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The biology of tissue forms and other asexual stages in mammalian plasmodia

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Key words. Malaria; Plasmodia; asexual stages; sporozoite, exoerythrocytic form; sporozoite-hepatocyte interaction.

To children, those perfect parasites; to parents, those humble hosts.

A concealed conception

It is a remarkable fact that all organisms in the suborder of Haemosporina are transmitted by bloodsucking Diptera and that after the introduction of the infective stage into the circulation, always a schizogonic development occurs in tissues. The next stage is a sexual one which parasitizes the red cells, and after a bloodmeal of the vector these forms continue their development by fertilization and asexual development to the sporozoite stage.

It is only in the genus *Plasmodium* that an extra schizogonic cycle occurs in red blood cells immediately after the tissue stage. These asexual blood forms can perpetuate themselves or, in some avian species, give rise to new tissue forms, or generate sexual forms.

To us it seems that the tissue form is the basic stage of multiplication in the vertebrate host, whereas asexual blood forms merely increase and prolong the gametocytogenesis, to guarantee the shift to mosquitoes. There is no ground for the hypothesis[10] that plasmodia are on their evolutionary way to skip over the tissue phase. In fact, this stage is so central in the development of the parasite that it is being used for species differentiation[11,35]. Functionally they form a bridge-head through which massive numbers of daughter cells are generated and released into the blood.

The stage that kept malariologists busy mostly has been and still is the asexual blood form, both because of its clinical relevance and its ready availability. That has led to the usage of the asexual development in the blood as a model for other phases and extrapolating the acquired knowledge to mosquito- and tissue forms. This choice of a highly specialized form may not be the most ideal starting point for comparing cell-biological features. On practical grounds it is understandable because relatively little is known of the tissue forms discovered more than half a century after the first description of malaria parasites: those of avian plasmodia in 1937, primate plasmodia in 1948 and rodent plasmodia in 1965. The bulk of information on ultrastructure has become available only during the past 10 years.

In this review an attempt is made to decide why the tissue form (for which we use the term 'exoerythrocytic'[11]) is the central and exemplary phase. We first summarize the recent findings on fine stucture, development and interaction with tissue cells and use this as a starting point for comparison with both the sporogonic development in the mosquito and the merogonic development in the vertebrate hosts. In this respect it is revealing to recall what Professor Garnham exclaimed when he first saw an exoerythrocytic schizont: "This is an oocyst in the liver" (Sinden, personal communication). Contrary to some existing reviews we emphasize the unifying aspects in the ultrastructural morphology and the conservative nature of the malaria parasite, despite its repeated changes of environment and host. The

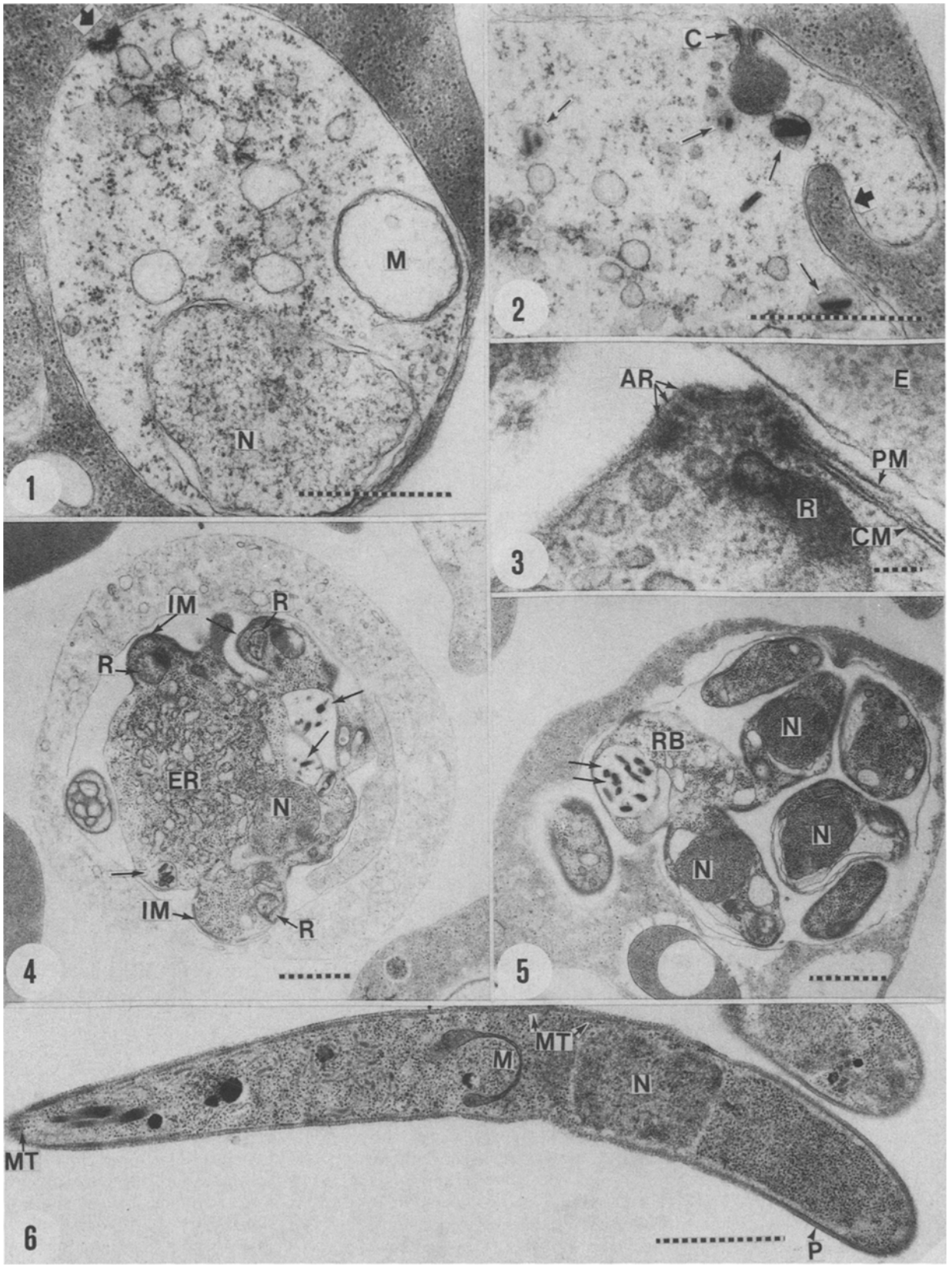

Figure 1. Young intraerythrocytic trophozoite with a nucleus (N) and a large acristate mitochondrion (M) enclosed by a parasitophorous vacuole membrane. Arrow points to a beginning cytostome. There is no malarial pigment visible. × 27,000, bar 1 μm. Figure 2. Trophozoite with an active cytostome (c). Malarial pigment is distributed throughout the parasite (thin arrows). Compare the cytostome with the ordinary invagination of the red cell into the parasite (arrow). × 32,000; bar 1 μm. Figure 3. Detail of the anterior part of a merozoite still inside the erythrocyte (E). Note the 3 apical rings (AR), the 3 pellicular membranes consisting of an outer plasmalemma (PM) and 2 cytomembranes (CM) ending in the apical rings and the rhoptry (R). Subpellicular microtubules are absent or very rare. × 80,000, bar 0.1 μm. Figure 4. Segmenting intraerythrocytic

tissue forms of mammalian plasmodia are the most perfect parasites and in this review the rodent species *P. berghei* and *P. yoelii* are taken as a pars pro toto. Needless to say, the unavoidable extrapolations that are used for the sake of argument will override sometimes the peculiarities of other plasmodial species.

The seeds of proliferation

The sporozoite (fig. 6) which eventually gives rise to one exoerythrocytic schizont in the liver has a structure that is grossly comparable to that of the ookinete and the merozoite (fig. 22). These free stages also precede a division, notably the oocyst and erythrocytic schizont.

In order to appreciate the transition from a free to a parasitic life and the accompanying change of becoming intracellular, some structural details of the sporozoite and other motile stages are summarized. They all contain one nucleus, a mitochondrion, endoplasmic reticulum and an apex where glandlike organelles (the micronemes and rhoptries) end and may empty (figs 3, 6). The invasive stages have a rigid structure which consists of a trilaminar pellicle, the inner one of which is sutured in a wavy line. In the pellicular membranes a pattern of proteinaceous particles occurs showing a longitudinal arrangement which is in association with underlying microtubules[3]. A connection is likely to exist between the outer membrane and the inner membranous complex through which movement and attachment to (cell)surfaces is governed by the microtubules[57]. These have a specific arrangement of 10 to 16+1 and enable the parasite to make corkscrew movements[64]. The microtubules are connected at the apical end with polar rings. The whole architecture of the pellicular complex and apical end is designed for movement towards and into cells of the mosquito and of the vertebrate host.

Until now there are no reports available on the fine structure of sporozoites penetrating cells in vitro and we thus have only the in vivo experiments to consider. Here, we meet with a controversial question as to how sporozoites reach the hepatocytes so quickly and we have to first elaborate on this item before we come back to the invasion process itself.

The delivery of sporozoites

When the sporozoite-containing blood enters the liver, either through the hepatic artery or portal vein, it is forced into the branching system of portal venules and arterioles. There it flows through the sinusoids and is directed via the terminal venules into the inferior vena cava. It is at the sinusoids that the sporozoites come close to their destiny; only the endothelial lining separates them from the underlying hepatocytes.

It is attractive to assume that sporozoites can find their way through the endothelial layer via gaps between cells or holes or fenestrae in the stretched endothelial cell plates[62]. However, one might ask as to what the function of the irregular gaps in the endothelial lining is, if the cell plates have their numerous small fenestrae as well, for the supply of plasma nutrients to the hepatocytes. Indeed, it appears that after careful fixation by perfusion and under low pressure no such gaps are to be seen in the endothelium and occur only as artefacts if unphysiological pressure is applied[79]. The fenestrae (0.1 µm) are far too small for sporozoites (12 × 1 µm) to pass and the sieve plates themselves are tightly linked with each other, with only the Kupffer cells in between[80].

We must conclude that neither the blood cells nor the sporozoites can reach the hepatocytes directly. Thus the question arises as to how sporozoites can overcome this barrier and reach the space of Disse behind the endothelium, where they can invade hepatocytes.

In 1979 we presented a hypothesis on the role of Kupffer cells in this process, because we observed that blockade of the phagocytic activity and killing of Kupffer cells in vivo reduced the number of EEF (exoerythrocytic forms) in hepatocytes and kept the sporozoites in the circulation for an extended period of time[77,78]. Sporozoites injected into intact animals are readily taken up by Kupffer cells, very shortly after their introduction into the circulation[62,68]. Ultrastructural findings on the first contact of sporozoites with Kupffer cells indicate that long pseudopods reach out into the sinusoidal lumen and grasp sporozoites from between the blood cells (fig. 7)[46]. Local accumulations of microfilaments are seen in the pseudopods at the places of contact regardless of the position of the sporozoites, indicating an active phagocytosis[50], and the clearing of 95% of them during the first 10 min after inoculation working irrespectively of their maturity of infectiousness also points to a non-specific removal[16,71].

When the sporozoites are completely engulfed by Kupffer cell cytoplasm, they lie inside an endocytotic vacuole (figs 8, 9). In this situation, their only option is to leave the phagocytic cells before they are killed and digested by lysosomal enzymes. There are strong indications that some strains of rats and mice allow many more sporozoites to reach hepatocytes than others, which may well have its origin in the variable ease of triggering the oxidative burst of Kupffer cells (Smith, personal communication). The 'successful' sporozoites may have evaded this killing mechanism in the Kupffer cells and worked their way out. In fact, after exiting they are not confronted by other barriers since the endothelial cell plates do not continue under the Kupffer cells.

Another indication for the capacity of sporozoites to leave Kupffer cells might be the significant increase in density of EEF in dexamethasone-treated rats (unpublished observation). We found repeatedly a 3-fold in-

schizont. An inner pellicular membrane (IM) is laid down just beneath the future budding point of merozoites. The rest of the parasite is still enclosed by a single membrane. Malarial pigment (arrows), nucleus (N), ER and developing rhoptries (R) are clearly visible. × 13,000, bar 1 µm. Figure 5. Advanced stage of a segmenting schizont. Partially budded merozoites are still attached to the residual body (RB) containing malarial pigment (arrows). N, nucleus. × 13,000, bar 1 µm. Figure 6. Longitudinal section through a sporozoite showing its sickle shape. Rhoptries and micronemes together with a nucleus (N) and a dumb-bell shaped mitochondrion (M) are visible in the anterior part. The 3 layered pellicle (P) and subpellicular microtubules (MT) are apparent in this free parasite. × 23,000, bar 1 µm.

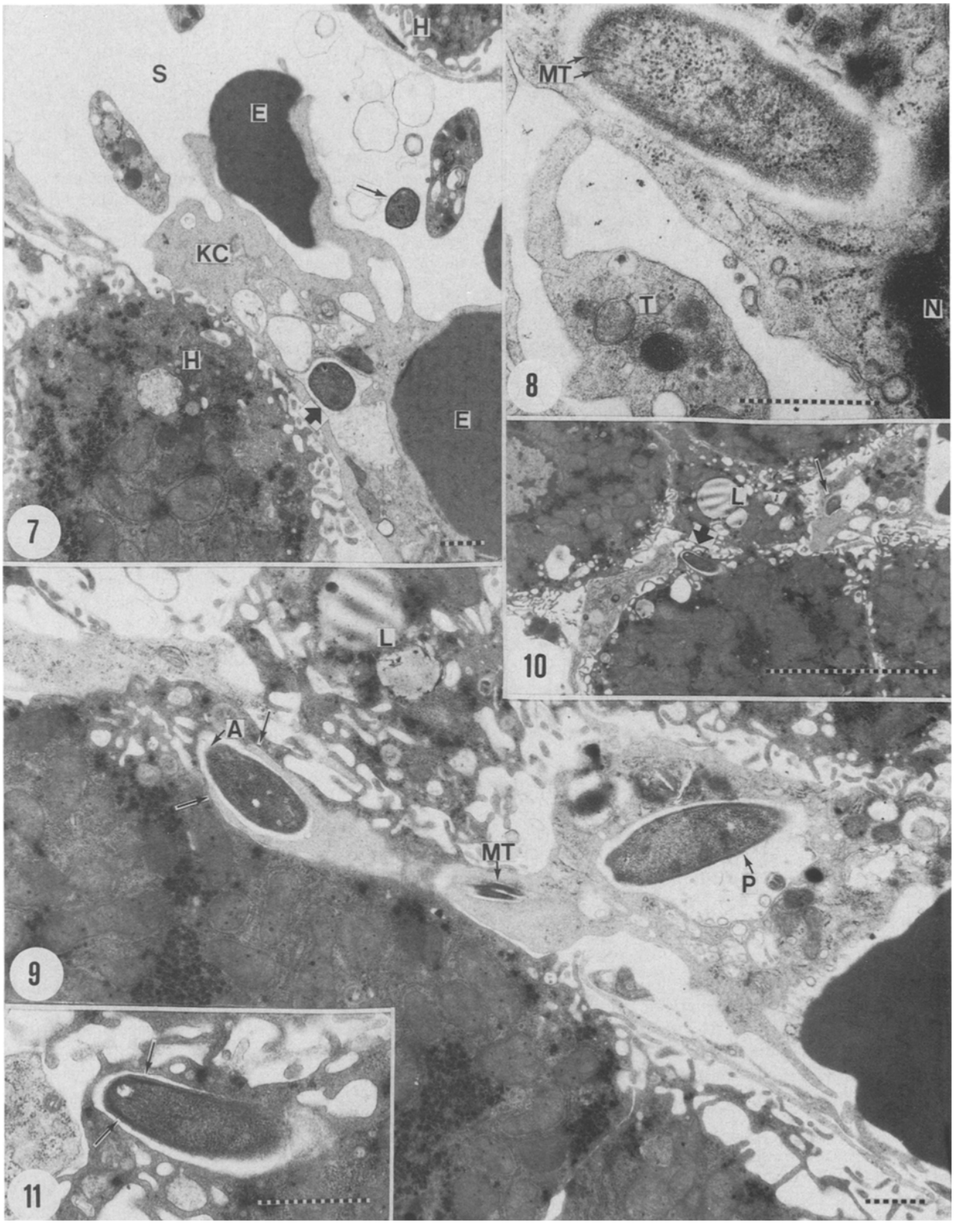

Figure 7. Liver sinusoid (S) with a lining Kupffer cell (KC) bordering the hepatocytes (H). The Kupffer cell has phagocytozed a sporozoite (arrow) and an erythrocyte (E). Another erythrocyte (E) and sporozoite (thin arrow) are being engulfed. × 7,600, bar 1 µm. Figure 8. Detail of a sporozoite in a Kupffer cell demonstrating clearly the longitudinal section through the subpellicular microtubules (MT). N, Kupffer cell nucleus; T, thrombocyte. × 24,500, bar 1 µm. Figure 9. A sporozoite with its posterior end (P) localized in the Kupffer cell body and the anterior part (A) in a protrusion and in free contact with the hepatocyte. Arrows point to the retracting Kupffer cell philopods. Note the section through the subpellicular microtubules (MT) localized in the Kupffer cell protrusion. L, Lipid droplets. × 11,000, bar 1 µm. Figure 10. Low power magnification of

crease of EEF, which may be brought about by a membrane-stabilizing effect of this drug on macrophages and the inhibition of the fusion of lysosomes and phagocytic vacuoles. If the intracellular killing is thus delayed, and none of the other effects of this synthetic corticosteroid influences the parasite-liver cell interactions, the sporozoites may leave the Kupffer cells more easily.

Further to the role of Kupffer cells as mediators for the admission of sporozoites to hepatocytes is the distribution of mature EEF in the liver acini coinciding with that of Kupffer cells. The basal liver units show their Kupffer cells in highest numbers at the areas of entrance of the blood flow (zone 1) and low at the exit areas (zone 3)[63]. Similarly the majority of EEF are in zone 1, and measurably closer to the afferent vessels than to the efferent ones[49].

Thus there is an increasing body of indirect evidence pointing to the Kupffer cells as 'porte d'entrée' to the space of Disse and the bordering hepatocytes. To this we have recently added direct ultrastructural proof of Kupffer cell passage[49]. A sporozoite was observed in an endocytotic vacuole of a Kupffer cell, lying in a cytoplasmic projection at the undersurface (i.e. in the space of Disse). The latter was embedded in and attached to an adjacent hepatocyte. Serial sections made clear that the sporozoite was protruding out of its vacuole and penetrated the hepatocyte cytoplasm with its apical end (figs 9, 10, 11).

At least in the case with *P. berghei*-rat models, we consider that the hypothesis on the dual role of Kupffer cells in the fate of sporozoites is significantly substantiated.

Invading forms and forces

Sporozoites of rodent plasmodia present themselves to hepatocytes with their apical end forward, after actively leaving the Kupffer cells[49, 52]. The attachment to hepatocytes may be facilitated by galactose receptors on the hepatocyte membrane, as sporozoites have acquired glycoproteins in the plasma, which contain galactose moieties[28, 59] and as the penetration is inhibited in vitro by ligands that compete for the galactose receptor.

The apical organelles, which may generate surface antigen[21] and other substances[34] to facilitate the penetration process are clearly visible but do not empty. Yet, the apical end attaches and remains fixed to the hepatocyte membrane. Invagination of this membrane takes place as the sporozoite moves inside (fig. 11). There is no point of attachment visible between the hepatocyte membrane, and the more distal parts of the sporozoite and the latter is nowhere constricted. The membrane probably seals as the caudal end of the sporozoite is far enough inside, thereby creating a parasitophorous vacuole (fig. 12).

There are no signs of microfilament-accumulations in the hepatocyte cytoplasm around the invading sporozoite, which suggests activity only by the parasite[52]. Russell and Sinden[55, 56, 65] proposed that the motile stages are able to penetrate actively, because a membrane-associated actin-based contractile system is present which connects the ligands with the helically coiled microtubules and brings about a capping reaction through which the parasite moves into a cell, with a rotating locomotion. Cytochalasin B, which interferes with locomotion through dismantling of microfilaments, inhibits the penetration of sporozoites in cultured cells[15, 27].

The whole sequence of events in the sporozoite-hepatocyte interaction strongly resembles that of the merozoite-erythrocyte[2, 5]. This small oval stage is free in the bloodstream and therefore needs a more avid recognition system than the sporozoite. It is now clear that various species use different receptors on the erythrocyte: merozoites of *P. vivax* and *P. knowlesi* make use of the Duffy blood group[5] and *P. falciparum*-merozoites use the oligosaccharide part of glycophorins on the erythrocyte surface as receptors[54].

After recognition and attachment the merozoite orients its apical end to the erythrocyte membrane and a junction is established through fine fibrils. Here again the content of the rhoptries and micronemes deforms the erythrocyte membrane[34]. Remaining attached to the host membrane the apex moves inward, thus creating an indentation. This thickening zone of contact becomes circumferential and may represent contractile protein aggregates, which flow away from the apex. The band remains in contact with the merozoite only as a narrow junction which is resolved at the caudal end of the parasite[6].

Meanwhile the surface coat is stripped from its surface. The erythrocyte membrane reseals and the merozoite has entered a parasitophorous vacuole, the membrane of which is of outer membrane origin but greatly expanded, protein poor and lipid rich[2].

The parallels with the hepatocyte-penetrating sporozoite are obvious, but the details of the merozoite-erythrocyte interaction are overwhelmingly more abundant.

Engrossment in the host cells' womb

Intact sporozoites can be observed inside hepatocytes from a few minutes to several hours after inoculation[45, 52, 62] (fig. 12). At 4 h sporozoites begin to dedifferentiate; the part containing nucleus and mitochondrion becomes bulbous and the long extension degenerates after losing the rhoptry-microneme complex (fig. 13). Subpellicular microtubules are still visible at places where the inner membranes are intact (fig. 14). 10 h after inoculaton parasites have lost most of the characteristics of a sporozoite. They have about doubled in volume, are more or less spherical and enveloped partly by a unimembranous pellicle. The cytoplasm has become more electron-lucent with scattered ribosomes, few polysomes and scantly endoplasmic reticulum[51].

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another section of the same area as shown in figure 9. The Kupffer cell protrusion has disappeared. The apical end of the sporozoite is laying in contact with the hepatocyte (thick arrow), while the posterior end is still visible in the Kupffer cell body (thin arrow). × 3000. Compare the position of the lipid (L) with figure 9. Figure 11. This is a detail of figure 10 demonstrating the anterior tip of the sporozoite with the polar rings (arrows). × 17,000, bar 1 µm. (Figures 9, 10, 11 reproduced from Meis et al.[49], with permission of Cambridge University Press and the authors).

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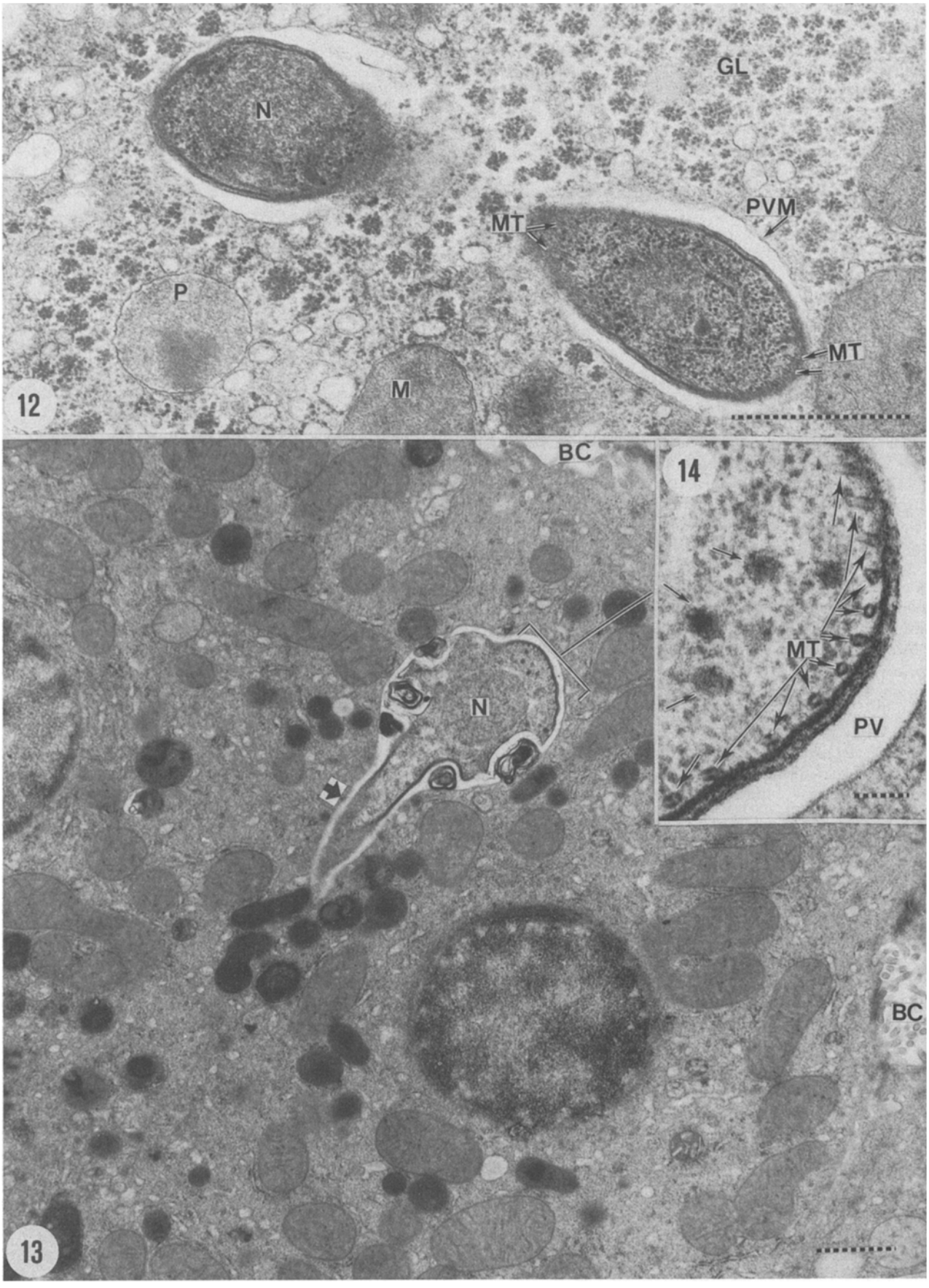

The structural changes find their parallel in the erythrocytic phase of *P. berghei*[40] and other species[42]. The breakdown of organelles typical of the merozoite and rounding-up of the parasite take place after rapid degradation of the inner structures of the pellicular complex. Once inside an erythrocyte the merozoite dedifferentiates into a structurally simple trophozoite, which remains so for several hours (fig. 1). The ookinete-oocyst transformation undergoes a similar dedifferentiation[66, 69, 73], but in oocysts the apical tip of the former ookinete is retracted within the parasite and later on the remnants of ookinete microtubules are found deep in the cytoplasm[14, 18].

Unlike the motile, invasive stages, the major need of sessile forms is nutrients in order to fulfill their goal of multiplication. EEF feed by diffusion of substances through the parasite membrane[44, 47]. Oocysts use this mechanism as well; moreover, parts of the surrounding capsule are pinched off and subsequently disppear in a process of digestion[1]. Quite differently, the erythrocytic stages maintain a cytostome in their plasmalemma through which the hemoglobin is drawn into a cavity (fig. 2). What remains in food vacuoles, after digestion, is the hemozoin or malarial pigment (figs 2, 4, 5).

The organelles involved in metabolism, digestion and excretion are mitochondria, endoplasmic reticulum (ER), Golgi complexes and particularly in the EEF, various types of vesicles[47].

The mitochondria are slender and double-membraned, assuming various shapes. With the growth and consecutive nuclear divisions, the mitochondria assume a dumb-bell shape and through fission their numbers increase. Simultaneously with the nuclear and mitochondrial multiplication, there is an increase in the number of ER-aggregates (figs 17, 18), which are continuous with the perinuclear space, very similar to the development in oocysts[8, 44, 60]. On the other hand ER in erythrocytic forms consists of a single aggregate (fig. 4), which again reflects the much reduced replication potential of the latter.

A distinct type of unimembranous vesicles originates at the ER of the liver form. These are filled with fluffy material and become bigger the closer they lie to the periphery of the parasite where they fuse with each other. Both in vivo and in vitro, after fusion with the parasite's enveloping membrane they discard their content in the narrow parasitophorous vacuole, often causing identations into the host membrane and cytoplasm[44, 47] (fig. 19). This phenomenon has been observed in EEF of *P. yoelii*[41, 60], and *P. falciparum*[13], but also in oocysts[8]. Their function may be related with the excretion of material that digests either the host cell, or in case of the oocyst, its outer wall. We have observed an increased permeability of the outer membrane of host hepatocytes for ruthenium red[32]. The lysosomal enzyme acid phosphatase was particularly associated with these vesicles[53]. This enzyme might play a role in the change of apperance of host hepatocytes[32, 41] and the increased leakiness of the outer host cell membrane. It may facilitate the diffusion of nutrients through the narrow rim of host cytoplasm to the maturing schizont. Pinocytotic vesicles are very frequent along the outer membrane of the infected hepatocyte, both in vivo and in vitro. The content of the parasitophorous vacuole is shown to include antigens that occur on sporozoites and on blood stages[25]. Some maturing EEF are rapidly changing and degraded upon attack by macrophages after the release of parasitic material (figs 15, 16).

Associated with the ER complex in EEF, groups of small bristle-coated vesicles are present that arise by budding from the cisternal membranes. They group together in rows or circles which occur abundantly and randomly throughout the growing EEF[48]. Such supposed Golgi complexes were observed earlier in oocysts[58] and sporozoites[76] where they occur in relation to the nuclear envelope and the Golgi bodies might secrete the constituents of the thick oocyst capsule. In asexual erythrocytic stages Golgi complexes have rarely been found[42] which is remarkable considering the high intake of saccharides and output of glycoproteins.

Feeding and fostering

All stages of the malaria parasite are dependent on exogenous glucose as a source of energy. Both extra- and intracellular stages rely on the host supply for their carbohydrates.

Glycogen is not observed in EEF (and other stages), but it is abundant in surrounding hepatocytes. We did find host-hepatocytes depleted of glycogen in our EM studies, but other cells showed the characteristic rosettes of glycogen (fig. 12). Though EEF and glycogen may not be mutually exclusive in one hepatocyte, it was shown that the general distribution of EEF in liver acini is in the afferent zones where the storing of glycogen is minimal[49]. We have evidence that EEF-density can be increased more than 2-fold after 48 h of starvation, when hepatocytic glycogen is depleted.

Similar results have been obtained with *P. cynomolgi* in starved monkeys[10]. In the latter experiments the drug phlorhizin was used, which further depletes the liver of glycogen. Parasite density increased 5-fold without a corresponding change in size or alteration of organelles, from which it was concluded that glycogen may act as a barrier for intrahepatocytic growth and glucose being acquired directly from the blood. This in turn would fit with our observation that rats are more susceptible for sporozoite infection at the end of their daily resting period, when the glycogen content in the liver of nocturnal rodents is minimal. When the active feeding period is over, i.e. towards the end of the night, infectivity is lowest and glycogen content at its peak. Whatever the

Figure 12. A sporozoite in a hepatocyte 1 h after inoculation, surrounded by glycogen rosettes (GL), mitochondria (M) and a peroxysome (P). The parasite is enclosed by a host cell derived parasitophorous vacuole membrane (PVM). Note the subpellicular microtubules (MT) and the nucleus (N). × 35,000, bar 1 μm. Figure 13. A partially transformed intrahepatocytic sporozoite 4 h after inoculation. The part of the sporozoite around the nucleus (N) is enlarged and rounded. At places where no subpellicular membranes are situated the inner pellicular membranes are lost. The remaining part of the sporozoite (arrow) probably is not involved in the differentiation process. × 14,000, bar 1 μm. Figure 14. Detail of the indicated part of figure 13. At the places where subpellicular microtubules (MT) are situated the inner pellicular membranes are still intact. 5 electron-dense granules (50 nm) are also visible (arrows). PV, parasitophorous vacuole. × 105,000, bar 0.1 μm.

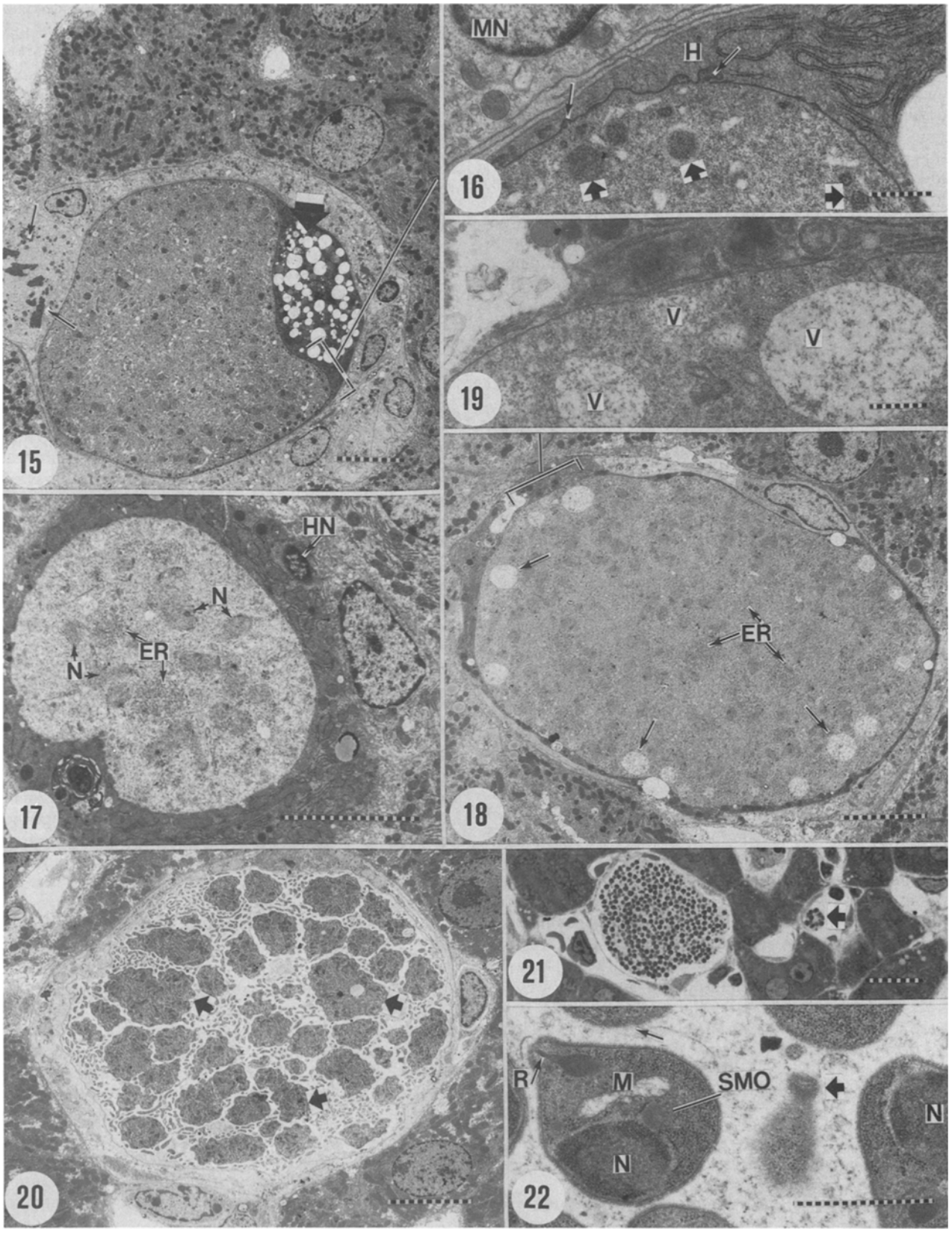

Figure 15. A degenerating almost mature parasite 49 h after inoculation of sporozoites. This parasite is completely surrounded by mononuclear cells. The parasite has become electron-dense with dilated ER. The peripheral vacuolization is reduced to electron-dense granular vacuoles (see figure 16). The host hepatocyte is vacuolated (arrow). Note the dividing Kupffer cell with condensed chromosomes (thin arrows). × 1250, bar 1 μm. Figure 16. Detail of the indicated area in figure 15. The condensed appearance of the vacuoles is visible (arrows). The same material is in the parasitophorous vacuole (thin arrow). The host hepatocyte (H) fits closely to a mononuclear cell (MN). × 11,000, bar 1 μm. Figure 17. Exoerythrocytic form 33 h after inoculation. About 10 nuclear fragments (N) and few ER patches are visible. Note the tangential section through the host

mechanism of this circadian fluctuation in sensitivity to infection, the parasite adapts itself at various levels of development to the condition of its host(s).

Histochemistry of EEF revealed no enzymes that could play a role in the generation of glycogen or glucose[53]. The exogenous glucose, acquired by the parasite, appears to be degraded in different ways.

First, it became evident that glucose is broken down via pyruvate to lactic acid, as the enzyme lactate dehydrogenase can be demonstrated histochemically in EEF[53]. The erythrocytic stages are, however, more dependent on this enzyme than the EEF, as they grow under relative anaerobic conditions. More importantly, suggestive evidence was found that the EEF have an active Krebs cycle, by demonstrating three enzymes of the citric acid pathway, among others succinate dehydrogenase (SDH). Both oocysts and sporozoites do have an active Krebs cycle, whereas the blood forms do not. This is reflected in the architecture of the mitochondria of the various stages. In oocysts and sporozoites they are clearly cristate, EEF have less, but demonstrable cristae[44,74] and the erythrocytic forms have acristate mitochondria (fig. 1).

Howells[29] has given an elegant demonstration of the congruency of SDH activity and cristae in mitochondria, the erythrocytic forms lacking both. Unfortunately, both histochemical and ultrastructural data on EEF, suggesting a lack of SDH and cristae, needs correction[8,31]. EEF take an intermediate position in both respects which makes the change of metabolism at transition from invertebrate to vertebrate host less sudden than was thought before[53]. The utilization of oxygen may well be indicated by the presence of cytochrome oxidase in the mitochondria, irrespective of the presence of cristae, as was shown repeatedly for oocysts, sporozoites, EEF and blood forms[30]. But the latter, because of their micro-aerophilic nature might not use the oxygen for the generation of energy but for the synthesis of pyrimidines[61].

These findings suggest a system for aerobic energy generation in both the mosquito stages and the EEF, and a glycolytic metabolism for the erythrocytic forms. Part of the glucose taken up by the EEF is converted to ribose via the pentose phosphate pathway. The demonstration of glucose-6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase activity in EEF[53] sets this tissue form again apart from the blood forms in their catabolism. The former enzyme is almost always lacking in erythrocytic development and debate continues as to how these stages acquire their NADPH and pentoses. Reduced coenzyme is a key product for the synthesis of fatty acids and sterols, whereas the pentoses are used for the generation of nucleic acids. Both are of prime importance for the dramatic growth, formation of membranes and nuclear division in EEF. As the multiplication rate is so enormous[35] (from 1 sporozoite to 10,000 merozoites in 50 h) the parasite needs, in order to meet the much higher demands, metabolic pathways more special than those likely to exist in oocysts and blood schizonts.

Dying for daughter cells

The young EEF of *P. berghei* remains uninuclear until about 22 h, and at 25 h the first signs of nuclear division become apparent. In the following 25 h the nuclear complex grows into a syncitium with no nuclear fission occurring (figs. 17, 18). The continuity of the nuclear envelope remains intact as the number of nuclear profiles increases. Multiple spindle figures in one nuclear profile can be demonstrated[44], a situation which is similar to that in oocysts[73,76] and, to some extent, in erythrocytic schizonts[64].

As spindles are seen all the time and no classic interphase occurs, nuclear division is a continuous process and not an activity that takes place in waves. The nuclear division is clear at metaphase: spindle fibers originate at the nuclear envelope and radiate in a shower-shaped fashion towards the middle of the nucleoplasm, where they meet other fibers. The shorter fibers probably end in pentalaminate kinetochores, as was seen in oocysts[58] and blood schizonts. The nucleoplasm is finely granular and, similar to that of the sporozoite, it shows no heterochromatin. Chromosomes do not condense and thus are not visible in any stage of nuclear division of malarial parasites and chromosome numbers may only be derived from counting kinetochores in serial EM-sections, assuming a 1:1 relationship. In oocysts this number amounts to eight[58].

The spindle fibers originate in a unique type of centriolar plaque, which is electron-dense and transverses the nuclear envelope at a pore. This is particularly clear in oocysts, and probably finds its parallel in the similarly designed EEF. In oocysts the plaques are ring-like and consist of 9 triplets, the whole functioning as microtubule organizing centers (MTOC) from where the long and short fibers are polymerized and fan out. Centriolar plaques duplicate themselves, and the new spindles move along the nuclear envelope to an opposing position, initially keeping contact by an electron-dense bridge along the inner nuclear membrane[58].

In asexual blood forms only 4 or 5 nuclear divisions occur, while the number in oocysts and EEF is of a completely different order. In the case of *P. berghei* these latter stages both give rise to 10,000 daughter cells, and thus the number of nuclear divisions must be at least 14. Particularly in EEF of rodent species the dynamics mount to the unimaginable, since it takes only 25 h as compared with oocysts that take more than

nucleus (HN). × 2500, bar 10 μm. Figure 18. A normal developing exoerythrocytic parasite 48 h after inoculation of sporozoites. Note the clear peripheral vacuolization (arrows), patches of ER and nuclei. × 1500, bar 10 μm. Figure 19. Detail of the indicated area in figure 18 showing peripheral vacuoles (V) with fluffy granular content. × 10,000, bar 1 μm. Figure 20. A mature exoerythrocytic form segmented in pseudocytomeres (arrows), from which merozoites bud. × 1500, bar 10 μm. Figure 21. Semithin section showing a mature exoerythrocytic form with fully formed merozoites. No host cell reaction is apparent. Note the small cluster of merozoites free in the sinusoid (arrow). × 1500, bar 20 μm. Figure 22. Detail of figure 21 demonstrating exoerythrocytic merozoites. Note the pellicular membranes with the clear surface coat (thin arrow). The rhoptry (R) ends in the apical complex. A tangential section through the apical rings is demonstrated at arrow. Nucleus (N) with heterochromatin, acristate mitochondrion (M) and smooth membraned organelle (SMO) are visible. × 24,500, bar 1 μm.

8 days for their nuclear multiplication (i.e. approximately half of their life span).
Only during the very last maturation division a differentiation into merozoites occurs. These events are preceded by the formation of large vacuoles that fuse at the periphery, thus enlarging the parasitophorous vacuole in which the budding of merozoites can take place. The spaces grow into the cytoplasm through cleft formation and subdivide it into peninsulae or pseudocytomeres (sporoblasts and meroblasts respectively) (fig. 20). The endoplasmic reticulum assumes the appearance of concentric whorls[19].
Along the limiting membrane of these pseudocytomeres interrupted strands of an inner layer are formed under which an electron-dense mass becomes visible. This is an MTOC which (at least in avian plasmodia) generates the future sub-pellicular microtubules. The polymerization of these structures keeps pace with the outgrowth of the inner layer into a collapsed vacuole, thus creating the pellicular complex of the budding merozoite. These structures are found opposite to the centriolar plaques and only at this time a real nuclear fission takes place. While the growing merozoites protrude out of their pseudocytomeres rhoptry and microneme precursors appear and lateron the cells are provided with a nucleus, a mitochondrion associated with a spherical body, endoplasmic reticulum and presumably also a Golgi complex (fig. 22). Shortly thereafter the spherical pear-shaped merozoites move free in the parasitophorous vacuole (fig. 21), together with a remnant cytoplasm that contains the organelles not intended for daughter cells, the socalled residual body. They remain lying in a spongy stroma that enters the sinusoid, and Kupffer cells may engulf clusters of these merozoites[22, 75]. Our own observations do not confirm suggestions[1, 64] that exoerythrocytic merozoites are bigger than erythrocytic ones; both measure about 1.5 µm in length. Whether some of these develop into gametocytes remains to be confirmed[35].
The new merozoites have a feeding organelle, the cytostome, which starts functioning once the parasite has settled in an erythrocyte[9]. In the nuclei a clear difference is visible between euchromatin and heterochromatin (fig. 22). In merozoites and trophozoites of *P. gallinaceum* such clumps of chromatin are also marked; they resolve before schizogony. Chromatin in nuclei of blood stage trophozoites of *P. berghei* can be made visible only with cytochemical techniques[1, 7].
The daughter merozoites of tissue stages in avian plasmodia are much fewer in number, more elongated in shape and contain a more obvious system of subpellicular microtubules, which determines their cell shape[4]. Erythrocytic and exoerythrocytic merozoites of *P. berghei* do not have subpellicular microtubules (figs 5, 22). A microfibrillar coat was seen to adhere on the outer pellicular membrane (fig. 22); analogous structures are seen on erythrocytic merozoites of primate plasmodia[42], but not on those of *P. berghei* (fig. 5).
In the avian parasite *P. lophurae* a unique surface glycoprotein was identified for exoerythrocytic merozoites[33] but these have the ability to reinvade tissues, unlike merozoites of mammalian plasmodia. The target cell of both types of merozoites of the latter is the erythrocyte and therefore major differences in surface ligands may not be required. Moreover, the antigenic homology of blood stages and mature EEF in rodent and human plasmodia, as found with immunofluorescent or immunoperoxidase antibody techniques is striking[17, 20, 25]. Yet, merozoites of EEF still show some sporozoite antigen, whereas blood stage merozoites do not.

Dormant and born-again plasmodia

Tissue forms are clearly a way of establishing high infection rates in the blood of vertebrates and for survival sake some species like *P. yoelii* in its original rodent host have additional liver forms with retarded development, just like in other genera of Haemosporina[41]. Bird plasmodia have a system of rapid multiplication through 3 consecutive schizogonies that vary in tissue-location and morphology, and yield restricted numbers of daughter cells. The versatility of that system is great, as daughter cells and cells of distant generations may re-enter the tissues. Similarly, it was thought for a long time that merozoites from exoerythrocytic schizonts of *P. vivax* and related species gave rise to secondary exoerythrocytic development. This hypothesis was put forward to explain the notorious relapses that occur after primary attack and cure of the parasitemia. It was known since the late 1920's that relapses had something to do with the development of sporozoites, as blood-inoculated *P. vivax* (for the treatment of neurosyphilitic paralysis) could be readily eliminated by drugs, unlike the mosquito-borne infections.
Recently, the morphological basis for these relapses was demonstrated and the existence of secondary liver forms disproved. In a combined effort of several research groups it was elegantly shown that sporozoites of the relapsing monkey parasite *P. cynomolgi* give rise to both primary schizonts and small resting forms in hepatocytes[12, 38, 39]. The dormant forms measure about 4–5 µm in diameter, contain 1 nucleus and were named hypnozoites. They were supposed to resume growth and multiplication in their host-hepatocytes at a much later stage, giving rise to new outbreaks of parasitemia: the true relapses. Despite meticulous screening, no hypnozoites could be demonstrated earlier than 3 days after inoculation of sporozoites, which makes a temporary stay elsewhere worth considering.
They may well lurk inside Kupffer cells in some sort of antigenic disguise. The existence of hypnozoites makes the understanding of biological characteristics of *P. vivax* from the (sub)tropical and temperate zones easier. The former give rise to the primary attack within 2 weeks, relapse shortly thereafter and may continue to do so quite frequently at short intervals. Strains from temperate climates often fail to give a primary attack until 8 months after inoculation of sporozoites and may relapse thereafter, once or twice.
An excellent example of dormant malaria was the formerly ill-famed *P. vivax* of The Netherlands, and the existence of its long latency was already put forward in 1902 by the eminent Dutch malariologist P.C. Korteweg[36]. The regular epidemiological pattern turned out to be based on transmission during the autumn by few semi-hibernating *Anopheles atroparvus* and clinical cases

that peaked in numbers during the next May-June period, when no infected mosquitoes were around. Experimental proof of this long latency was obtained when he and several colleagues exposed themselves to one infected mosquito: they all experienced their first attack 8½ months thereafter[72]. Feeding of greater numbers of mosquitoes on volunteers or neurosyphilis patients was followed in about 60% of the cases by a primary attack with 1–2 weeks. Chance of relapse in these patients was about 13%, although naturally infected persons relapsed in about 2–50% of the cases[37]. A possible relationship between the number of relapses and biting mosquitoes was not worked out further. Only recently, dilution experiments with sporozoites of temperate-zone *P. vivax* confirmed the old observations and showed also a direct relationship of sporozoite numbers and numbers of relapses[12]. It was suggested that sporozoites are pre-programmed to develop either into immediate schizogony and parasitemia or to stay dormant for varying periods of time. The predictability of latency periods and relapse patterns in the various strains of *P. vivax* suggest inbuilt differences in sporozoites and not a certain reactivation signal from the host. This is supported by the fact that experimental infections throughout the year always led to latency of about 8 months. At any rate, it is clear that the presence of reactivated hypnozoites is of utmost advantage for survival of the parasite.

Recent breakthroughs in infecting primary human hepatocytes and hepatoma cells in vitro with *P. vivax*-sporozoites, and maintaining these cells for long periods, will be of major importance for the study of hypnozoites[24, 43]. The longevity of hepatocytes in vivo must be, however, of a completely different order since we have to presume that hypnozoites giving rise to relapses after more than a year, must have remained inside their particular host-cells all along this period.

Expected development

The present possibilities for in vitro cultivation of the liver forms of mammalian plasmodia will undoubtedly augment the burst of interest that is presently being shown in this unobtrusive and non-pathogenic stage[23, 26, 27, 43, 47, 67].

The biology, fine structure, biochemistry and metabolism of the primate parasite species will be studied and a tool for estimating the viability of sporozoites will become available.

The culture possibilities might also provide a means for isolating EEF and testing them as a possible immunogen or vaccine. The metabolic state of hepatocytes and their influence on resting hypnozoites and growing schizonts is another field of interest that can be exploited in vitro. Particularly the findings that hunger would make the recipients of sporozoites more vulnerable and expose them to higher initial blood infections may be of practical value for human malaria. Also the well known age-related susceptibility in rodents might be studied in models using human plasmodia and human liver cells.

Animals that experience parasitemia at the moment of inoculation of sporozoites develop reduced numbers of EEF[78]. The background of this phenomenon is not completely known. Apart from parasite-related hepatic disfunction the presence of activated macrophages in the liver might give rise to elevated levels of oxygen radicals that promote killing. Moreover, parasitemias probably induce acute phase responses in the liver, which result in fever with the increased release of prostaglandins and of hormones that are required for the restoration of the animal's homeostasis. The resultant changes in hepatocytes and possible influence on their fitness to sustain growth of EEF can be studied in vitro.

Research in the field of causal prophylactic and tissue schizontocidal drugs on in vitro grown EEF of primate plasmodia will drastically decrease the use of monkeys, apes and human volunteers for screening purposes. The system might also provide a means for testing anti-relapse drugs on in vitro grown hypnozoites.

The availability of a very susceptible rodent model may be used for the application of lipsomes charged with primaquine derivatives and other drugs to the liver as particularly the involvement of Kupffer cells in the uptake and release of liposome content and sporozoites is interesting[70].

We express the hope that more of the weak spots of this perfect parasite will be discovered and that the control of malaria is facilitated by new means that attack the parasite in its tissue phase. It would mean higher survival and a better quality of life for children and parents in malarious areas.

Addendum

It may be noted that recently two research groups have reported on the in vitro growth of EEF of *P. falciparum* in primary human hepatocytes[42a, 69a]. Furthermore, suggestive evidence for the appearance in vitro of *P. vivax* – hypnozoites has been presented[24a].

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