kinetosomes are surrounded by a helical basket of the tubules which extend down from the plasmalemma. Altough previously considered to be made of microtubules, the basket tubules are almost certainly membranes. The membranes may contribute significantly to the highly antigenic surface of the rapidly emerging microgamete. Microgamete formation is dependent upon de novo protein synthesis[139], and is sensitive to actinomycin D, however, the reason for this sensitivity has yet to be established.

At exflagellation, the axonemes become activated within the cytoplasm independently driving each microgamete, kinetosome first, out of the cell surface[117, 121]. The kinetosome drags the spindle pole and a condensed haploid genome into the gamete. The 'mature' gamete usually contains a single kinetosome, axoneme, and nucleus, however numerous aberrant forms have been described. The microgametes are torn from the microgametocyte surface and rapidly move away into the bloodmeal with a characteristic alternating activity pattern[7, 122] – an activity that in vitro is dependent upon a glucose source. Viability persists for 60 min in vitro, fertilization however often occurs within 20 min. No directed migration of the male to the female gamete has yet been demonstrated.

The gamete, and other sexual vaccines

The extracellular micro- and macrogametes, zygote and ookinete have been shown to be susceptible to the action of antibodies, and vertebrate phagocytic cells, in the mosquito bloodmeal. These actions are now being exploited in the development of the so-called 'altruistic' vaccines.

Immunisation of hosts with gametocytes[60], microgametes[19,87], mixed gamete preparations[19,21], macrogametes or zygotes[152] or ookinetes[78] induces an immunity which does not inhibit parasitemia but blocks transmission to the mosquito. Depending upon the antibody class this activity may involve complement factors[100], but usually involves agglutination or surface adhesion of the gametes[4,21]. Monoclonal antibodies produced against the gametes recognize high molecular weight molecules including one of approximately 230 kilodalton (kd)[99]. It is not known at present what the relationship is between these molecules and those of similar electrophoretic mobility (Mr) in the zygote[75], they can be detected in the macrogamete and the mature gametocytes[115,150]. Recent observations indicate significant differences in the protein composition of different isolates of *P.falciparum,* some differences being detectable in the 'transmission blocking' (surface) antigens[18,115]. Whilst of practical significance in the development of the altruistic vaccines these observations might suggest the presence of distinct gene pools within the *P.falciparum* population despite the suggestions of Walliker to the contrary[156]. Ultrastructural studies suggest the antigens are distributed over the entire surface of the gamete[4], nevertheless monoclonal antibody action is synergized if 2 or more are used in combination[99]. The exact biological role of the 'transmission blocking' antigens remains a mystery, however, they are the subject of intense study and are of interesting potential application.

Zygote formation and ookinete development (figures 6–8)

Fertilization is rapid, the plasmalemmas of the 2 gametes fuse and the male axoneme and nucleus enter the macrogamete cytoplasm. The male nucleus dissociates from the axoneme, becomes enlarged and the chromatin decondensed, it comes to lie against a channel of endoplasmic reticulum and ultimately fuses with the female nucleus at a point where the nuclear envelope is thrown into extensive folds. The first recorded change in the zygote is the polarization of the nucleus and the appearance of perinuclear microtubules. In *P.gallinaceum* these microtubules originate from a centriole-like MTOC but are not apparently associated with an intranuclear spindle. Adjacent to the MTOC electron dense masses are laid down under the single zygote plasmalemma, these masses act as the focus for the development of the collar and polar rings – which in turn are the MTOC for the 55–65 subpellicular microtubules. The gradual assembly of this apical complex in part determines the gross changes in shape in the developing ookinete from sphere to 'retort-form', thence to the leaf or banana shaped mature ookinete. Between the plasmalemma and the apical complex a double membrane system is laid down. It is formed by the fusion of short segments of vacuoles, which it may be predicted will form petal-like sacs. Characteristic linear arrays of intramembranous particles (IMP) will lie on the outer- and inner-most surfaces of the sacs.

Staining with acridine orange[8] suggested nuclear fusion may not have occurred in the ookinete, this suggestion is probably incorrect because Mehlhorn et al.[86] have shown the presence of bipolar intranuclear spindles in the zygote at or about the time of budding of the apical complex in the retort-form parasite. By comparing the number of kinetochores on this initial spindle with the number on spindles of known haploid stages it has been suggested there is an immediate reduction division which inevitably excludes the possibility of chiasma formation and intrachromosomal recombination. Within the ookinete nucleus a prominent nucleolus may readily be labeled with ^{3}H-adenosine suggesting that, despite the rapid nuclear division, nucleic acid synthesis is significant[118]. Subsequent labeling of the cytoplasm indicates that RNA transcripts are rapidly transported from the nucleus. Other radiolabeling of the ookinete achieved includes the incorporation of ^{35}S-methionine into some 19 proteins and glycoproteins, 2 of which (mol.wt 35 kd and 27 kd) are components of the distinct glycocalyx on the ookinete plasmalemma[78] – a structure readily detected by ruthenium red staining[55]. The proteins so far radiolabeled can only represent a small fraction of the massive synthesis required for construction of the complex ookinete. Interestingly it has been shown that development of the parasite ceases at the ookinete stage unless a macromolecular (non-dialyzable) component is present in the bloodmeal[42,43]. Our ability to culture the ookinete in vitro[5,22,23,48,102,162,163,165] now allows more penetrating studies on the cell- and molecular biology of these stages.

The description of highly glycosylated proteins on the ookinete[78] is compatible with the ultrastructural observations showing a massive development of the endoplasmic reticulum and Golgi apparatus in the young ookinete. Intracellular vesicles develop in association with the golgi; these vesicles are the crystalloid – a collection of regular membrane bound electron-dense spheres which may be lipoprotein in nature and be an energy reserve for the parasite. Other vesicular structures to develop include the rhoptries and micronemes, situated between the nucleus and apical complex. These organelles become connected by ducts to the plasmalemma at the apical tip – probably to a rosette of IMP's which can be located at this site by freeze-fracture techniques[37,98]. Mitochondria in the ookinete are more cristate than in the macrogamete, a change which may correlate with the reappearance of succinic dehydrogenase activity in the mosquito stage[66].

The ookinete is a motile cell which, like the sporozoite, glides across the substratum[44], there is at present no evidence for any 'directed' or purposeful migration of the parasite out of the bloodmeal. The structural basis of this motility lies in the complex pellicle, the helically coiled microtubules of which dictate both the shape[116,128] and pattern of locomotion of the cells[44]. The theoretical model for this suggests that there is a microtubule directed, microfilament mediated capping of substratum binding ligands in the ookinete plasmalemma[104,106].

The ligands involved must be non-specific and include components of the erythrocyte surface, the peritrophic membrane and the plasmalemma of the midgut epithelium – the possibility of developing ligand blocking agents, as proposed for the merozoite receptors, has yet to be investigated.

Invasion of the midgut epithelium is achieved by the

induction of a vacuole, however, there is no evidence for the persistence of this vacuole around the ookinete as it moves through the cells to the basement membrane[17, 31, 86]. Ultimately the ookinete penetrates the basement membrane, but cannot pass through the basal lamina of the midgut – suggesting the microneme and rhoptry secretions interact with lipoprotein membranes but not glycoprotein matrices.

The oocyst (figures 9–15)

The overall organization of sporogony is, at the cellular level, very similar to that of both erythrocytic, and more particularly, pre-erythrocytic schizogony. Initial ultrastructural studies were made in 1960 but the subject has not since been vigorously pursued. Extensive early efforts were made to culture the sporogonic stages but despite the success in culturing parasites for overlapping 3–4-day periods[9, 10, 110, 155] direct culture of the entire mosquito stages still eludes us. Nevertheless such cultures have been used to study successfully the uptake of radiolabeled compounds.

Despite the usual though not invariable growth of the oocyst under the basal lamina of the midgut, oocyst development is not site specific. Weathersby[158, 159] showed that development could occur in the hemocoele if gametocytes were inoculated there directly. Similarly Beaudoin et al.[13] found oocysts developing ectopically within the midgut epithelium following normal infection. Shute[113] observed the distribution of oocysts in the midgut of infected mosquitoes, he noted a markedly clumped distribution particularly influenced by the effect of gravity on the erythrocytes in the stomach of the resting and engorged anopheline. Whilst the clumped distribution leads to competition for nutrients and a consequent variability in oocyst size, in low density infections the growth of oocysts is predictable both in size and stage of development, e.g. mature oocysts are found on the 10th day in *A. atroparvus* infected naturally with *P. falciparum,* at the same time infective sporozoites are found in the salivary glands where they may persist for up to 59 days[25, 50].

In vivo the ookinete rapidly rounds up on reaching the midgut basal lamina (e.g. 18–24 h after bloodmeal). This is accompanied by the retraction of the collar and microtubular cytoskeleton into the oocyst cytoplasm, and the separation and fragmentation of the inner pellicle membranes. The skeletal microtubules however do not depolymerize and can be detected for many days in the young oocyst. In contrast the crystalloid quickly disappears during the rounding up of the oocyst, suggesting its function is indeed confined to the ookinete stage. Synthetic organelles proliferate rapidly, e.g., the cristate mitochondria, dispersed Golgi, distinct islands of endoplasmic reticulum and ribosomes. Expansion of the ribosome population occurs in the absence of a detectable nucleolus in 'mammalian' parasites although this organelle is present in the 'avian' plasmodia. Biochemical observations in the oocysts are very fragmentary but most observations are consistent with the increase in synthetic apparatus observed. Cultured oocysts, like other stages of the life cycle, readily incorporate ^{3}H-adenosine but not thyimidine[33]; similarly amino-acids and ^{32}P-phosphate are avidly incorporated[11, 73, 118]. Enzyme activities detected include acid phosphatase, β-glucuronidase, aryl sulphatase[32], and succinic dehydrogenase – an enzyme associated with the development of the cristate mitochondria[66]. Terzakis has shown that the morphology of the mitochondria is affected by the napthoquinones[133]. The same study indicated that pyrimethanine in the mosquito bloodmeal blocked nuclear division. Not surprisingly, numerous antigens are common to the oocyst and the asexual erythrocytic parasite as revealed by the indirect fluorescent antibody test[77].

The interpretation of early cytological studies on the oocyst[12] which described meiotic chromosomal configurations is incorrect. Ultrastructural studies have shown the nucleus to undergo accelerated mitosis[17, 67, 111] within a persistent nuclear envelope producing a highly lobed polyploid syncytical nucleus. Spindles (0.5–1 µm long) form on typical 'gastrula-like' invaginations of the nuclear envelope. A single MTOC, which initated the polymerization of both cytoplasmic and spindle microtubules, splits. The 2 daughter poles are separated by a 'couche-fibrillaire'[111] and opposed across short reaches of the nuclear envelope. Each spindle contains about 25 microtubules of which 5–14 bear kinetochores[17, 126], no chromosomes are visible by electron microscopy although the area around the kinetochores is DNAse sensitive[118]. At the time of formation of the circa 10,000 sporozoites, small nuclear lobes divide into adjacent sporozoites. The location of the final spindle MTOCs dictates the site of sporozoite development. The haploid nucleus having been incorporated into the sporozoite bud soon undergoes changes, the DNA condensing in a herochromatic state around the inner face of the nuclear envelope.

Prior to sporozoite formation the oocyst undergoes considerable cytoplasmic subdivision producing sporoblasts. Formation of these lobes differs between species. In the 'mammalian' parasites vacuoles appear between

Figure 7. *P. berghei* zygote partially transformed into ookinete. The tripartite pellicle is recognisable as an electron dense thickening of the plasmalemma. Two widely separated spindle hemispindles indicate the initial nuclear division has occurred in this parasite 5 h after fertilization. × 13,700.

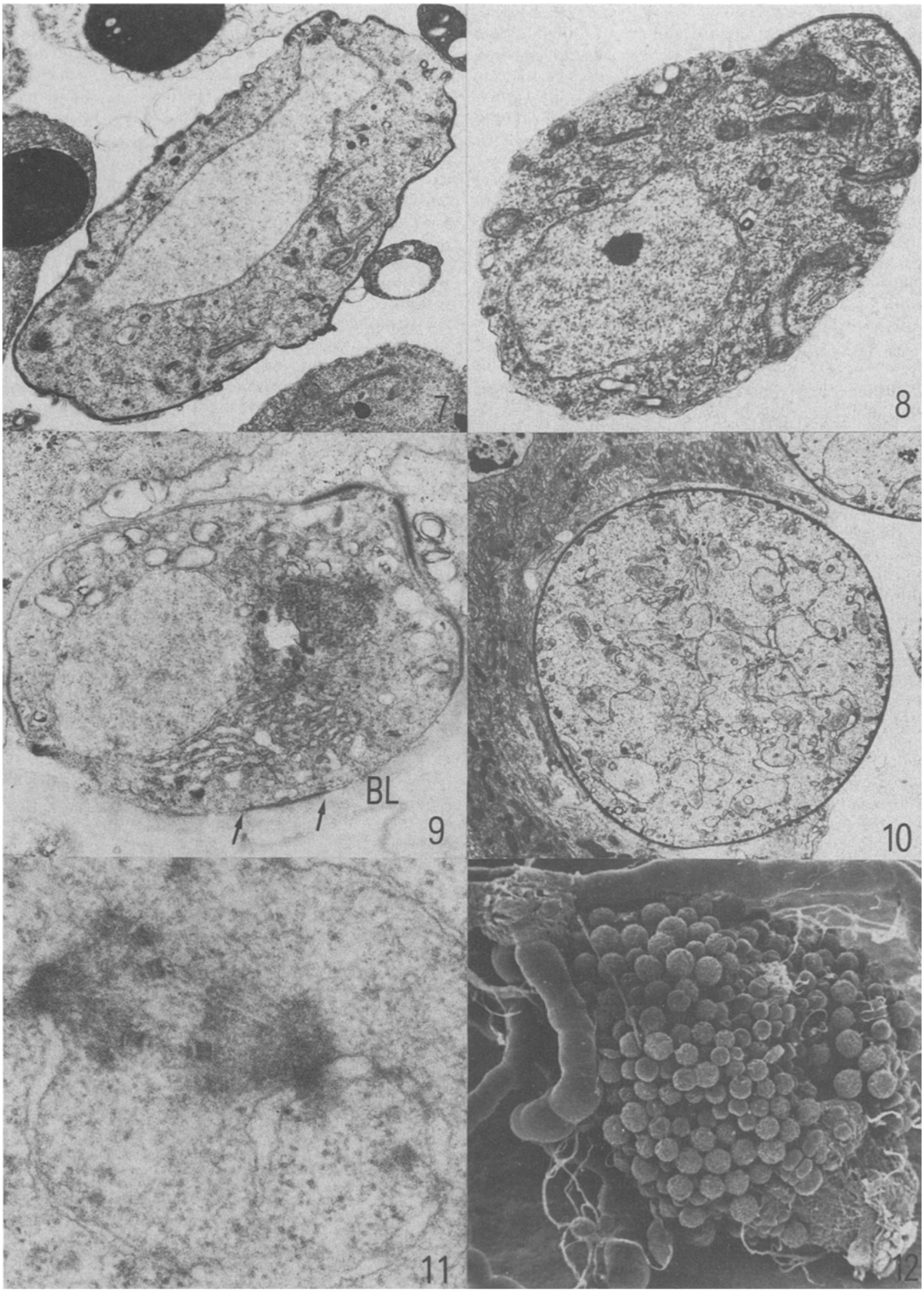
Figure 8. Section of retort form ookinete of *P. berghei* showing the protrusion of the developing apical complex as the motile parasite is assembled from the zygote. × 17,300.

Figure 9. Following penetration of the midgut epithelium the ookinete is halted by the basal lamina (BL). Here the body of the parasite rounds up and the tripartite pellicle becomes fragmented (arrows) as the parasite increases in size. The nucleus, crystalloid and endoplasmic reticulum are prominent in this section. × 17,500.

Figure 10. Section of 6.75-day oocyst of *P. falciparum* showing the developing dense extracellular cyst wall, large lobed nucleus and extensive cytoplasmic proliferation. × 5200.

Figure 11. TEM of one of the numerous small spindles found in the syncytial, polyploid oocyst nucleus, the kinetochores are clearly visible but the chromosomes do not condense during mitosis. *P.* berghei 13-day oocyst in *Anopheles stephensi.* × 76,900.

Figure 12. SEM of 6-day oocyst infection of *P. berghei* in *Anopheles stephensi.* × 132.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the plasmalemma and the oocyst wall, these vacuoles coalesce and penetrate ever deeper into the parasite cytoplasm, thus subdividing the cell[132, 135, 136, 149]. In the 'avian' parasite e.g. *P. falciparum* the cytoplasmic clefts are formed by the direct expansion of the endoplasmic reticulum[126]. The site of sporozoite formation parallels the method of cytoplasmic fission, in the 'mammalian' parasites (e.g. *P. berghei, P. cynomolgi*) they form over the entire plasmalemma surface, but in the 'avian' parasites (e.g. *P. falciparum*) sporozoites develop on the expanded surface of the endoplasmic reticulum and lie within a network of large interconnected vacuoles.

The sporozoites form 9–14 days after the bloodmeal. Individual membrane bound vesicles form under the plasmalemma and beneath these radiate the newly forming subpellicular microtubules. The suggested origin of these vesicles from capsid-like membrane from virus-like particles as early as 48 h after the bloodmeal[134] is not plausible. Polar rings form under the membrane, and are closely associated with the spindle MTOC. These rings in turn act as the MTOC for the subpellicular microtubules. The elongate sporozoite is formed by the extension of this modified area of the plasmalemma.

The microtubules and inner pellicular vesicle never extend into the cytoplasm of the sporoblast suggesting the assembly of both is controlled at the point of emergence[105]. The subpellicular microtubules, which are typically arranged in an asymmetrical (11–17)+1 circumferential pattern, may be attached along their entire length to the inner pellicular membranes by the linear arrays of IMPs found on the P_1 face of the pellicle found in both the immature and mature sporozoite[2, 38, 88]. The single inner membrane vacuole is wrapped around the sporozoite its tightly apposed edges forming a single longitudinal suture which must lie on the microtubule depleted side of the parasite. Sporozoites of most plasmodia have at least 1 micropore in the pellicle, although none have been detected in the rodent malarial parasites[123]. This 60–80 nm diameter cylinder is centrally situated, it is attached to the plasmalemma and penetrates 100 nm through the inner membrane vesicle. Micropores are formed on the surface of the sporoblast and drawn into the budding sporozoite.

Between the pellicular envelope and the nucleus of the budding sporozoite 2 clear vacuoles form by the fusion of Golgi-derived coated vesicles. These vacuoles become progressively more electron dense following the initial appearance of flocculent electron dense material. Ultimately these electron dense vacuoles – the rhoptries – are connected by small ducts which pass through the polar ring to the plasmalemma. Following the release of the sporozoite from the oocyst the rhoptries continue differentiation whilst in the salivary glands becoming branched and 'beaded' forming the micronemes[123, 129].

Other organelles incorporated into each sporozoite include endoplasmic reticulum and mitochondria. The sporozoite is now an elongate (circa 9–12 μm long; 1.5 μm diameter) rigid cell, which has a limited capacity for movement and can be seen bending from side to side. Metabolic energy for this movement is obtained from amino-acids and sugars by glycolysis and an active Kreb's cycle[82]. Amino acid incorporation into the sporozoite is rapid and is heavily biassed to the surface or circumsporozoite (CS) proteins.

At this time SEM studies show the oocyst to be wrinkled and sporozoites can be seen pressing against the pliable thin cyst wall[116, 130]. The cyst wall remains of relatively constant thickness throughout development but the basal lamina becomes attenuated from 1.0 μm to 0.1 μm by the enlarged oocyst (now 30–50 μm in diameter); ultimately the previously impenetrable lamina is extensively ruptured, thereby removing the barrier to sporozoite escape[116].

The sporozoite (figures 16–18)

The mature cylindrical sporozoite was the first stage of the malarial parasite to be examined by electron microscopy[40] and was the subject of extensive studies by Garnham et al.[51–53]. Dependent upon species, the sporozoite varies from 9 to 16.5 μm in length and from 0.4 to 2.7 μm in diameter; aberrant forms have been described up to 40 μm long. The helically coiled, or crescentic sporozoite has a smooth plasmalemma, in which lies the depression of the micropore. The parasite pellicle[131] is further reinforced by the single membrane vacuole lying immediately beneath the plasmalemma, below which lie the assymmetric sub-pellicular microtubules. The microtubules are anchored anteriorly on the polar ring within which lie 2 more apical rings. The rhoptries and micronemes are found between the slanting anterior pole and the centrally situated nucleus. Within the nucleus the chromatin is in a heterochromatic state against the nuclear envelope. The mitochodrion is found against the elongate nucleus, posterior to which lie the bulk of the endoplasmic reticulum and ribosomes[118].

The motile sporozoites emerge into the hemocoele from holes in the oocyst wall; holes possibly produced by a combination of the muscular action of the gut wall and the activity of the sporozoites[116]. Sporozoites are weakly motile, they glide forward and backward, rotate, and

Figure 13. TEM showing parts of two 9-day oocysts of *P. falciparum* on midgut of *A. gambiae*. Sporozoites are seen budding from the internal membrane systems. × 6000.

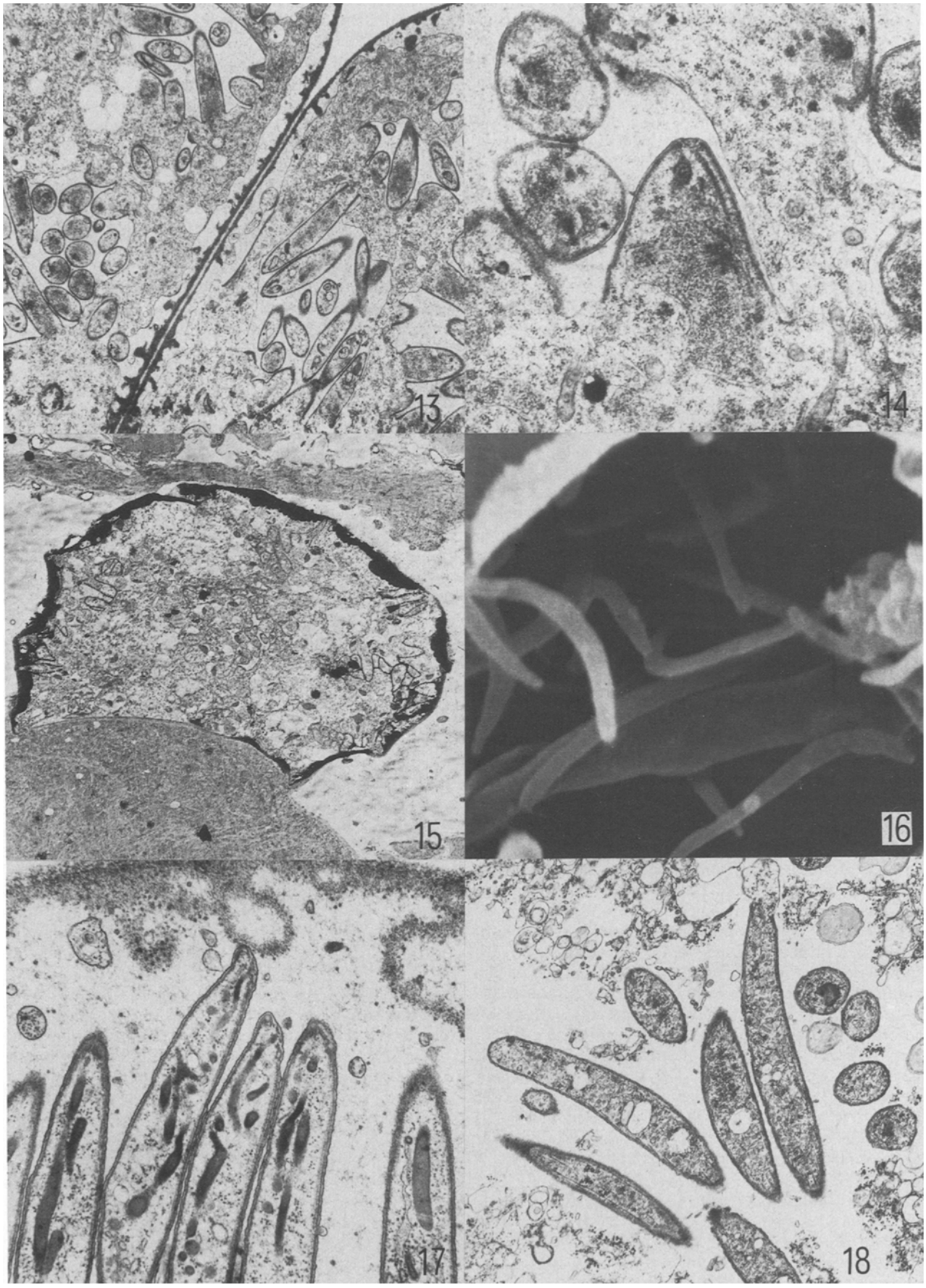
Figure 14. TEM of a single budding sporozoite of *P. falciparum*. The tripartite pellicle is seen to be confined to the conical projection of the developing 'zoite. Within the indistinct nucleus lies an electron dense nuclear spindle. The spindle pole is connected to the developing apical rings by an electron dense band (right side). The primordial vacuole of a developing rhoptry lies between the nucleus and apical ring. × 20,700.

Figure 15. TEM of Ross's black spore showing extensive melanisation of the oocyst wall and some internal membrane systems. *P. berghei* in *A. stephensi*. × 2600.

Figure 16. SEM of completed oocyst sporozoites of *P. falciparum*, in broken oocyst. × 5000.

Figure 17. TEM of apical poles of oocyst sporozoites of *P. yoelii*, clearly showing the tripartite pellicle, electron dense micronemes and rhoptries. The oocyst wall is visible at the top of the micrograph. × 27,200.

Figure 18. Oblique and transverse sections of intracellular sporozoites of *P. yoelii* in the salivary glands of *A. stephensi*. × 13,100.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occasionally bend[106, 143, 164]. Within the hemocoele the sporozoites are carried throughout the mosquito. Sporozoites penetrate the basal lamina covering the salivary glands and enter the acinar cells and collect therein. Sporozoites are seen lying the tight bundles in the distal portions of the glands, where interestingly the salivary duct is not chitinised. No chemotactic gradient to the glands has been found, yet sporozoites are only rarely found in aberrant sites (e.g. the midgut wall or haemocytes[118]). Infective sporozoites may persist for 50–59 days in the salivary glands, a gradual loss of infectivity may be related more to a reduction in number as opposed to a reduction of innate infectivity of individual sporozoites. Sporozoites in the salivary glands are rarely abnormal, those in the oocyst are frequently so, suggesting selection of the sporozoite population has occurred. This selection could explain the observed failure of some heavy oocyst infections to invade the salivary glands. The efficiency of salivary gland invasion is poorly understood, whilst it is often argued there is an 'optimum' number of oocysts there is no real evidence for this. Excessive sporozoite burden could cause the pathological vesiculation and cytoskeletal changes which have been reported in infected tissues[129] and the infected glands are unquestionably more fragile. When an infected mosquito takes a second feed 0.1–17.8% of the salivary gland burden is injected into the tissue or capillary of the vertebrate[145], suggesting an enormous infective potential lies in each infected mosquito.

Sporozoite vaccines

Probably the most exciting recent studies on the cell biology of the mosquito stages of *Plasmodium* relate to the surface coat of the sporozoite. Techniques for the mass isolation of sporozoites[64, 83, 89, 95, 151] have allowed rapid advances in our understanding of molecular biology of the surface coat. Many differences between the oocysts and salivary gland-forms of the sporozoite have been described. Infectivity of the salivary gland form is circa 10^3-fold higher[144], and this change is time rather than tissue dependent[30]. The electrophoretic mobility of the oocyst form is lower (i.e. more negative) than the salivary gland form[140, 142]. The salivary gland form is a more antigenic[94, 148] and stage specific antibodies can be produced[90, 91, 94, 148] of which only those against the salivary gland form are protective. Protective antibodies induce a capping reaction on the viable sporozoite – the circumsporozoite (CSP) reaction[147], which is sensitive to metabolic and microfilament inhibitors[26, 106]. The CSP reaction is accompanied by the agreggation of some of the IMPs in the sporozoite plasmalemma[2], IMPs which have increased in density from the oocyst to the salivary gland form[38]. Sporozoite infectivity and the surface coat is destroyed by trypsin[26, 141] but not neuraminidase, simultaneously the electrophoretic mobility of the cell is reduced. The sporozoite coat binds lectins with very low efficiency[112, 142].

The above changes are related to the expression of one of a family of immunologically and structurally related proteins (i.e. of common genetic ancestry in the genus *Plasmodium*)[27]. These molecules are synthesized as high molecular weight precursors (*P. knowlesi* 52 and *P.k.* 50; *P. berghei* 54 and *P.b.* 52) found in the oocyst sporozoite molecules which are processed to *P.k.* 42[27] and *P.b.* 44[166] (= *P.b.* 53[153, 154]) which are expressed in the salivary gland form of the parasite. cDNA has been prepared from mRNA coding for these proteins and fusion proteins expressed in *E. coli* using pBr 322 as vector. Fusion proteins with β lactamase successfully compete for specific monoclonal antibodies[39]. The major epitope in the CS protein is repeated[167]. This epitope is in fact present 12 times and is a short amino acid sequence (Ala_3, Gln_3, Gly_3, Pro, Asp, Asn)[57]. The purpose of these repeats is unlikely to be a specific receptor, they may therefore act as an immunodominant shield which can be shed (capped) at will.

An important application of anti-CS protein Mab's is the development of enzyme-linked and radioimmunoassays[16, 28, 168] to determine the number and species of sporozoites present in batches of field caught mosquitoes; assays which surpass tedious individual mosquito dissection methods.

Factors modifying parasite development in the mosquito

a) Deleterious factors

Conversion of gametocytes to oocysts is biologically expensive. Eyles[41–43] indicated that 315 gametocytes (252♀) of *P. gallinaceum* are required for each oocyst produced; for *P. cynomolgi* the estimate is 40 macrogametocytes for each oocyst[108]. The efficiency of infection by membrane fed cultured gametocytes of *P. falciparum* is also low requiring between 4000 and 780,000 gametocytes for each oocyst[97, 119]. Rosenberg et al.[103] found that only 10 zygotes/mm^2, i.e. 15–36 per mosquito were required to produce an average of 1 *P. gallinaceum* oocyst in *Aedes*, whereas to produce 1 ookinete in vitro required only 1.4–2.5 zygotes. This loss of parasites is therefore multifactorial and multifocal and may depend upon properties of the parasite, vertebrate host or mosquito. In the case of the mosquito these properties are considered innate and not acquired[161], although the presence of other 'acquired' infections e.g. virus and microsporida *(Nosema algerae)* have been reported to reduce the success of malarial transmission[47, 71].

1. Inhibition of gametogenesis

Not all gametocytes recognized as morphologically mature in the peripheral blood are infective (capacitated, but are either immature or senile[34, 79] and are either incapable of recognizing, or fail to respond to, the stimulus to exflagellation. Recently it was shown that infectivity declined with increasing blood passage, and that this was accompanied by a reduction in repetitive DNA from 18% to 3% in the parasite population[15, 36].

Assuming gametocyte numbers were unchanged, it still remains to be shown if the repetitive DNA is found exclusively in the gametocyte, and whether it is significant in zygote transformation[120].

Acquired factors in the host blood considered to influence gametogenesis include: the depletion of essential nutrients and the appearance of toxic factors[20, 119, 146].

Mosquito factors inhibiting gametogenesis are few if any, exflagellation is commonly found in bloodmeals of refractory mosquitoes[65].

2. Inhibition of fertilization

Antibodies directed against the surface of the macro- and microgamete will inhibit gamete dispersal by agglutination and surface fixation[21], this activity in some instances being complement dependent. Additionally phagocytic cells of the vertebrate will ingest extracellular parasites both in vitro and in vivo[107, 125].

3. Inhibition of ookinete development and function

Mosquito insusceptibility to malaria may involve the degeneration of the zygote, e.g. *P. cathemerium* and *P. relictum* in strains of *Culex pipiens*[70], and *P. vinkei* in *Anopheles*[65]. In some combinations of *P. cynomolgi* and *Anopheles* it is the ookinete which degenerates[14]. Ookinetes were shown to be susceptible to mosquito enzymes in elaborate double feeding experiments[54] which indicated that parasites 0–10 h old were most susceptible (i.e. zygote to ookinete transition). This was confirmed in vitro and trypsin-like enzymes secreted on the endoplasmic reticulum of the midgut[63] were incriminated[56], their major effect being to change the permeability of the parasite plasmalemma[55]. From these data it was suggested that parasites in the center of the bloodmeal are most likely to survive. The peritrophic membrane (PTM) is secreted within 2 h of the bloodmeal, yet offers little physical barrier before 15 h[24]. Subsequent losses include ookinetes which cannot penetrate, or are trapped in the PTM (indeed if dissected out the PTM is an excellent source of ookinetes for histological studies). Further ookinete losses have been recorded in *P. cathemerium* infections of *C. pipiens* within the intestinal wall[70].

The role of the entire midgut wall has been summarized by Weathersby[161] thus: 'All stages of *Plasmodium*, endogenous and exogenous, are capable of development to the infectious stage when the stomach wall was bypassed by means of injections directly into the hemocoele of susceptible mosquitoes'. Additionally Weathersby demonstrated[159] an inhibitory (antiblastic) factor in refractory mosquitoes transferable by parabiotic twinning[160]. Humoral activity against the oocyst is clear, resulting in the melanisation of the cyst wall and internal membranes to produce Ross's black spores[123]. Other humoral 'poisons' have been suggested by Garnham[49] which kill sporozoites of *P. gonderi* or *P. brasilianum* in the hemocoele of *A. aztecus* or *A. maculipennis*.

It has been suggested[161] that neither the oocyst nor the sporozoite are attacked by the mosquito's haemocytes, evidence to the contrary suggesting that sporozoites at least are attacked has however been presented[118].

Antibodies against the ookinete, if present in the bloodmeal of an immunized chicken will reduce subsequent infections by *P. gallinaceum*[78].

b) Factors increasing parasite numbers in mosquito

Extraneous factors enhancing parasite transmission are poorly represented. Drug treatment of the vertebrate host reportedly increases infectivity, such drugs include sulphamethazine and quinine at subcurative levels[114]. Terzian[138] suggested that sulphanilamide and sulphadiazine at 0.1–0.2% in the mosquito diet raised oocyst numbers, and that this was inhibited by p-amino-benzoic acid. This study did not distinguish whether the effects were direct on the parasite, or indirect by the elimination of competitive fauna in the mosquito gut. Conversely Peters and Ramkaram[96] demonstrated a clear increase in oocyst rates in mosquitoes receiving, p.a.b.a. prior to the infective feed, an effect inhibited by sulphadoxine**.

Innate mosquito susceptibility

The breadth of this subject is beyond this review, the reader is directed to other comprehensive reports[29, 50, 157]. Briefly summarized: – following initial studies[68–70] Denhofer[35] demonstrated refractoriness to *P. cathemerium* in *Culex quinquefasciatus* controlled by an incompletely dominant gene in the 3rd linkage group. Kilama and Craig[76] showed a monofactorial autosomal recessive gene in linkage group 2 in *Aedes aegypti* conferring refractoriness to *P. gallinaceum*. Susceptibility of *Anopheles* to mammalian malaria is variable: susceptibility to *P. berghei* is reportedly controlled by a single incompletely dominant gene and is expressed by the failure of ookinetes to penetrate the PTM[72, 74]. A line of *A. gambiae* refractory to *P. berghei* has been produced where susceptibility is incompletely dominant to refractoriness (which was controlled by interacting polygenes) – refractoriness being expressed as oocyst degeneration on the midgut wall[6].

Mosquito physiology modified by malarial infection

A number of studies have shown that plasmodium may have deleterious effects upon its mosquito host in terms of its longevity and fecundity[45, 109]. The latter effect may be related to changes in the composition of the haemolymph of an infected mosquito[46, 80, 81, 84], differences which may be due more directly to variation in the infective bloodmeal, rather than the growing parasite in the vector. Notable reductions were found in the hemolymph sugars, and variously amongst the amino-acids[46, 80] including valine, histidine, methionine, alanine, aspartic acid and glycine.

Future studies

Areas poorly understood at present are all too obvious; parasite biochemistry in the mosquito has not been examined with any real success. Our understanding of drug sensitivity of the sporogonic stages lags far behind studies on the vertebrate phases of the life cycle although the value of the insect stages in any screening[137] has recently been re-emphasized[61]. Mosquito defence reactions in relation to susceptibility and refractoriness and the metabolic and developmental interaction between mosquito and malarial parasite all deserve far greater attention, although the absence of in vitro culture methods will continue to hinder advances.

Nevertheless a recognized area of particular interest and of rapid and potentially useful application is the development of transmission blocking agents, particularly the sporozoite and the 'altruistic' vaccines which may contribute to the eventual limitation of malarial transmission.

Notes added in proof

* Evidence has been presented that one of these species (*P. berghei*) is infective for prolonged periods, e.g. 26 h (Mons et al., Parasitology, in press).

** Increased oocyst rates have been described in infected mosquitos maintained on 0.005–0.5% p.a.b.a. (Li, C., and Gorg, G., J. Parasit. parasit. Dis. *2* (1984) 9).

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Malaria: immunity, vaccination and immunodiagnosis

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Key words. Malaria; immunity; immunodiagnosis; immunization.

The range of investigations in the field of immunology of malaria is immense and covers various areas from immunopathology to both natural and specific immunity, immunodiagnosis and vaccination trials in experimental animal models. Over the last few years, a number of excellent reviews have been published on several of these aspects[5, 15, 16, 18, 44, 54, 70, 74]. The present review will therefore be restricted to major concepts, will mention more specialized reviews and concentrate on recent developments, particularly those concerning *P. falciparum*, the most lethal species for man.

I. Immunity

The development of resistance to malaria in man depends on the frequency and duration of exposure to the parasite[49, 55]. In non-immune human volunteers, *P. falciparum*, *P. vivax* and *P. ovale* infections have a duration of more than 2 years and *P. malariae* may persist for over 30 years. In endemic areas, babies from immune mothers are resistant to malaria during their first 3 months of life. Later, they suffer from severe and recurrent attacks and most deaths due to malaria occur in young children[49]. From adolescence to adulthood, there is a progressive decrease in the severity and frequency of malaria attacks and clinically significant malaria becomes infrequent in adults, except pregnant women. Resistance in humans therefore builds up slowly following the course of successive infections, but sterile immunity is probably never achieved and low grade parasitemia is still observed from time to time in adults living in endemic areas[49, 55]. These observations suggest that humans and, more generally, vertebrate hosts can raise a protective immune response but that plasmodia,