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MÉMOIRES ORIGINAUX

Ultrastructural observations on the merocyst and gametocytes of *Hepatocystis* spp. from Malaysian squirrels

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Summary.

An immature merocyst of *Hepatocystis malayensis* and gametocytes of *H. brayi* were studied with the electron microscope. The merocyst consisted of a highly complex cytoplasmic reticulum ramifying through an amorphous matrix: the entire complex was enclosed by a simple unit membrane. The host cell was apparently destroyed completely during growth of the cyst.

Immature gametocytes were highly amoeboid and showed extensive vacuolisation or attenuation of the cytoplasm. The nucleus contained one or two prominent nucleoli.

Mature gametocytes had compact cytoplasm and contained pyriform osmiophilic bodies which were believed to function in the release of the parasites from the host cells. Macrogametocytes were distinguished from microgametocytes by cytoplasmic differences in numbers of ribosomes, and cristate mitochondria and in the extent of development of the smooth endoplasmic reticulum. The compact nuclei of the macrogametocytes had inconspicuous DNA but prominent nucleoli whereas those of the microgametocytes were irregular and showed a central aggregate of DNA.

In microgametogenesis karyokinesis of the parent nucleus was delayed until axoneme formation was complete. Then the nuclear buds were extruded into emerging microgametes. At fertilisation the plasmalemmas of the two gametes fused and the single axoneme and nucleus of the microgamete moved into the cytoplasm of the macrogamete.

Résumé.

Observations sur l'ultrastructure des mérocystes et des gamétocytes d'*Hepatocystis* spp. des Ecureuils de Malaisie.

Un mérocyste immature d'*Hepatocystis malayensis* et des gamétocytes d'*H. brayi* ont été étudiés en microscopie électronique. Le mérocyste était formé par un réticulum cytoplasmique très complexe se ramifiant à travers une matrice amorphe : l'ensemble était entouré par une simple membrane unique. La cellule hôte avait apparemment été entièrement détruite au cours de la croissance du kyste.

Les gamétocytes immatures étaient très amiboïdes et présentaient une vacuolisation étendue ou une atténuation du cytoplasme. Le noyau contenait un ou deux nucléoles bien visibles.

Les gamétocytes mûrs avaient un cytoplasme compact et renfermaient des corpuscules piriformes osmiophiles que nous pensons jouer un rôle dans l'émergence des parasites de la cellule hôte. Les macrogamétocytes ont été différenciés des microgamétocytes par des variations dans le nombre de ribosomes, celui des mitochondries en cristaux et l'étendue du développement du réticulum endoplasmique régulier. Les noyaux compacts des macrogamétocytes avaient un DNA peu visible, mais des nucléoles bien apparents, alors que ceux des microgamétocytes étaient irréguliers, avec un amas central de DNA.

Chez les microgamétocytes, la karyocinèse des noyaux parents était retardée jusqu'à ce que l'axonème soit complètement formé. Les bourgeons nucléaires ont alors poussé et donné des microgamètes. Au cours de la fécondation, les plasmalemmes du microgamète se sont déplacés dans le cytoplasme du macrogamète.

The ultrastructure of exoerythrocytic stages of Haemosporina has been little studied relative to that of erythrocytic stages, owing to the difficulty of locating the rather scarce schizonts in thin sections of the host's tissues. The first observations were made on avian Plasmodiidae in tissue cultures (Meyer and Mussachio, 1960, 1965 ; Hepler, Huff and Sprinz, 1966 ; Beaudoin and Strome, 1973) and in avian embryonic liver (Aikawa, Huff and Sprinz, 1968). Observations on tissue stages of the avian parasites *Haemoproteus columbae* (Haemoproteidae) and *Leucocytozoon simondi* (Leucocytozoidae) in their natural hosts were reported by Bradbury and Gallucci (1971, 1972) and Desser (1970 *a*, 1973) respectively. The only studies on tissue stages of mammalian Haemosporina are those on *Plasmodium berghei* (Garnham, Bird, Baker and Killick-Kendrick, 1969 ; Desser, Weller and Yoeli, 1972) and *Plasmodium cynomolgi* (Sodeman, Schnitzer, Durkee and Contacos, 1970).

Gametocytes and gametogenesis have been studied in a wider range of these blood parasites. Principal studies on the Plasmodiidae are those of Aikawa, Huff and

Sprinz (1969) on several species of avian, reptilian and mammalian malaria parasites and of Sinden, Canning and Spain (1976) on *Plasmodium yoelii*. The following avian and reptilian Haemoproteidae and Leucocytozoidae have been studied ; *H. columbae* (Bradbury and Trager, 1968 *a, b* ; Gallucci, 1974), *L. simondi* (Aikawa, Huff and Strome, 1970 ; Desser, Baker and Lake, 1970), *Parahaemoproteus velans* (Desser, 1972) and *Haemoproteus metchnikovae* (Sterling, 1972).

A notable omission from this series of ultrastructural studies is one on a haemoproteid parasite of mammals for, apart from an early study of microgametogenesis of malaria parasites (Garnham, Bird and Baker, 1967) which included *Hepatocystis kochi*, no observations have been made on the fine structure of these parasites.

The easy maintenance of *Hepatocystis*-infected squirrels from Malaysia in the authors' laboratories provided an opportunity to study *Hepatocystis* in its merocyst and gametocyte stages. As certain ultrastructural differences have been recorded between the avian and mammalian Haemosporina, notably in the presence or absence of functional mitochondria and centrioles and in surface structure, it was interesting to ascertain whether *Hepatocystis* more closely resembles the mammalian Plasmodiidae or the avian Haemoproteidae.

Materials and methods

Merocysts and gametocytes of two species of *Hepatocystis* were present in natural infections of *Callosciurus nigrovittatus* and *Callosciurus notatus* captured near Kuala Lumpur, Malaysia. The squirrels were held for several months in the authors' laboratories in London and Paris.

Merocysts were taken from the squirrels at biopsy. Cysts of different sizes surrounded by a minimal amount of host tissue were dissected from liver slices into fixative at room temperature. Blood, containing immature and mature gametocytes during a recrudescence of parasitaemia, was either withdrawn directly into a syringe containing fixative or was dripped into a deep watch-glass containing a drop of heparin (4 I.U. per ml of Grace's medium), from which samples were withdrawn for fixation at 1 minute intervals. After fixation the blood was spun at 1,500 rpm for 5 min, the supernatant removed and the concentrated cells were sucked into a piece of 1 mm diameter catheter tubing. This was folded in half, centrifuged at 2,500 rpm. for 10 min and the packed cells were expressed in 1 mm lengths into molten agar and allowed to cool. The agar was trimmed around the blood to form a block for further processing.

Merocysts and some blood samples were fixed in Karnovsky's fixative and were processed as described by Sinden, Canning and Spain (1976). Other blood samples were fixed in 1.25 % gluteraldehyde in 0.05 M phosphate buffer, pH 7.4 containing 4 % sucrose, for 10 min. at 20° C and for a further at 0° C. After 3 washes in 0.05 M phosphate buffer, the agar pellet was post-fixed in 1 % osmium tetroxide in phosphate buffer at 0° C. Dehydration was in ascending concentrations of ethanol followed by propylene oxide. All blocks were embedded in Araldite CY212.

The gametocytes studied by electron microscopy were those of *Hepatocystis brayi* described by Miltgen, Landau, Le Bail and Yap (1976) while the merocysts were those of *Hepatocystis malayensis* described by Landau, Miltgen, Yap and Le Bail (1976).

Observations

1. Merocysts.

Only one of several cysts processed for electron microscopy was an active, developing parasite. The others proved to be lesions consisting of masses of macrophages such as are commonly found after invasion and destruction of old merocysts. The intact cyst measured 600 μm in diameter and corresponded to the half grown cysts of *H. malayensis* of the type which provoked no host reaction as described by Landau et al. (1976).

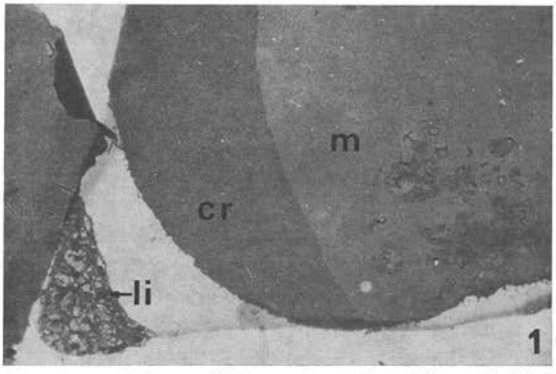

FIG. 1. — 0,5 μm thick section of araldite-embedded merocyst of *Hepatocystis malayensis*. The cyst is sharply divided into matrix (m) and the peripheral crescents of cytoplasmic reticulum (cr). The cyst is separated by a gap from the liver parenchyma (li) (X 175).

Briefly, the cyst consisted of a wall, which had no precise inner boundary but gave way imperceptibly to a largely amorphous matrix occupying the cavity of the cyst. The cytoplasm of the parasite, organised as a highly complex reticulum, ramified through the peripheral layers of the matrix and appeared as two dark crescents on opposite sides of the cyst (*fig. 1*).

a. WALL. The outline of the cyst appeared smooth in the light microscope but the electron microscope revealed an irregular boundary with numerous finger-like projections. Although the cyst was separated by a space 40-70 μm tick from the adjacent liver cells this was certainly created by tearing of the tissue during dissection and in life the projections would probably have interdigitated with irregularities in the liver cells, providing a close contact between host tissue and parasite.

The surface was bounded by a single unit membrane to which small external papillae with electron lucent contents were connected by a narrow neck (*fig. 2*). Beneath the plasmalemma was an homogenous electron dense layer ca. 140 nm thick which merged with the underlying matrix. The outer part of the matrix between the surface and the crescents of true cytoplasm, contained small vesicles with dense boundaries and a mass of amorphous dense material similar to that immediately beneath the plasmalemma.

b. MATRIX. The matrix filled the cyst, even occupying the interstices of the true cytoplasmic network (*fig. 3*). For the most part it consisted of an amorphous material of uniform electron density but contained islands of disorganised cellular components, including fragments of unit membrane, large vacuoles and mitochondria with tubular cristae, associated with dense amorphous material similar to that at the cyst surface (*fig. 4*).

c. CYTOPLASM. The cytoplasmic reticulum was bounded by a plasmalemma whose surface was sculptured into parallel dense lines (*fig. 6* insert). The reticulum formed a finely divided maze packed with monoribosomes, with areas ca. 17 μm wide containing nuclei and other organelles joined by strips narrowing to ca. 100 nm. Only this region of the cyst showed true cellular organization (*fig. 3, 6*).

There were numerous nuclei, oval or elongate in shape measuring ca. 1 μm in section. Many were undergoing endomitotic division, showing kinetochores on radiating microtubules and dense centriolar plaques inserted into nuclear pores in the nuclear envelope (*fig. 5*). Nucleoli and well-defined accumulations of chromatin were not seen either in interphase or in dividing nuclei. Mitochondria with tubular cristae were dispersed throughout the network, while some branches showed cysternae of smooth endoplasmic reticulum (*fig. 6*). At intervals the reticulum was expanded to enclose large vacuoles (*fig. 3*) bounded by unit membrane and containing electron dense material, possibly lipid, which was labile in the electron beam.

Vesicles intermediate in size between mitochondria and nuclei and bounded by doubles membranes were quite commonly encountered. They were usually rounded and occurred singly or in groups of up to five, which shared a common outer membrane (*fig. 6*). The inner membrane formed a packet around strongly electron dense material which usually appeared amorphous but occasionally contained spherical or perhaps tubular structures (*fig. 7*). Similar structures which occurred as branching tubes with less dense contents may have been stages of development or dissolution of the same organelles. Their nature and function is unknown though some aspects suggested a mitochondrial origin.

Merozoite formation was not observed and was not expected since the small size of the cyst and the dividing nuclei suggested that further growth and development would take place before maturity.

2. Immature gametocytes.

The earliest intra-erythrocytic parasites were highly irregular, 'amoeboid' stages (*fig. 8*). Sometimes several pseudopodia could be cut in a single section. They were surrounded by a unit membrane which itself was closely applied to a second membrane of equal thickness believed to be the vacuolar membrane of host origin. A typical micropore was observed in one case in the process of ingestion of erythrocytic cytoplasm (*fig. 8*).

Extensive vacuolisation of the cytoplasm was a striking feature which would have suggested poor fixation but for the presence of well-fixed mature gametocytes in the

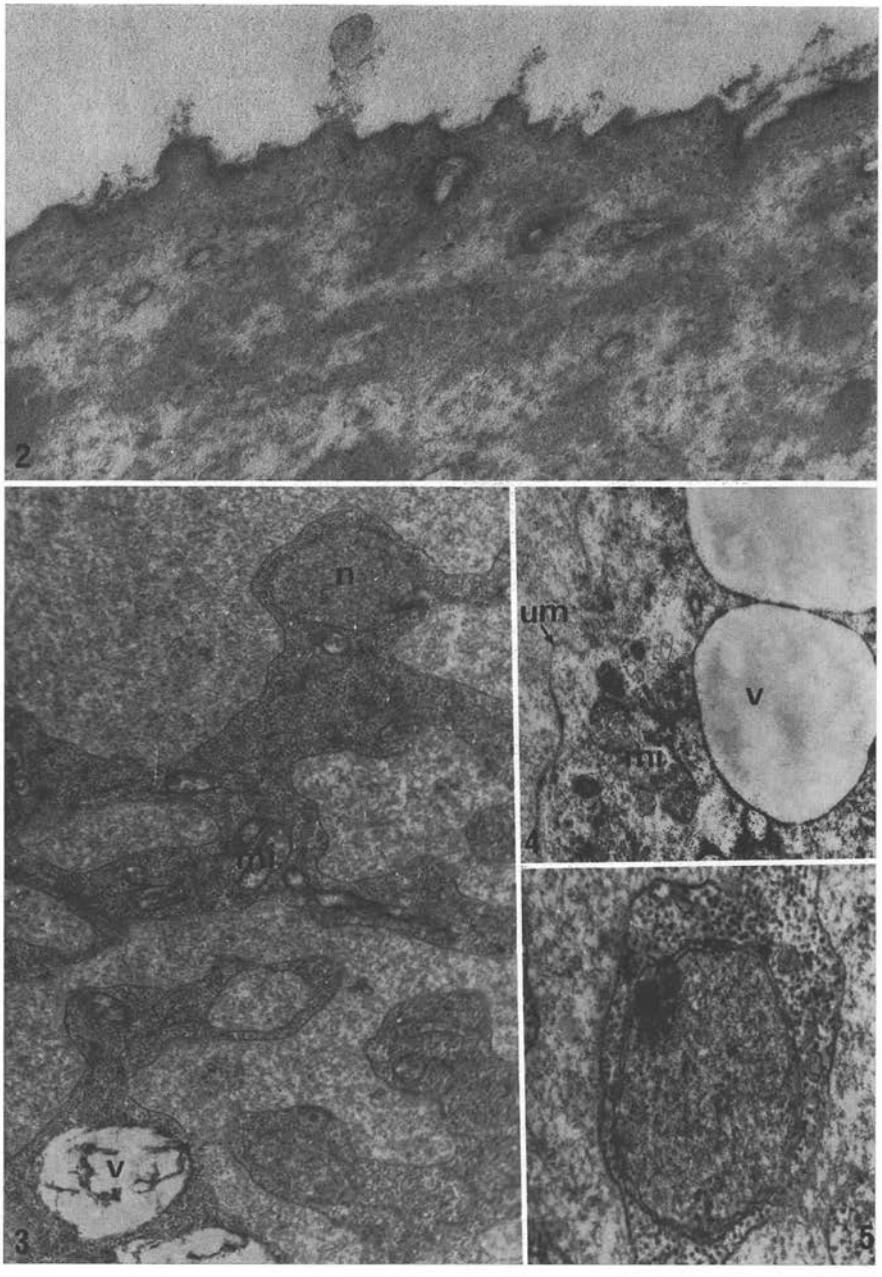

FIG. 2. — Peripheral layers of merocyst. The surface membrane with papillae overlies electron dense material (X 85,500).

FIG. 3. — The cytoplasmic reticulum of the merocyst contains nuclei (n), mitochondria (mi) and large vacuoles (v). The matrix occupies the interstices (X 20,500).

FIG. 4. — The edge of an island of disorganised cytoplasm in the merocyst matrix. The islands show unit membrane fragments (um), mitochondria with tubular cristae (mi) and large vacuoles (v) (X 16,000).

FIG. 5. — Endomitotic division in a nucleus of the cytoplasmic reticulum. A centriolar plaque inserted into a pore of the nuclear envelope and several kinetochores are visible (X 38,500).

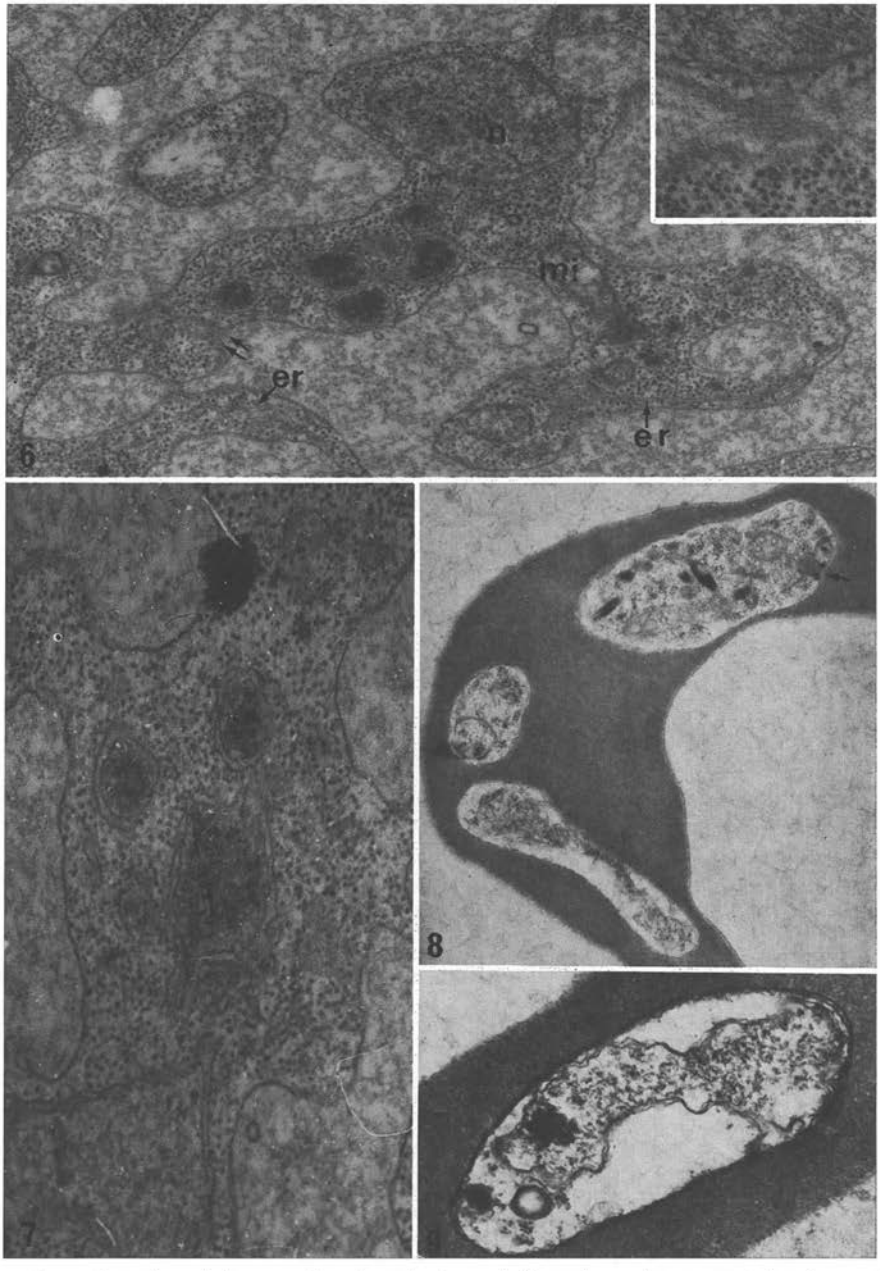

FIG. 6. — A region of the cytoplasmic reticulum of *H. malayensis* merocyst showing endoplasmic reticulum (er arrowed) mitochondria (mi) nucleus (n) and a group of 5 double-membraned vesicles with dense contents (X 38,000). An enlargement of an area such as that arrowed in fig. 6 showing surface sculpturing of the membrane of the cytoplasmic reticulum is inserted at top right (X 92,000).

FIG. 7. — Dense vesicles in the merocyst reticulum showing internal spherical or tubular structures (X 48,000).

FIG. 8. — Highly amoeboid intra-erythrocytic young gametocyte of *Hepatocystis brayi*. Cristate mitochondria, pigment granules and a micropore (arrowed) are visible (X 17,000).

FIG. 9. — *H. brayi*. Young gametocyte with vacuolated cytoplasm and nucleus containing a prominent nucleolus (X 20,500).

same block. The effect was achieved in some places by the presence of large and small membrane-bound vacuoles and in other places by attenuation of the cytoplasm especially in the immediate vicinity of the nuclear envelope. Compact cytoplasm, where present, contained cristate mitochondia, a few cysternae of rough endoplasmic reticulum, small vesicles, pigment crystals and abundant ribosomes. The ribosomes occurred as mono- or poly-ribosomes, the latter being more abundant or more clearly seen in attenuated regions. Clear distinctions between macro- and microgametocytes were not detectable at an early stage of development.

The nucleus occupied a considerable proportion of the cell volume and was characterised by the frequent occurrence of one or even two nucleoli, close to the nuclear envelope in an otherwise uniformly granular nucleoplasm (*fig. 9*). These dense accumulations of ribonucleic acid appeared as conspicuous dots at the edge of the nucleus in blood smears.

3. Mature intra-erythrocytic gametocytes.

During growth the attenuated and vacuolated state of the cytoplasm was lost, the parasites came to occupy almost the entire volume of the erythrocyte leaving only a narrow strip of cytoplasm outside and the features characteristic of the macro- and microgametocytes were differentiated (*fig. 10, 12*). The mature gametocytes possessed an extensive but incomplete system of membranes internal to the plasmalemma. These consisted of flattened sacs continuous with the endoplasmic reticulum or with the nuclear envelope. Surrounding the parasite, the vacuolar membrane was closely applied to the gametocyte plasmalemma and there were sometimes two parallel dense membranes which ran a wavy course in the narrow strip of erythrocyte cytoplasm (*fig. 11*). These membranes continued into folds which occasionally extended from the erythrocyte in the manner of the « apron » described for cells infected with *Plasmodium falciparum* (Kass et al., 1971).

The macrogametocytes (*fig. 10*) were characterised by extensive smooth-membraned endoplasmic reticulum, close packed monoribosomes and numerous cristate mito-

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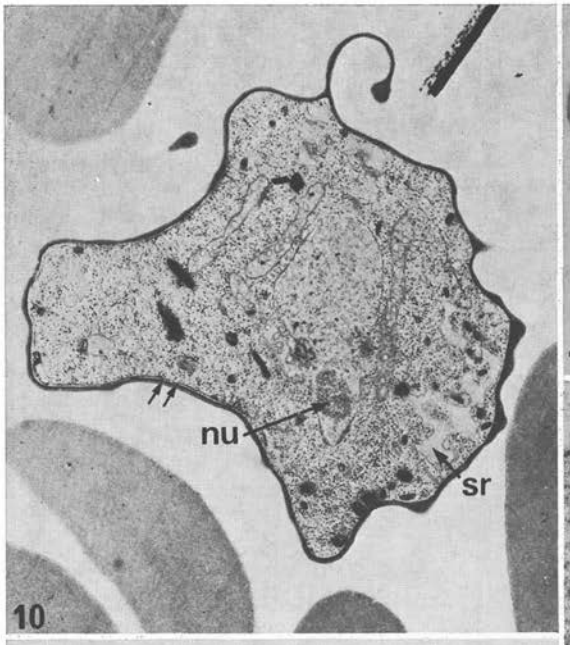
FIG. 10. — Mature macrogametocyte. The characteristic features — dense monoribosomes extensive smooth endoplasmic reticulum (sr), abundant mitochondria, pigment and small nucleus show. A pocket of the nucleus contains a nucleolus (nu). Flattened cysternae appearing as double membranes beneath the plasmalemma are indicated by double arrows (X 18,250).

FIG. 11. — Enlargement of part of fig. 10. Dense membranes in the erythrocyte cytoplasm are continued into a curved extension of the erythrocyte (X 49,000).

FIG. 12. — Mature microgametocyte showing large nucleus with dense DNA ; scattered polyribosomes, pigment and osmiophilic bodies. An " atypical " centriole (9 + 1 microtubules is arrowed (X 21,500).

FIG. 13. — Laminated crystalline pigment granules lying next to a mitochondrion in a microgametocyte (X 92,000).

FIG. 14. — Piriform osmiophilic bodies amongst polyribosomes in microgametocyte cytoplasm (X 61,250).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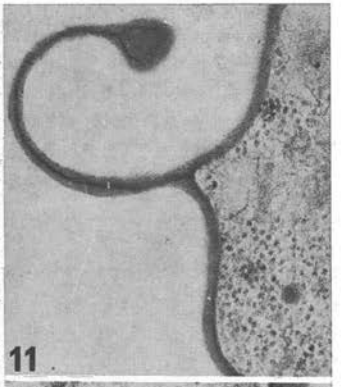
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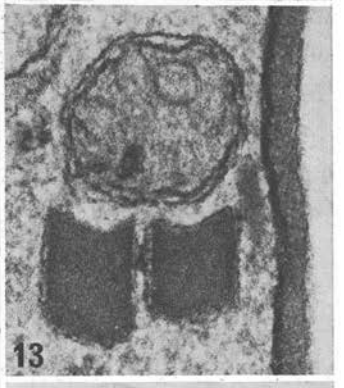
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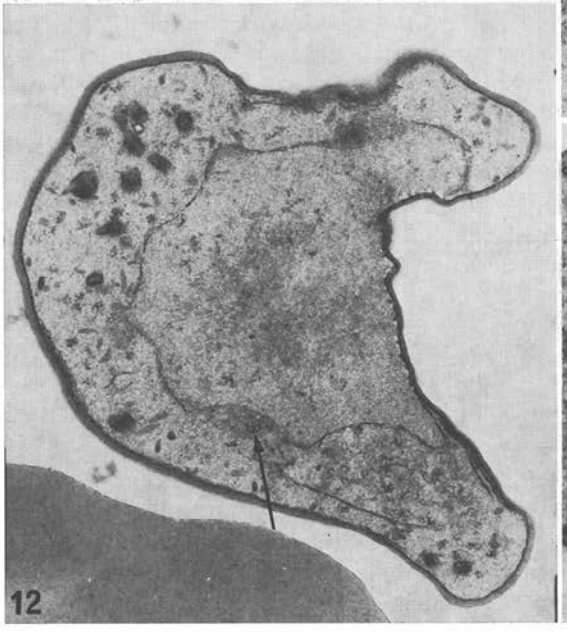
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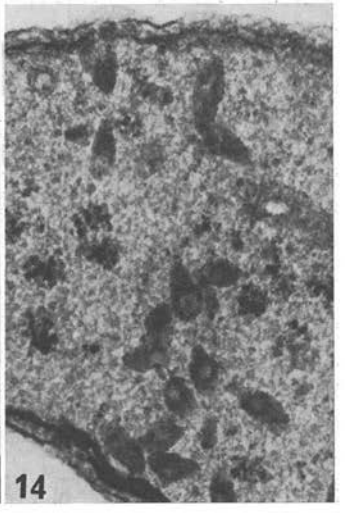
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chondria which often closely followed the nuclear envelope. The nucleus was small and usually of simple outline. One or two nucleoli were found just within the nuclear envelope and there were inconspicuous, 5 nm fibrils, probably of DNA and 60 nm particles dispersed or in small aggregates in the nucleoplasm.

In the microgametocyte (*fig. 12*) there were sparse ribosomes in groups of polyribosomes, inconspicuous endoplasmic reticulum and fewer mitochondria, though those present possessed well-developed tubular cristae. The nucleus was large and irregular in outline, sometimes highly irregular with more than one profile in a single section. A large area of electron dense material in the centre of the nucleus probably represented the DNA. There were dispersed particles and small aggregates but no well-defined nucleoli.

Nuclear pores were numerous in both types of gametocyte and other features, common to both were irregularly distributed pigment occasionally seen in its laminated crystalline structure (*fig. 13*) and piriform osmiophilic bodies (*fig. 14*) of medium electron density with a less dense area at the broader end.

An « atypical centriole » consisting of 9 single peripheral microtubules around a single central microtubule was observed within an electron dense mass adjacent to the nuclear envelope of one mature microgametocyte (*fig. 12*).

4. Gametogenesis.

Removal of the blood from the host initiated gametogenesis, which involved the dissolution of the remnants of the erythrocyte, a decrease in the number of osmiophilic bodies and rounding off of the gametocytes, which had previously often been elongate and flattened within host erythrocytes. The inner sub-pellicular sacs of endoplasmic reticulum were much reduced or had passed deeper into the cytoplasm so that the surface covering was reduced to a simple plasmalemma almost everywhere.

There were no radical changes in the organisation of the macrogamete. The nucleus remained compact, retained the granular appearance and the nucleoli, and occupied a central or peripheral position.

Microgametogenesis was initiated almost immediately when blood was removed from the host and could be completed in as little as one minute though it usually took several minutes. It was thus difficult to find extracellular microgametocytes which had not undergone some alteration. Many showed conspicuous peripheral vesicles open to the exterior (*fig. 15*).

The early stages of nuclear spindle and axoneme formation were not seen. Fully formed axonemes, some of which appeared to originate in dense kinetosomes, lay coiled in the cytoplasm (*fig. 15, 16*). The nucleus first assumed a more regular shape. Complete spindles were not seen, though sections were found of spindle termini each consisting of a centriolar plaque in a nuclear pore. There was also a group of 30 or more microtubules (*fig. 15*), which were shown in serial sections to extend into an elongate pocket of the nucleus (*fig. 16*). Evidence for the occurrence of several spindles simultaneously was provided in another microgametocyte where at least 3 unrelated groups of microtubules were seen in different parts of the nucleus. Chromosomes

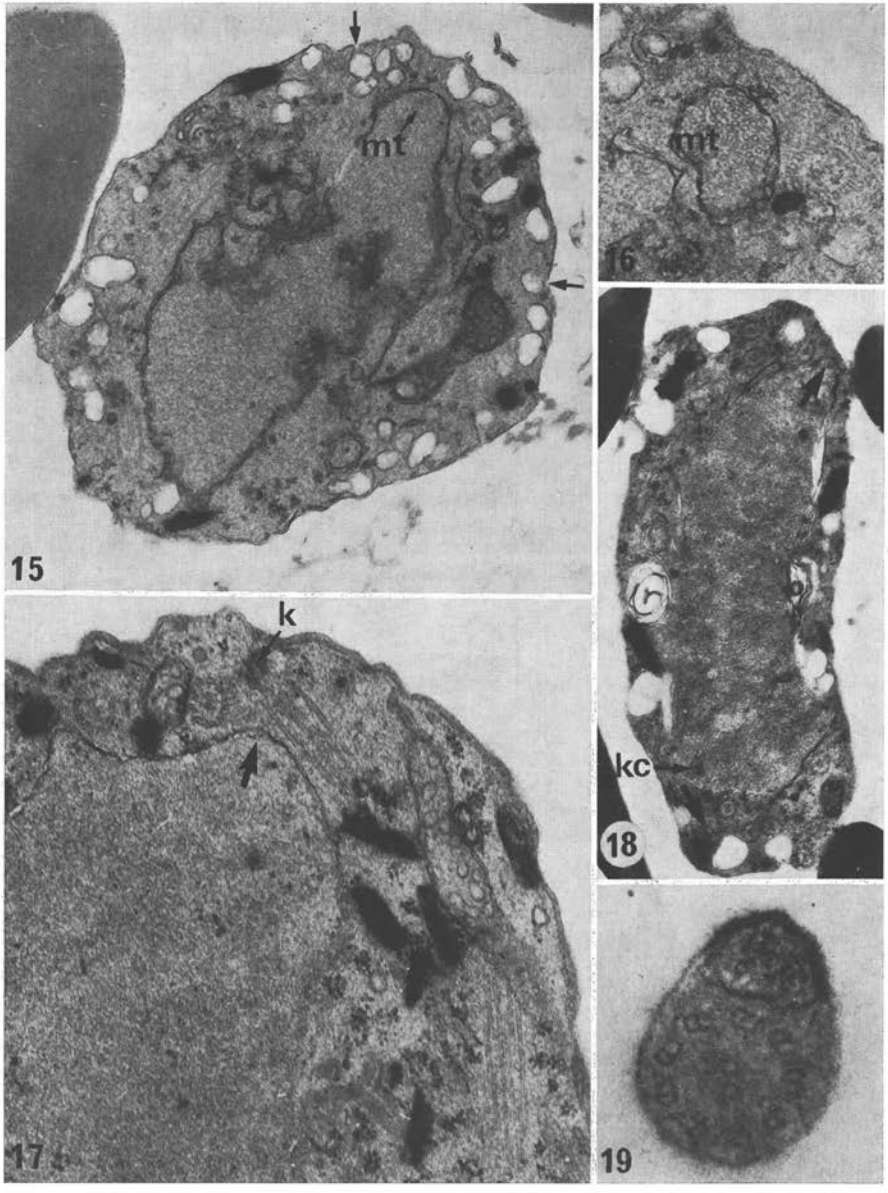

FIG. 15. — Microgametocyte early in gametogenesis. Peripheral vesicles communicate with the exterior (arrows). Several sections of axonemes are visible and a pocket of the nucleus contains numerous microtubules (mt) (X 21,400).

FIG. 16. — The same nuclear pocket illustrated in fig. 15 is shown at a different level in the gametocyte. Microtubules (mt) (X 50,500).

FIG. 17, 18. — Microgametocytes just before exflagellation. Nuclear buds are pushing towards the surface (arrows). Axonemes are fully formed: the dense area at the base of one axoneme my be a kinetosome (k). A kinetochore (kc) of the last nuclear segregation is visible near a nuclear bud (X 41,000 and X 16,000).

FIG. 19. — Transverse section of a microgamete showing axoneme and nucleus (X 120,000).

were not recognised in association with the microtubules and kinetochores which were seen occasionally (*fig. 18*) were not associated with any specific condensations of DNA.

At the time of separation of the microgametes the nucleus extruded buds, containing dense chromatin fibrils, towards the surface (*fig. 17, 18*). The axonemes possibly with their kinetosomes directed forwards as in *Plasmodium yoelii* (Sinden et al., 1976) pushed towards the same points. The full sequence of gamete emergence was not

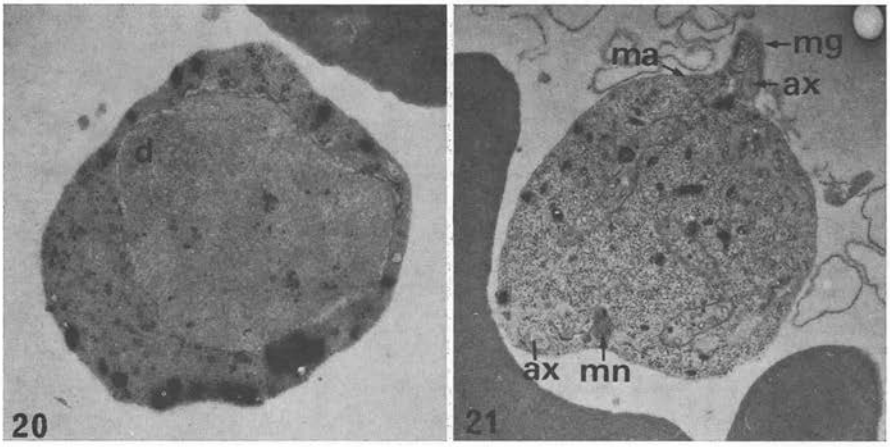

FIG. 20. — *Hepatocystis brayi.* Microgametocyte residuum. The dense cytoplasm and absence of axonemes are characteristic. Frequently one or more masses of DNA (d) which fail to enter an emerging gamete, remain in the nuclear residuum (X 21,000).

FIG. 21. — *H. brayi.* Fertilisation. The plasmalemmas of the microgamete (mg) and macrogamete (ma) have fused and the naked axoneme (ax) and dense microgamete nucleus (mn) have entered the macrogamete. The diffuse macrogamete nucleus is not visible in this section (X 14,500).

observed. The microgamete contained an elongate nucleus of condensed chromatin and a single axoneme (*fig. 19*). The microgametocyte residuum (*fig. 20*) after emergence of the microgametes was recognised by its dense cytoplasm, absence of anoxemes and rounded nucleus which sometimes contained a pocket of condensed chromatin which had failed to pass into an emerging gamete.

5. Fertilisation.

Fertilisation was achieved by fusion of the plasmalemmas of the micro- and macrogametes. The cytoplasm of the macrogamete flowed up into the base of the attached microgamete during the passage of the naked axoneme and compact microgamete nucleus into the body of the macrogamete (*fig. 19*). Fusion of the two nuclei and subsequent development of the zygote were not seen.

Discussion

Features of special interest about the merocyst of *H. malayensis* are the apparent complete destruction of the host cell and the sharp demarcation of the parasite into matrix and cellular components.

Landau et al. (1976) described two types of schizont of *H. malayensis :* those not stimulating a host cellular response and those surrounded by a halo of macrophages. Young forms resembled pre-erythrocytic schizonts of *Plasmodium* in the uniform distribution of cytoplasm and nuclei. The host cell was an hepatocyte which was slightly enlarged and had a slightly hypertrophied nucleus. Host cell cytoplasm and nucleus were not visible at the stage when the amorphous material was differentiated from the true cytoplasm. The schizont found in the present study corresponded with this stage.

The mechanism by which the merocyst becomes sharply defined into cytoplasm and matrix could not be determined from the half-developed cyst examined by electron microscopy. Possibly the original plasmalemma of the invading sporozoite or merozoite is expanded as the surface covering of the merocyst, while surface invagination or development and fusion of endoplasmic reticulum cysternae might bring about the formation of an internal system of membranes which partition the cytoplasm from the contents of the cysternae. The latter may accumulate as matrix. The islands of membrane fragments and cytoplasmic organelles in the matrix may represent areas of cytoplasm originally partitioned but in the process of dissolution in the heart of the matrix.

An alternative explanation is that the merocyst itself represents the modified host cell and that the parasite changes from a compact multinucleate body into a complex reticulum within the modified cell. The islands of degenerate parasite tissue in the heart of the matrix are difficult to explain on this hypothesis.

In most Haemosporina, where a parasitised cell becomes grossly hypertrophied the nucleus is retained. In the megaloschizonts of *Leucocytozoon simondi* (Desser, 1970 *a*) phagocytic cells engulf incompletely divided cytomeres of hepatic schizonts and become grossly enlarged as the cytomeres continue to develop within them. The host cell nucleus lies at the centre of the cell cytoplasm and also enlarges, while a capsule is formed around the cell, consisting of the plasmalemma and a thick, external fibrous layer. The parasitised cell is thus modified by the presence of the parasite to form a complex which is itself parasitic on host tissue. Other species of *Hepatocystis* namely *Hepatocystis kochi* and *H. brayi* both cause cell changes including gross hypertrophy and multiplication of the cell nucleus. Even in the case of *Plasmodium berghei* (Desser, Weller and Yoeli, 1972) and *Haemoproteus columbae* (Bradbury and Gallucci, 1972) the tissue schizonts develop in membrane-bound vacuoles within enlarged host cells and, though the host cell is reduced to a narrow band, it persists even at schizont maturity.

As with other Haemoproteidae the stages in erythrocytes were developing and mature gametocytes. Bradbury and Roberts (1970) believed that the swollen « empty » appearance of young gametocytes of *H. columbae* was due to poor fixation. We have found that cytoplasmic attenuation and vacuolisation are also features of immature gametocytes of *Hepatocystis* and are present in dry-fixed blood smears as well as in material fixed for electron microscopy. There is reason to believe that the condition is normal as other species of *Hepatocystis* fixed in high osmotic strength fixatives display a similar vacuolar appearance (unpublished results), though it may possibly be due to differences in osmotic properties of the young stages.

The pellicle of the gametocytes resembles that of *Plasmodium yoelii* (Sinden et al., 1976) in that the layer of flattened sacs beneath the plasmalemma are incomplete and in some cases nearly absent. *Hepatocystis* thus differs from the avian Haemoproteidae and Plasmodiidae, in which there is an almost complete double layer of membranes beneath the plasmalemma. In *Leucocytozoon* the pellicle is even thicker consisting of two complete thick layers described as pentalaminate (Desser et al., 1970). The pentalaminate appearance is probably derived from two closely apposed unit membranes. The complications of microgametogenesis due to the pellicular structure of the avian parasites, involving dumb-bell formation in *H. columbae* (Bradbury and Trager, 1968 *b*) and protrusion of primary flagellar buds through gaps in the inner membranes in *L. simondi* (Aikawa, Huff and Strome, 1970), contrast with the simple extrusion of gametes from the surface of the gametocyte in the mammalian *Haemosporina*.

The pair of parallel membranes undulating in the erythrocyte cytoplasm of *Hepatocystis*-infected cells resembles the multilaminate structure surrounding mature gametocytes of *Parahaemoproteus velans* (Desser, 1972).

The osmiophilic bodies of *Hepatocystis* resemble those described from mammalian malaria parasites in being elongate, compared with the round type in avian parasites (Aikawa, Huff and Sprinz, 1969). Their disappearance during emergence of the gametocytes from the host cells further supports the view, discussed by Sinden et al. (1976), that they play a role in the dissolution of the overlying erythrocyte cytoplasm. The presence of numerous cristate mitochondria in *Hepatocystis* is a feature in common with avian rather than mammalian parasites.

Macrogametes after emergence from erythrocytes remained apparently inert. Gallucci (1974) found evidence for nuclear reduction in *H. columbae :* there were centrioles — the so-called « atypical centrioles » — consisting of 9 single outer microtubules round a single central tubule, intranuclear spindles converging on centriolar plaques and maturation bodies which were belived to be of nuclear origin but had no connection with the main body of the nucleus. She suggested that the presence of atypical centrioles in *Haemoproteus metchnikovae, P. gallinaceum* and *L. simondi* might also signify that nuclear reduction took place in these macrogametocytes. In support of this Canning, Killick-Kendrick and Garnham (1974) and Canning and Morgan (1975) reported on the presence of pale-staining and dark-staining macrogametocytes of *Hepatocystis* sp. which showed bodies resembling kinetic centres on the nuclear envelope and apparently separate nuclei. They did not however believe that

such divisions could be reductional. *H. brayi* also shows similar dark bodies at the nuclear envelope but the present ultrastructural studies have revealed them as nucleoli. Furthermore, though fully mature macrogametocytes showed compact rounded nuclei, slightly immature forms, not quite filling the erythrocyte, showed highly irregular nuclei with separate profiles which could often be shown to be connected by a narrow isthmus. It is now thought that the pale macrogametocytes represented immature forms and that in common with macrogametocytes of *P. berghei* (Sinden et al., 1976) there are no significant nuclear changes in *Hepatocystis* after emergence from the erythrocytes.

Information on microgametogenesis of *Hepatocystis* was incomplete. An atypical centriole, embedded in electron dense material, similar to those found in *P. gallinaceum* and *P. cathemerium* (Aikawa, Huff and Sprinz, 1969), *L. simondi* (Aikawa, Huff and Strome, 1970) and *Parahaemoproteus velans* (Desser, 1972), was found close to the nuclear envelope in an intraerythrocytic microgametocyte of *H. brayi*. The association of this centriole with an intranuclear body as found by Aikawa, Huff and Strome (1970) was not demonstrated. If the electron dense material round the centriole corresponds to a microtubule organising centre (MTOC) as discussed by Sinden et al. (1976) then the kinetosomes of the axonemes are probably formed from the centriole/MTOC complex during microgametogenesis. The presence of a centriole in the inactivate gametocyte is another feature in common with avian rather than mammalian parasites.

As in *P. berghei* (Sinden et al., 1976) condensed chromatin was not seen on the nuclear spindles and appeared only in the nuclear buds as they pushed towards the surface. Though there was evidence that the microgamete nucleus was separated as a bud and passed with the axoneme into the emerging gamete, there was no sign of the complex apparatus involved in this emergence in *P. berghei* (Sinden et al., 1976), namely the peri-kinetosomal basket and juxta kinetosomal sphere and granule. There was equally no sign of sub-pellicular tubules from which the basket tubules could have been derived, though we note that different fixation techniques were used in these two investigations.

The microgametes contain a single nucleus and axoneme and resemble those of mammalian Plasmodiidae. Microgametes have been recorded with two axonemes in *H. columbae* (Bradbury and Trager, 1968 *a*) and *L. simondi* (Desser, 1970 *b*) though a single axoneme was reported for *L. simondi* by Aikawa, Huff and Strome (1970). There is a single axoneme also in *Parahaemoproteus velans* (Desser, 1972).

In gametocyte structure and gametogenesis, *Hepatocystis* has features in common with mammalian and avian malaria parasites. Landau, Miltgen and Chabaud (in press) has used gametocyte characters to argue for a polyphyletic origin of malaria parasites and has linked *Hepatocystis* with the *vivax* group of *Plasmodium*. The similarities of *Hepatocystis* with avian Haemosporina revealed in the present study would suggest that this stock branched off early in the evolution from the avian parasites. Studies on the ultrastructure of a wide range of Plasmodiidae and Haemoproteidae, including *Polychromophilus*, should provide useful insight into the affinities of the species.

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