# *Advances in*
# PARASITOLOGY

VOLUME 27

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# PREFACE

In this volume, as in others, we hope that there will be something to interest everyone. The volume starts with two reviews concerning protozoa; that by Dr Meis and Dr Verhave, distinguished by its superb electron micrographs, presents a very full picture (literally) of the erstwhile "cryptic" exoerythrocytic schizont of *Plasmodium*. Dr Tzipori's contribution is particularly timely because of the recent surge of interest in *Cryptosporidium* as an "opportunistic" parasite of immunocompromized persons, including those unfortunate enough to be infected with human immunodeficiency virus. Then follow two chapters of more general interest, though with a leaning towards helminthology—that by Dr Leid on the interaction between parasites and their hosts' complement system, and that by Dr Haseeb and Dr Fried dealing with the problems of chemical communication between parasitic worms. Finally, there is Dr Thompson's and Dr Lymbery's contribution on a rather more restricted, though no less interesting, subject—subspecific variation in *Echinococcus*. This is a topic of growing interest with many parasitic genera, helminths and protozoa, because of the availability now of a range of new techniques of what is rather loosely referred to as "molecular taxonomy". These techniques are introducing a whole new dimension into parasite taxonomy, and it seems likely that this will not be the last contribution concerning this subject to appear in *Advances in Parasitology*.

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J. R. BAKER
R. MULLER

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# CONTENTS



## Exoerythrocytic Development of Malarial Parasites

J. F. G. M. MEIS AND J. P. VERHAVE



## Cryptosporidiosis in Perspective

S. TZIPORI



## Parasites and Complement

R. W. LEID

## Chemical Communication in Helminths

M. A. HASEEB AND B. FRIED



## The Nature, Extent and Significance of Variation Within the Genus *Echinococcus*

R. C. A. THOMPSON AND A. J. LYMBERY

# Exoerythrocytic Development of Malarial Parasites*

J. F. G. M. MEIS AND J. P. VERHAVE

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## I. Introduction

The life-cycle of *Plasmodium* species is completed when, in the stomach of the insect vector, a uninucleate zygote is formed from male and female gametes present in the bloodmeal. The insect vector is normally a female mosquito (many species of *Anopheles* and, for lizard and bird malaria, culicine mosquitoes). The zygote, now called an ookinete, is active and moves through the stomach or midgut wall. The parasite becomes localized under the lining of the gut and transforms into an oocyst.

This multiplication stage produces thousands of infective sporozoites which become concentrated in the salivary glands and so are in a favourable position to enter the vertebrate host when the mosquito takes a new

*Dedicated to P. C. C. Garnham FRS, Emeritus Professor of Medical Protozoology in the University of London.

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bloodmeal. After introduction of the sporozoites into the bloodstream, a series of cycles begins that involves different cells and tissues depending on the parasite species and host. The mammalian malaria parasites may enter various tissue cells, but develop solely in liver parenchymal cells, whereas the sporozoites of avian species develop in fixed macrophages. It is now recognized that sporozoites of mammalian species produce one exoerythrocytic (EE) multiplication cycle in hepatocytes, thereby delivering merozoites into the bloodstream. These may enter erythrocytes and start the erythrocytic phase of the life-cycle. In avian malaria, however, the EE development takes place in mesodermal tissues and the EE merozoites may re-enter the tissues and repeat the tissue cycle. This repetition definitely does not occur in the cycle of mammalian forms. Until recently, the latter option was retained as a hypothetical possibility to explain relapses characteristic of, e.g., *P. vivax*, *P. ovale* and *P. cynomolgi*. Recent research has revealed the existence of a dormant form in hepatocytes, the hypnozoite (Krotoski, 1985). This form is responsible for relapses of vivax and ovale malaria after curing of the primary attack. Two types of sporozoites are thought to exist, one of which undergoes immediate liver schizogony and subsequent blood infection while the other, held back by an as yet unknown cause in its development, persists as a small uninucleate form of 4–5 μm in the host hepatocyte. These dormant forms are larger than the diameter of sporozoites and are therefore thought to have rounded up but not differentiated beyond the uninuclear phase.

The cyclic multiplication in the blood is manifested in repeating episodes of chills and fever. The length of a single cycle in the blood varies with the species of *Plasmodium*. Some of the merozoites in the red blood cells develop into sexual forms that grow into male microgametocytes or female macrogametocytes. When a mosquito bites the vertebrate host at this stage of the life-cycle, the gametocytes are taken into the insect's midgut and there they quickly mature into the microgametes and macrogametes. Fertilization now can take place, and the zygotes thus formed complete the sexual phase (Fig. 1). In recent years many problems and gaps in our knowledge of the biology of *Plasmodium* have been solved. Extensive reviews on the sporogonic stages in the mosquito and the erythrocytic stages, including the sexual stages, in the vertebrate host are available (Aikawa, 1971, 1977; Aikawa and Miller, 1983; Brown *et al.*, 1986; Sherman, 1983, 1985; Sinden, 1978, 1983). The present review attempts to summarize our knowledge of the EE form, a stage in the life-cycle of *Plasmodium* last discovered and least well known (Bray, 1957; Bray and Garnham, 1982). In describing the development of the intracellular EE stage it should be realized that, at the moment of fixation, the parasites are not resting stages but actively growing forms. As Yoeli and Most (1965) truly stated about this form: "We must not forget that we are dealing with a three-dimensional body, the growth of which resembles in many ways a balloon increasing in dimensions within another living structure

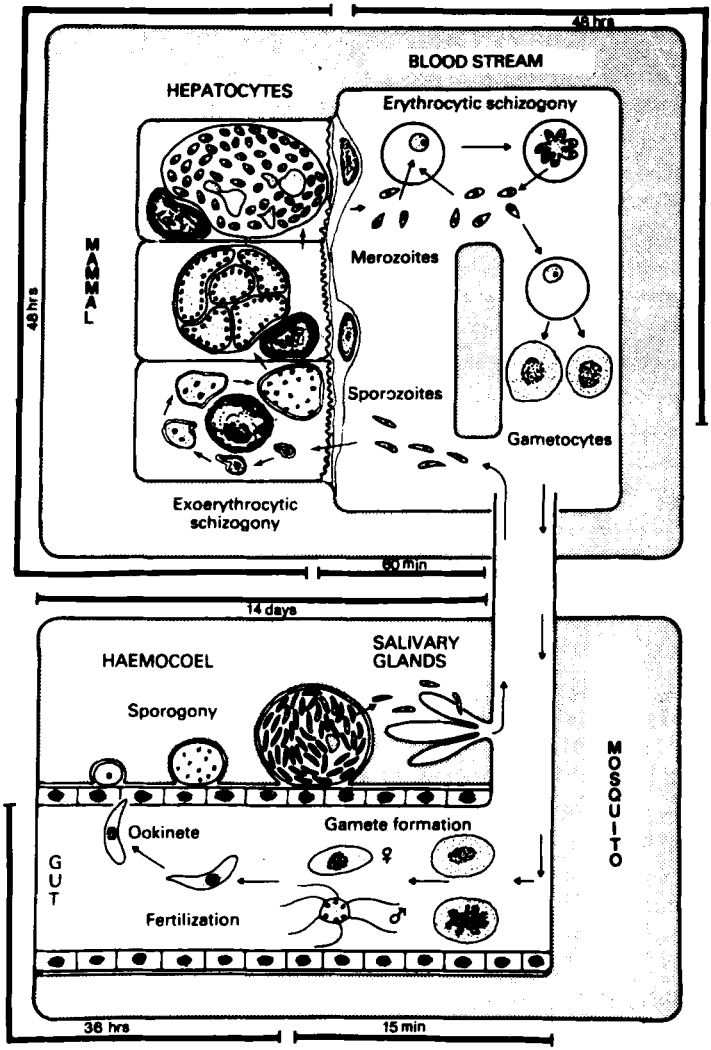


FIG. 1. Life-cycle of the mammalian malaria parasite, *P. berghei* (modified after Wilson, 1979).

and being affected not only by its own growth but by forces of physical resistance within the invaded and occupied cell and space". Because of the importance of the EE cycle in the establishment of a blood infection we consider the EE form the original or primitive stage, a bridgehead by which *Plasmodium* captures the vertebrate host. Furthermore, the tissue forms of mammalian species, which as such cause no harm to the host, are the target

of drug prophylaxis of relapsing malaria which still deserves further refinement. Thus knowledge of their development and metabolism should facilitate studies on the activity of new prophylactic drugs and on their ways of administration.

We will include in this review a historical survey; the morphology of the exoerythrocytic liver stage; the metabolism of the exoerythrocytic liver stage; and the cultivation of the exoerythrocytic liver stage.

Among the avian, rodent and primate EE forms there are many structural similarities, while many differences also exist among avian parasites between the host cells and tissue cycles. This review attempts to concentrate on the new findings of EE development. The fine structure of avian parasites has been known for several years, and excellent reviews dealing with these parasites have been published (Aikawa, 1971, 1977). The rodent EE forms and their development from sporozoites in hepatocytes have been studied more extensively in recent years, and only very recently have the primate EE parasites yielded to fine structural studies, allowing more knowledge to become available.

## II. Historical Survey

In northern Europe, where intermittent fevers (due to *P. vivax*) were common, it was known that long periods of latency could occur. This was particularly clear when a person, having left a malarious area, developed the disease long after having settled elsewhere. To explain this phenomenon, the Dutch professor of internal medicine P. K. Pel stated in 1886: "During the latent period, the germ is fixed somewhere or not able to reproduce, until by some cause its conditions for life becomes more favourable. Then the germs can multiply or shift to another more active stage of development, reach the blood and cause particular disease symptoms". This was the very first, and a very correct, description of the question of incubation, latency and relapses. It remained unappreciated, and a lot of non-coherent searches for parasitic forms in various tissues or cells of the host followed.

Some years later, Italian workers suggested that parasites might lurk in white blood cells. Pel's collaborator Tanja reported in 1898 that crescents of pernicious malaria were not touched by mononuclear leucocytes, but the spheric forms with their flagella were approached; then the movements ceased and the parasites were ingested. In line with Laveran, who called these forms, now known to be activated microgametocytes with their male gametes, "Dauerformen", Tanja thought that they could survive inside the phagocytes until their "resurrection" and thus cause relapses. The idea that gametocytes were a source of recrudescing malaria was very appealing, though Grassi postulated in 1900 that sporozoites would not directly enter

the red blood cells, a postulate which he based on the difference in nuclear architecture between sporozoite and merozoite.

This latter proposal was over-ruled by the description by Schaudinn (1903) of *P. vivax* sporozoites allegedly invading erythrocytes. Moreover, Schaudinn reported that female gametocytes could give rise parthenogenetically to a new crop of relapsing parasites, which explained why gametocytes were not sensitive to quinine. The authority of this eminent parasitologist diverted research in other directions, and gametes continued to be considered as the source of relapses until about 1925.

The incidental descriptions of tissue schizonts in related parasites, the discrediting of parthenogenesis, the failures to demonstrate sporozoite invasion of erythrocytes and the observations that only sporozoite inoculations of *P. vivax* resulted in long latency and relapses, gradually started to shake Schaudinn's theory. In particular, the experimental inoculation of neurosyphilis patients and volunteers with sporozoites of *P. vivax*, which started in 1921, yielded useful information. In the Netherlands, Korteweg and van Assendelft were involved, and the latter postulated a third area of initial development which he believed to be at the site of the mosquito bite (van Assendelft, 1931). The British malariologist James (1931) proposed similar ideas, but thought of reticulo-endothelial cells of the lungs and other organs, where parasites would go through a cycle of growth and sporulation. In 1931, Korteweg confirmed the difference in the response to quinine between blood and sporozoite-induced infections, and the lack of relapses in the former.

The Malaria Commission of the League of Nations drew attention in 1933 to the theory of a third site of development (cited by Bray, 1957), and the question of the fate of the sporozoite became an important subject of research and theorizing.

The next year brought an observation by Raffaele (1934) in Italy on schizonts of *P. elongatum* in bone marrow of sporozoite-infected canaries. A host of observations on several malaria parasites of birds followed, from which a very complicated scheme of development was finally compiled. One thing became certain: sporozoites entered the reticulo-endothelial system for a first schizogony in histiocytes. Therefore, the presence of a tissue development in mammalian *Plasmodium* was postulated by many workers. Korteweg in 1933 had already pointed to the different lengths of prepatent periods in Dutch *P. vivax* (2–3 weeks or 8.5 months) and the Madagascar strain (1 week). Another Dutch parasitologist, Brug (1940, 1941a), published the presumed discovery of EE schizonts in lung endothelial cells of a patient who had been given a therapeutic *P. vivax* infection (despite the warnings of Swellengrebel, expressed in the discussion of his papers, that in blood-inoculated *P. vivax* no relapses, and thus no EE forms, were to be expected).

Later, Brug (1941b) corrected his views because such inclusions were also found in non-malarious patients. Many other searches in bone marrow, skin, spleen, liver and lung were reported from various laboratories, but the presumed tissue forms were not found. Several reports emphasized the disappearance of sporozoites from the blood very quickly after inoculation. Fairley (1946) carried out experimental blood transfusions from human volunteers who had received a sporozoite inoculum, and clearly confirmed in recipients the existence of a negative phase of the blood from 1 hour to 6 days after inoculation of *P. falciparum* sporozoites, and from 1 hour to 8 days when he used *P. vivax* sporozoites. In 1947, Garnham discovered schizonts of *P. (=Hepatocysis) kochi* in a monkey liver (Garnham, 1948). These observations paved the way for a breakthrough when, in 1948, Shortt and Garnham discovered schizonts of *P. cynomolgi* in the liver of monkeys which had been inoculated with sporozoites. Then, with other colleagues, they demonstrated similar forms of *P. vivax* in liver parenchymal cells of a human volunteer, 8 days after exposure to mosquitoes (Shortt *et al.*, 1948). Some years thereafter followed the discovery of similar stages of *P. falciparum* (Shortt *et al.*, 1951). Much later, after using several human volunteers, Garnham and colleagues (1955) succeeded in demonstrating *P. ovale* in human liver and *P. malariae* in chimpanzee liver (Bray, 1960). The latter parasite developed much more slowly (i.e. in 15 days).

Finally, in 1948, Belgian workers isolated *P. berghei* from rodents in Congo (Vincke and Lips, 1948), but it was another 17 years before Yoeli *et al.* (1964) discovered that low temperatures were essential for mosquito infection. This led them quickly to the description of liver forms in hamsters; development in hepatocytes appeared to take about 2 days. Surprisingly little research was carried out with these liver forms during the next decade.

The EE forms that develop from sporozoites and immediately continue their development have recently been described at the fine structural level, over the whole duration of their development (Verhave and Meis, 1984). This is particularly true for *P. berghei*, in rats and *in vitro*, and for *P. falciparum in vitro*. The route of the sporozoite in the liver was also elucidated, and Kupffer cells in rats have been described as a route of passage from the sinusoids to the space of Disse and the bordering hepatocytes. It is a historical curiosity that the initial approach, using *P. berghei* in rats, was to try to eliminate the role of Kupffer cells in the liver (Verhave *et al.*, 1980). Instead of finding the expected large numbers of parasites in rats whose reticulo-endothelial system had been "blocked" by the administration of silica, we found virtually none (Table 1). This led us to the hypothesis that Kupffer cells deal with sporozoites in two ways: either they digest them or they let them pass on their way to a hepatocyte. This idea triggered a wave of ultrastructural and histochemical studies that form the basis of this review.

TABLE 1 *Effect of silica treatment of Wistar rats on* P. berghei *sporozoite circulation time and EE stage density*

| Time between giving silica and sporozoites | Number of EE stages per mm² (mean ± standard error) | Sporozoites present in (+) or absent from (−) blood at times shown | |
|---|---|---|---|
| | | 15 min | 60 min |
| 1 day | 0 | + | + |
| 2 days | 0.3 ± 0.9 | + | + |
| 3 days | 0 | + | − |
| Control | 6.3 ± 3.4 | + | − |

The issue of the route of the sporozoite was now switched from "where does it go?" to "how does it get there?". Recent evidence has been put forward that hepatocytes are liable to changes in receptiveness, brought about by hormonal (i.e. corticosteroid) fluctuations of the host (Verhave *et al.*, 1985). The finding of "relapse bodies" induced a hypothesis of secondary generations within the liver. This was later proven to be void, and a theory of dormant forms in addition to straightforward EE development took its place. This was finally proven by Krotoski and his British and American colleagues, who demonstrated hypnozoites and schizonts of *P. cynomolgi* in the liver of apes (Krotoski *et al.*, 1982a,b). It also made it likely that the long latency *P. vivax*, of northern Europe and Asia, remains within hepatocytes as hypnozoites. The ultrastructure of these forms and the nature of the signal for their revival await description. The small size of hypnozoites and young, growing EE stages, and their scarcity in liver tissue, have been major obstacles to studies in the past. The former were discovered by applying immunofluorescence, while young forms of *P. berghei* were seen only after a number of attempts to increase their numbers in the liver.

## III. MORPHOLOGY OF EXOERYTHROCYTIC DEVELOPMENT

### A. AVIAN MALARIA

Knowledge of the fine structure of EE forms has been greatly expanded after the introduction of a tissue culture system for *P. gallinaceum* and *P. fallax* (Meyer and Musacchio, 1959; Huff *et al.*, 1960). Although many similarities exist with the erythrocytic stages, food vacuoles and malaria pigment are absent from EE stages.

The avian EE merozoites (Fig. 2) bud from pseudocytomeres in a manner

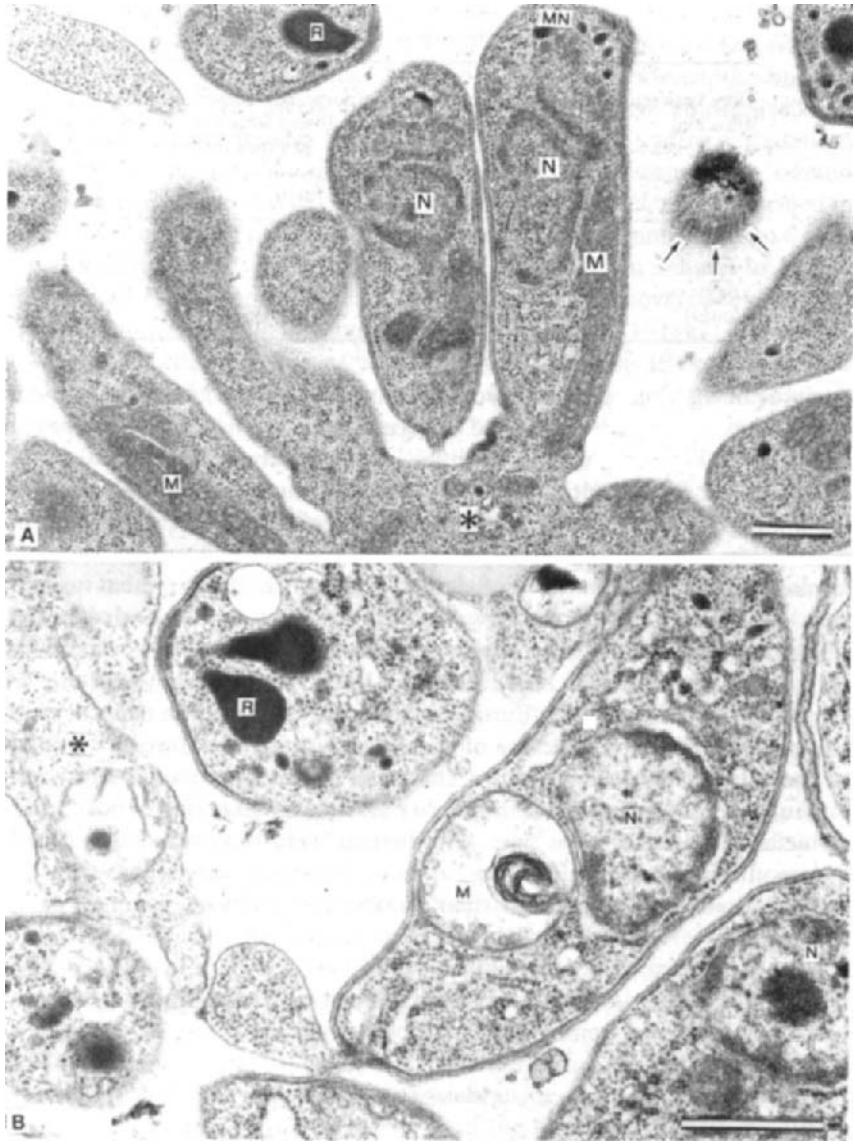


FIG. 2. (A) EE stage of *P. gallinaceum*. Elongated merozoites bud from the pseudocytomeres (asterisk). The merozoites have a typical layer of subpellicular microtubules (arrows), a nucleus (N), elongated mitochondrion with tubular cristae (M), rhoptries (R) and micronemes (MN). Bar = 0.5 μm. (B) EE merozoites of *P. fallax* and pseudocytomeres (asterisk). Bar = 0.5 μm. Courtesy of Dr M. Aikawa, Cleveland.

that is similar in every malarial schizogony, but they are larger than the erythrocytic merozoites. With its dimensions of 3–4 μm in length and 1–2 μm in width, the EE merozoite ranges in size between the sporozoite and erythrocytic merozoite (Aikawa, 1971). All invasive stages of malaria parasites possess rhoptries and micronemes; sporozoites have greater numbers than EE merozoites, and these in turn have greater numbers than erythrocytic merozoites. While the cytostome in erythrocytic merozoites is clearly designed to ingest host cytoplasm, the cytostome in EE merozoites is not functional in all species (Aikawa *et al.*, 1966; Hepler *et al.*, 1966). In contrast to EE stages of *P. fallax*, in which the cytostome is inactive, those of *P. elongatum* (Aikawa *et al.*, 1967) and *P. lophurae* (Beaudoin and Strome, 1972) are involved in the ingestion of host cell cytoplasm. It has been suggested that food vacuoles are lacking from other EE stages because of the low density of the host cell cytoplasm compared to that of the erythrocyte cytoplasm containing haemoglobin (Aikawa, 1971). Avian culture systems have been extensively used for studies on the influence of drugs on the parasites (Aikawa and Beaudoin, 1969). Despite the suitability and efficiency of the system (Davis *et al.*, 1966), no details of penetration and early development in host cell cultures are known (Beaudoin and Strome, 1973).

### B. RODENT MALARIA

The fine structure of avian EE stages has been described extensively, while that of mammalian parasites was elusive until the first report in 1969 by Garnham and colleagues (Garnham *et al.*, 1969). A schizont of *P. yoelii* already containing merozoites was described in the liver of a mouse 51 hours after the inoculation of sporozoites. Somewhat younger stages of *P. berghei* and *P. vinckei* were described in the liver of the natural host, *Thamnomys surdaster* (Bafort, 1971; Desser *et al.*, 1972). Light microscopy had already shown that the younger the stage, the smaller the EE form and hence the more difficult it was to localize these scarce parasites for ultrastructural studies. We have tried to overcome this problem by increasing the numbers of developing EE stages in a given liver volume (Meis *et al.*, 1981). By ligating the vessels supplying blood to the median and left lateral lobes, EE stages were concentrated in the small right and caudate lobes (Fig. 3).

The choice of a very susceptible rat strain (Brown Norway) for *P. berghei* infection further increased the numbers of developing schizonts (Table 2). The distribution of *P. berghei* parasites through the liver acinus appeared not to be random. Most developing EE stages were found around afferent portal tracts (Meis *et al.*, 1983c; Verhave *et al.*, 1985), which again increased the chance of encountering parasites in thin sections.

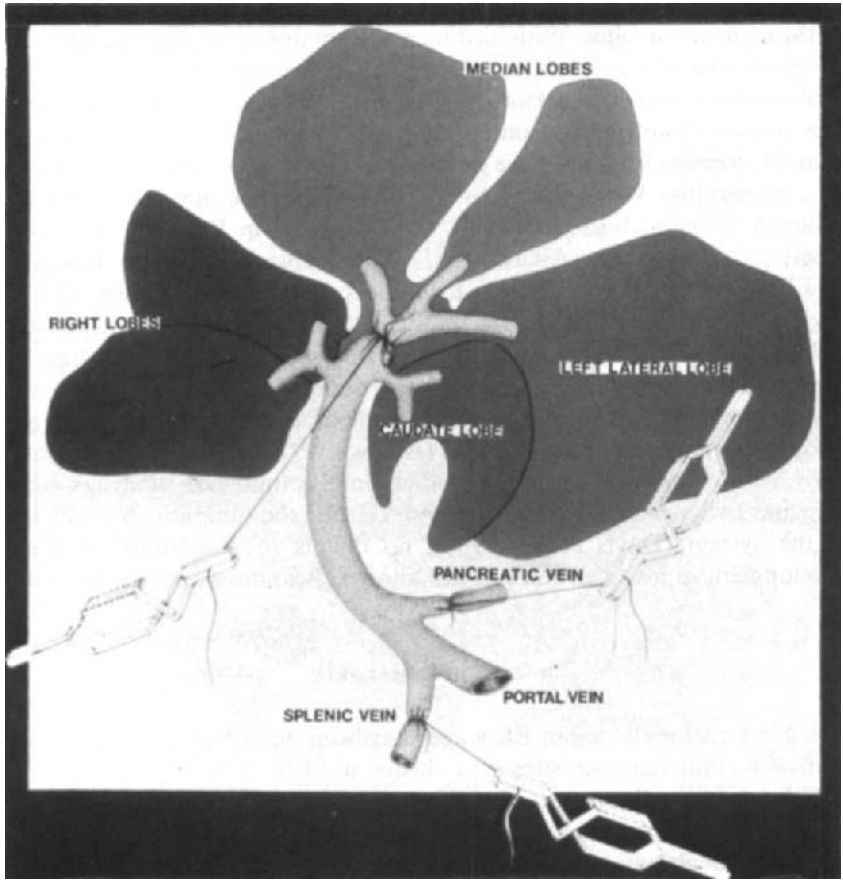


FIG. 3. The median and left lateral lobes of rat liver were cut off from their blood supply by ligating the portal vein and hepatic artery that feed these lobes. The pancreatic and splenic veins were ligated in order to reduce the increased portal flow and pressure.

TABLE 2 *Susceptibility of three strains of rats to infection with* P. berghei *sporozoites*

| Rat strain | Number of EE stages per $mm^2$ (mean) | Diameter (μm) |
|---|---|---|
| Sprague Dawley | 0.05 | 24.7 ± 5.6 |
| Wistar | 0.35 | 28.6 ± 2.8 |
| Brown Norway | 1.60 | 30.1 ± 2.7 |

The least understood aspect of the malarial life-cycle was the behaviour of the sporozoite after injection into the bloodstream by the mosquito. Until recently, there was no information on the process of infection of the vertebrate host and the subsequent intrahepatocyte development. It had long been assumed that the sporozoite had direct access to the hepatocyte. However, no evidence was available to support this viewpoint, and recent ultrastructural studies of the liver architecture plead against it. Sinusoids in the liver lobule are completely covered with a unique type of endothelial lining, comprising endothelial cells with large flattened plates perforated by small fenestrae of about 0.1 μm in size, and phagocytic Kupffer cells (Fig. 4). Proper perfusion fixation is highly critical in the preservation and visualization of the endothelial fenestrae. In careful studies by Wisse and colleagues

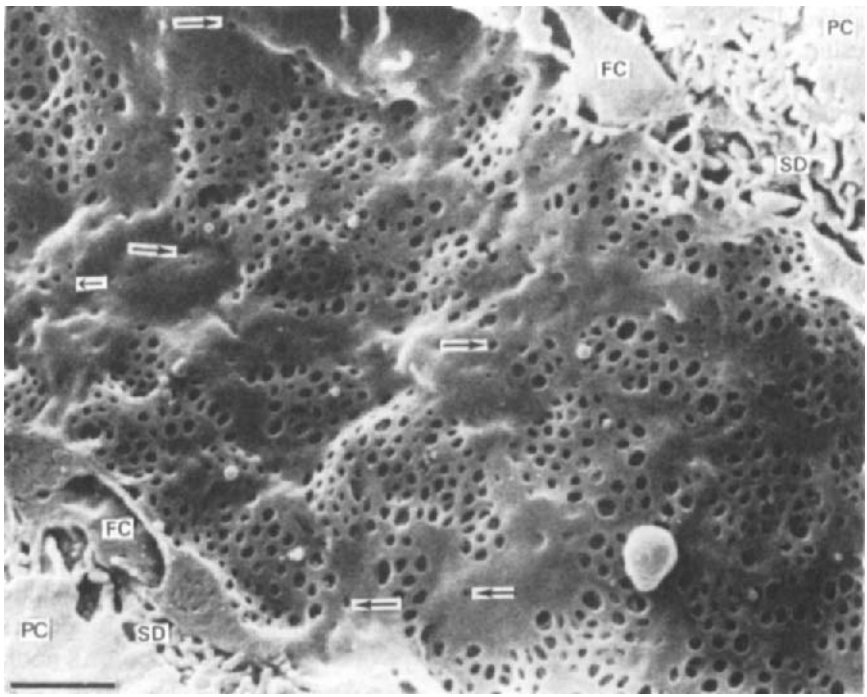


FIG. 4. Scanning electron micrograph of a rat liver sinusoid. Note the clustering of fenestrae in sieve plates. The sieve plates are separated by cytoplasmic arms of endothelial cells, in which depressions of micropinocytotic vesicles (coated pits) can be observed (arrows). In the space of Disse (SD) processes of fat-storing cells (FC) and numerous microvilli of parenchymal cells (PC) can be seen. These endothelial cell plates prevent a direct interaction of sporozoites with hepatocytes. Bar = 1 μm. Courtesy of Dr E. Wisse, Brussels.

(Wisse *et al.*, 1982, 1983, 1985) it was established that there are no large gaps in the endothelial lining, only the small fenestrae. Others before them had failed to recognize the open spaces between endothelial cell plates as fixation artefacts, and thus malariologists never considered it a problem to explain the easy access of sporozoites to hepatocytes.

Considering that sporozoites measure 15 × 1 μm they cannot pass through the fenestrae, and in fact we have never seen any attempt to take this route to the space of Disse. How then do they circumvent or pass the endothelial barrier to get at their target cells, the hepatocytes? In a study on silica-impaired Kupffer cells and the subsequent effect on sporozoite infection, it was demonstrated that the usual short circulation time of sporozoites was considerably extended in silica-treated rats (Table 1) (Verhave *et al.*, 1980). Furthermore, the numbers of developing schizonts were significantly reduced. The former results were confirmed by Sinden and Smith (1982). By using isolated perfused liver it was shown that over 95% of injected sporozoites were retained in the liver after 15 min of perfusion (Sinden and Smith, 1982; Danforth *et al.*, 1982). Mechanical trapping accounted for a large part of this retention. However, a significant proportion could also be due to a biologically active retention of sporozoites in the liver. Non-viable sporozoites and immature non-infective sporozoites derived from oocysts were as effectively retained in the liver as viable parasites (Sinden and Smith, 1982; Danforth *et al.*, 1982). However, mature viable sporozoites were able to evade the oxidative burst when taken up by peritoneal macrophages *in vitro*. Immature, heat-inactivated or trypsin-treated sporozoites, on the other hand, triggered the respiratory burst (Smith and Alexander, 1986).

Retention of sporozoites in perfused intact rat liver does not seem to be influenced by the source of serum (Smith and Sinden, 1982). However, *in vitro* attachment and interiorization into peritoneal macrophages is promoted after incubation of sporozoites in serum of the same host species (Danforth *et al.*, 1980; Schulman *et al.*, 1980; Holmberg *et al.*, 1981). It is impossible to find sporozoites or early post-sporozoite stages with light microscopy. Therefore we have specifically screened sinusoids in the periportal regions of the liver lobules. In these areas most of the developing EE stages (Verhave *et al.*, 1985) and active Kupffer cells (Sleyster and Knook, 1982) were found.

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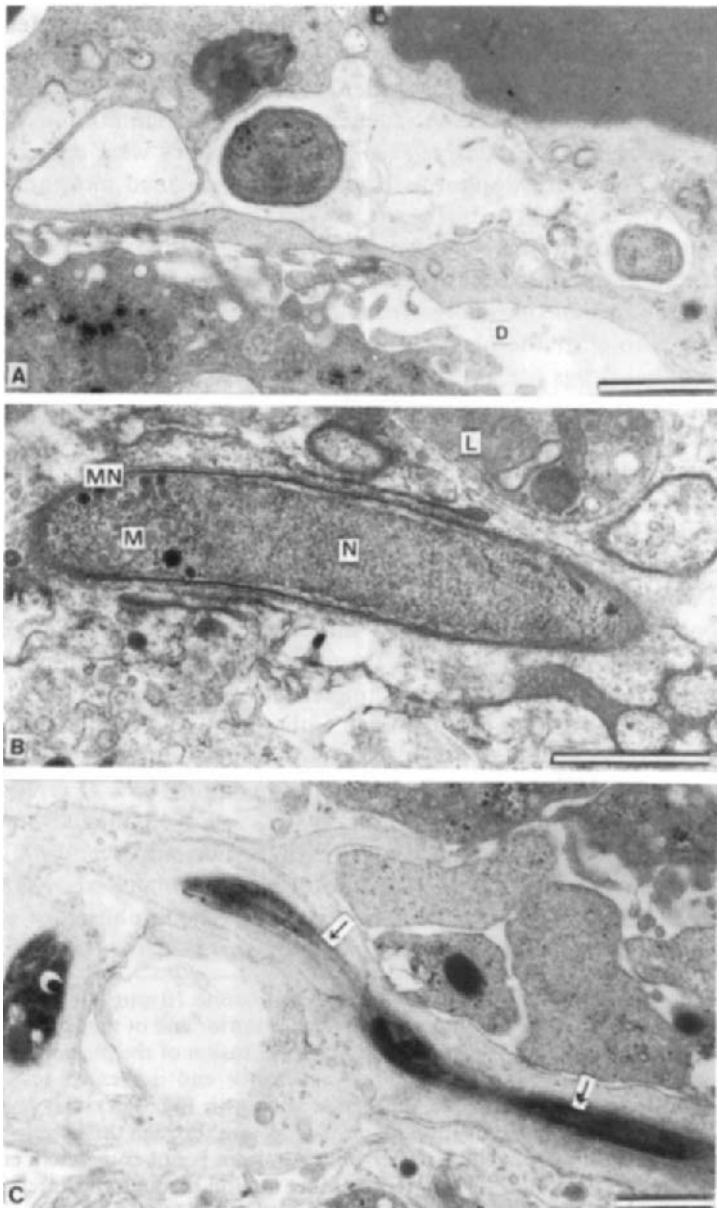
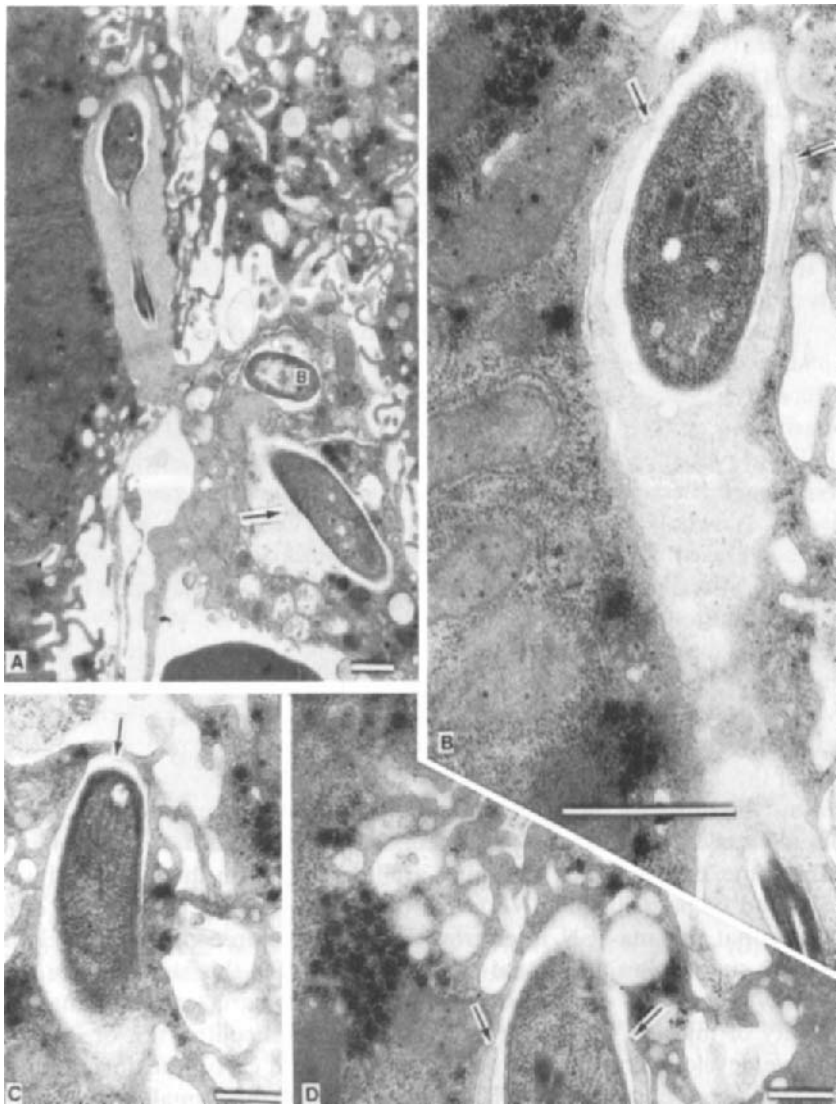
FIG. 5. (A) A sporozoite of *P. berghei* in an endocytotic vacuole of a Kupffer cell 10 min after inoculation. The Kupffer cell borders the space of Disse (D) and the adjacent hepatocyte. Bar = 1 μm. (B) A sporozoite in the cytoplasm of a Kupffer cell 1 hour after inoculation. The sporozoite still has well preserved cytoplasmic structures such as nucleus (N), micronemes (MN) and a mitochondrion (M). Although the phagocyte's membrane is closely apposed to the sporozoite, the parasite is morphologically intact. L = lysosome. Bar = 1 μm. (C) Remnants of a degenerated sporozoite in a Kupffer cell 1 hour after inoculation with only the subpellicular microtubules still intact (arrows). Bar = 1 μm.


A
D
B
MN
M
N
L
C

We have shown, by electron microscopy in a rat model, that many sporozoites were rapidly engulfed by Kupffer cells (Fig. 5) which were thus responsible for the removal of sporozoites from the circulation (Meis *et al.*, 1982, 1983c). Whereas the invasion of the hepatocyte appeared to be an active process by the sporozoite (Sinden, 1981; Verhave, 1982), their uptake by Kupffer cells was passive and independent of the orientation or position of initial contact (Meis *et al.*, 1985d). The sporozoites were enclosed by a membrane-bound parasitophorous vacuole and remained morphologically intact during the first 30 min (Fig. 5). By that time, primary lysosomes had fused with the parasitophorous vacuole membrane and parasites had started to degenerate (Fig. 5). It is hardly surprising that Kupffer cells play a major role in the removal of sporozoites from the circulation, since it is one of their major duties to clear the blood of foreign and degenerating material. It is interesting that drugs (i.e. primaquine) encapsulated in large liposomes also are taken up by Kupffer cells and finally end up in hepatocytes, where they can stop EE development (Alving, 1986).

More important than the retention of the sporozoites is the question of how they circumvent the endothelial barrier to reach the target cells. In an earlier study (Verhave *et al.*, 1980) it was hypothesized that Kupffer cells play a dual role in the processing of sporozoites. Kupffer cells in immunized animals would degrade sporozoites and thus prevent infection. These sporozoites, coated with antibody on their short journey, are taken up efficiently via the Fc-receptor on Kupffer cells, thereby triggering the oxidative burst. In our experience this is how the protecting effect of anti-sporozoite antibodies works *in vivo*. Sporozoites preincubated with an anti-sporozoite monoclonal antibody (3D11) (Yoshida *et al.*, 1980) and subsequently injected into host rats were almost completely neutralized, although not 100% (Ferreira *et al.*, 1987). When the Fc-portion of the antibody was removed, the Fab fragments neutralized only 60% of the injected sporozoites (Ferreira *et al.*, 1987). These results suggest the destruction of some of the inoculated sporozoites by Kupffer cells that are activated via Fc-receptors. The inhibition of penetration of hepatocytes by antibody-coating of sporozoites, as seen *in vitro* with

FIG. 6. Four different sections of a *P. berghei* sporozoite 10 min after inoculation, apparently escaping from a Kupffer cell. (A) The posterior end of the sporozoite is in the Kupffer cell body, and its anterior part is in a protusion of the phagocyte in close contact with the hepatocyte. Note that the posterior end is located in a similar endocytotic vacuole (arrow) as the sporozoite shown in Fig. 5(A). B = bacterium. Bar = 1 μm. (B) Higher magnification of another section through the anterior part of the sporozoite, showing that the Kupffer cell cytoplasm is not completely enclosing the sporozoite (arrows). Bar = 1 μm. (C) Another section through the anterior tip of the sporozoite (arrow), demonstrating that this part is in free contact with the hepatocyte. Bar = 1 μm. (D) Intermediate section between (B) and (C) demonstrating the distinct Kupffer cell filopods (arrows). Bar = 1 μm. Reprinted from Meis *et al.* 1983c, with permission from the authors and Cambridge University Press.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D

*P. berghei* (Hollingdale *et al.*, 1982; Leland *et al.*, 1984) and *P. falciparum* (Hollingdale *et al.*, 1984; Mazier *et al.*, 1986), works differently *in vivo*. Stewart *et al.* (1986a) showed, by elegant studies *in vitro*, that anti-sporozoite antibodies interfere with sporozoite motility. In our opinion these *in vitro* experiments with antibodies cannot be taken as a model for *in vivo* phenomena. The intervention of Kupffer cells in preventing active movement through the sinusoidal barrier cannot be mimicked *in vitro*. Thus the *in vitro* inhibition of hepatoma cell invasion by antibodies is less critical than the *in vivo* mechanism.

With electron microscopy it was shown that a sporozoite, after being taken up by a Kupffer cell, can escape from the phagocyte on the side that borders the space of Disse and the hepatocyte (Fig. 6). The mediator role of Kupffer cells in the initial infection process should be considered as an alternative to the "direct infection" theory, which was proposed in the absence of a better explanation (Shin *et al.*, 1982).

Recently, the effect of gamma-interferon (IFN) on sporozoite-induced malaria was studied using *P. berghei* (Ferreira *et al.*, 1986b). A very significant reduction in the number of parasites in the liver, measured with a DNA probe (Ferreira *et al.*, 1986a), was found when the gamma-IFN was injected a few hours before sporozoite infection. This effect was found to be less pronounced if the gamma-IFN was administered 18 hours before, or a few hours after, sporozoite infection. It is well known that gamma-IFN plays an important role in macrophage activation (Nathan *et al.*, 1980). We think it likely that specific activation of the Kupffer cells greatly diminishes the number of EE stages (Verhave *et al.*, 1980), and also that the Kupffer cells are involved in the infection of hepatocytes (Meis *et al.*, 1983c). Therefore it is highly likely that the influence of gamma-IFN on malaria infection is at the level of the activated Kupffer cells, which degrade the sporozoites instead of passing them on to the hepatocytes. Innate production of IFN in non-immune rats exposed to infective sporozoites does not seem to be significant at that time, or when the merozoites are liberated from the liver, or during the period of parasitaemia (Verhave, 1975). The finding by Ferreira *et al.* (1986b) that gamma-IFN interferes with the development of intracellular parasites suggests an additional but distinct role for this substance *in vivo*. The low infectivity of sporozoites of rodent malaria suggests that the majority of injected sporozoites do not reach the hepatocytes, or undergo abortive development. Studies on the invasion and early transformation of sporozoites presented great difficulties because of their small size and low density in the liver. By using the previously mentioned method of ligation of liver lobes and the choice of a sporozoite-sensitive rat strain, we could follow the transformation and development of *P. berghei in vivo*. This development, ending in the generation of thousands of merozoites, takes about 50 hours with *P. berghei* (Killick-Kendrick, 1974).

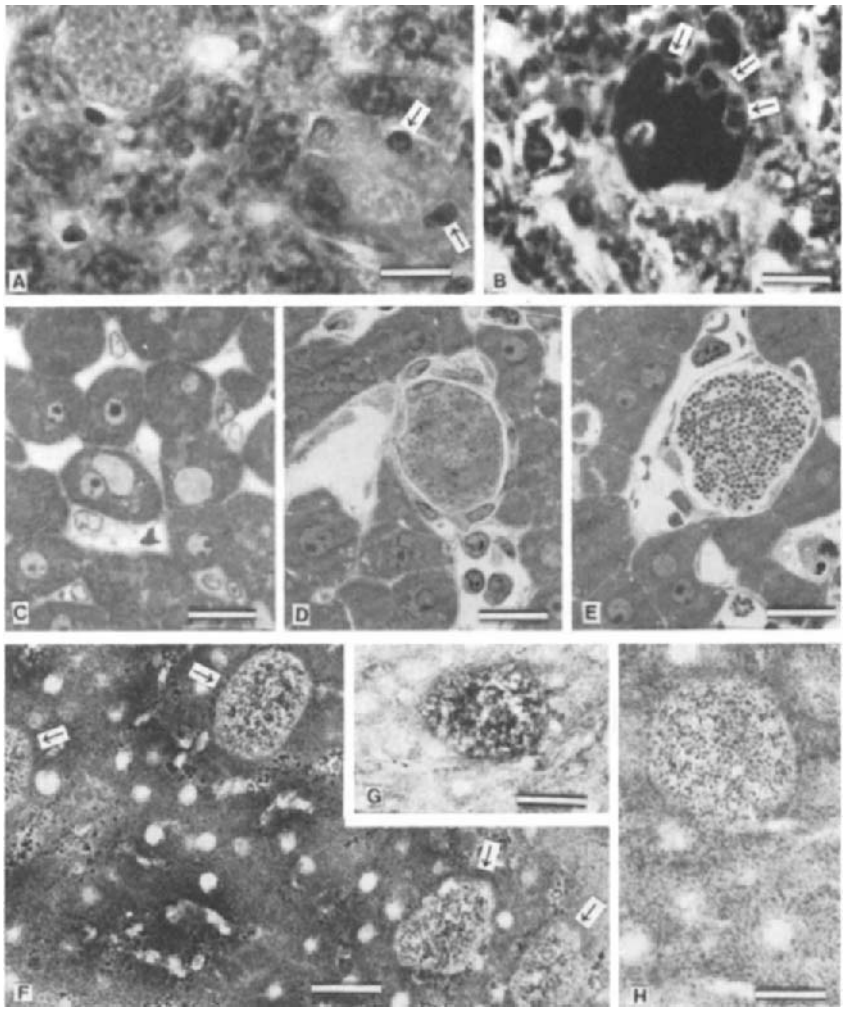



FIG. 7. Light micrographs of sections of *P. berghei* EE stages in rat liver. (A) Carnoy-fixed Giemsa stained paraffin section of EE stages at 48 hours. The parasite on the right is attacked by mononuclear cells (arrows). Bar = 25 μm. (B) Giemsa-colophonium stained parasite with infiltrating polymorphonuclear and mononuclear cells (arrows). Bar = 25 μm. (C–E) Semi-thin toluidine-blue stained Epon sections. EE stages expand gradually during development until the mass of formed merozoites comes to lie in the sinusoids: (C) = 28 hours; (D) = 51 hours; (E) = 54 hours. Bar = 20 μm. (F–H) Histochemically stained frozen sections of liver infected 48 hours previously with *P. berghei* sporozoites: (F) shows four EE stages (arrows) in one field. The parasites react positively for glucose 6-phosphate dehydrogenase. Note the absence of reaction product above the hepatocyte nuclei. Bar = 20 μm. In (G) the EE stage reacts strongly for the enzyme α-glycerophosphate dehydrogenase. Bar = 20 μm. In (H) the parasite reacts positively for 6-phosphogluconate dehydrogenase. Bar = 15 μm.

## 1. *Light microscopy*

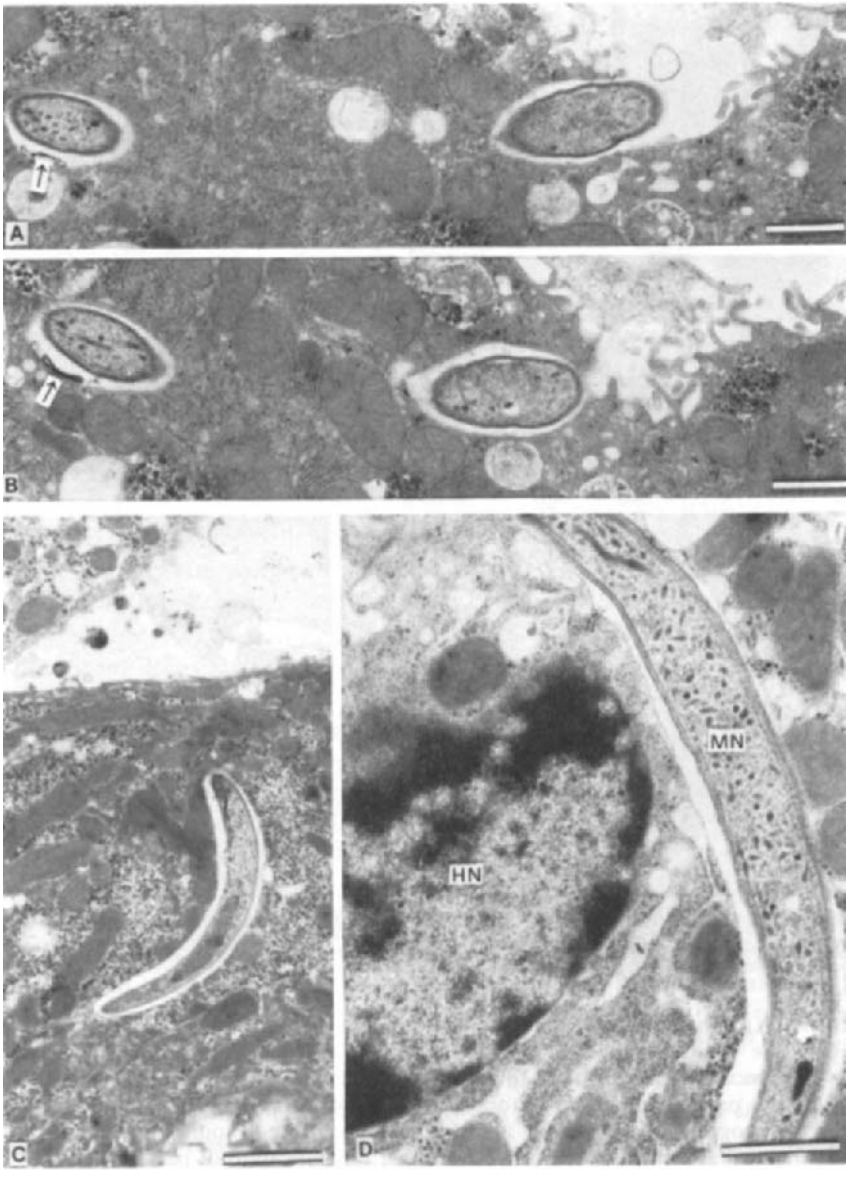
Before 1969, several light microscope studies (Fig. 7 (A–E)) had been made on the EE development of rodent malaria parasites (Yoeli and Most, 1965; Landau and Killick-Kendrick, 1966). In these studies the infected liver tissue was fixed in Carnoy's fluid and sections were stained with Giemsa-colophonium (Garnham, 1966) (Fig. 7 (A, B)). With this staining method only stages beyond 25 hours of development were detectable. The densely staining basophilic clumps in parasites from 30 hours onwards appeared similar to the large patches of rough endoplasmic reticulum (RER) seen by electron microscopy in developing schizonts. In a recent study, we were able to localize young EE schizonts, in semi-thin sections of liver embedded in epoxy resin and stained with toluidine-blue, from 15 hours onwards. The parasites measured from 3 μm (15 hours), via 7 μm (24 hours) to 10 μm (28 hours). At 33 hours parasites of about 15 μm were found, at 43 hours they were 30 μm, and at 48 hours they measured about 40 μm in diameter (Fig. 7 (C–E)).

In normal toluidine blue-stained sections no parasite could be detected younger than 15 hours. After removal of the embedding medium from the sections (Meis *et al.*, 1985a) and reaction with anti-sporozoite antibodies, younger parasites could be found with indirect immunofluorescence (IF). Similar results were obtained using IF on parasites in cryo-sections of liver tissue, and, by Danforth *et al.* (1978), with sections of Carnoy-fixed liver tissue. The latter investigators found that schizonts aged from 14 hours to 30 hours reacted with anti-sporozoite serum, but after 30 hours the parasites reacted strongly with anti-erythrocytic stage serum. These results suggested a profound change in antigenicity of liver stages at about 30 hours.

## 2. *Electron microscopy*

Sporozoites invade hepatocytes by moving into a parasitophorous vacuole created by the invagination of the outer plasmalemma (Meis *et al.*, 1983b) (Fig. 8 (A,B)). *P. berghei* detected in hepatocytes up to 2 hours (Fig. 9 (A,B)) still had all the morphological characteristics of an elongated, sickle-cell

FIG. 8. (A, B) Two sections of a sporozoite of *P. berghei* fixed in the process of invading a rat hepatocyte. The parasite is localized in a membrane-bound parasitophorous vacuole, which is generated by the invagination of the outer hepatocyte plasmalemma. This membrane reseals when the caudal end of the sporozoite is far enough inside the hepatocyte. Note the membrane whorls in the parasitophorous vacuole (arrows). Bar = 1 μm. (C) A sporozoite of *P. falciparum* in a parasitophorous vacuole in a human hepatocyte. Bar = 3 μm. (D) Another *P. falciparum* sporozoite in a human hepatocyte. The sporozoites are always closely associated with the host nucleus (HN). Note the abundance of micronemes (MN) in the sporozoite. Bar = 0.5 μm.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B
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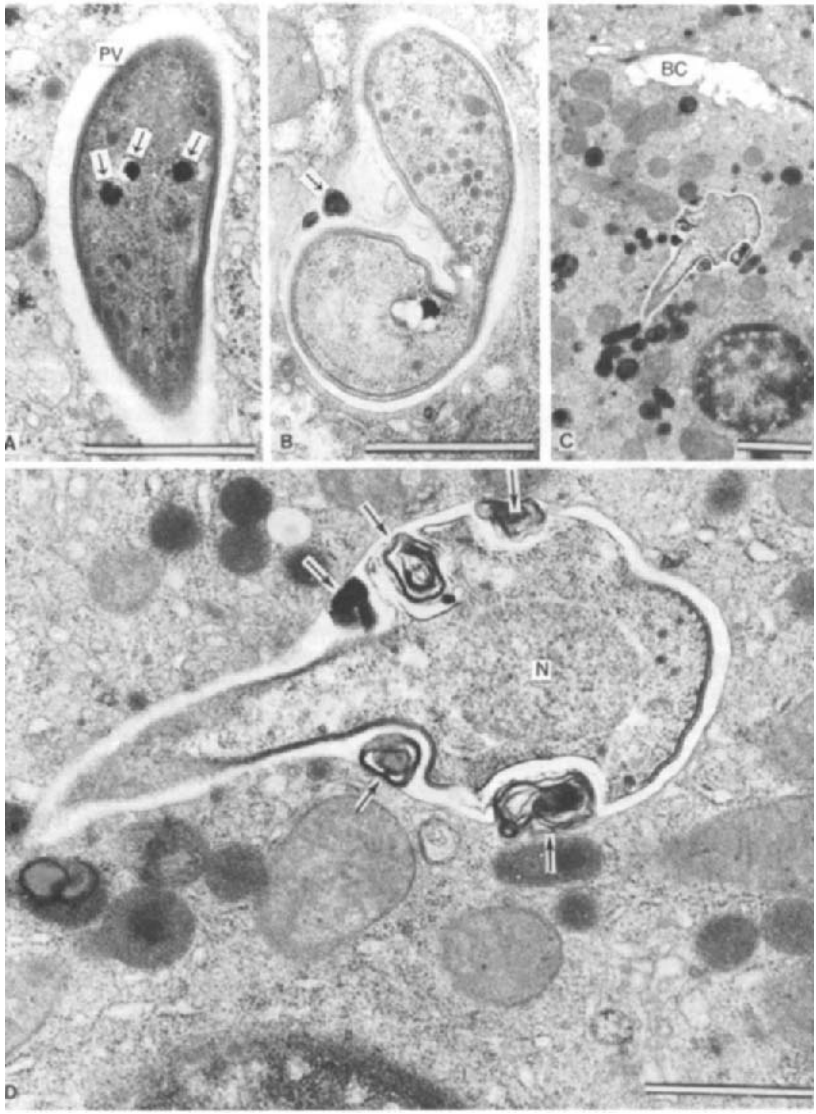


FIG. 9. (A) Sporozoite of *P. berghei* in the parasitophorous vacuole (PV) of a rat hepatocyte 1 hour after inoculation. Note the osmiophilic bodies (arrows). Bar = 1 μm. (B) Sporozoite 2 hours after inoculation. Osmiophilic membrane material is present in the parasitophorous vacuole (arrow). Bar = 1 μm. (C) Low power micrograph of a partially transformed sporozoite 4 hours after inoculation. BC = bile canaliculus. Bar = 2 μm. (D) Higher magnification of part of (C). Part of the sporozoite around the centrally located nucleus (N) has become enlarged. At places where subpellicular microtubules are seen, the triple layered sporozoite pellicle remains visible. Note the abundant osmiophilic membranous whorls (arrows). Bar = 1 μm.

shaped sporozoite, with a rhoptry–microneme complex and pellicular membranes with subpellicular microtubules. The parasites were localized in a membrane-bound parasitophorous vacuole of host cell origin. Membrane whorls in the parasitophorous vacuole (Figs 8 (A,B); 9) are thought to be rhoptry secretions (Stewart *et al.*, 1985). Similar material was seen with *P. falciparum* merozoites (Stewart *et al.*, 1986b) and *P. falciparum* sporozoites, inoculated on to human hepatocyte monolayers, which were localized in parasitophorous vacuoles (Fig. 8 (C,D)). Sporozoites of *P. berghei* (Aikawa *et al.*, 1984; Meis *et al.*, 1984c; Suhrbier *et al.*, 1986) and *P. falciparum* (Fig. 8 (D)) became closely associated *in vitro* with the host nucleus after penetration of the host cell.

The intracellular parasite has to start a process of dedifferentiation in order to enter its growth (vegetative) phase. In the region of its nucleus the parasite becomes enlarged and rounded (Figs 9 (C,D); 10). The rigidity and shape of the sporozoite's anterior complex is still visible (Meis *et al.*, 1983d). Parts of the inner membranes, especially where no microtubules are located, appear to be gradually broken down during development and in these regions the parasite becomes amoeboid and expanded.

The apical complex of the sporozoite remains visible until 15 hours (Fig. 10 (B,C)), and the pellicular membranes with the attached subpellicular microtubules remain visible until at least 33 hours (Meis *et al.*, 1985a, 1986b). We have not found this persisting sporozoite pellicle in more mature stages of *P. berghei*.

The young EE stages remain unicellular until about 20–22 hours. From this time onwards, the first signs of nuclear division become apparent (Fig. 11 (A, B)). This nuclear division continues, without nuclear fission, until a large nuclear reticulum is formed, appearing in sections as a number of nuclear profiles. In one profile multiple spindle figures can be observed, similar to those seen in oocysts (Schrével *et al.*, 1977).

The two major benefits which the hepatocyte confers on the parasite are a stable environment and a supply of nutrients. In contrast to the motile invasive stages, the intracellular form needs nutrients in order to grow and multiply. Mammalian EE stages probably acquire their nutrients by diffusion through the parasite membrane. Bulk feeding of host cytoplasm via a cytostome, as clearly seen in the erythrocytic stage, has never been observed in mammalian EE stages. We have never observed cytostomes in *P. berghei*, nor have others in *P. yoelii* (Seureaux *et al.*, 1980). This accords with observations that this organelle appears to be rare or absent in rodent-infective sporozoites (Sinden and Garnham, 1973)—a striking difference between sporozoites of the rodent parasite *P. berghei* and those of the human parasite *P. vivax*. In the latter parasite, cytostomes can be readily observed and they have recently been described for the first time in *P. vivax* EE stages

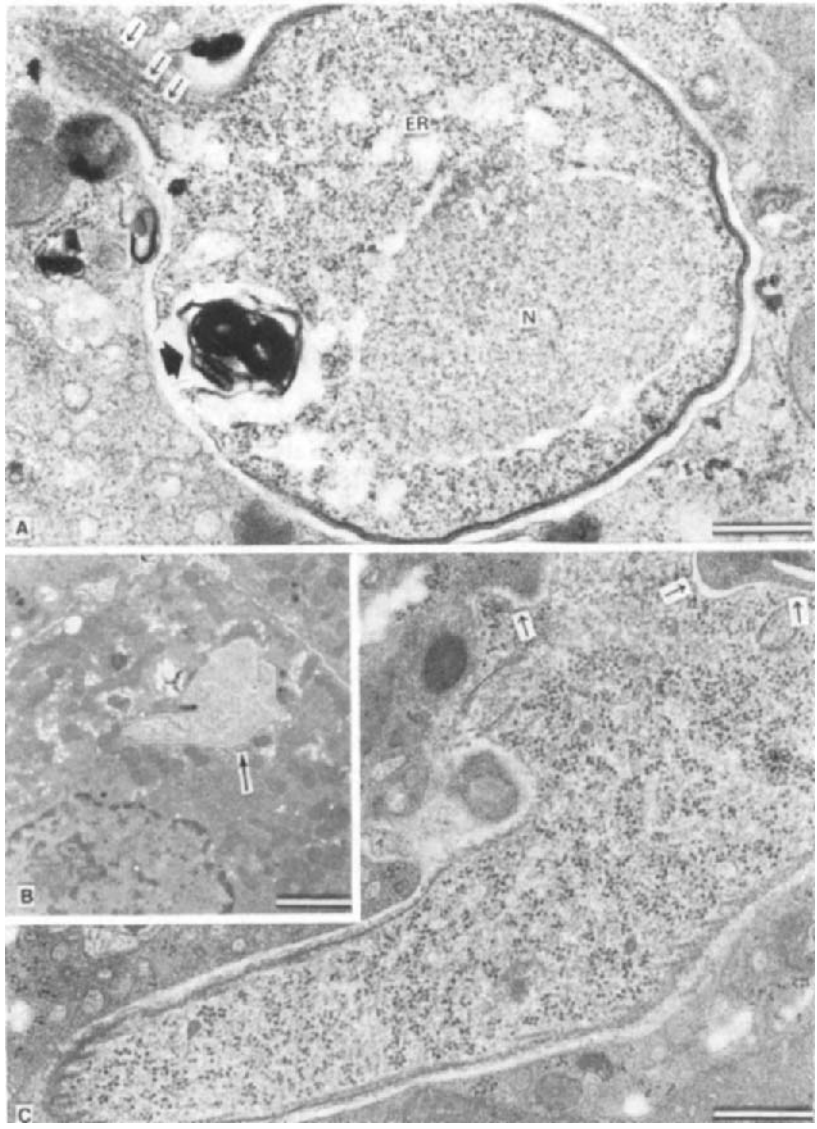


FIG. 10. *P. berghei.* (A) Rounded intrahepatocytic parasite 10 hours after inoculation. A large part of the parasite still has the typical triple layered sporozoite pellicle with subpellicular microtubules (arrows). Osmiophilic membranous whorls (thick arrow) are visible in the parasitophorous vacuole. With a large nucleus (N), some endoplasmic reticulum (ER) and packed ribosomes, the parasite has not started any differentiation. Bar = 1 μm. (B) Low-power micrograph of a partially transformed intrahepatocytic sporozoite (arrow). Bar = 2 μm. (C) Higher magnification of another section of (B). Part of the parasite still has the sporozoite pellicle with subpellicular microtubules, while other parts have a single plasmalemma (arrows). Bar = 0.5 μm.

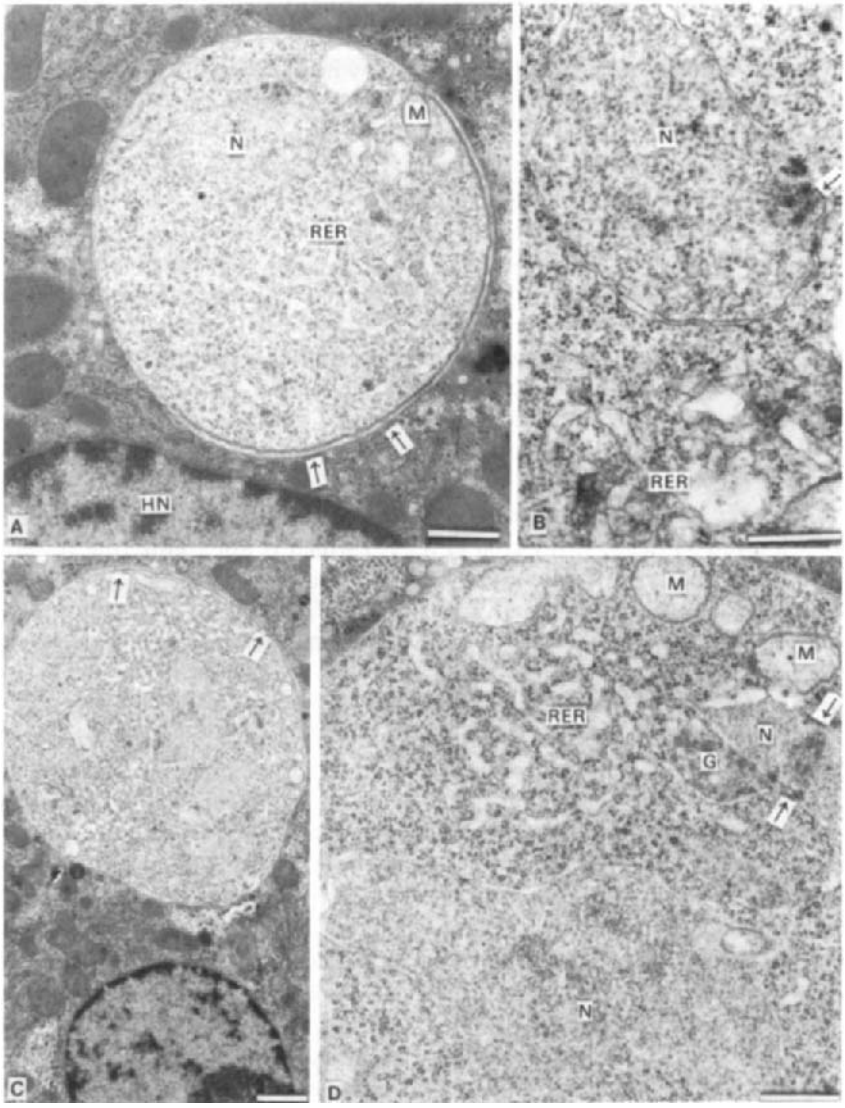


FIG. 11. *P. berghei.* (A) An exoerythrocytic form 21 hours after infection. The inner pellicular membranes still cover half of the parasite's surface area (arrows). A nuclear fragment (N), rough endoplasmic reticulum (RER) and a mitochondrion (M) are visible. HN = host nucleus. Bar = 1 μm. (B) Higher magnification of part of (A). A mitotic spindle (arrow) with intranuclear microtubules is the first sign of nuclear division. Bar = 0.5 μm. (C) An exoerythrocytic schizont 24 hours after infection. Although the parasite is now largely expanded, a strand of inner membrane is still present (between arrows). Bar = 1 μm. (D) Detail of (C) showing a large nuclear fragment (N), mitotic spindle (arrows), rough endoplasmic reticulum (RER) with a Golgi area (G), and mitochondria (M). Bar = 1 μm.

grown *in vitro* (Uni *et al.*, 1985). Feeding by ingestion of host cytoplasm has not been seen, and thus it is not clear whether the cytostome in the EE stage is functional.

From 21 to 28 hours the intracellular *P. berghei* parasites expand from about 4 to 10 μm. At 28–33 hours many nuclear profiles, RER and tubular elongated mitochondria (Fig. 12) are present. The latter preferentially localize just beneath the plasmalemma. The parasite plasmalemma is very intimately associated with the parasitophorous vacuole membrane in the young EE stage. From about 40 hours onwards, peripherally localized vesicles in the parasite fuse with the plasmalemma, creating a space between the membranes. By this fusion, the contents of the vesicles are discharged into the parasitophorous vacuole. In more mature EE stages these vesicles coalesce, forming large vacuoles which are arranged bead-like at the periphery (Fig. 13).

The inner surface of the parasitophorous vacuole membrane is covered by a thick electron-dense fuzzy coat (Fig. 13) which is apparently the result of previous discharges of the contents of the vesicles into the parasitophorous vacuole. At about 51 hours it is possible to find different developmental stages of *P. berghei* in the same liver. Maturation proceeds with the majority of the parasites in the same phase, but some parasites invariably develop more rapidly or are retarded (Vanderberg, 1982; Meis *et al.*, 1985b).

A dramatic appearance in the differentiation of the EE stages is the partial subdivision of the solid cytoplasm by invaginations of the parasite plasmalemma at multiple sites (Fig. 14). Branching clefts form a continuous network of spaces, and highly irregular invaginations at the periphery of the EE stage adopt a labyrinthine configuration (Fig. 14). The latter structures were also found in EE stages of *P. y. yoelii* by Landau *et al.* (1982), who assumed that the labyrinthine networks were generated by the dilation of RER cisternae. Although this mechanism was also proposed for the subdivisions of *P. falciparum* oocysts (Sinden and Strong, 1978), we have not found evidence that cleft formation in EE stages of *P. berghei* follows the same process. The absence of vacuoles from the already segmented EE stages, and their presence in immature parasites, suggest an involvement of the vacuolar membranes in cleft formation. Furthermore, the RER remains constantly visible during division of the EE stages. The cisternae are also visible in the cytoplasmic strands of the labyrinth. These points make it unlikely that the labyrinthine structures are due to expansion of RER cisternae.

In addition to producing the long clefts from which the merozoites start to bud, the highly convoluted labyrinth provides the parasite with an enormous increase in surface area, which facilitates the transport of nutrients. At the surface of the long clefts a highly synchronized process of merozoite development starts. The homogeneous nucleoplasm in nuclei of the

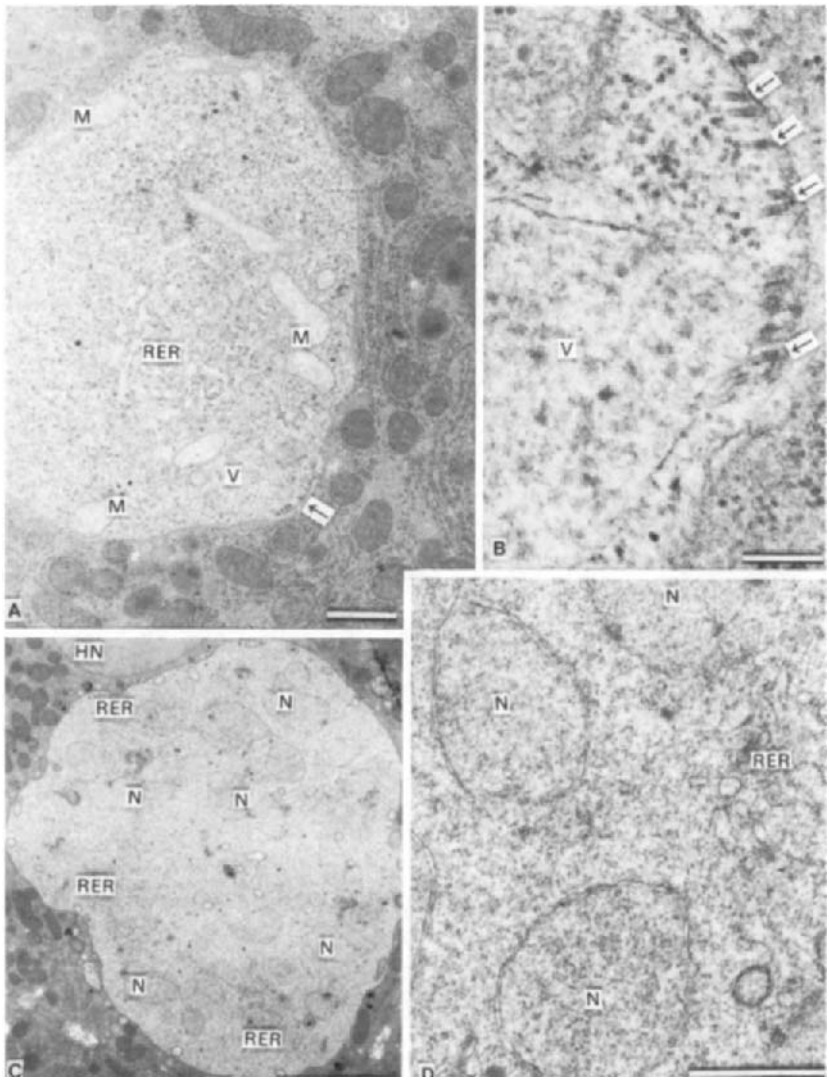


FIG. 12. *P. berghei.* (A) Exoerythrocytic form at 28 hours showing mitochondria (M) and rough endoplasmic reticulum (RER). Part of the parasite pellicle still has double membranes with attached microtubules (arrow). Bar = 1 μm. (B) Higher magnification of (A) demonstrating the persisting subpellicular microtubules (arrows). V = vacuole with fluffy content. Bar = 200 nm. (C) Exoerythrocytic form at 33 hours showing several nuclear profiles (N) and rough endoplasmic reticulum (RER). Bar = 5 μm. (D) Detail of (C) showing nuclear profiles (N) which form a three-dimensional nuclear reticulum. Bar = 1 μm.

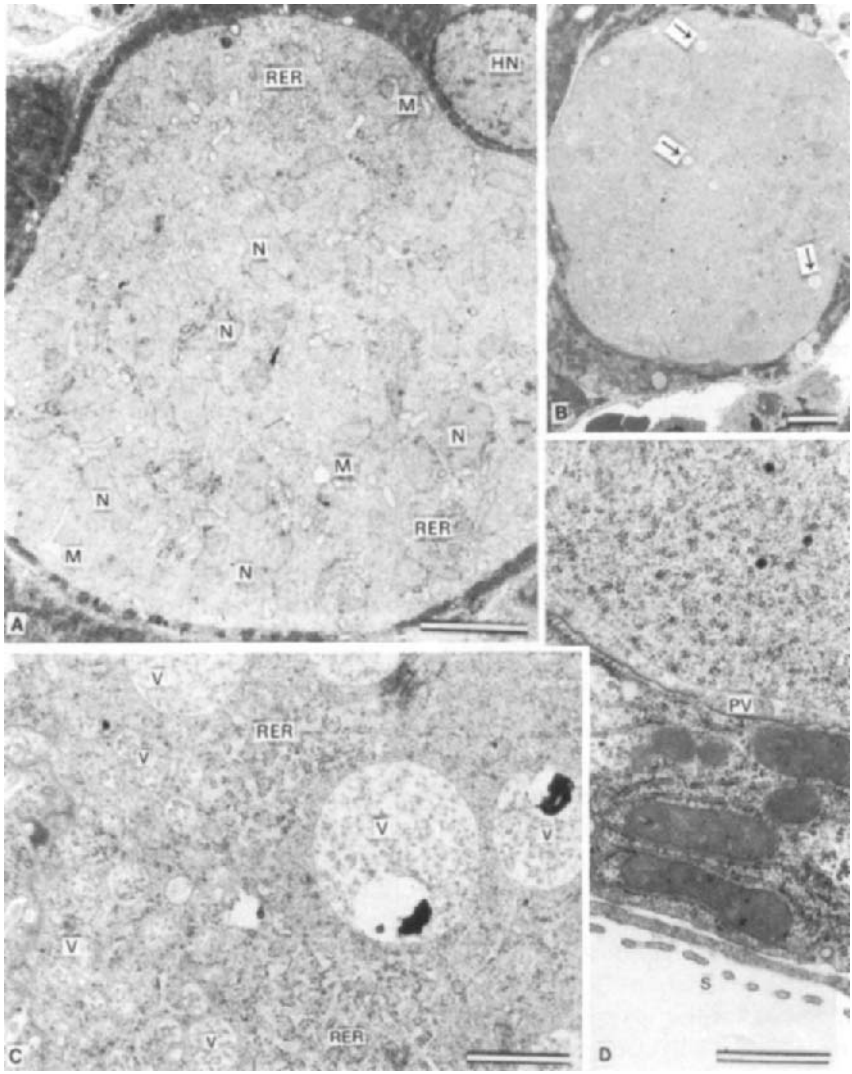


FIG. 13. *P. berghei.* (A) A largely expanded exoerythrocytic form at 43 hours has displaced the host nucleus (HN) to the periphery. Numerous nuclear profiles (N) are visible with, between them, patches of rough endoplasmic reticulum (RER) and mitochondria (M). Bar = 5 μm. (B) A parasite at 51 hours. It is hardly possible to discriminate organelles in the solid cytoplasm; only some lipid droplets are visible (arrows). Bar = 5 μm. (C) Part of the periphery of a parasite at 48 hours. Many single membrane-bound vesicles (V) of different sizes with similar contents fuse with each other and with the parasite plasmalemma. The fluffy material is thus deposited in the parasitophorous vacuole. Bar = 1 μm. (D) Part of the periphery of a parasite at 51 hours. The parasitophorous vacuole (PV) has become widened and is filled with similar material to that in the vesicles. Note the sinusoid (S) with fenestrated endothelium. Bar = 1 μm.

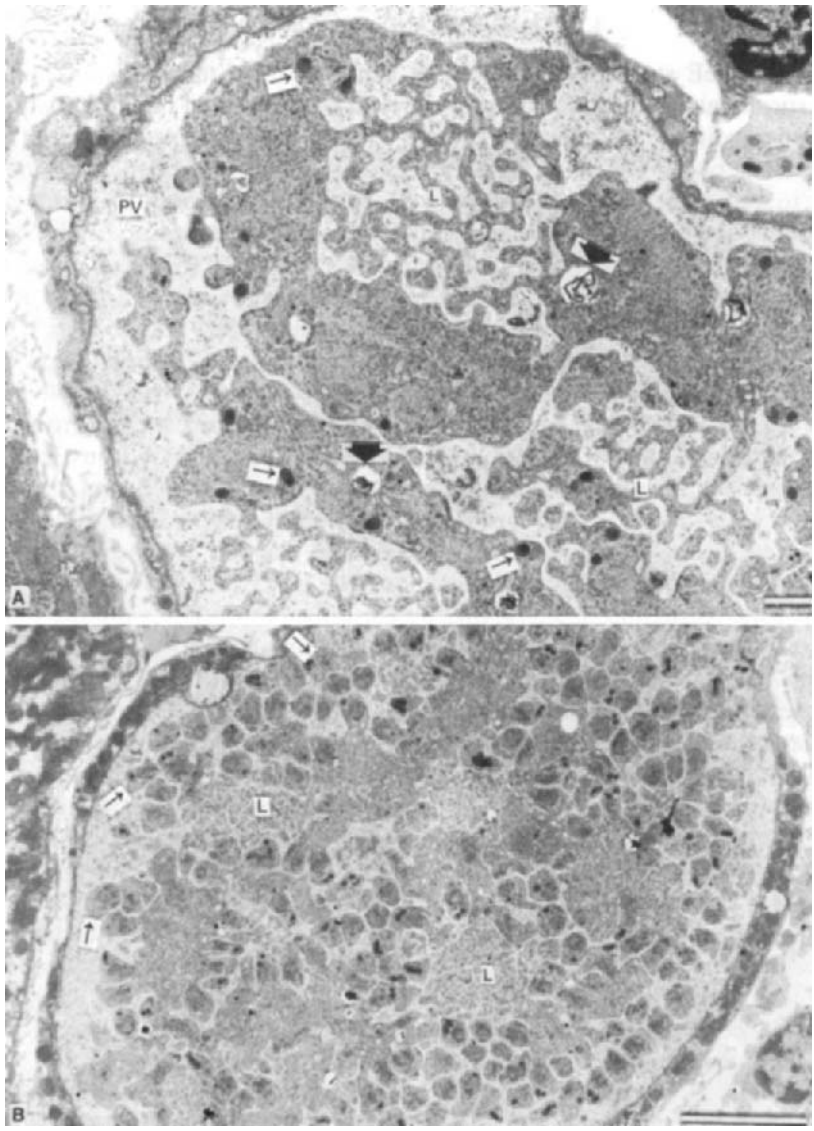


FIG. 14. *P. berghei.* (A) Subdivision of the formerly solid parasite cytoplasm has occurred by long tubular cleft-like invaginations and branching networks of tubules resembling a labyrinth (L). The parasitophorous vacuole (PV) is connected with the labyrinthine spaces and both contain the fluffy material. Along the clefts beneath the plasmalemma, strands of inner osmiophilic layers and electron-dense rhoptries (arrows) are formed. At this stage the parasite's lipid droplets have disappeared, leaving only some remnants (thick arrows). Bar = 1 μm. (B) A more advanced stage of development. The labyrinthine structures (L) are clearly visible, although their spaces are smaller. Merozoites are almost fully formed and some have already separated from the meroblast (arrows). Bar = 5 μm.

immature EE stage, indicative of an active state of metabolism, changes drastically at budding. Chromatin starts to condense and remains visible in the merozoite. Dense linear areas are formed beneath specific sites of the meroblast plasmalemma. At these places membrane-bound bodies of moderate to very dense texture arise between the inner membrane deposits and nuclei (Fig. 14 (A)). In the nuclei mitotic spindles are always visible. Small bristle-coated vesicles (50 nm) bud from the nuclear envelope; these are similar to the Golgi vesicles associated with nuclear membranes and RER cisternae previously described in EE stages (Meis *et al.*, 1983a). The dense linear areas develop into a set of lateral inner membranes during the process of budding, and conical protrusions into the parasitophorous vacuole form and eventually separate from the residual cytoplasm to become merozoites.

After completion of merozoite formation, the parasitophorous vacuole membrane may disrupt and a mixture of hepatocyte organelles and parasites occurs (Fig. 15). This sequence of events is different from the generally assumed idea that merozoites are released by the rupture of the host cell (Sinden, 1978). A similar phenomenon was described previously for EE merozoite formation in *P. berghei* by Desser *et al.* (1972). Collapse of the parasitophorous vacuole membrane was also reported in erythrocytes containing *P. falciparum* merozoites (Langreth *et al.*, 1978). It has been suggested that the motility of merozoites may contribute to the rupture and collapse of the parasitophorous vacuole membrane (Sinden, 1978). This motility of merozoites was confirmed by observations in tissue culture by phase-contrast illumination (M. R. Hollingdale, personal communication). In an advanced stage of development, the merozoites are embedded in a spongy stroma consisting of remnants of the hepatocyte cytoplasm (Fig. 15 (B,C)). This mass of merozoites, kept together by stroma, bulges out into the sinusoid and groups of merozoites are released. The merozoites have to liberate themselves and enter erythrocytes before being taken up from the bloodstream by Kupffer cells (Terzakis *et al.*, 1979). After penetration into the red cell, the EE merozoite develops into an asexual trophozoite or transforms into a gametocyte (Killick-Kendrick and Warren, 1968).

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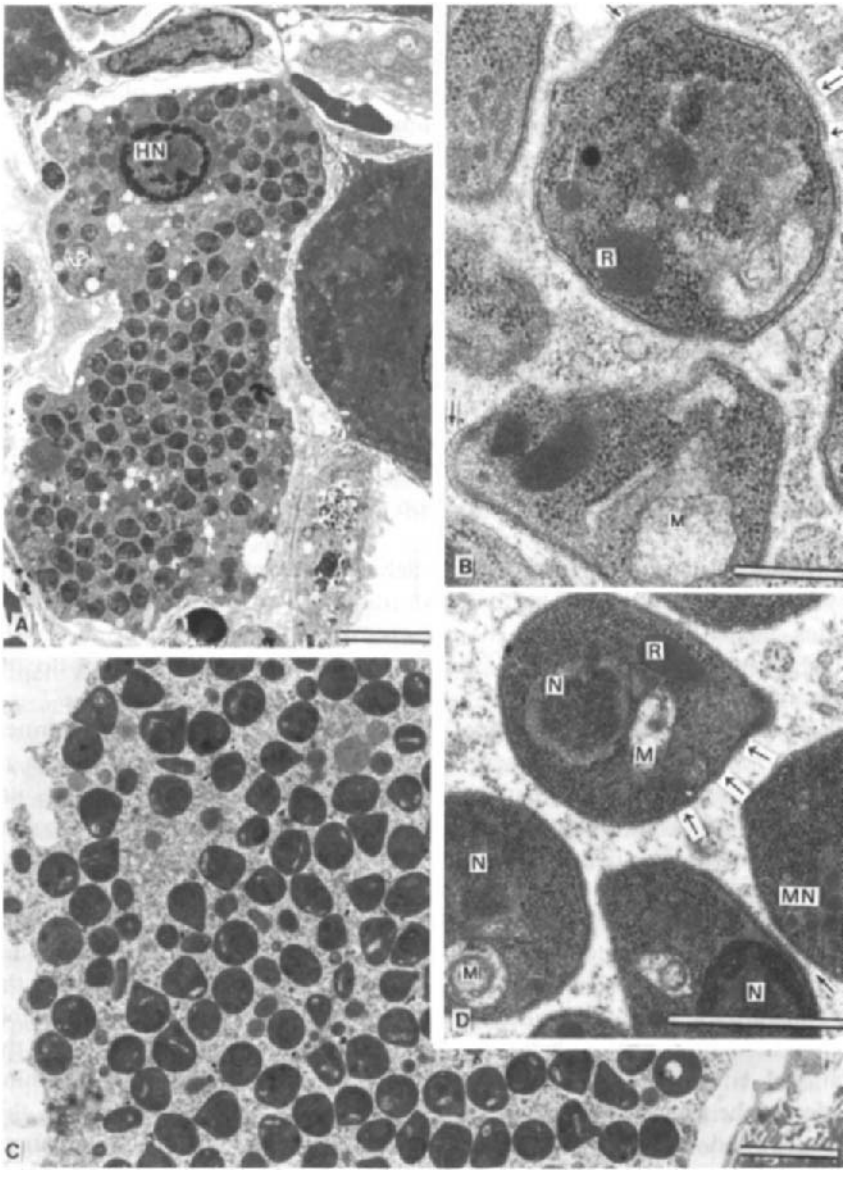
FIG. 15. *P. berghei.* (A) When the parasitophorous vacuole membrane has collapsed, the newly formed merozoites become mingled with the remaining hepatocyte cytoplasm. This mass of parasites, hepatocytic organelles and the host cell nucleus (HN) now lie in a sinusoid. Bar = 5 μm. (B) Detail of (A), demonstrating merozoites surrounded by the hepatocytic cytoplasmic matrix. Note the presence of the typical fibrillar surface coat on the parasites (arrows). R = rhoptries, M = mitochondrion. Bar = 0.5 μm. (C) Large cluster of merozoites still embedded in, and kept together by, a stroma derived from the degenerated hepatocyte cytoplasm. Bar = 3 μm. (D) Higher magnification of (C) showing the pear-shaped merozoites with a central nucleus (N), mitochondrion (M), rhoptries (R) and micronemes (MN). The regular bristle-like surface coat (arrows) on the fully mature merozoites is clearly visible. Bar = 1 μm.


HN
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The fine structure of *P. berghei* tissue-stage merozoites (Fig. 15 (D)) had already been described by Garnham *et al.* (1969). In these merozoites no multi-membraned structures, as described previously for erythrocytic merozoites of *P. knowlesi* (Bannister *et al.*, 1977), *P. falciparum* (Langreth *et al.*, 1978) and *P. berghei* (Verhave and Meis, 1984), are visible; they may be fixation artefacts. The mature pear-shaped EE merozoites of *P. berghei* (length 1.5–1.7 μm, diameter 1.0–1.2 μm), invested by a trilaminar pellicle, contain a large central nucleus, a single acristate mitochondrion and a lateral cytostome. The cytostome, which measures 70 nm in inner diameter (Meis *et al.*, 1985b), is the same size as that of *P. yoelii* in the mouse (Garnham *et al.*, 1969) and *P. berghei* in the tree rat (Desser *et al.*, 1972).

Although we have studied many EE stages of *P. berghei* in several laboratory rats, we have not seen polymorphism of the schizonts and nuclei of merozoites, as demonstrated for *P. v. vinckei* (Bafort, 1971) in one tree rat 74 hours after inoculation of sporozoites. Landau and her colleagues (see Landau and Boulard, 1978) also found curious small persistent schizonts of *P. yoelii* in the liver of a wild-caught tree rat after 8 months in captivity. These were not seen in laboratory infections, but could be mimicked by keeping the tree rat over long periods on a low dose of ethionine (Landau *et al.*, 1975, 1982).

Mammalian tissue-stage merozoites definitely have the same dimensions as erythrocytic merozoites. This is in contrast with avian tissue-stage merozoites, which are longer than the erythrocytic ones (Aikawa *et al.*, 1968). Furthermore, avian EE stages produce far fewer merozoites than do the mammalian EE stages.

The merozoite pellicle consists of an outer, and two closely aligned inner, membranes. Beneath the inner membrane, in some species, a row of subpellicular microtubules can be found. In *P. berghei*, microtubules are absent or rare (Meis *et al.*, 1985b). This contrasts with avian EE merozoites, which have 24–26 subpellicular microtubules (Aikawa, 1967). The merozoite's outer membrane is covered with a typical 20 nm microfibrillar surface coat, which was rarely observed in this form on erythrocytic merozoites of rodent malaria parasites. Bannister *et al.* (1975, 1977) described a similar surface coat, consisting of regularly spaced T- or Y-shaped bristles, on the cell membrane of *P. knowlesi* erythrocytic merozoites, which was visible throughout merogony. They suggested that this coat originated from the schizont, either as part of the merozoite's intrinsic structure or as something added in the parasitophorous vacuole (Bannister *et al.*, 1977). Recently, this group found evidence that the cell coat is indeed a regularly structured component of the merozoite surface, and that its formation commences early in the erythrocytic schizont (Bannister *et al.*, 1986). The surface coat on tissue-stage merozoites, which is also already present on the plasmalemma of

the meroblast, might be derived from the floccular material occupying the parasitophorous vacuole. The presence of this surface coat on intra-erythrocytic (Bannister *et al.*, 1977, 1986) and intra-hepatocytic merozoites (Meis *et al.*, 1985b) is in opposition to the suggestion of Aikawa and Seed (1980) that this coat is formed when merozoites are released from the red cell or hepatocyte, respectively.

The surface coat of both erythrocytic and tissue-stage merozoites is involved in red cell invasion, and therefore a prime target for the host immune system (reviewed by Brown *et al.*, 1986), and surface proteins are potential candidates for a vaccine (Sherman, 1985). Antigens shared by both types of merozoites are of especial interest. Kilejian *et al.* (1982) found cross-reactivity between surface antigens of EE and erythrocytic merozoites of *P. lophurae*, although each stage penetrates different host cells. How the various antigens on the two types of merozoites of mammalian malaria parasites are related remains to be clarified, although preliminary results *with P. vivax* seem promising (Aley *et al.*, 1988).

A cellular response against EE stages is generally believed not to occur as long as the parasites remain intracellular; only when merozoites are formed, and released into the circulation, are phagocytes attracted (Garnham and Bray, 1956; Terzakis *et al.*, 1979). Contrary to this, we have found evidence that nearly mature EE stages (47–51 hours) could evoke a local tissue reaction, manifested by encompassing infiltrations of mono- and polymorphonuclear cells (Fig. 7 (A,B)) (Jap *et al.*, 1982). With electron microscopy, there is a profound difference visible between normal EE stages and those attacked by phagocytes (Fig. 16). A reduction of the prominent peripheral vacuolization is the most pronounced change. The original peripheral vesicles become smaller and more condensed, with packed electron-dense granules (Fig. 16 (B)). The host hepatocyte, which is reduced to a small rim, becomes electron-dense and is in very close contact with the parasite. The rim of host cytoplasm is penetrated by the surrounding phagocytes and eventually disappears (Meis *et al.*, 1987) (Fig. 17).

EE stages in more advanced stages of development can be seen penetrated by filopods of a neutrophil and monocyte (Fig. 17). The parasite fluffy material, which is always present in the parasitophorous vacuole and in the peripheral vacuoles, is ingested by the neutrophil (Fig. 17 (C)). Eventually, the EE stage is completely infiltrated by phagocytes (Fig. 17 (D)).

These observations clearly show that macrophages, neutrophils and monocytes recognize a proportion of the intracellular parasites, which are subsequently destroyed, and support speculations about the importance of cell-mediated immunity against the tissue stage of the malaria parasite. Some points can be considered which may explain the mechanism by which the infiltrating cells are attracted. From 47 hours onwards, some EE stages start

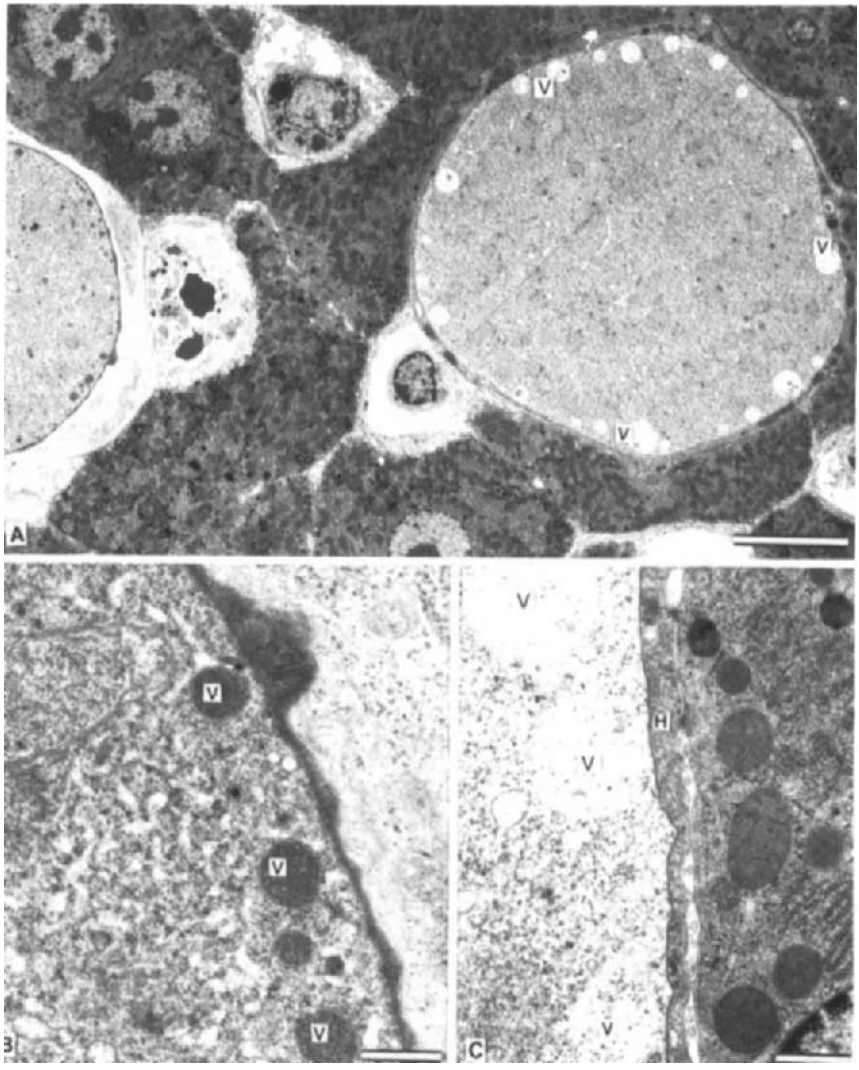



FIG. 16. *P. berghei.* (A) Low-power micrograph of two exoerythrocytic stages 49 hours after inoculation of sporozoites. The parasite at the right appears normal, with clear peripheral vacuolization (V) and no infiltrating cells. The parasite on the left is degenerating, with a thin electron-dense rim of host cytoplasm and dense peripheral vacuoles; the parasite is surrounded by macrophage-like cells with the typical lightly staining cytoplasm. Bar = 10 μm. (B) Detail of the degenerating EE stage. The peripheral vacuoles (V) and the remaining host cytoplasm have become electron-dense. Bar = 1 μm. (C) Detail of a normally developing EE stage with large peripheral vesicles (V) and a morphologically unaltered host hepatocyte (H). Bar = 1 μm.

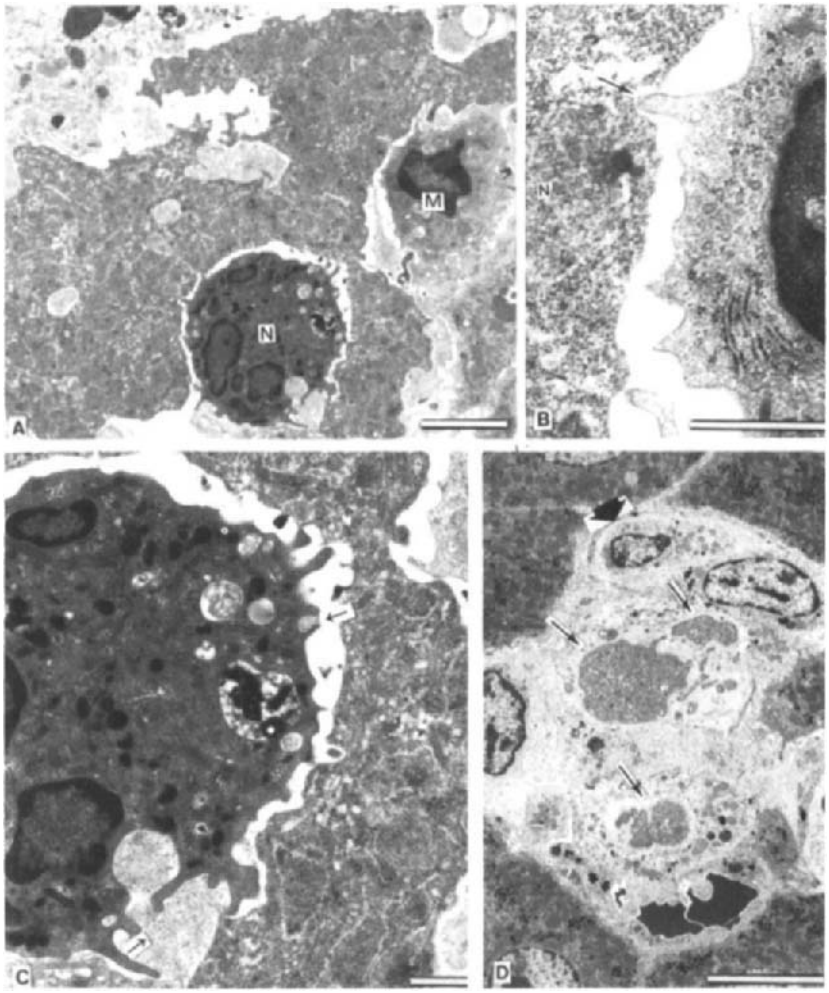



FIG. 17. *P. berghei*. (A) An EE stage, already degenerate, attacked by infiltrating cells. The host hepatocyte is destroyed in most places, leaving spaces for the penetrating neutrophil (N) and monocyte (M). Bar = 5 μm. (B) Detail of (A) showing the monocyte's filopods (arrow) penetrating into the naked EE stage. N = parasite nuclear fragment. Bar = 1 μm. (C) The aggressive neutrophil is lying almost completely in the EE stage, thereby penetrating the parasite with short filopods and ingesting the fluffy parasitic material (arrows) from the parasitophorous vacuole. Bar = 1 μm. (D) The cytoplasm of the degenerating parasite (arrows) becomes divided by the extensions of the infiltrated macrophage-like cells. The uppermost cell is a pit cell (thick arrow), the function of which is not yet established. Bar = 10 μm. Reprinted from Meis *et al.*, 1987, with permission of the American Society of Tropical Medicine and Hygiene.

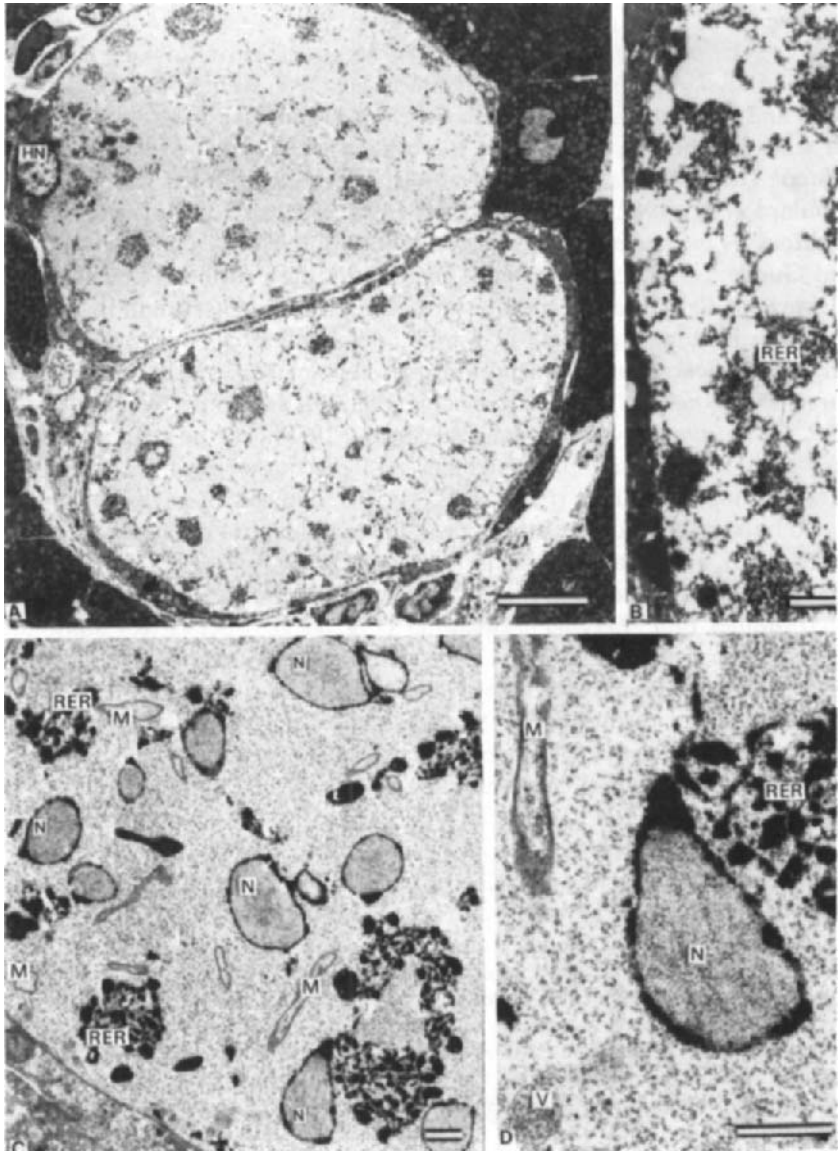
to rupture. This process attracts phagocytes, which might also act upon some of the nearby mature EE stages. Infected hepatocytes are permeable (Fig. 21 (C)), and parasitic material (especially that from the parasitophorous vacuole) might leak out into the circulation, thus attracting phagocytes. With metal impregnation techniques the internal structure of mature EE stages has been further clarified. Osmium–zinc iodide specifically stained the RER and nuclear envelopes, thus improving the visualization of the compartments in the mature EE stage (Fig. 18 (A)). Prolonged osmium impregnation not only improved visualization of nuclei and RER (Fig. 18 (C)) but also leached out the ground matrix, thereby displaying a system of intermediate-sized filaments (Fig. 18 (D)) (Meis *et al.*, 1986b). Parasites treated with the detergent Triton X-100 revealed a coarse filamentous pattern (Fig. 18 (B)), reminiscent of RER and nuclear membranes.

### C. PRIMATE MALARIA

The first description by electron microscopy of a primate malaria parasite in the liver was published in 1970 by Sodeman *et al.* The fine structure of the EE stage of *P. cynomolgi* was found to be similar to that of the other mammalian parasites. Sodeman *et al.* (1970) noted two types of vacuoles around the periphery of the EE stage, which were thought to play a role in the subdivision of the cytoplasm, or in transporting nutrients from the host, or both. The hepatic stages of human parasites cannot be studied routinely in natural infections in man (Shortt *et al.*, 1951; Jeffery *et al.*, 1952) and only with great difficulty in primate models (Sodeman *et al.*, 1969; Druilhe *et al.*, 1982, 1984).

Boulard *et al.* (1982) were the first to describe the fine structure of an EE stage of *P. falciparum* from the liver of a *Cebus* monkey. The vacuolar system, which was well known from the rodent parasites, appeared to be

FIG. 18. *P. berghei.* (A) Two developing parasites 48 hours after sporozoite inoculation. The material was stained with osmium–zinc iodide mixture, giving a specific staining of rough endoplasmic reticulum and nuclear envelopes. HN = host nucleus. Bar = 10 μm. (B) Parasites treated with Triton-X100 followed by osmium–zinc iodide staining. All soluble components of the cytosol are removed, leaving patches of coarse filamentous material (RER) and nuclei with extracted nucleoplasm. Bar = 1 μm. (C) Osmium impregnation of EE stages for 48 hours results in a dense staining of the nuclear (N) envelope and the rough endoplasmic reticulum (RER). Mitochondria (M) remain unstained. Bar = 1 μm. (D) Detail of (C). The cisternal contents of the nuclear envelope and rough endoplasmic reticulum (RER) are covered with a coarse dense deposit. Peripheral vacuole (V) and mitochondrion (M) remain unstained. The parasite's ground matrix has been leached out, leaving a network of fine filaments with attached polysomes. Bar = 1 μm. Reprinted from Meis *et al.* (1986b) with permission from the authors and publishers.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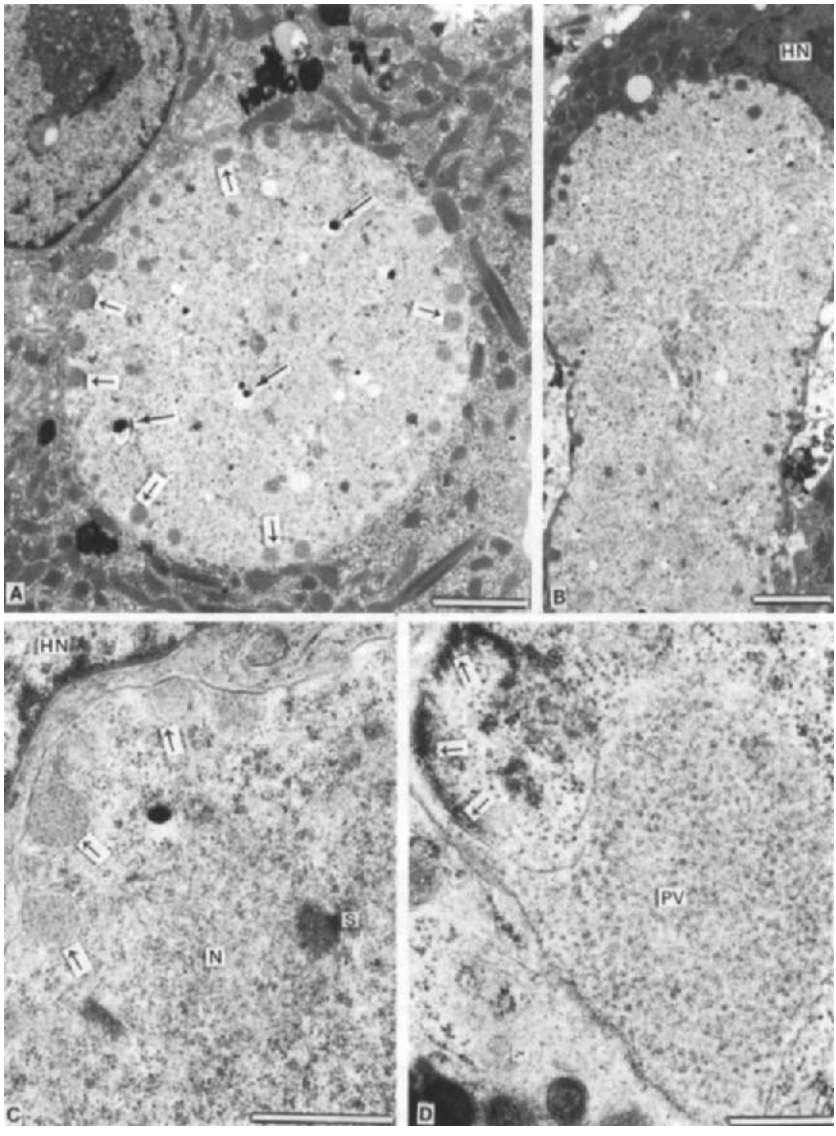
present abundantly. However, many characteristics of avian malaria parasites were seen in this human parasite. Mitochondria with tubular cristae in an electron-dense matrix, prominent nucleoli and "fenestrated buttons" were reminiscent of avian parasites, and confirmed earlier suggestions of the close phylogenetic relationship of *P. falciparum* with avian malaria parasites (Landau *et al.*, 1976). Recently, methods for the culture of EE stages of *P. vivax* and *P. falciparum* have been developed, and used to provide material for studying the fine structure of these stages (Uni *et al.*, 1985; Meis *et al.*, 1986a). These studies confirm the results obtained from the *Cebus* monkey (Boulard *et al.*, 1982). *P. falciparum* EE stages cultured in primary human hepatocytes *in vitro* appear to grow slightly more slowly than in man (Bray and Gunders, 1963). The material enclosed in the peripheral vesicles of the EE stage of *P. falciparum* (Fig. 19 (A,C,D)) is much denser than that seen in rodent parasites (Fig. 13 (C)). Similar dense material is observed in the peripheral vacuoles and parasitophorous vacuole of EE stages of the primate parasites *P. knowlesi* and *P. cynomolgi* (Fig. 20), and in peripheral vacuoles of *P. falciparum* oocysts (Sinden and Strong, 1978). The peripheral vacuoles fuse with the parasite plasmalemma and discharge their content in the parasitophorous vacuole (Figs 19 (C,D); 20), as in rodent parasites. "Fenestrated buttons", which are also present in avian malaria parasites (Garnham *et al.*, 1960), are always abundant in *P. falciparum* (Fig. 19 (A)) (Meis *et al.*, 1986a; Ponnudurai *et al.*, 1986). The inner membranes and subpellicular microtubules of *P. berghei* sporozoites (Fig. 12 (B)) persist for some time during the transformation into EE forms. In *P. falciparum* EE stages *in vitro* (Fig. 19 (D)), they can be seen until day 7, which indicates that the sporozoite pellicle does not disintegrate in the EE stage. In a recent study of mature cultured *P. falciparum* EE stages, 15 cross-sections of subpellicular microtubules could be found, corresponding to the 15 ($\pm$ 1) microtubules of the sporozoites (Meis *et al.*, 1986a).

No microtubule was detected in a mature *P. vivax* EE stage cultured in a human hepatoma cell line (HepG2) (Uni *et al.*, 1985). Many cytostomes were present in the *P. vivax* EE stage, and are also clearly present in avian *P. fallax*

FIG. 19. (A) Electron micrograph of an exoerythrocytic form of *P. falciparum* grown in a human hepatocyte in culture. Peripheral vesicles are clearly visible (arrows). Also arrowed are electron-dense vesicles of different sizes ('fenestrated buttons'), distributed throughout the parasite. Bar = 3 μm. (B) Another exoerythrocytic parasite pushing the host nucleus (HN) to the periphery. Bar = 3 μm. (C) Detail of a host/parasite interface showing peripheral vesicles (arrows) fusing with the parasite plasmalemma. A nuclear fragment (N) with a mitotic spindle (S) indicates division. HN = host nucleus. Bar = 1 μm. (D) Detail of the host/parasite interface. A peripheral vesicle has just fused with the parasite plasmalemma, thereby widening the parasitophorous vacuole (PV). Note remnants of inner membranes with attached subpellicular microtubules (arrows). H = host hepatocyte. Bar = 0.5 μm.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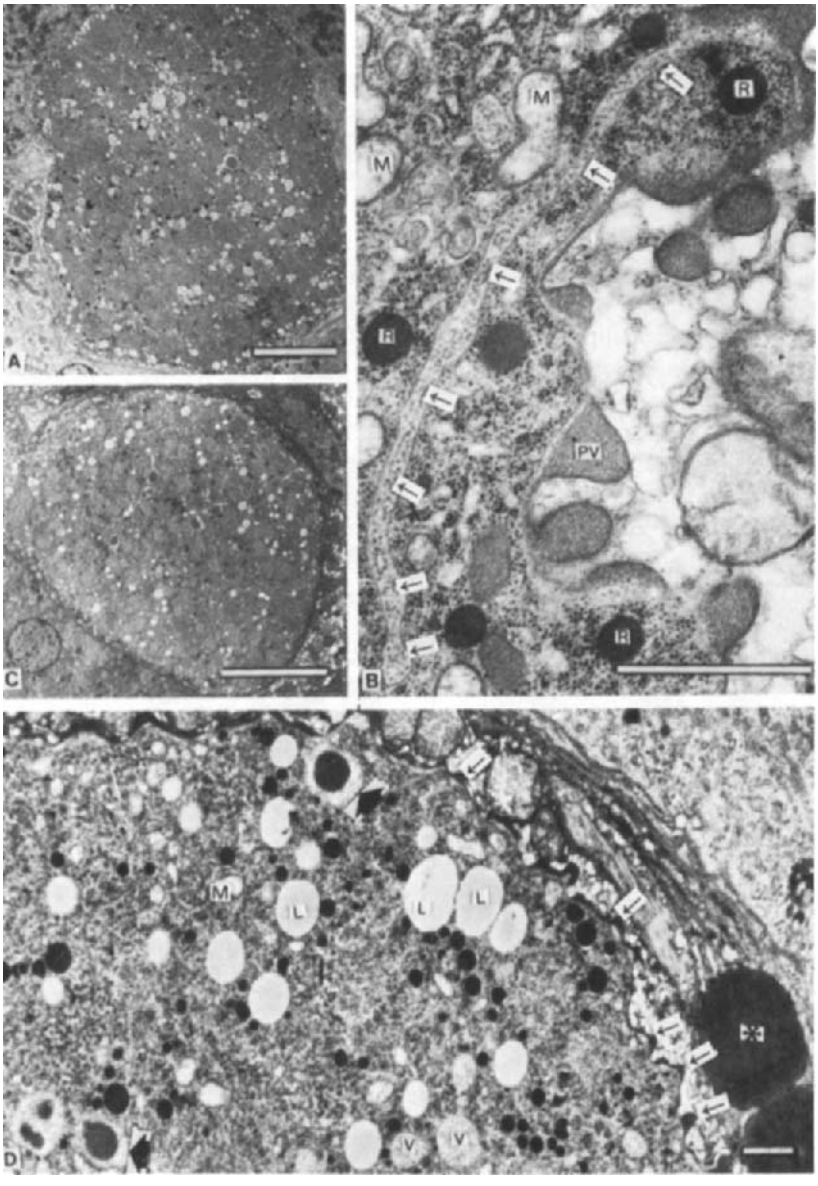
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EE stages (Hepler *et al.*, 1966). This contrasts with *P. falciparum* and rodent malaria EE stages, in which cytostomes have never been described (Verhave and Meis, 1984). It is hardly possible that the failure to detect a cytostome in the latter EE stages is because of the paucity of material for examination. *P. vivax* and *P. gallinaceum* sporozoites also possess cytostomes, in contrast to sporozoites of the rodent parasites (Sinden and Garnham, 1973). It is possible to correlate these findings with infectivity, as the rodent parasites are poorly infective compared to *P. vivax* and *P. gallinaceum*.

The development of methods to culture *P. vivax* in a cell line has recently permitted the demonstration of hypnozoites in culture (Hollingdale *et al.*, 1985c). Of considerable interest is the ultrastructure of these hypnozoites, from which *P. vivax* relapses originate. By using a strain of *P. vivax* which inherently produces a high proportion of hypnozoites (e.g. North Korean), it should be possible to describe the fine structure of hypnozoites in the near future (Hollingdale *et al.*, 1986).

## IV. Metabolism of Exoerythrocytic Forms

The EE stages are destined to produce an enormous number of new parasites in a relatively short period. Their fast multiplication requires the production of a lot of membrane and nuclear material, with accompanying demands for energy production.

Many papers have been published on the biochemistry of malaria parasites (see reviews by Homewood, 1978; Sherman, 1979; Homewood and Neame, 1980). Almost all studies dealt exclusively with intra-erythrocytic stages, while the other stages were relatively neglected. The main reason was that the erythrocytic stages are relatively easy to get in large quantities. The isolation

FIG. 20. (A) EE stage of *P. knowlesi* in a rhesus monkey's liver, 5 days after infection. Many electron-dense rhoptries, lipid droplets and vesicles are visible. Bar = 10 μm. (B) Detail of (A) showing the host/parasite interface. The host hepatocyte appears degenerated with swollen mitochondria. The parasite plasmalemma has a wavy appearance enclosing electron-dense material in the parasitophorous vacuole. From the parasitophorous vacuole membrane long tubular clefts, filled with granular fluffy material (arrows), run into the parasite, dividing its cytoplasm. Along the clefts electron-dense rhoptries (R) are localized. M = parasite mitochondria. Bar = 1 μm. (C) EE stage of *P. cynomolgi* from the liver of a rhesus monkey 7 days after infection. As in the *P. knowlesi* schizont, many vacuoles, vesicles and lipid droplets are apparent. Bar = 10 μm. (D) Detail of (C) showing lipid droplets (L) and vesicles with granular fluffy material (V), sometimes condensed in the centre (thick arrows). Similar material is visible in the parasitophorous vacuole (arrows), as in the *P. knowlesi* EE stages (B). Membrane-bounded electron-dense rhoptries are distributed throughout the parasite. Note the difference in staining of the parasite lipid (L) and host lipid (asterisk). Bar = 1 μm. Courtesy of Dr R. E. Sinden, London.

of the EE stage from the liver is not yet possible, and therefore its biochemical characterization is impossible. Identification of enzymes specific for the parasite was recently started (Meis *et al.*, 1984b); further research may lead to a rational development of drugs against the liver stage.

Glycogen has never been found in malaria parasites by histochemical methods or electron microscopy. *Plasmodium* sporozoites appear to depend on exogenous glucose (Garnham, 1966; Mack and Vanderberg, 1978). The enzymes α-glucan phosphorylase and glucose-6-phosphatase are not detectable in the parasite (Meis *et al.*, 1984b).

One end-product of glucose catabolism, among others, is lactic acid. The enzyme lactate dehydrogenase is histochemically demonstrable in EE stages of *P. berghei*, although in smaller quantities than in the erythrocytic stages, which grow under relatively anaerobic conditions.

Furthermore, suggestive evidence was found that the EE stages have an active citric acid cycle. Three of its enzymes were found, among them succinate dehydrogenase (SDH). Both oocysts and sporozoites have an active citric acid cycle, whereas the blood forms lack this cycle. This can be correlated with the morphology of the mitochondria in the various parasitic stages. In oocysts and sporozoites the mitochondria are clearly cristate; EE stages have fewer cristae in their mitochondria, and blood stages have acristate mitochondria. The mitochondrial changes during the life-cycle of *P. berghei* have been studied, and a correlation was shown between SDH activity and cristate mitochondria (Howells, 1970a,b). In that study it was found that EE stages as well as erythrocytic stages appeared to lack SDH activity.

However, ultrastructural studies (Terzakis *et al.*, 1974; Meis *et al.*, 1981) on EE stages have shown the presence of some cristae in their mitochondria. The histochemical detection of SDH in EE stages (Meis *et al.*, 1984b) makes it plausible that these stages occupy an intermediate position between the other vegetative stages, the oocysts and erythrocytic schizonts.

The membrane-bound enzymes adenosine triphosphatase and 5′ nucleotidase were detected neither histochemically in cryostat sections (Meis *et al.*, 1984b) nor cytochemically by electron microscopy (Fig. 21 (A)).

Cytochemical studies of malaria parasites have mostly been done using the asexual stages (Aikawa and Thompson, 1971; Aikawa *et al.*, 1972). Cytochrome oxidase can be detected histochemically in the mitochondria of oocysts, sporozoites, EE stages and erythrocytic stages (Howells, 1970a,b; Howells *et al.*, 1969, 1972). In our hands, electron microscopic cytochemistry on *P. berghei* EE stages has not resulted in localization of cytochrome oxidase on parasite mitochondria, while host cell mitochondria clearly revealed the presence of the reaction product (Fig. 21 (B)). In the erythrocytic stages this enzyme might be involved in pyrimidine synthesis rather than energy generation (Sherman, 1983).

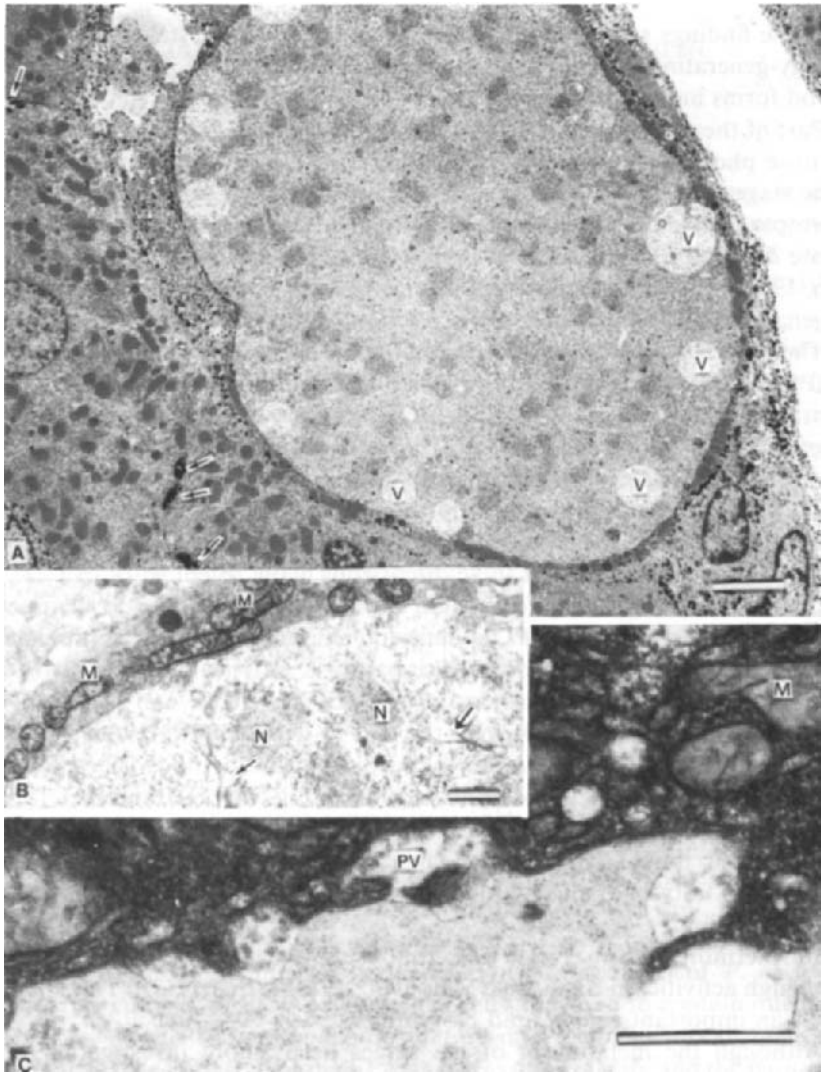



FIG. 21. *P. berghei.* (A) Cytochemical reaction to demonstrate 5′-nucleotidase activity. No activity is shown in the EE stage, while sinusoidal cells and hepatocytic surfaces, especially the bile canaliculi (arrows), react positively. Note the large peripheral vacuoles (V) in the EE stage. Bar = 5 μm. (B) Cytochemical reaction to demonstrate mitochondrial cytochrome oxidase. The host mitochondria (M) react positively, while no activity was detected in the parasite's mitochondria (arrows). N = parasite nuclei. Bar = 1 μm. (C) The host hepatocyte cytoplasm, harbouring mature EE stages, is readily permeable by ruthenium red. The stain does not permeate host mitochondria (M) or the parasitophorous vacuole (PV). Bar = 1 μm.

These findings suggest that, in the mosquito and liver stages, an aerobic energy-generating system is predominant, while in the micro-aerophilic blood forms an anaerobic glycolytic metabolism is present.

Part of the glucose taken up by the EE stage could be metabolized via the pentose phosphate pathway. The existence of this pathway in the erythrocytic stages has been disputed because there have been conflicting results of attempts to demonstrate the presence of the first enzyme, glucose-6-phosphate dehydrogenase (G6PDH) (Homewood, 1978; Hempelmann and Wilson, 1981). The second enzyme of this pathway, 6-phosphogluconate dehydrogenase (6PGDH), has been detected by most investigators.

The presence in erythrocytic stages of 6PGDH and the undetectability of G6PDH could be explained by the fact that the parasite obtains the substrate, 6-phosphogluconic acid, from the host erythrocyte, without the intervention of a parasite G6PDH. In favour of this explanation is the fact that G6PDH-deficient erythrocytes support parasite growth poorly. Another explanation is that the regeneration of NADPH, which is necessary for protection against, and repair of, oxidative damage, is impaired.

Of considerable interest in these discussions is the recent work of Usanga and Luzzatto (1985). They found that *P. falciparum* erythrocytic stages can produce G6PDH when G6PDH-deficient red cells are used as host cells. Adaptation by gene expression of a parasitic enzyme might explain the variability in results of studies on the erythrocytic stages.

Howells and Bafort (1970) were the first to detect G6PDH and 6PDGH inside the EE stages of *P. berghei* by histochemical methods. These results have been confirmed by us (Meis *et al.*, 1984b) (Fig. 7 (F,H)), and therefore it is likely that the EE stage has the ability to carry out the pentose phosphate shunt at all times. The reduced coenzymes are key substances for the synthesis of fatty acids and steroids, whereas the pentoses are used for nucleic acid formation.

Furthermore, the enzyme α-glycerophosphate dehydrogenase was found with high activities in EE stages (Meis *et al.*, 1984b) (Fig. 7 (G)). This enzyme plays an important role in lipid biosynthesis.

Although the metabolism of EE stages needs more investigation, one might conclude that the liver stages have metabolic pathways resembling glycolysis and the citric acid cycle, as is the case with oocysts and sporozoites in the mosquito. Furthermore, the enormous multiplication of *P. berghei* between 21 and 48 hours after sporozoite inoculation is reflected in high enzyme activities in the intermediary and pentose phosphate pathways.

## V. Culture of Exoerythrocytic Forms *in vitro*

### A. AVIAN MALARIA

Avian EE stages can be maintained easily in continuous cell cultures, mainly due to their less stringent host specificity and the ability of formed merozoites to reinvade other cells of the same tissue (or tissue culture). Infected tissue (Hawking, 1945; Davis *et al.*, 1966), merozoites (Beaudoin *et al.*, 1974) or sporozoites can be used to inoculate cell monolayers.

The first report of continuous cultivation was published over 30 years ago by Oliveira and Meyer (1955). Cultures of *P. gallinaceum* were maintained in the first instance for one year, and in a later report continuation of cultures for 4 years was described (Meyer and Musacchio, 1959). Despite a slight decline in virulence of the strain, infection of chicks remained possible.

Other species of avian malaria have been grown in primary cultures for varying lengths of time (Hawking, 1946; Tonkin and Hawking, 1947; Huff, 1964). By using embryonic chick cells, which were better hosts for the parasites, continuous cultivation of *P. fallax* in large amounts became possible (Davis *et al.*, 1966). This particular *in vitro* system for EE forms (Huff, 1964) made possible a thorough study of parasite biology and drug action in the late 1960s and early 1970s, which otherwise would have been very difficult or impossible.

### B. RODENT MALARIA

Attempts to initiate *in vitro* cultures of mammalian EE stages from sporozoites were, in contrast to the avian systems, long unsuccessful. Foley *et al.* (1978a,b) were able to prepare suspensions of parenchymal cells from livers of rats that had been infected previously with sporozoites of *P. berghei*. Monolayer cultures of these parenchymal cells appeared to contain infective EE stages, as was shown by subinoculation into recipient rats or mice. In these studies the initiation of the EE stages was done *in vivo*, and the parasites were then maintained *in vitro*.

Strome *et al.* (1979) reported that isolated *P. berghei* sporozoites added to cultures of rat embryonic liver and brain cells entered these cells and developed into multinucleated parasites. Embryonic cells were chosen because of previous success with avian parasites in these cells. The embryonic cells appeared at first not ideal for rodent parasites (Sinden and Smith, 1980). However, in 1981 the first successful *in vitro* culture of mammalian EE stages was made with primary human embryonic (WI-138) lung cells as hosts and *P.*

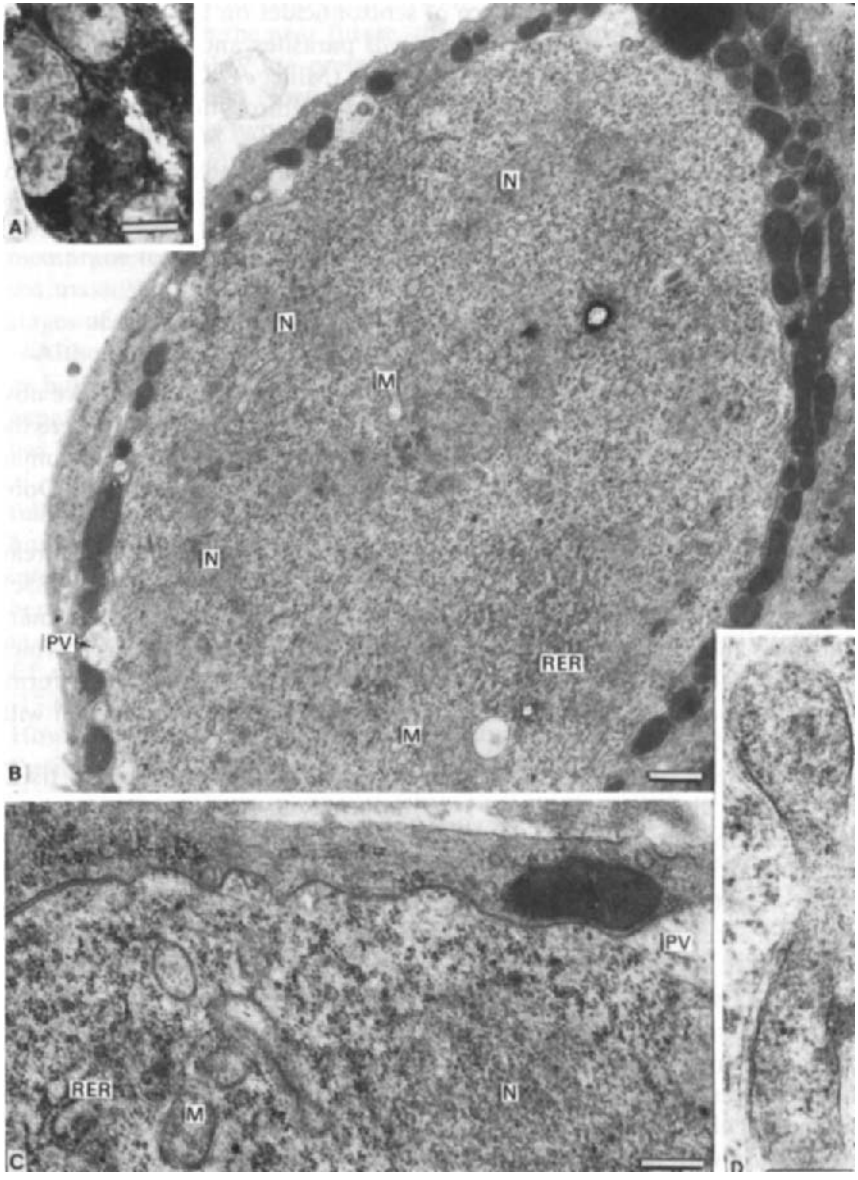
*berghei* parasites (Hollingdale *et al.*, 1981). Sporozoites were obtained from the salivary glands of *Anopheles stephensi* mosquitoes and seeded on to the cell cultures. The parasite development, although slower, was comparable with that occurring *in vivo* in the rat. While *in vivo* merozoites were formed after about 50 hours, in tissue cultures merozoites were seen 68 hours after inoculation of sporozoites. Merozoites collected from the cultures were infective for mice upon inoculation. Hollingdale *et al.* (1983d) found, additionally, that the cell type used was the most important factor in merozoite formation, not the culture medium.

The penetration and development of parasites in tissue culture was studied with IF, immunoperoxidase techniques (Hollingdale and Leland, 1982; Hollingdale *et al.*, 1983a), and immunoradiometric assays (Zavala *et al.*, 1985), which all clearly showed that young schizonts (up to 28 hours) reacted with anti-sporozoite antibodies, whereas mature schizonts reacted with antibodies against the erythrocytic parasite. A similar reaction pattern was demonstrated previously *in vivo* for *P. berghei* (Danforth *et al.*, 1978; Hollingdale and Leland, 1982) and the human parasite *P. vivax* (Cogswell *et al.*, 1983).

The initial successful infection of WI-138 cells with *P. berghei*, and complete development of the cycle *in vitro*, followed demonstrations that sporozoites could develop successfully in a human hepatoma cell line (HepG2 clone A16) (Hollingdale *et al.*, 1983c, 1985a) and in primary rat hepatocytes (Lambiotte *et al.*, 1981; Mazier *et al.*, 1982; Pirson, 1982; Meis *et al.*, 1984a).

Serological properties of the developing EE stages closely resemble those grown *in vivo* in rat liver (Meis *et al.*, 1981). The ultrastructure of parasites cultured *in vitro* is largely comparable to parasites grown *in vivo* (Aikawa *et al.*, 1984; Meis *et al.*, 1984a). Only the parasite's mitochondria appear to have a denser internal matrix (Fig. 22). Also, the host-cell hepatocyte mitochondria differ in size and electron-density from those of uninfected hepatocytes (Meis *et al.*, 1984a). *In vitro*, the parasite vesicles with flocculent material appear to bud from the RER cisternae (Meis *et al.*, 1984a). Although it is highly likely that this same mechanism occurs *in vivo*, it has never been observed. The entry of *P. berghei* sporozoites into cultured red cells and their

FIG. 22. (A) Semithin (1 μm) section of an EE stage of *P. berghei* at 48 hours, grown *in vitro* in a rat hepatocyte, stained with toluidine blue. Bar = 10 μm. (B) Electron micrograph of an EE stage *in vitro* at 48 hours. Rough endoplasmic reticulum (RER), nuclei (N), mitochondria (M) and, at some places close to the extracellular medium, a widened parasitophorous vacuole (PV), are visible. Bar = 1 μm. (C) Detail of the host/parasite interface. The fine structure is essentially similar in parasites grown *in vivo*. However, parasite mitochondria (M) appear to have a denser matrix than do those in parasites grown *in vivo*. Bar = 0.5 μm. (D) Detail of a mitochondrion showing the denser internal matrix. Bar = 250 nm.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transformation therein has been described using light microscopy by Hollingdale *et al.* (1983b) and, incompletely, using electron microscopy by Aikawa *et al.* (1984). The influence of schizonticides on EE development is now being studied *in vitro* using *P. yoelii* parasites and isolated primary hepatocytes of the laboratory rat and tree rat (Millet *et al.*, 1985). Inhibitors of ornithine decarboxylase appear to block exoerythrocytic schizogony of *P. berghei* in cultured HepG2 cells (Hollingdale *et al.*, 1985b). These approaches, using infected cell cultures, provide a standardized model for drug studies on EE development in preference to the *in vivo* system (Peters *et al.*, 1984).

### C. PRIMATE MALARIA

Applications of the techniques of cultivating rodent EE parasites have now enabled the growth of human parasites *in vitro*. Mazier *et al.* (1984) were the first to demonstrate the development of *P. vivax* sporozoites in human hepatocytes. Similar but less conclusive results have been reported by Doby and Barker (1976).

Very recently, the full cycle of EE development of *P. vivax* (North Korean strain) has been confirmed using HepG2-A16 cells (Hollingdale *et al.*, 1985c). Of particular interest in the latter study was the descriptions of both primary schizonts and small non-dividing parasites (5–6 μm in diameter), which persisted after the completion of primary schizogony. The latter forms resembled the hypnozoites detected in chimpanzee liver after infection with relapsing *P. cynomolgi* (Krotoski *et al.*, 1982a,b).

The *P. vivax* sporozoites transform within 24 hours into primary tissue trophozoites and grow slowly to 5–10 μm at day 3. By day 9 they have an average diameter of 37 μm; parasites as large as 80 μm are also present. With the use of an immunoperoxidase technique and monoclonal antibody against the *P. vivax* CS-protein, between days 5 and 9 similar numbers of large and small parasites have been observed. The serological reaction against the small forms on days 5 to 9 was similar to that shown by the 24 hour trophozoite stage. It may be noted that a similar number of large schizonts and small resting stages is formed *in vivo* when a *P. vivax* strain from a tropical country is used. If the strain originated from a temperate region, many more sporozoites change into hypnozoites and few develop directly into merozoites generating EE stages (Krotoski *et al.*, 1986). The latter observation with temperate *P. vivax* was confirmed using a similar strain in culture (Hollingdale *et al.*, 1986). This work could result in considerable advances in our understanding of hypnozoite activation into secondary schizonts, which is responsible for the relapse pattern.

Recently, an ultrastructural investigation of *P. vivax* in cultured HepG2 cells has demonstrated that the parasites are morphologically similar to forms grown *in vivo* (Uni *et al.*, 1985). This study has demonstrated the likelihood that, in the near future, the ultrastructure of hypnozoites will be described, since they are present in culture in the same numbers as large schizonts. Using the North Korean strain of *P. vivax* should increase the chance of finding hypnozoites and describing their ultrastructure (Hollingdale *et al.*, 1986). The development of techniques for *in vitro* culture of the asexual (Trager and Jensen, 1976) and sexual (Ifediba and Vanderberg, 1981) stages of *P. falciparum*, and for the subsequent infection of mosquitoes by membrane feeding techniques (Ponnudurai *et al.*, 1982), has made possible the massive supply of sporozoites for the initiation of cultures of the EE stages of this parasite.

Although *P. vivax* sporozoites invade and develop the complete EE cycle in both HepG2-A16 cells (Hollingdale *et al.*, 1985c) and primary human hepatocytes (Mazier *et al.*, 1984), to date EE development of *P. falciparum* has been achieved in human hepatocytes only (Smith *et al.*, 1984; Mazier *et al.*, 1985; Meis *et al.*, 1986a). *P. falciparum* EE parasites appear to develop fully in fresh and frozen–thawed human hepatocytes (Meis *et al.*, 1985c); however, definitive evidence of merozoite formation is still lacking. *In vitro* infectivity of such merozoites can be tested by adding human red cells to the hepatocyte cultures and then examining the erythrocytes for merozoite invasion and development. A report of *in vitro* invasion of erythrocytes by EE merozoites of *P. falciparum* released from cultured hepatocytes relied on IF antibody techniques without Giemsa-staining (Mazier *et al.*, 1985). However, the red blood cells were not cultured further, and thus proof of the feasibility of establishing the full vertebrate cycle *in vitro* is still lacking. Complete development *in vitro* of the vertebrate cycle, of *P. berghei* has been achieved recently (Suhrbier *et al.*, 1987). Development of a technique permitting the complete cycle of development of a human malaria parasite, without the need of its vertebrate host (cells), would be extremely useful, particularly for molecular biological studies on genetic variability (Ploeg *et al.*, 1985) and the influence of mosquito passages on the production of sexual forms.

## VI. Final Remarks

The exoerythrocytic development of malaria parasites has now been well documented for avian and rodent species. In contrast to these, the species infecting primates have been little studied either in primate models or, recently, in culture systems. The *in vitro* culture of EE stages (Hollingdale

and Leland, 1986; Mazier, 1986) may offer an alternative approach to the study of the numerous biological questions associated with EE development, particularly the hypnozoite mystery and the action of new prophylactic drugs. Culture systems can be used to investigate the role of gamma-IFN and other lymphokines in host defence against the malarial liver stages (Ferreira *et al.*, 1986b).

Tissue forms of the malaria parasite are clearly a means of establishing high infection rates in the blood of vertebrate hosts and, for survival's sake, some species like *P. yoelii* in *Thamnomys* rats have additional liver forms with retarded development (Landau *et al.*, 1975). It was thought for a long time that EE merozoites of primate malaria parasites producing relapses were able to re-enter other hepatocytes and give rise to secondary EE development. Avian *Plasmodium* species can initiate secondary and subsequent EE development. The existence of secondary liver forms in mammalian species was later disproved in favour of small resting forms in the liver, the hypnozoites. First for *P. cynomolgi* and later for *P. vivax*, it was demonstrated that sporozoites give rise to both primary EE stages and hypnozoites, measuring 4–5 μm.

Although it is well established that hypnozoites (or hypnozoite-like forms) are not present in rodent malaria parasites, we note that the hypnozoites of *P. cynomolgi* (Krotoski *et al.*, 1982a,b) and *P. vivax* (Krotoski *et al.*, 1982c, 1986) are comparable in size with a *P. berghei* EE form at about 15–20 hours. The latter parasite is uninuclear and still has many sporozoite-like characteristics. It is reasonable to assume that the hypnozoite has also retained some sporozoite characteristics. The *P. berghei* EE form uses the first 24 hours for dedifferentiation only with no nuclear division occurring in this period (Meis *et al.*, 1985a). This is consistent with IF observations on irradiated sporozoites, which developed normally during the first 24 hours. Thereafter, growth appeared to cease following alteration of the nuclear division process (Ramsey *et al.*, 1982; Sigler *et al.*, 1984). Ferreira *et al.* (1986a), on the contrary, claimed that, at 20 hours, *P. berghei* had already undergone several nuclear divisions.

Recent breakthroughs in infecting tissue culture cells with malaria sporozoites will be of the utmost importance in the study of EE stages, and the mechanism of attachment and entry into the cells. Inhibition of sporozoite invasion (ISI test) of cultured cells represents the only *in vitro* assay of functional antibodies. Whether the antibodies only block attachment and penetration (Hollingdale *et al.*, 1982), or additionally affect intracellular development (Mazier *et al.*, 1986), remains to be elucidated. Humoral protection against EE stages and sporozoites alone seems not enough to block a malaria infection totally. Moreover, we have as yet unpublished evidence that non-specific components in sera from healthy blood donors, and other factors such as gamma-IFN and prophylactic chloroquine, may

interfere to some extent with penetration of *P. falciparum* sporozoites. Thus, in evaluating "protective" anti-sporozoite antibodies, results of the ISI test have to be judged with care.

Immunity to sporozoites is correlated with production of antibody to an immunodominant protein on the surface of the sporozoite, the circumsporozoite (CS) protein. Monoclonal antibodies to the CS protein block infection with sporozoites *in vitro* and protect animals *in vivo* (Hollingdale, 1985). Therefore, suggestions were made that the CS protein was used as a ligand for a specific receptor on the hepatocyte membrane. Very recently, evidence was presented (Aley *et al.*, 1986) that a highly conserved region of the CS protein of *P. falciparum* and *P. knowlesi* recognizes receptors on a human cell line (Hep-G2-A16) under conditions where invasion by sporozoites can occur. These results remain to be confirmed with primary human hepatocytes. Anti-CS monoclonal antibodies can react with young EE stages, indicating that this protein is still distributed on the liver stages. Cryo-ultramicrotomy, combined with immunogold techniques, could be used for the antigenic localization of the CS protein after penetration and during development (Fine *et al.*, 1984; Posthuma *et al.*, 1987).

Development of vaccines based on malaria antigens has so far been concerned only with the sporozoite, red blood cell and sexual stages of the parasite. Largely because of difficulties in obtaining sufficient material, research on the vaccine potential of the EE stages of malaria has been hampered. The hepatic stages offer a number of potential advantages for vaccine research, both in concert with blood stages and as an independent target. The antigenic homology of erythrocytic stages and mature EE stages of rodent and human malarial parasites, as shown by IF or immunoperoxidase antibody techniques, is striking (Danforth *et al.*, 1978; Hollingdale *et al.*, 1983a; Druilhe *et al.*, 1984; Aley *et al.*, 1988).

The EE merozoite invades the same target cell (the erythrocyte) as the blood stage merozoite and probably uses a similar mechanism. Analysis of common surface proteins necessary for erythrocyte invasion would be helpful in vaccine studies.

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# Cryptosporidiosis in Perspective

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## I. Introduction

Acute diarrhoea remains one of the most important health problems; it is a major contributor to illness and death in children of the developing world. A review by the World Health Organization of 24 community-based surveillance studies in 18 developing countries illustrated the high mortality rates caused by diarrhoea, with a rate of 20 deaths per 1000 children per year in the

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under one year age groups (Black, 1985). Cryptosporidiosis, which is emerging as a human disease, is undoubtedly responsible for some of these mortalities.

In the concluding remarks made in an earlier review (Tzipori, 1983), I wrote "Humans are probably susceptible to disease as some evidence exists to suggest that it [cryptosporidiosis] may be a cause of transient, mild, or acute diarrhoeal illness in immunologically normal individuals. The significance of cryptosporidiosis in humans, however, depends on the severity of the disease it produces and the incidence in the population, both of which are unknown". Within a relatively short time, over 80 communications including letters, commentaries, editorials, case-reports, epidemiological surveys, which included clinical observations, age distribution, seasonality, and many other aspects of the disease in the general population, have since been published in the scientific literature. Consequently, a major portion of this review is devoted to the analysis of these epidemiological observations which, I hope, should provide firm answers to earlier questions about severity and incidence in the human population. More accurate information regarding the morphology and the life-cycle is now available and a detailed outline is included. However, the information available is not yet sufficient to permit full understanding of the nature and the unique and variable behaviour of *Cryptosporidium* in different classes of vertebrates, different hosts, and their various body systems and organs. Although undoubtedly a coccidian, *Cryptosporidium* has numerous characteristics which set it apart from the rest of that subclass. The parasite's ability to maintain persistent infection in certain individuals and its astonishingly stubborn resistance to chemotherapy are serious medical problems that need to be addressed in the future.

## II. Classification and Natural History

*Cryptosporidium* is a genus in the family Cryptosporidiidae, suborder Eimeriina, order Eucoccidiida, subclass Coccidia, class Sporozoa, phylum Apicomplexa (Levine, 1980). At present, the suborder Eimeriina contains 13 families with over 1500 named species. Most of these species, however, belong to the genera *Eimeria* and, less commonly, *Isospora*. Both are intracellular parasites which primarily infect the intestinal tract of vertebrates. *Toxoplasma* and *Sarcocystis*, tissue cyst-forming coccidia, are two other important members of the suborder Eimeriina.

*Cryptosporidium* was so named by Tyzzer in 1907 to signify that it is a sporozoon (belonging to the class Sporozoa) in which spores are indistinguishable, absent or concealed (*crypto* in Latin) in the oocyst. The genus was subsequently established in a family of its own by Léger in 1911. The

occurence of more than one species within the genus was proposed in 1912 by Tyzzer (1912) on the basis of transmission experiments which he conducted in mice, and the assignment of a new specific name to each additional new animal isolate continued up to 1980. By 1985 *Cryptosporidium* had been reported in more than 20 species of animals. Cross-transmission experiments, conducted earlier using organisms obtained from guinea-pigs (Vetterling *et al.*, 1971a) and cats (Iseki, 1979), supported the notion of speciation. But since 1980 evidence has been accumulating which suggests that host species specificity is not a characteristic shared by all, or even most, isolates of *Cryptosporidium* (Tzipori *et al.*, 1980a; Reese *et al.*, 1982). Consequently Levine (1984), in a review of the taxonomy of the genus *Cryptosporidium*, tentatively nominated four species representing isolates from mammals (*C. muris*), birds (*C. meleagridis*), reptiles (*C. croteli*) and fish (*C. nasorum*), to which he assigned all other known isolates. The nomination of these species is largely based on insufficient information rather than on experimental and extensive epidemiological evidence. Therefore, further studies are required before *Cryptosporidium* isolated from various sources can be firmly allocated to different species. Because they appear to be morphologically and, from limited serological studies, antigenically (Tzipori and Campbell, 1981) indistinguishable, assignment to species is probably premature. In other species of coccidia, species within the same genus often show some morphological and biological variation from each other, which has presumably evolved through adaptation to a particular group of hosts. However, *Cryptosporidium*, presumably because of its ability to exist in different species, has acquired no peculiar features unique to a particular host.

Lack of host specificity, at least among domestic, or even all, mammalian species, is one of the major characteristics that sets *Cryptosporidium* apart from the rest of the coccidia. Unlike *Toxoplasma*, the only other well known member of Eimeriina that infects a wide range of mammals, which requires two hosts to complete its life-cycle, *Cryptosporidium* can completely do so in one host. *Sarcocystis*, on the other hand, consists of numerous species—often three or four within a single host. The lack of specialization—adaptation to a single cell type, location or host—in the evolutionary sense, is usually interpreted as a lack of sophistication. However, adaptation to a broad host range may represent an advanced stage of evolution.

Although the case for a monospecific genus (Tzipori *et al.*, 1980a) remains valid for the time being, there is sufficient evidence to indicate that some subtle biological differences exist in terms of preference for a particular host or particular location within the host. However, this may reflect "strain" differences. There is little doubt that the organism which infects the intestine can also infect the trachea, as demonstrated in both immunocompromised and immunocompetent patients with concurrent infections (Forgacs *et al.*,

1983; Kocoshis *et al.*, 1984; Harari *et al.*, 1986), and by experimental inoculations of animals (Tzipori, 1983; Heine *et al.*, 1984b; Lindsay *et al.*, 1986). Yet, under natural conditions, some birds suffer mostly from respiratory infections (Hoerr *et al.*, 1978), while others have infections of the gut (Tyzzer, 1929). Some strains of *Cryptosporidium* appear to infect one site consistently, often in the same host, while others prefer a different site. Tyzzer (1910) distinguished *C. muris* when he observed the parasite in the gastric mucosa and was able to demonstrate its predilection for this site by transmission experiments. Two years later (Tyzzer, 1912), he did a similar experiment with an organism which had a preference for the ileal mucosa of the mouse, instead of the stomach. He subsequently called the ileal species, which also had slightly smaller oocysts, *C. parvum*. Upton and Current (1985), who also identified two morphologically similar oocysts of different size in cattle, recommended that two distinct species, as described by Tyzzer (1910, 1912), be recognized. However, they provided no further distinguishing biological features, e.g. site predilection, variation in pathogenicity or antigenic variation. One suspects that Tyzzer (1912), in making the distinction, may have been impressed by site predilection as much, if not more, than by variation in the size of oocysts. Upton and Current (1987) warned, appropriately, against naming new species based on host specificity in the absence of adequate cross-transmission data, or careful examination of endogenous development, particularly when oocysts are structurally indistinguishable.

There is little doubt that some isolates of *Cryptosporidium* infect some species of animals more readily than others, suggesting again a degree of adaptability. *Cryptosporidium* isolated from adult mice readily infected other adult mice (Tyzzer, 1910); similarly, isolates from cats (Iseki, 1979) and guinea-pigs (Vetterling *et al.*, 1971a) infected young adults of the same species. In my experience, and that of others, *Cryptosporidium* isolated from humans, calves, deer, lambs and goat kids could infect infant mice but not adults (Sherwood *et al.*, 1982; Tzipori, 1983).

The nature of the differences between *Cryptosporidium* isolates requires further investigation. *Cryptosporidium* from sources other than domestic animals or humans should be examined in newborn laboratory animals, free of specific antibody, and in cell culture for evidence of biological and morphological differences. Experimental studies on organisms found in unusual hosts such as fish or snakes, or from peculiar sites such as the conjunctival sac, trachea, or kidney of infected birds, will no doubt help to identify the range of infectivity of *Cryptosporidium*. Sophisticated studies with monoclonal antibodies, possibly one-dimensional fingerprint analysis, and iso-enzyme studies, will help to define differences between isolates.

*Cryptosporidium* isolates endemic among humans and domestic animals may prove to be more closely related and more interchangeable than those found in wild animals which exist in greater isolation.

## III. Life-cycle and Ultrastructure

The life-cycle of *Cryptosporidium* has been outlined by several investigators. Broadly speaking, it follows closely the pattern characteristic of other coccidia; asexual followed by sexual endogenous stages resulting in production and discharge of oocysts in the faeces (Fig. 1).

The most detailed study was the very first, by Tyzzer in 1910 and 1912. With minor exceptions he established the outline accepted today without the aid of sophisticated equipment now available. He described the existence of a minute oocyst with four naked sporozoites (without sporocysts) and identified the parasite's unique potential for autoinfection, which plays a crucial role in the pathogenesis of the infection in immunologically compromised hosts. For the next 75 years, various investigators have re-examined the life-cycle, latterly with the aid of electron microscopy. Vetterling *et al.* (1971b)

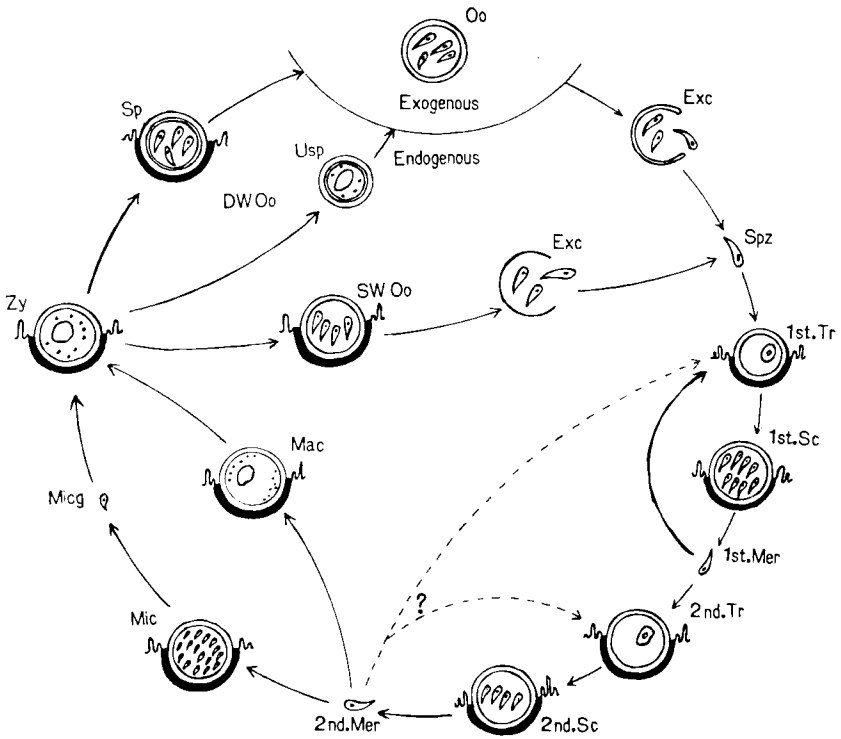


FIG. 1. Schematic representation of the life-cycle of *Cryptosporidium*. 1st. = first generation, 2nd. = second generation, DW = double-walled, Exc = excystation, Mac = macrogamont, Mer = merozoite, Mic = microgamont, Micg = microgamete, Oo = oocyst, Sc = schizont, Sp = sporulated, Spz = sporozoite, SW = single-walled, Tr = trophozoite, Usp = unsporulated, Zy = zygote.

described second-generation schizogony in the guinea-pig and suggested that these schizonts were structures previously described by Tyzzer (1910) as sporulated oocysts. Pohlenz *et al.* (1978a) confirmed their existence, showing that second-generation schizonts produced four merozoites. They also identified, as oocysts, structures similar to those described by Tyzzer (1910). Pohlenz *et al.* (1978a) and Iseki (1979) demonstrated that oocysts in the faeces can be used for diagnosis of cryptosporidiosis in calves and cats. In studies in mice, chicken embryos and cell culture, Current and co-workers (reviewed by Current, 1985) observed two kinds of oocysts which differed in the consistency of the wall; thin-walled oocysts were identified which may hold the key to autoinfection.

The issue of whether *Cryptosporidium* is intracellular or extracellular appears to have been resolved, and the term "intracellular-extracytoplasmic" was coined by Goebel and Braendler (1982), who, in addition, provided useful details on the gametogony of the parasite.

The life-cycle of the parasite begins with the intake of oocysts orally, and possibly by inhalation. Oocysts undergo excystation which, *in vitro* at least, requires the combined action of trypsin and bile salts (Current and Haynes, 1984). Excystation releases four naked, non-flagellated sporozoites which are morphologically indistinguishable, as yet, from two types of merozoites. They approach and enter the microvillous epithelial border in the small intestine by flexing and twisting movements to initiate infection (Fig. 2). The sporozoite (and later merozoites) indents the microvillous membrane, invaginating it in a glove-like fashion (Fig. 3). The double unit membranes of the host cell extend along the surface of the parasite, finally covering it entirely and forming a parasitophorous envelope which encapsulates the parasite within a parasitophorous vacuole. Electron-dense bands are formed in the host cell cytoplasm opposing the parasite (Fig. 3(B)).

(*a*) *Host cell invasion and the host parasite interface.* This process was studied by Marcial and Madara (1986), employing high resolution electron microscopy and freeze-fracture techniques on ileum from infected guinea pigs. Their study was confined to early and mature trophozoites but presumably similar processes apply to other intracellular forms. The redundant folds of host cell membrane envelop the organism, resulting in the intracellular localization of the parasite within a sac of internalized microvillous membrane. The plasma membrane of the parasite subsequently fuses towards its base with the invaginated host membrane. The two membrane domains isolated by this process subsequently undergo drastic alteration. The host membrane dissolves, and the isolated parasite plasma membrane, which is now in direct contact with the host cell cytoplasm, becomes amplified. During this process, the inner unit membrane of the parasite

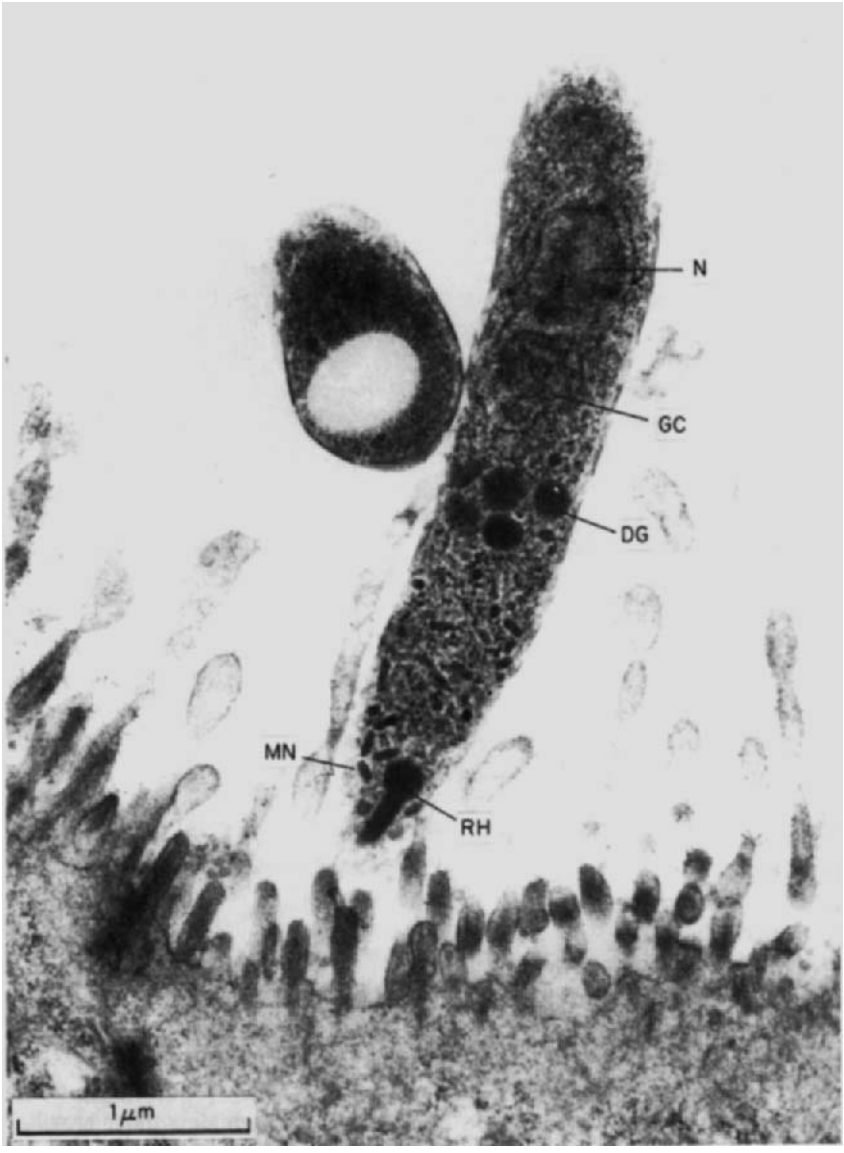


FIG. 2. Two sporozoites, one in longitudinal section, approaching the microvillous border of an enterocyte. Note micronemes (MN), Golgi complex (GC), dense granules (DG), nucleus (N), and rhoptries (RH). *Note.* This and all following transmission electron micrographs (Figs 2–10, 14, 15) illustrate a bovine isolate of *Cryptosporidium*, studied in the ileum of specific-pathogen-free lambs.

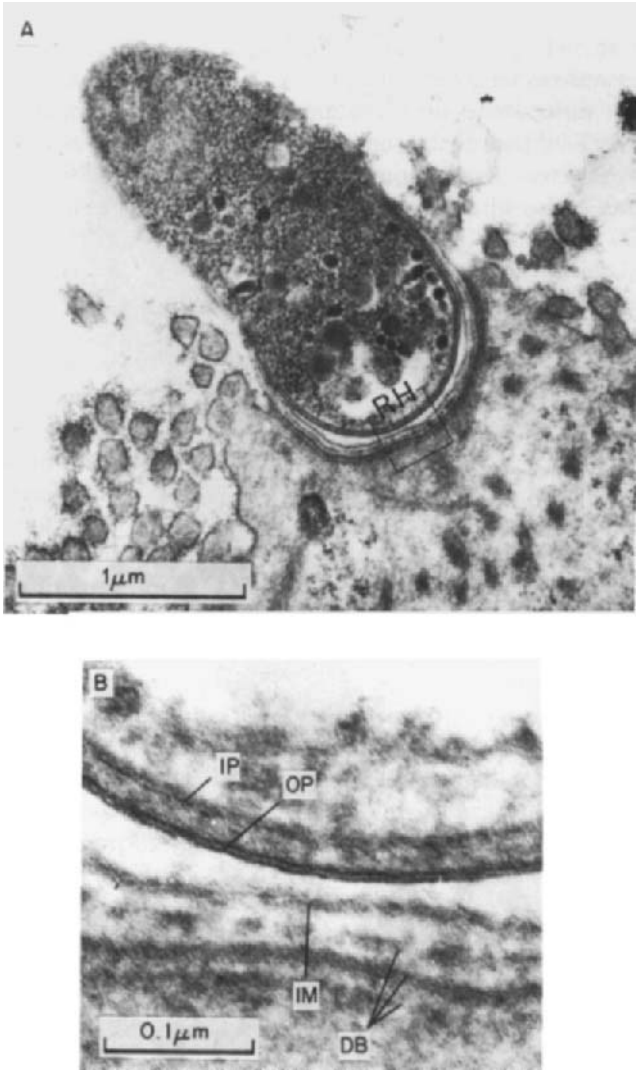


FIG. 3. (A) Merozoite penetrating mucosal surface by invaginating the microvillous membrane. A constriction by the infolded microvillous membrane, which has not yet been lysed, is evident around the trophozoite. The nucleus is poorly defined but the electron-lucent appearance of the rhoptries (RH) suggests that they have released their contents. (B) Enlargement of marked area, showing other membranes that can be distinguished at this early stage, including the inner microvillous (IM) membrane, and inner (IP) and outer (OP) parasite or pellicle membranes. Three dense bands (DB) in the cell cytoplasm are distinguished which in later stages appear as one band.

pellicle disappears, particularly toward the base of the parasite. Membrane invagination is the method of host cell entry for other coccidia (Long and Speer, 1977). The outer membrane of the pellicle at the attachment zone is thrown up into numerous folds at the base of the parasite to form the "feeder organelle" from which the parasite derives its nutrients directly from the host cytoplasm (Goebel and Braendler, 1982). The dense bands underlying the parasite attachment site are areas of modified host cell cytoskeleton, the function of which, it is thought, is to help anchor the parasite to the host cell, or resist further invasion into the absorptive cell cytoplasm, or both (Marcial and Madara, 1986). The vesicles formed next to the feeder organelle have the function of pinocytosis (Goebel and Braendler, 1982).

(*b*) *Trophozoites.* After penetration, the merozoite rounds into a spherical body and undergoes differentiation until all organelles are resorbed except the nucleus, Golgi anlagen, pellicle and cytoplasmic ribosomes. Concurrently, the nucleus and nucleolus enlarge. Redifferentiation includes development of the endoplasmic reticulum adjacent to the nucleus, and derivation of ribosomes from the nucleolus. The vacuolated zone with interdigitated membranous folds develops adjacent to the cell cytoplasm (Fig. 4). Trophozoites are surrounded by five unit membranes, of which the outer two are of host origin—being the inner and outer membranes of the envelope—and the other three correspond to the pellicle; one is the parasite plasma membrane and the remaining two are the inner, double, unit membrane, intrinsic to the parasite, which is not always distinguishable (Fig. 5).

(*c*) *Schizogony.* The trophozoite nucleus divides either three times, to form eight merozoites characteristic of first-generation schizogony or twice, to give rise to four merozoites which is typical of second-generation schizonts. During these divisions the size of the nuclei decreases. As schizogony progresses, the pellicle of the schizont invaginates deeply and covers each of the divided nuclei and the cytoplasmic mass containing the rough endoplasmic reticula. Each merozoite is surrounded by a pellicle composed of three membranes—an outer and two inner (Iseki, 1979; Marcial and Madara, 1986). Merozoites have an internal structure similar to that described for other coccidia, which includes dense granules, micronemes, nucleus, Golgi complex, conoidal complex, rhoptries, endoplasmic reticulum and polar ring (Vetterling *et al.*, 1971b). At the end of the process of schizogony, the parasitophorous vacuole contains eight or four free merozoites, depending on the generation of schizont, a small mass of residual cytoplasm of the schizont, a round body or a vacuole, and the attachment zone at the base (Fig. 6).

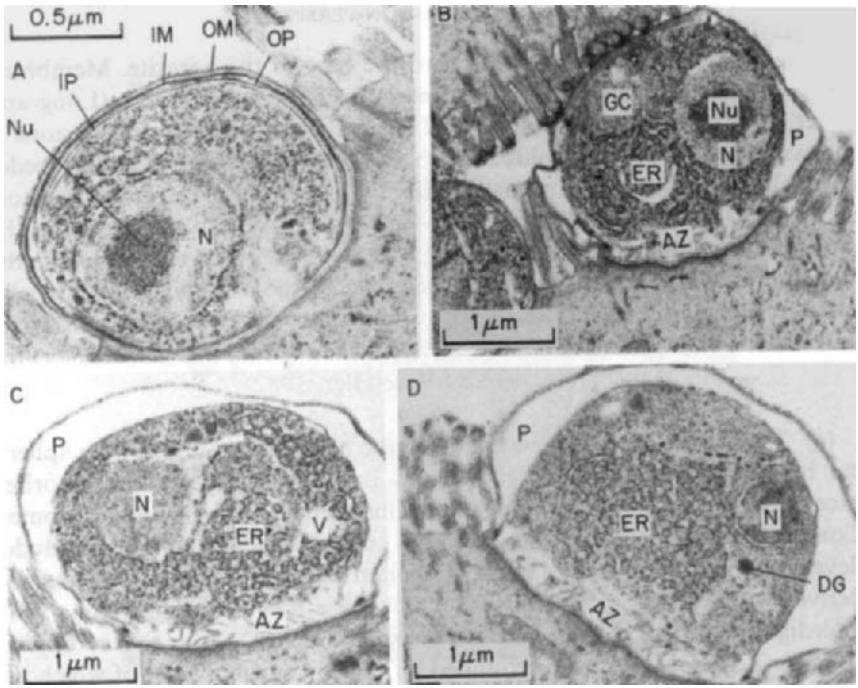


FIG. 4. Trophozoites. Four different stages of maturation can be seen, from soon after attachment and rounding up (A), to first nuclear division (D). There is no telling whether they are destined to be first- or second-generation schizonts. (A) Note characteristic nucleus (N) and large nucleolus (NU) within, a double membrane pellicle and not yet fully formed attachment zone. There is no endoplasmic reticulum at this early stage. There is a clear separation zone between the host cell and parasite pellicle, and the parasite membrane has not yet begun to resolve. The inner (IM) and outer (OM) microvillous membranes and inner (IP) and outer (OP) pellicle membranes are apparent; note that IP here appears as a double unit membrane. (B) The rough endoplasmic reticulum (ER) has begun to fill the cytoplasm, the Golgi complex (GC) is visible, and the inner membrane of the pellicle has been absorbed along the area of attachment. The parasitophorous vacuole (P) is enlarged. (C) Fully developed trophozoite before schizogony. The nucleolus has disappeared, a vacuole (V) has appeared, and dense granules, which are later seen in the merozoites, are also present. The attachment zone (AZ) has become vacuolated with interdigitated membranous folds forming the feeder organelle. (D) One nucleus is visible after the first nuclear division, and the ER fills the cytoplasm.

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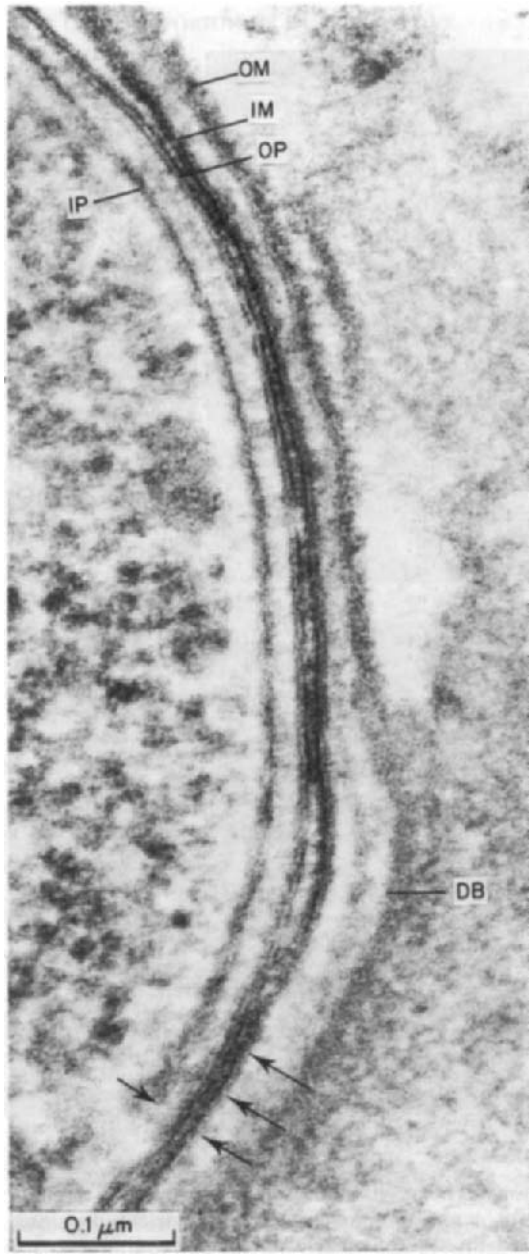
FIG. 5. An enlargement of part of Fig. 4(A), showing an area at the interface below and above the terminal web. Note that the outer microvillous membrane (OM) is covered by glycocalyx of the same consistency as, and continuous with that of, the cell microvilli. The inner microvillous membrane (IM) is internalized (by invagination) and is also covered by glycocalyx; there is a thin layer of cell cytoplasm between OM and IM. At the base, the two units of the inner pellicle (IP) have begun to regress (single arrow), and the IM and outer pellicle (OP) are becoming fused (three arrows).




Later, the IM dissolves leaving the OP which forms infoldings and is the only structure separating the cell cytoplasm from the parasite. The "dense band" (DB) is of host origin with no unit membrane structure and therefore is not a true interface between parasite and host cell.

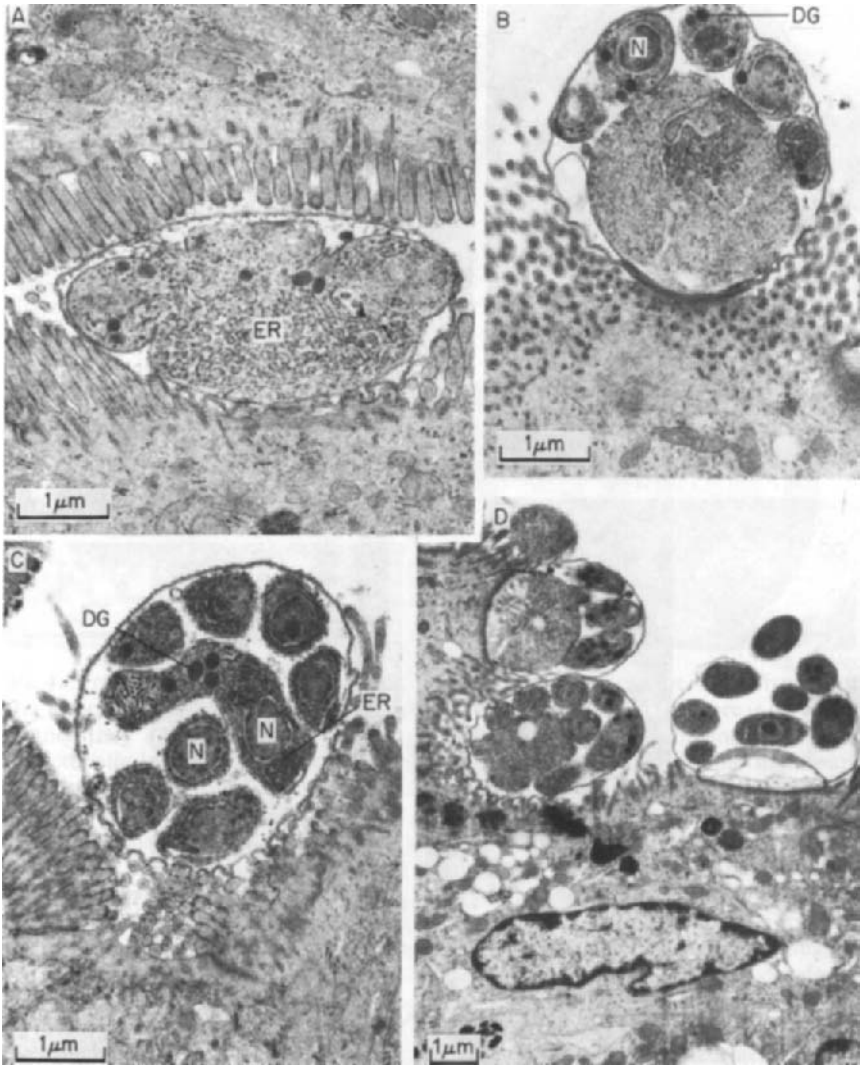



FIG. 6. Schizogony. The asexual multiplication phase of the life-cycle includes three (first-generation), or two (second-generation), nuclear divisions and the release of eight or four merozoites, respectively. (A) Three budding merozoites which retain the double membrane pellicle. Dense granules (DG) within the newly formed merozoites and a large residuum filled with ER can be seen. (B), 5, and (C) 8 merozoites are visible, each with a nucleus (N), double pellicle, ER and DG. (D), 3 schizonts, 2 first-generation and one second-generation (top left). Apart from the number of merozoites, there is little difference in size and shape between schizonts and merozoites from the 2 generations.

(*d*) *Gametogony.* Macrogamonts, the female forms of which measure 4–5μm in diameter, are found in abundance in the brush border, second in number only to trophozoites. The macrogamete is found within a parasitophorous vacuole. It has a large nucleus, situated eccentrically, with a distinct nucleolus; a round membrane-layered lipid vacuole is normally found next to the nucleus. In addition, the macrogamete typically contains in the cytoplasm a large number of polysaccharide granules, electron-dense bodies which are thought to be products of the reduction division (Tyzzer, 1907) or maturation bodies, wall-forming bodies, and rough endoplasmic reticulum. As with all other forms, a vacuolated membranous attachment zone is found at the base (Fig. 7).

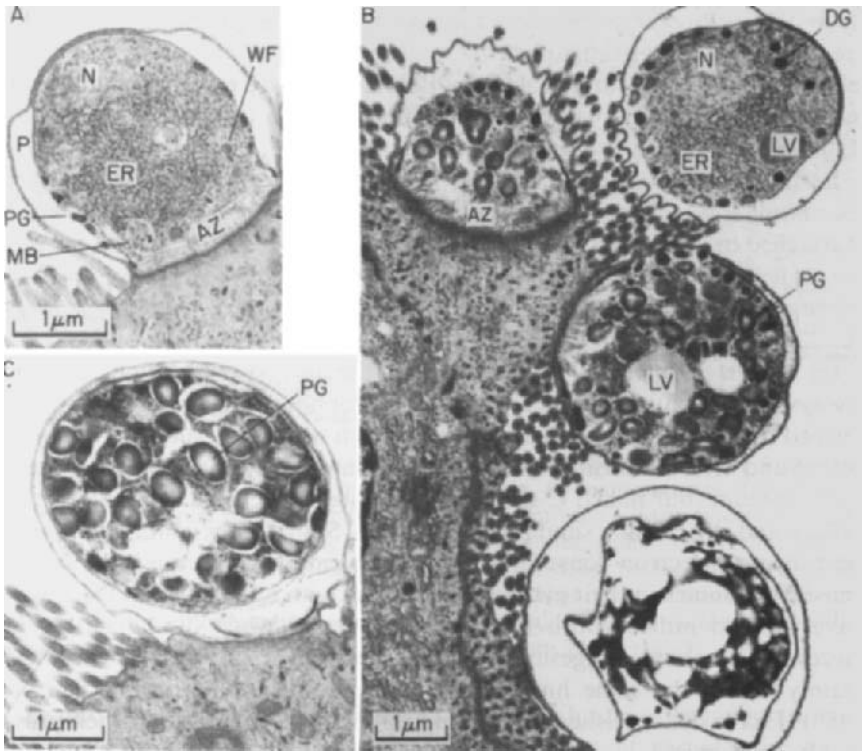



FIG. 7. Macrogametogony, the formation and maturation of the female form. (A), a maturing macrogamete within the parasitophorous vacuole, distinguished from a trophozoite by the presence of polysaccharide granules (PG), wall forming bodies (WF), maturation bodies (MB), and double membrane pellicle. The nuclear material is not distinct in this micrograph. (B), 3 macrogametes at different stages of development, top right being the most immature before fertilization. Note lipid vacuole (LV), nucleus (N) and ER. Two forms have visible, functional feeder organelles at the attachment zone (AZ). (C), fertilized macrogamete 'walled off' and free within the parasitophorous vacuole, shortly before differentiation.

Microgamonts are found less frequently than other forms, with the possible exception of sporulated oocysts. They measure 4–5 μm, are similar to schizonts in shape and may contain 14–16 microgametes and a residual body. The microgametes are rod-shaped and the nucleus dominates the small amount of the cytoplasm. During microgametogenesis the nuclei of the microgametes escape into the parasitophorous vacuole by budding. They are thereby covered by the pellicle of the microgamont, which, after the fissions of the nuclei, evaginates into the parasitophorous vacuole. The freed microgamete is wedge-shaped, measuring just under 1 μm in length with a thickened apical pole. The microgametes of *Cryptosporidium* most closely resemble those of other Eimeriina, except that they lack flagella. This is interpreted by some (Goebel and Braendler, 1982) to indicate a primitive stage of development. Therefore the microgametes are either immobile and are passed to the macrogamete by intestinal flow, or the intracytoplasmic microtubules (Goebel and Braendler, 1982) may have a role in locomotion by inducing flexing and twisting in a manner similar to that of sporozoites (Fig. 8).

Fertilization is achieved by protrusion of parts of the macrogamete membrane towards the microgamete. The adhesion zone of the microgamete is attached to the parasitophorous vacuole of the macrogamont, indenting all the wall membranes. Fertilization is most probably by penetration of the microgamete into the macrogamete (Fig. 9).

(*e*) *Oocyst formation.* Formation of the oocyst and often, but not always, sporogony take place in the parasitophorous vacuole. Oocysts are formed from the fertilized macrogamete which undergoes successive changes before and after fertilization (Fig. 7). Sporulated oocysts, which measure 4–6 μm, contain four naked C-shaped sporozoites, which are surrounded by a pellicle and structurally similar to merozoites. Sporozoites contain numerous micronemes, electron-dense bodies, electron-pale vacuoles and highly condensed ribosomes in their cytoplasm. Oocysts with both thin and thick walls have been identified. Both types are sporulated when discharged in the faeces. It has been suggested that the thin-walled oocysts, which excyst mainly within the same host, are responsible for autoinfection (Current, 1985). I have distinguished single- and double-walled oocysts, which presumably correspond to the thick- and thin-walled forms (Fig. 10). More recently, larger oocysts similar in size to those of *C. muris* (7.4–5.6 μm) described by Tyzzer (1907), have been identified in a small proportion of bovine faeces. Upton and Current (1985) considered the difference in size of these oocysts sufficient to regard them as belonging to a separate species.

The duration of the life-cycle seems to vary from a minimum of 48 hours to as long as 10–14 days before the first appearance of oocysts in the faeces of

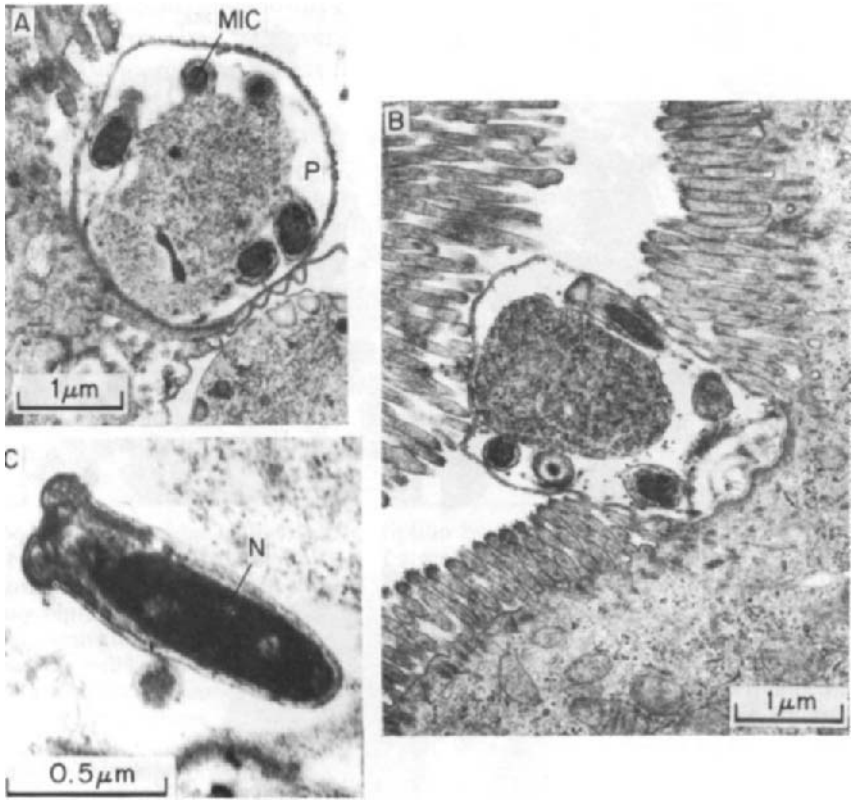
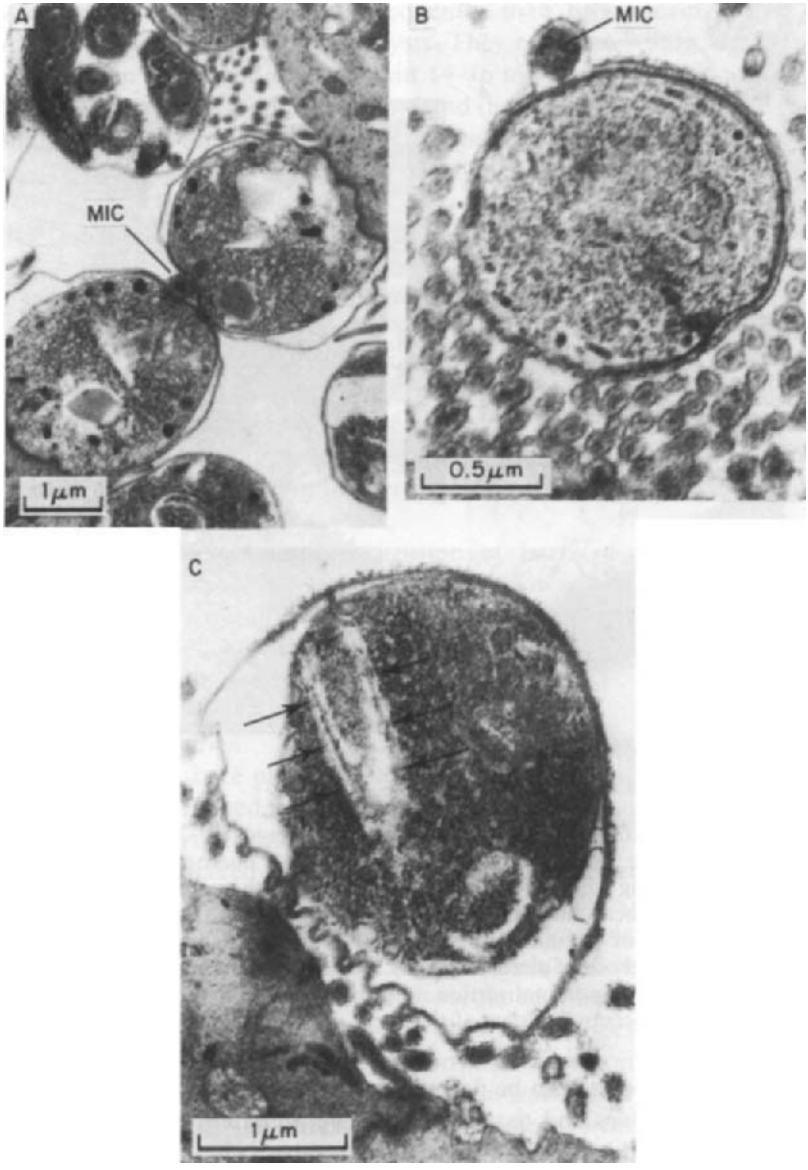



FIG. 8. Microgametogony. Microgametogenesis proceeds in 2 phases; a growing phase with multiple fissions of nuclei resulting in 16 microgametes, followed by differentiation during which the gametes evaginate into the parasitophorous vacuole (P). (A), 5 microgametes budding from the microgamont, pellicle of which forms the basis of the pellicle of the microgamete (MIC). Note dense nuclei which are readily distinguished from those of merozoites. (B), free microgametes sectioned in different planes with one in longitudinal section. (C), free wedge-shaped microgamete with a dense nucleus (N) occupying most of the space within 2-unit pellicle.

some animals. Oocysts can be detected within 48 hours after inoculation of piglets, lambs, calves and tissue culture. The prepatent period is longer in rodents and in chicken embryos, the shortest being normally 4 days. Duration of the cycle seems therefore to depend on the host, but it is not clear whether some or all steps of the life-cycle are more rapid in a susceptible host, or whether there is a fundamental difference in the sequence. It is also possible that the earlier appearance of oocysts in faeces of clinically affected animals is due to the diarrhoea. It has been established through sequential


A
MIC
1μm
B
MIC
0.5μm
C
1μm

studies that sporozoites always give rise to first-generation schizonts containing eight merozoites. However, it is not clear what proportion, if any, of first-generation merozoites repeat the first-generation schizogony—thus producing more of the same type—and for how long, and how many go on to produce second-generation schizonts containing four merozoites. Nor is it known whether all second-generation merozoites directly form micro- and macrogamonts, or recycle, as do the first generation. Trophozoites of unknown outcome are the predominant forms seen under the microscope in a given infected intestinal section, followed by macrogamonts, and then schizonts containing eight merozoites. This may merely indicate a more prolonged association of these forms with the host rather than their relative prevalence in the gut. However, if the duration of both schizogony generations is similar, it is suggested that first-generation merozoites produce both first- and second-generation schizonts simultaneously, while second-generation merozoites form only micro- and macrogamonts.

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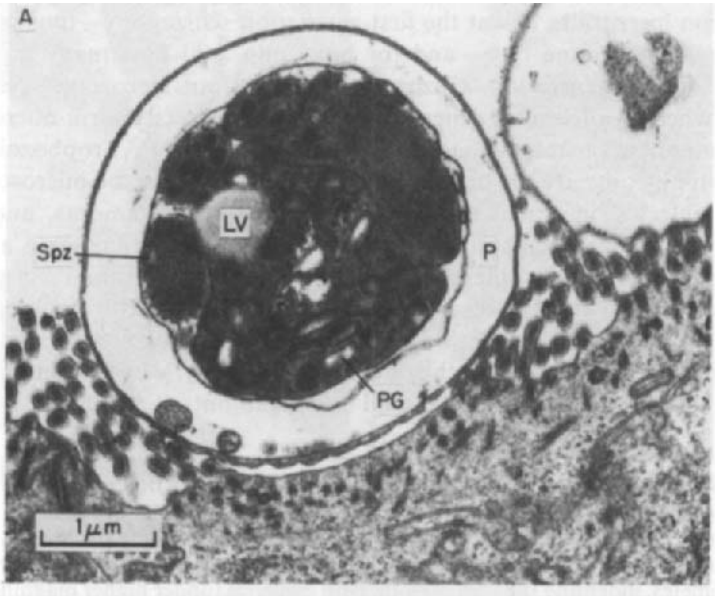
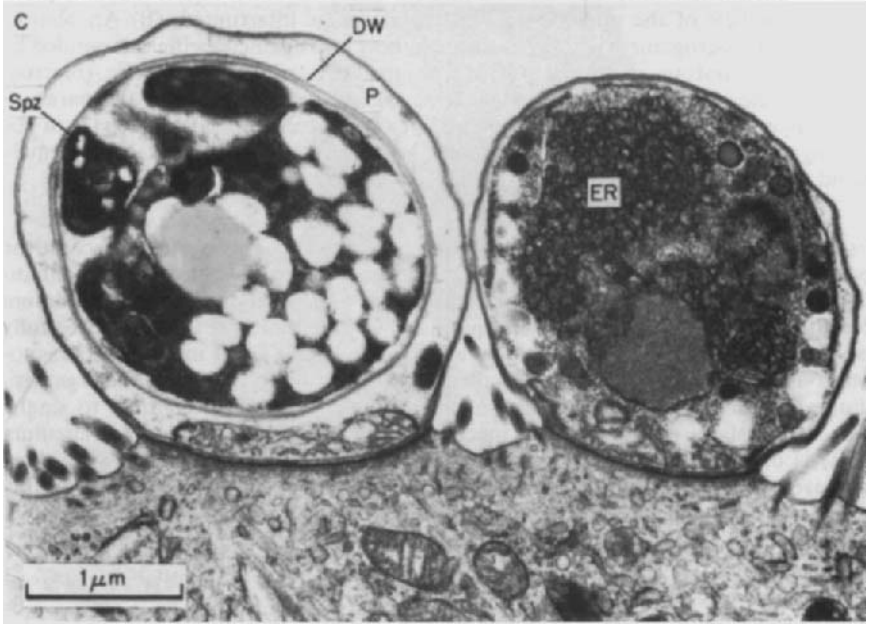
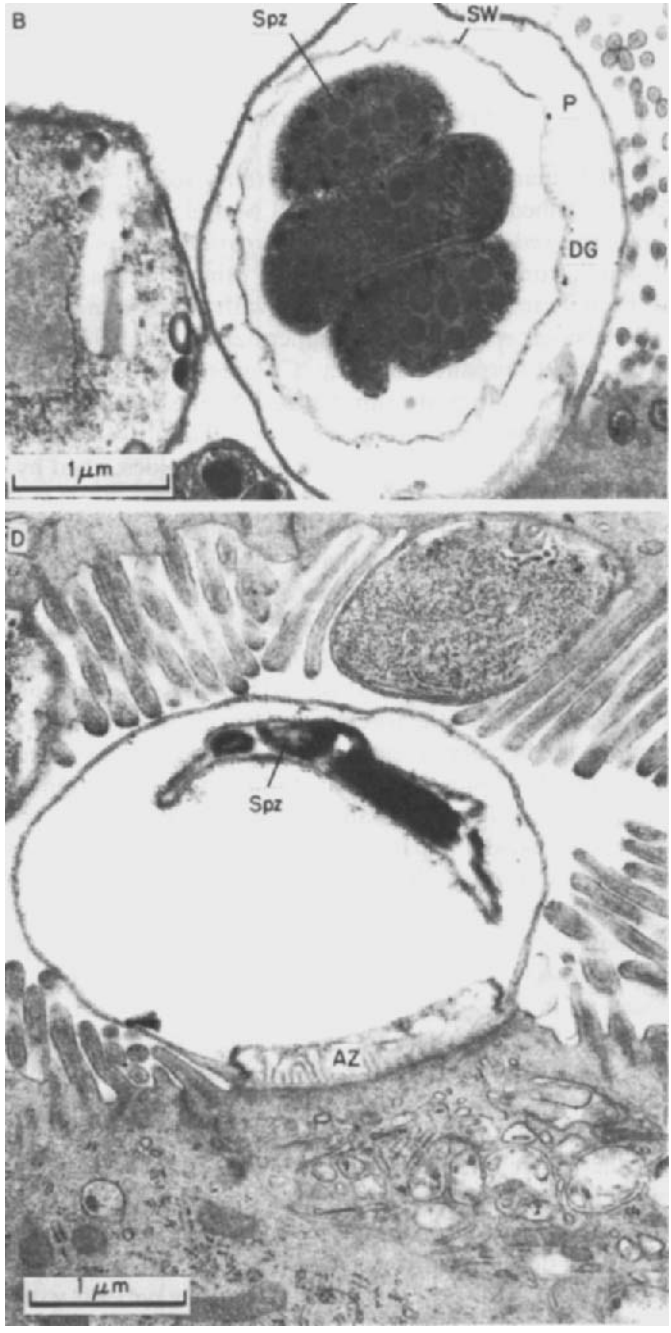
FIG. 9. Fertilization. Unlike the description by Goebel and Braendler (1982), who elegantly demonstrated fertilization of a macrogamete by attachment of the microgamete with the blunt end (A) and (B) depict probable fusion along the long axis; they may represent a more advanced stage. (A) A microgamete is trapped between two macrogametes, indenting the wall of both, with evidence (under higher magnification) that the pellicle of the upper one, which is closer, is interrupted. (B) An oblique section of a macrogamete with what seems to be a microgamete being surrounded by the parasitophorous envelope. (C) Macrogamete in which a probable (blurred) microgamete can be distinguished (six arrows). A thickened area in the parasitophorous envelope opposite suggests a recent "reinforcement" after entry(?) of the microgamete. Penetration and dissolution are rarely seen and must be extremely rapid.

FIG. 10 (overleaf). Sporogony. Oocyst formation and sporogony take place in the parasitophorous vacuole shortly after fertilization. (A) Single-walled oocyst containing four newly formed sporozoites (Spz) with a still relatively large residiuum containing a lipid vacuole (LV) and polysaccharide granules (PG). (B) Three fully developed sporozoites within a single-walled oocyst (SW) still inside the parasitophorous vacuole (P). (C) Double-walled sporulating oocyst (DW) with large residiuum within the parasitophorous vacuole. Note rigid wall, in contrast to single-walled oocysts; a newly fertilized macrogamete containing endoplasmic reticulum (ER) is on the right. (D) A fully sporulated double-walled oocyst showing three sporozoites (Spz) with no apparent trace of the residium. The double wall invariably collapses with processing; AZ = attachment zone.


A
LV
Spz
P
PG
1μm


C
DW
Spz
P
ER
1μm


B
Spz
SW
P
DG
1 μm
D
Spz
AZ
1 μm

## IV. The Infection in Humans

### A. EPIDEMIOLOGICAL OBSERVATIONS

The history of human cryptosporidiosis is fairly recent, the first two cases having been described by Nime *et al.* and Meisel *et al.* in 1976. Bird and Smith (1980) showed that human *Cryptosporidium* was morphologically indistinguishable from forms described from other animals. By 1980 further cases had been described in individuals with some clinical evidence of acquired (Weisberger *et al.*, 1979; Stemmermann *et al.*, 1980) or congenital (Lasser *et al.*, 1979) immunodeficiency. Cryptosporidiosis in these patients was confirmed by histological examination of intestinal biopsies. The first case of cryptosporidiosis in an immunocompetent adult, who suffered severe, watery but self-limiting diarrhoea and vomiting, was described by Tzipori *et al.* (1980b). This patient was the first in whom the diagnosis was made by detection of oocysts in faecal smears. The transmission of this human isolate to mice and lambs not only showed that it was biologically indistinguishable from *Cryptosporidium* of other mammals, but also indicated indirectly the zoonotic potential of this parasite (Tzipori *et al.*, 1980a, 1982a), as subsequently confirmed by Reese *et al.* (1982). The emergence of acquired immunodeficiency syndrome (AIDS) and AIDS-related infections promoted human cryptosporidiosis to the forefront of interest in 1982. The prevalence of the infection in the general population also became apparent with the publication of the first epidemiological studies in 1983 (Jokipii *et al.*, 1983; Tzipori *et al.*, 1983b), although it had been suspected as early as 1980 from a limited study conducted on patients with gastroenteritis in a hospital in Newcastle, UK (S. Tzipori, R. Madeley and K.W. Angus, unpublished observations). Ironically, at the time the information was considered by two medical journals not to be of particular relevance. The zoonotic potential of cryptosporidiosis was confirmed by accidental infections of humans who had close contact with infected calves (Anderson *et al.*, 1982; Current *et al.*, 1983; Rahaman *et al.*, 1984). The early epidemiological studies were followed by numerous reports from around the globe. The following aspects of the infection in humans will be discussed: the frequency of diarrhoea attributed to it in various regions; clinical manifestations; seasonal variation; association with other infections and travel; the mode of transmission; and asymptomatic infections.

Tables 1, 2 and 3 summarize the frequency with which *Cryptosporidium* oocysts have been detected in faeces in various studies of human diarrhoea; they indicate a worldwide prevalence of 1–4% among patients with diarrhoea in most developed countries, and up to 16% in some less developed countries.

TABLE 1 *The frequency of human cryptosporidiosis reported in Asia and the Pacific*

| Location | Number of patients Tested | Positive (%) | Children | Peak season | Asymptomatic carriage (number tested) | Reference |
|---|---|---|---|---|---|---|
| Australia | | | | | | |
| Victoria | 884 | 36 (4.1) | mostly | Summer | 0 (320) | Tzipori *et al.* (1983b) |
| S. Australia 1 | 9056 | 11 (< 1) | < 50% | Not stated | | Lumb *et al.* (1985) |
| 2[a] | 94 | 9 (9.6) | only | Spring–summer | | Lumb *et al.* (1985) |
| W. Australia | 2164 | 29 (1.3) | only | Summer–autumn | | Anonymous (1986) |
| New Zealand[a] | 36 | 8 (22) | only | Summer | | Te Wiata and Lennon (1985) |
| Thailand (Bangkok) | 410 | 13 (3.2) | only | Not stated | 1 (104) | Taylor and Echeverria (1986) |
| Bangladesh (Dacca) | 578 | 25 (4.3) | only | Spring | | Shahid *et al.* (1985) |
| India (Lahore) | 682 | 89 (13.1) | only | All year | 41 (418) | Mathan *et al.* (1985) |

[a]Studied during a limited season only.

TABLE 2 *The frequency of human cryptosporidiosis reported in America*

| Location | Tested | Number of patients Positive (%) | Children (%) | Peak season | Asymptomatic carriage (number tested) | Reference |
|---|---|---|---|---|---|---|
| Canada | | | | | | |
| British Columbia | 7300 | 46 (0.6) | > 50 | Summer | | Montessori and Bischoff (1985) |
| Newfoundland | 1621 | 19 (1.2) | > 50 | Summer–autumn | | Ratnam *et al.* (1985) |
| United States | | | | | | |
| Massachusetts | 1703 | 47 (2.8) | > 50 | Summer–autumn | 3[c] | Wolfson *et al.* (1985) |
| S. Carolina | 582 | 25 (4.3) | < 50 | Spring | 1[c] | Holley and Dover (1986) |
| Texas[a] | 843 | 5 (0.9) | only | Summer–autumn | | Wiedermann *et al.* (1985) |
| Michigan | 1752 | 53 (3.5) | < 50 | Summer–autumn | | Bossen and Britt (1985) |
| Haiti | 702 | 116 (16.5) | only | All year | | Pape *et al.* (1985) |
| Costa Rica 1 | 946 | 46 (4.9) | only | Summer | 0 (116) | Mata (1986) |
| 2[b] | 280 | 10 (3.6) | only | Summer | 0 (89) | Mata *et al.* (1984) |
| 3[b] | 278 | 8 (4.3) | only | Summer | 0 (90) | Mata *et al.* (1984) |
| Brazil | 117 | 9 (8) | only | Summer | 0 (22) | Weikel *et al.* (1985) |
| Venezuela | 120 | 13 (10.8) | only | Not stated | | Perez-Schael *et al.* (1985) |
| Chile | 100 | 4 (4) | only | Not stated | | Weitz *et al.* (1985) |

[a]Studied during a limited period.
[b]2 is urban and 3 is rural.
[c]Asymptomatic contacts of index case.

TABLE 3 *The frequency of human cryptosporidiosis reported in Europe and Africa*

| Location | | Tested | Number of patients Positive (%) | Children (%) | Peak season | Asymptomatic carriage (number tested) | Reference |
|---|---|---|---|---|---|---|---|
| Finland[a] | | 4545 | 119 (2.6) | < 50 | All year | 0 (120) | Jokipii *et al.* (1985b) |
| Denmark[a] | | 900 | 16 (2) | < 50 | Spring | | Holten-Andersen *et al.* (1984) |
| United Kingdom | 1 | 6580 | 140 (2.1) | mostly | All year | | Casemore *et al.* (1985b) |
| London | 2 | 213 | 7 (3.2) | only | Autumn | 1 (112) | Isaacs *et al.* (1985) |
| Bristol | 3 | 867 | 43 (5) | < 50 | Autumn–winter | | Hunt *et al.* (1984) |
| France | | 190 | 4 (2.1) | only | Autumn | | Arnaud-Battandier and Naciri (1985) |
| Liberia | | 278 | 22 (7.9) | only | Not stated | | Hojlyng *et al.* (1984) |
| Ghana | | 474 | 61 (12.9) | only | Not stated | | Addy and Aikins-Bekoe (1986) |
| Rwanda | | 293 | 23 (7.6) | mostly | October–January | | Bogaerts *et al.* (1984) |

[a]High proportion associated with recent travel.

Most of the figures are presented as the yearly incidence. There are two important factors influencing the frequency in a given study; the age distribution of the population studied and the season of the year. The incidence is higher in children and during the wet, warmer months of the year. For instance, the study in Australia by Tzipori *et al.* (1983b) (Table 1) revealed an overall frequency of 4.1% compared with 7% for the three summer months, and 8.2% for children under 5 years of age. Similarly, the yearly average in British Columbia was 0.6% (Table 2), but during the summer months it was recorded as 2.9% (Montessori and Bischoff, 1985). A distinct seasonal trend was not observed in all countries. For instance, in the UK and in India no seasonal variation was documented (Casemore *et al.*, 1985b; Mathan *et al.*, 1985). Indeed, Hunt *et al.* (1984) recorded a prevalence of 5% (Table 3) during late autumn and winter in a predominantly adult group. The study by Mathan *et al.* (1985) illustrated the relationship between temperature and rainfall; while oocyst excretion was detected during all seasons, shedding was reported to be more frequent during the months with rainfall and was lowest in January and February, which are cool dry months.

The frequency can also vary depending on the age distribution of the children under study, as it has been shown to be much higher among children aged 12 months or less. In a Liberian study in which the total prevalence was 7.9%, 23% of cases were in children aged between 6 and 12 months (Hojlyng *et al.*, 1984), and of a total prevalence of 12.9% in Ghana, 21.6% were children in that age group (Addy and Aikins-Bekoe, 1986). Similar findings were reported in the UK (Hunt *et al.*, 1984), India (Mathan *et al.*, 1985), and in Massachusetts (Wolfson *et al.*, 1985).

### B. TRANSMISSION OF INFECTION

Cryptosporidiosis is transmitted by various means: person-to-person, animal-to-human, human-to-animal, and human and animal faecal contamination of the environment, including water, food and possibly air, are proven routes of transmission. Person-to-person spread is probably the most important route as shown by sequential infections in families (Wolfson *et al.*, 1985), and clusters of cases and outbreaks in day-care centres (Table 4). The frequency of cryptosporidiosis was shown to be higher in urban areas of Costa Rica (Mata, 1986) and Liberia (Hojlyng *et al.*, 1984), where there is closer contact among humans than in rural villages. Infection can, undoubtedly, be contracted from contact with animals, particularly calves (Anderson *et al.*, 1982; Current *et al.*, 1983; Rahaman *et al.*, 1984), and possibly pets (Bennet *et al.*, 1985). Calves are an important source because, like humans, they develop diarrhoea and generate large quantities of oocysts which are

TABLE 4 *Frequency of shedding of* Cryptosporidium *oocysts among children attending day-care centres in the USA*

| Location | Number with diarrhoea: Children | (%) | Shedding oocysts | (%) | Asymptomatic carriage (number tested) | Season | Reference |
|---|---|---|---|---|---|---|---|
| Philadelphia | 23 | (43) | 13 | (65) | 3 (27) | Summer | Alpert *et al.* (1986) |
| Texas[a] | 24 | (30) | 18 | (75) | 4 (26) | Summer | Taylor *et al.* (1985) |
| Pennsylvania | 20 | (34) | 11 | (65) | 3 (28) | Summer | Anonymous (1984b) |
| California | 10 | (93) | 10 | (60) | 0 (1) | Summer | |
| Georgia 1[a] | 27 | (51) | 5 | (9.5) | 0 (26) | Spring | |
| Georgia 2[b] | 35 | (?) | 6 | (17) | Not clear | Spring | |
| Michigan[b] | 54 | (?) | 21 | (55) | Not clear | Summer | |
| New Mexico 1[a] | 18 | (47) | 5 | (29) | 0 (1) | Summer | |
| New Mexico 2[a] | 13 | (81) | 7 | (63) | Not reported | Summer | |

[a]Several index cases were concurrently shedding *Giardia lamblia*.
[b]The number of symptomatic patients was not stated.

shed into the environment, increasing the risk of infection of other animals and humans. Disposing of human effluent on to grazing land, the practice in a number of countries, presumably increases the risk of infection of grazing animals. Disposal of animal manure similarly can also contribute to further contamination of pasture (Casemore *et al.*, 1986). The period of survival of oocysts in the environment is unknown, and may not be long. Although oocysts are extremely resistant to the action of disinfectants (Campbell *et al.*, 1982; Angus *et al.*, 1982b), they do not appear to retain their infectivity for long under laboratory conditions (Tzipori *et al.*, 1981c). It will be shown later (Section VI, C) that oocysts require few special conditions for excystation, which may explain their rapid loss of infectivity since excystation may occur spontaneously during storage. Direct faecal contamination of water, from human or animal sources (D'Antonio *et al.*, 1985), or food, can lead to outbreaks or sporadic cases of cryptosporidiosis. Another potential source of transmission that requires evaluation is the respiratory tract. The rate of tracheal infection among immunologically normal humans, which may contribute to aerosol spread, is unknown. So far there has been one report of a laryngo-tracheal infection in a child (Harari *et al.*, 1986). The parasite readily infects the tracheal mucosa of birds (Hoerr *et al.*, 1978) and of experimentally infected piglets (Tzipori, 1983; Heine *et al.*, 1984b), and it may conceivably do so more frequently in humans, with or without causing symptoms. Other factors which facilitate spread are the ease with which *Cryptosporidium* infects a large number of host species with or without causing disease, and the prolonged shedding of oocysts in stools, often continuing long after clinical illness has resolved.

### C. CLINICAL MANIFESTATIONS

Cryptosporidiosis in humans is perceived as two distinct disease entities; a short and self-limited diarrhoeal illness in immunocompetent humans, and a persistent, often life-threatening, diarrhoea in immunodeficient patients (Anonymous, 1984c). Information generated by an increased awareness of infection worldwide indicates that there is a spectrum of human infection ranging from subclinical or mild illness of one day's duration, to illness which persists indefinitely. Most cases are at one or other end of the spectrum, but there are others which do not fulfill the criteria for either extreme, being neither of short duration nor associated with a distinct immunological abnormality. Some infections persist for several months without underlying immunodeficiency (Isaacs *et al.*, 1985), while remission, or spontaneous recovery, with or without treatment, has been reported in some individuals with AIDS (Berkowitz and Sidel, 1985). Indeed, asymptomatic carriage of

cryptosporidiosis in a child with AIDS has also been described (Zar *et al.*, 1985).

There are few, if any, features that distinguish gastroenteritis due to cryptosporidiosis in the immunocompetent patient from other enteric infections. A range of symptoms has been described with a wide spectrum of severity and duration, occurring in various combinations. The following features have been reported in a large number of published studies: diarrhoea varying from loose to watery and offensive stools, abdominal pain, vomiting, nausea, fever, anorexia, dehydration, weight loss, with symptoms and oocyst excretion persisting from one day to 2–8 weeks. The commonest pattern is one of non-inflammatory gastroenteritis, manifested by brown-green and offensive watery diarrhoea containing mucus but not blood or pus, lasting about one week. Diarrhoea, the most consistent symptom, is often preceded by one or more of the following: abdominal cramps, vomiting, low-grade fever or anorexia. The relative frequency of symptoms other than diarrhoea is extremely variable. For instance, in two separate studies in the UK, one in Bristol (Hunt *et al.*, 1984) and the second in Liverpool (Baxby and Hart, 1984), the frequencies of three symptoms predominating in children were: vomiting 17% and 63%, abdominal cramps 38% and 61%, and fever 21% and 34%. In one study, symptoms were more severe in adults, and lasted on average twice as long as in children (Hunt *et al.*, 1984). This could be due to the fact that adults would seek medical advice only when symptoms were severe. In developing countries the clinical illness is described as being more severe, with more frequent vomiting, fever, and a higher degree of dehydration (Bogaerts *et al.*, 1984; Shahid *et al.*, 1985). When diarrhoea occurs with malnutrition, the illness is prolonged and followed by further weight loss which may be fatal (Bogaerts *et al.*, 1984).

### D. SYMPTOMS IN IMMUNODEFICIENT PATIENTS

Cryptosporidiosis in the immunodeficient host has been extensively documented as case reports of individuals (Stemmermann *et al.*, 1980; Sloper *et al.*, 1982; Kocoshis *et al.*, 1984), and as group studies (Pitlik *et al.*, 1983; Whiteside *et al.*, 1984). In humans, clinical evidence indicates that both major branches of the immune system are required for recovery from cryptosporidiosis; impairment of either humoral or cellular immunity can lead to unremitting, profuse diarrhoea lasting for months with profound malabsorption and significant weight loss.

T-lymphocyte dysfunction in humans, resulting from infection with HIV virus, is the single most important disease syndrome predisposing to chronic diarrhoea due to cryptosporidiosis. Studies in nude mice have

strongly implicated the role of T-cells, either regulatory or effector or both, in recovery from experimental cryptosporidiosis (Heine *et al.*, 1984a), which may reflect the intracellular nature of the parasite. There have been only a few reported cases of chronic diarrhoea due to cryptosporidiosis in patients with congenital hypogammaglobulinaemia (Lasser *et al.*, 1979; Sloper *et al.*, 1982; Tzipori *et al.*, 1986). In individuals with severe combined immune deficiency, disseminated fatal cryptosporidiosis, affecting intestinal and pulmonary systems, has been reported (Kocoshis *et al.*, 1984). There have been several cases of chronic diarrhoea due to this infection which have coincided with patients receiving immunosuppressive chemotherapy (Meisel *et al.*, 1976; Weisburger *et al.*, 1979; Miller *et al.*, 1983), which have resolved upon withdrawal of therapy.

The incidence of diarrhoea among patients with AIDS is variable. In one hospital in the USA it was reported to be about 30%, and 7% of cases were attributed to infection with *Cryptosporidium* (Whiteside *et al.*, 1984). In Hawaii the incidence of cryptosporidiosis was estimated to be 10% of patients with confirmed AIDS, and in Haiti it was said to be 38% (Malebranche *et al.*, 1983). Detection of cryptosporidiosis in patients with chronic diarrhoea is often the first indication of the cell-mediated immune defect of AIDS (Cooper *et al.*, 1984; Whiteside *et al.*, 1984). The symptoms of chronic cryptosporidiosis are essentially the same as in self-limiting infection, except for duration, extent and outcome. Diarrhoea may be intermittent or continuous with bowel motions up to 25 times a day, and outputs of 2 to 12 litres per day have been reported. Other symptoms include vomiting, abdominal pain, weight loss from 10 to 50%, intermittent headaches, low-grade fever, diffuse abdominal tenderness and notable lymphadenopathy, malaise, anorexia and muscular wasting. Concurrent gastrointestinal tract infections such as amoebiasis, shigellosis and giardiasis can also occur (Pitlik *et al.*, 1983). Patients often require prolonged intravenous fluid therapy and parenteral nutrition.

As mentioned earlier, there are cases of cryptosporidiosis which do not fit into a distinct category. Prolonged diarrhoeal illness lasting 1–4 months, however, is not unique to cryptosporidiosis and has been described in relation to other enteric infections. Malnutrition in low socioeconomic groups, or multiple infections, are considered predisposing factors. Mild or moderate derangement of certain immune functions caused by underlying clinical or subclinical infection with viruses (e.g. measles), bacteria (e.g. mycobacteria) and other protozoa (e.g. *Toxoplasma*), have been documented. A higher incidence of cryptosporidiosis has been observed among children with measles (De Mol *et al.*, 1984). Influenza causes some impairment of humoral and cellular immune responses and reduces chemotaxis of polymorphonuclear phagocytes and macrophages (Anonymous, 1982;

Wainberg and Mills, 1985). Increased levels of hormones associated with a physiological state such as pregnancy can also influence the immune status of the host.

The occurrence of prolonged diarrhoea in patients under treatment with immunosuppressive drugs is perhaps the best indication of the role of certain immune functions. Diarrhoea gradually resolved after cessation of treatment of patients with bullous pemphigoid (Meissel *et al.*, 1976), lymphocytic leukaemia (Miller *et al.*, 1983; Lewis *et al.*, 1985), or graft-versus-host disease (Collier *et al.*, 1984). In one instance, diarrhoea resolved spontaneously in a child with rhabdomyosarcoma despite continuation of treatment (Miller, 1984).

## E. INFECTION IN YOUNG CHILDREN

Perhaps the best illustration of higher prevalence among young children is reports of outbreaks of diarrhoea in day-care centres (Table 4). Because more children are now attending day-care centres, more attention has been focused on the frequent transmission of pathogens there. The congregation of a large number of young children, not previously exposed to infection, is an ideal setting for outbreaks to occur. Enteric infections that have been the cause of outbreaks of diarrhoea in day-care centres include rotavirus, shigella, *Giardia* and *Clostridium difficile*. Two detailed studies, one in Philadelphia (Alpert *et al.*, 1986) and one in Texas (Taylor *et al.*, 1985), provide useful information. Alpert *et al.* (1986) found a higher prevalence of infection in household contacts (13 of 48 examined) of patients with symptomatic cryptosporidiosis, compared with only two of 56 contacts of asymptomatic children; these two were asymptomatic carriers. In contrast to the study by Hunt *et al.* (1984), the illness lasted longer in children (8.3 days) than in adults (3.4 days). Taylor *et al.* (1985), who carefully examined an outbreak of diarrhoea in which cryptosporidiosis and giardiasis were detected in almost equal numbers in a day-care centre (Tables 4 and 5), attributed the diarrhoea to the former. The different age distribution of the two infections was the key to discerning the cause of the diarrhoea in this outbreak. Cryptosporidiosis was commoner among children 6–12 months old and decreased with age towards 2 years, while giardiasis increased with age from 12 months onwards, peaking at 3 years. Eighteen children were infected with *Cryptosporidium* and 14 (78%) of them had diarrhoea; 5 of 35 (14%) children without diarrhoea were excreting *Cryptosporidium* oocysts. In contrast, only 5 of 18 (28%) infected with *Giardia* experienced diarrhoea, while 14 of 32 (44%) without diarrhoea were excreting *Giardia*. The occurence of asymptomatic giardiasis in day-care centres has previously been reported (Pickering *et al.*, 1984). In three of the

TABLE 5 *The relative frequency of detection of* Cryptosporidium *and* Giardia *in stools reported in some studies*

| Location | Total number of specimens | Number positive for *Cryptosporidium* | Number positive for *Giardia* | Reference |
|---|---|---|---|---|
| Australia | | | | |
| W. Australia | 2164 | 29 | 83 | Anonymous (1986) |
| Victoria | 980 | 13 | 46 | Tzipori (unpublished data) |
| UK (Liverpool) | 1947 | 27 | 23 | Hart *et al.* (1984) |
| Denmark | 800 | 16 | 18[a] | Holten-Andersen *et al.* (1984) |
| Finland | 4545 | 119 | 103 | Jokipii *et al.* (1985b) |
| Rwanda | 293 | 23 | 8 | Bogaerts *et al.* (1984) |
| Ghana | 474 | 61 | 52 | Addy and Aikins-Bekoe (1986) |
| Haiti | 702 | 116 | 7 | Pape *et al.* (1985) |
| Massachusetts | 1290 | 33 | 101 | Wolfson *et al.* (1985) |
| S. Carolina | 582 | 25 | 18 | Holley and Dover (1986) |
| Texas[b] | 50 | 18 | 18 | Taylor *et al.* (1985) |

[a]From a total of 265 tested.
[b]An outbreak in a day-care centre.

investigations reported in Table 4 (Anonymous, 1984b), some children were shedding *Giardia* cysts and *Cryptosporidium* oocysts concurrently, but the relative contribution of each to the illness was not clear.

Symptomatic infection among children is more frequent than in adults, not because of an innate age-related susceptibility, but because of the high prevalence of the infection in human and animal populations, shown by the accumulating epidemiological data and preliminary serological indications (Tzipori and Campbell, 1981). Thus most adults are immune because of frequent exposure throughout life. The relationship between the level of immunity and the size of the infectious dose determines, at each new exposure, whether infection occurs, remains subclinical or causes diarrhoea. Serological tests described so far appear to detect antibodies only from recent infections (Campbell and Current, 1983; Koch *et al.*, 1985; Casemore *et al.*, 1985a; Ungar *et al.*, 1986) and therefore are not sensitive enough for seroepidemiological studies. Faecal smears detect excretion of oocysts from either symptomatic patients or their contacts. Tables 1, 2 and 3 show that only two of 1067 randomly selected individuals without diarrhoea were excreting oocysts. Asymptomatic carriage has been detected mostly in contacts of symptomatic patients (Wolfson *et al.*, 1985; Isaacs *et al.*, 1985). Subclinical infections lead probably to a brief excretion, the time of which would be extremely difficult to determine without the occurrence of symptomatic contacts. Consequently, there has been an excellent correlation between shedding of oocysts in stools and, mostly, symptomatic cryptosporidiosis. The arguments for high prevalence of cryptosporidiosis in humans include (a) infection is commonest during the first 3 years of life (Hojlyng *et al.*, 1984; Mathan *et al.*, 1985; Addy and Aikins-Bekoe, 1986; Wolfson *et al.*, 1985; Baxby and Hart, 1984); (b) outbreaks are uncommon except in day-care centres for the reason mentioned in (a); (c) the disease is less common and occurs only sporadically in adults because of frequent previous exposure, not age-related innate resistance, since diarrhoea, when it occurs, can be at least as severe in adults as in children (Hunt *et al.*, 1984); and (d) the prevalence of diarrhoea appears to be less in neonates compared with infants of 6 months or older (Mata, 1986; Mathan *et al.*, 1985), because of protection by maternal antibody resulting from the mother's own frequent exposure. The youngest child reported with cryptosporidiosis was a three-day-old infant born to a mother who had had diarrhoea due to cryptosporidiosis several days before vaginal delivery (Bossen and Britt, 1985), but no clinical detail was given. A two-week-old child with cryptosporidiosis is the youngest so far reported with diarrhoea (Hart and Baxby, 1985).

The study by Mathan *et al.* (1985) deserves special attention; a high prevalence of cryptosporidiosis was reported in children with diarrhoea and in asymptomatic age-matched control children aged 5 months or younger. A

comparison between breast-fed and bottle-fed children showed no difference. Living conditions in rural southern India, as the authors described, were unsanitary—there was no protected water supply; animals were kept adjacent to, or inside, houses; and there were no proper waste or sewage disposal facilities. Babies (and their mothers) were regularly exposed to high infectious doses which occasionally overcame the high level of maternal protection to cause diarrhoea or asymptomatic infection. As maternal protection waned gradually, the incidence of symptomatic infection increased in children older than 6 months while the number of asymptomatic infections decreased. By the age of 2 years, no further case of asymptomatic infection was detected, compared with a rate of 2.7% symptomatic infections. The total prevalence remained low because, by this stage, most of the children had had cryptosporidiosis. It is difficult to understand the basis for the conclusion of Mathan *et al.* (1985) that *Cryptosporidium* was unlikely to have been a major cause of acute diarrhoea in that population, given the data presented in their study. It is unknown whether the high prevalence of subclinical infections reported in this study is unique to that population, as no similar study has been made of a large randomized group of asymptomatic neonates. The occurrence of subclinical enteric infections in this age group is not unique to *Cryptosporidium*; asymptomatic rotavirus infection has been reported on several occasions (Bishop *et al.*, 1979). Unlike Mata (1986), Mathan *et al.* (1985) found no difference in the prevalence of cryptosporidiosis among breast-fed or bottle-fed babies, which is interesting in light of the high proportion of asymptomatic infections. However, in experimental studies in animals, we showed that, while secretory antibodies are the key to protection against enteric infections due to rotavirus, high levels of circulating antibody of maternal origin are often sufficient to modify the clinical outcome (Tzipori and Williams, 1978).

In general, asymptomatic carriage of *Cryptosporidium* is less common than that of other enteric infections. For instance, in Bangkok asymptomatic infections with enteric bacteria can be as high as 25% compared to a rate of 0.25% reported for *Cryptosporidium* (Taylor and Echeverria, 1986).

### F. ASSOCIATION WITH OTHER PATHOGENS

Multiple infections with enteric pathogens are very common, particularly in developing countries, and *Cryptosporidium* is no exception (Mathan *et al.*, 1985; Taylor and Echeverria, 1986). Of all enteric pathogens, *Cryptosporidium* has been linked more closely with *Giardia*. They are similar in a number of ways. They are both intestinal protozoa with uncomplicated life-cycles.

Large numbers of infectious, resistant exogenous cysts (or oocysts) are shed into the environment in the faeces of infected individuals often long after recovery from illness. Both cause chronic diarrhoea and malabsorption. Both have been detected in animals, but *Cryptosporidium* infects a wider range of species, causing severe diarrhoea in some, thus generating and disseminating a large number of oocysts into the immediate domestic environment. Both infections have been linked with travel. Giardiasis, a well known and extensively documented infection, could not be distinguished clinically from cryptosporidiosis in adults by Jokipii *et al.* (1983), although the latter caused more severe abdominal pain, while anorexia, weakness and bloating were more common with giardiasis. Mata (1986), however, described cryptosporidiosis as being the more severe in children. Table 5 compares the frequency of detection of both parasites in stools of humans with diarrhoea submitted to laboratories in various countries. It is interesting that, in six of the 10 studies recorded in the table, the detection rate for *Cryptosporidium* oocysts was equal to, or greater than, that for *Giardia.*

Jokipii *et al.* (1983, 1985a) found a statistically significant association between infections with *Giardia* and *Cryptosporidium* in two studies. A similar association was reported by Wolfson *et al.* (1984), in the USA. However, in the studies by Jokipii *et al.* (1983, 1985a), both infections were associated with travel. Indeed, in a third study (Jokipii *et al.*, 1985b), there was a greater association betwen travel and infection with either parasite than between both organisms. This suggests a common source of infection rather than a predisposing effect of one infection for the other. The other interesting feature in this study, and that of Holten-Andersen *et al.* (1984) who also found a strong link with travel, particularly to Leningrad, was the fact that most of their patients were adults, presumably because more travellers are adults. Water source was suggested as the vehicle of transmision on the basis of earlier studies with *Giardia* (Jokipii, 1971). An outbreak of cryptosporidiosis linked to a common water source was reported from Texas, USA (D'Antonio *et al.*, 1985). Enteric pathogens that have been previously linked with traveller's diarrhoea include enterotoxigenic *Escherichia coli* (ETEC), *Entamoeba histolytica*, and occasionally *Giardia. Cryptosporidium* should now be added to the list. Regions that have been linked with travel and cryptosporidiosis are the Caribbean (Ma *et al.*, 1985), Mexico (Sterling *et al.*, 1986), Central Africa (Soave and Ma, 1985), the USSR (Jokipii *et al.*, 1983; 1985a,b), and south-east Asia (Tzipori *et al.*, 1983b).

### G. EFFECT ON INTESTINAL MUCOSA

The reaction of the mucosa is important in the pathogenesis of cryptospori-

diosis and may be one reason for variation in response to infection by different species of animals. While in animals experimental inoculations can be performed to examine and assess mucosal injury due to infections, information regarding the nature of mucosal response to cryptosporidiosis in humans is understandably fragmented. Very limited information is available on the histopathology of infection in immunocompetent patients despite the increasing number of epidemiological studies, particularly since diagnosis is now made by detection of oocysts in faecal smears, which has replaced earlier invasive techniques. Biopsies, when required, are taken mostly from either the duodenum/jejunum or from the large bowel. Animal studies indicate that these sites are probably the least affected, and so mucosal changes in these sites may not reflect the extent of injury. More information is available from detailed studies of individuals with immune defects. However, in such patients there are many complicating factors including other, coincidental infections. Moreover, the entire gastrointestinal tract can be examined only at the terminal stage of the infection, at necropsy.

The histopathology of the gastric mucosa, which is involved infrequently, varies from chronic gastritis associated with cytomegalovirus infection (Blumberg *et al.*, 1984), to little or no change (Andreani *et al.*, 1983; Modigliani *et al.*, 1985). Meisel *et al.* (1976) and Lasser *et al.* (1979) described moderate changes in the mucosa of the duodenal/jejunal biopsies, which included abnormal villous architecture with lengthening of the crypts, an increased number of plasma cells, polymorphs and lymphocytes, and occasional crypt abscesses. The surface epithelium was low and infiltrated with cells. However, most studies (Weisburger *et al.*, 1979; Weinstein *et al.*, 1981; Sloper *et al.*, 1982; Andreani *et al.*, 1983; Modigliani *et al.*, 1985) described normal or slightly blunted villi without degenerative change in the epithelium, normal crypts or occcasional cryptitis, some infiltration of the lamina propria with, mostly, plasma cells and a few polymorphs and macrophages. There were some subcellular changes in infected epithelial cells observed by electron microscopy (Weinstein *et al.*, 1981), which included sparse or abnormal microvilli, dilated endoplasmic reticulum, accumulation of lipid-like material, and increased density of the cytoplasm. Some bacteria were seen attached to the apical membrane of damaged cells.

In one study, a complete set of biopsies taken from a number of sites along the small and large intestines from an AIDS patient (Andreani *et al.*, 1983) showed that mucosal changes were minimal. The colonic and rectal mucosa were normal. Other reports have described colonic and rectal abnormalities (Nime *et al.*, 1976). Sequential studies (Sloper *et al.*, 1982) showed that, as the illness progresed, mucosal injury changed from mild to subtotal villus atrophy just before death. At necropsy, heavy infestation was reported in the jejunum and ileum and fewer organisms in the large bowel. Similar findings

were described in a child with combined immunodeficiency who contracted terminal cryptosporidiosis (Kocoshis *et al.*, 1984). Initial biopsies showed little mucosal change despite the presence of numerous organisms. Later biopsies showed subtotal villous atrophy and dense inflammatory infiltration by numerous polymorphs, eosinophils, histiocytes, and a few lymphocytes in the lamina propria and in crypts.

Cholecystitis associated with cryptosporidiosis has also been described and can vary from acute uncomplicated inflammation (Guarda *et al.*, 1983) to profound necrosis and gangrene, complicated by co-infection with cytomegalovirus (Blumberg *et al.*, 1984).

It is quite clear that in humans, as in other animals, *Cryptosporidium* can infect one, several, or all organs of the alimentary tract including pharynx, oesophagus, biliary system, and the entire gastrointestinal tract (Kocoshis *et al.*, 1984). Unlike most other enteric infections, *Cryptosporidium* may infect all or a portion of the alimentary tract with no clear indication as to the likely location of the infection. This suggests the occurrence of variable and often complex clinical symptoms involving one or more locations; the immunological status of the patient may, however, often determine the extent and clinical outcome of the infection.

### H. INFECTION OF THE RESPIRATORY TRACT

Vomiting may lead to the infection spreading from the gut to the upper respiratory tract. *Cryptosporidium* infection of the respiratory tract of certain species has been reported (Hoerr *et al.*, 1978). Similar infections in mammals were not known until recently, although suspected earlier on experimental grounds (Fig. 11) (Tzipori, 1983). Several cases of pulmonary cryptosporidiosis have been described in patients with AIDS or other forms of immunodeficiency (Forgacs *et al.*, 1983; Kocoshis *et al.*, 1984; Ma *et al.*, 1984). Pulmonary infection may be due to invasion from the gut in terminally ill patients, rather than being a primary site. The frequency of laryngotracheal infection in immunologically normal children (Harari *et al.*, 1986) is unknown. Infection of the respiratory tract has been confirmed by examination of sputum (Brady *et al.*, 1984), tracheal aspirate (Harari *et al.*, 1986), lung biopsies (Ma *et al.*, 1984), or lung tissue at necropsy (Kocoshis *et al.*, 1984). In the few reported cases, it is difficult to assess the contribution of cryptosporidiosis to the symptoms and overall lesions described, as all occurred in patients who had concurrent infections affecting the respiratory tract. Pulmonary cryptosporidiosis has been described together with generalized cytomegalovirus infection, tuberculosis, *Pneumocystis carinii* infection, and others. Symptoms described in association with pulmonary infection

include persistent sore throat (Brady *et al.*, 1984), dyspnoea, and diffuse rales associated with some lung markings in chest X-ray. Parasites were observed in the epithelium of the trachea (Ma *et al.*, 1984) and bronchioles (Kocoshis *et al.*, 1984), in alveolar exudates, and on, or inside, macrophages.

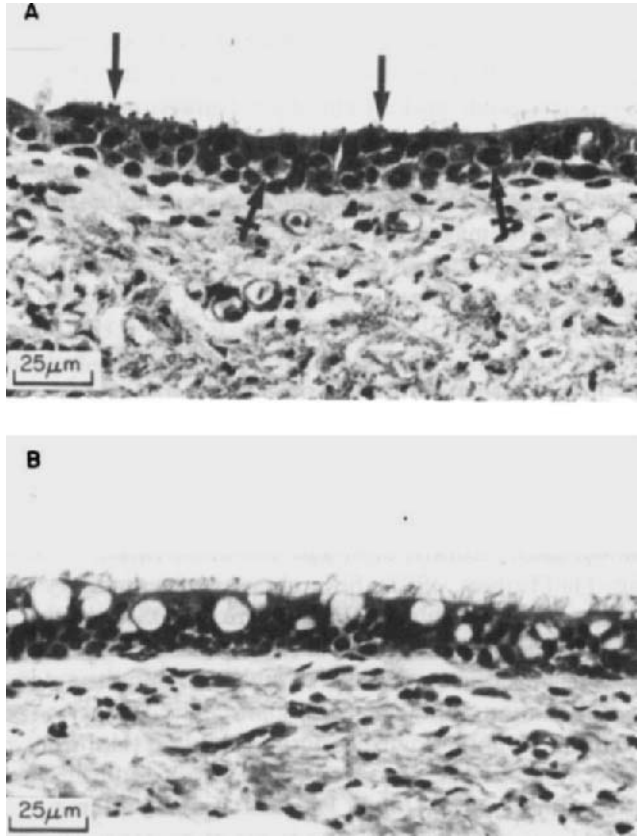



FIG. 11. Sections of trachea from a piglet infected with *Cryptosporidium* of human origin. (A) Organisms (arrows) are attached to the luminal surface of respiratory epithelium which shows loss of goblet cells, patchy loss of cilia, nuclear enlargement and mitotic figures (crossed arrows). (B) Neighbouring part of the trachea from the same animal showing normal pseudostratified ciliated columnar epithelium with goblet cells (haematoxylin and eosin staining).

## I. MECHANISMS OF DIARRHOEA

The mucosal reaction of the human gut to cryptosporidiosis, though far from fully understood, does not appear to be as severe as that observed in neonates of ruminants (Tzipori *et al.*, 1981c,e; Angus *et al.*, 1982c). The precise immunological and pathophysiological mechanisms underlying the disease in humans are also unclear. Cryptosporidiosis, whether causing self-limiting diarrhoea in immunologically competent hosts, or persistent diarrhoea in immunodeficient individuals, will probably prove to be peculiar to humans. Watery diarrhoea due to cryptosporidiosis is uncommon in adults of other animals. The increased susceptibility of adult humans to diarrhoea, in contrast to other animals, is not limited to cryptosporidiosis. While animals become rapidly resistant to most enteric viral (rotavirus), or bacterial (campylobacter, [ETEC]) infections, humans remain susceptible throughout life.

The proximal small intestine appears to be more predominantly involved in human cryptosporidiosis than in animals, and the infection causes mostly mild inflammation of the gut mucosa. Therefore, diarrhoea in the human appears to be mostly due to hypersecretion of fluid and electrolytes from the proximal small intestine into the lumen (Andreani *et al.*, 1983; Whiteside *et al.*, 1984; Modigliani *et al.*, 1985). Perfusion studies indicate profuse fluid secretion in the duodenum and proximal jejunum. More distally, large amounts of water and sodium were reabsorbed by the small intestine, to a greater extent than that observed in normal fasting subjects. The colonic reabsorption rates of water and sodium were near maximal absorptive capacity, which was consistent with the reported absence of mucosal abnormalities in the colon (Andreani *et al.*, 1983). Others observed that patients continued to have abdominal pain, vomiting and high stool output while on total parenteral nutrition (Kocoshis *et al.*, 1984), and the osmolality of faecal water was almost entirely accounted for by sodium, potassium and their accompanying anions in the proximal small intestine (Modigliani *et al.*, 1985). The rate of water and electrolyte reabsorption presumably is slower than normal in patients with heavy infection of the lower small and large bowels. Fat and carbohydrate malabsorption and loss of protein, as well as fluid and electrolytes, from the gastrointestinal tract, have occurred in the terminal stages of cryptosporidiosis when the mucosa of the entire gut is heavily infested and extensively altered (Kocoshis *et al.*, 1984). Under normal circumstances, infection was shown, in animal studies, to begin in the lower small intestine even in susceptible neonates, before spreading to the rest of the gut (Snodgrass *et al.*, 1984), and diarrhoea occurred if the proximal small intestine became infected. Infection of the proximal small intestine seems to occur much more readily in humans than in animals, which may explain the

greater susceptibility of humans, including adults, to this infection. Severity of certain enteric bacterial infections is often related to the efficiency of colonization of the proximal small intestine, which is more susceptible to the action of enterotoxin liberated by ETEC in piglets, compared with ETEC that colonize the lower small intestine (Tzipori *et al.*, 1982c). Colonization of the small intestine is extremely difficult because of physiological barriers such as extremes of pH, intense proteolytic activity and high flow rate. For an organism to become established in this part of the intestine requires special mechanisms of attachment or gut dysfunction, or it must become established during the immediate postnatal period, before effective physiological barriers have become fully developed.

Chronic cryptosporidiosis has been described as cholera-like secretory diarrhoea which responds favorably to chlorpromazine, indicating a possible cyclic-nucleotide-induced secretory diarrhoea (Kocoshis *et al.*, 1984). The role of parasite products released by *Cryptosporidium* into or adjacent to infected cells is unknown and requires investigation. Lysates of *Entamoeba histolytica*, a well known and extensively studied intestinal protozoon which also causes diarrhoea in man, have both cytotoxic and enterotoxic activities (Lushbaugh *et al.*, 1979). Strains of *E. histolytica* also possess a number of proteolytic enzymes, the function of which is not clear. In addition, hormone-like substances and a neurohumoral substance, similar to serotonin, have been detected in lysates of *E. histolytica* (McGowan *et al.*, 1982) and are thought to contribute to alteration in intestinal transport resulting in diarrhoea. Similar or related substances may yet be shown to occur in lysates of *Cryptosporidium*, which might explain the inducement of watery diarrhoea attributed largely to hypersecretion in the proximal small intestine.

## V. The Infection in Other Animals

The general perception of cryptosporidiosis in animals has changed little since previous reviews (Angus, 1983; Tzipori, 1983; Navin and Juranek, 1984), and readers are referred to them for further details. There have been several recent studies describing observations on the parasite in animal species, not previously reported. *Cryptosporidium* has now been detected in all classes of vertebrates; from all the domestic animals (cattle, pigs, sheep, goats, horses); domestic pets (dogs, cats); laboratory animals (rabbits, mice, rats, guinea-pigs, monkeys); wild mammals (squirrels, deer, artiodactyls, racoons); domestic fowls (turkeys, chickens); wild birds (peacock, red-lored parrots); reptiles (snakes); and fish. With a few exceptions, which will be mentioned below, the pattern of disease in mammals is essentially the same: enterocolitis in newborn mammals manifested by diarrhoea of variable

severity in some (bovine and small ruminants), asymptomatic in most, and presumably variable in a few (cats, horses, guinea-pigs, pigs, dogs). Upper respiratory tract infections have been described mainly in wild and domestic birds.

### A. CLINICAL OBSERVATIONS AND PATHOLOGY

Economically, cryptosporidiosis is significant in calves and is widespread among dairy and beef herds. It affects calves aged between one and four weeks with high morbidity and mostly low mortality (Tzipori, 1985a). A comparative study on the prevalence of the infection in herds in various regions showed that 22–40% of diarrhoeic calves discharged oocysts in their faeces (Tzipori, 1985b). Cryptosporidiosis is second to rotavirus as the most prevalent enteric pathogen causing diarrhoea in calves. It is more frequently isolated than bovine coronavirus and ETEC (Tzipori, 1985a). Mixed infections including *Cryptosporidium* are extremely common in calves (Morin *et al.*, 1978; Snodgrass *et al.*, 1980), and for a long time the concurrence of other organisms obscured the pathogenicity of the protozoon in this species (Pohlenz *et al.*, 1978b). The youngest affected calf reported was four days old, and the oldest was 26 days. Relapses after apparent recovery have also been reported (Tzipori *et al.*, 1980c). A study conducted over $2\frac{1}{2}$ years in a veterinary diagnostic laboratory in Canada (Sanford and Josephson, 1982) described more severe clinical signs of cryptosporidiosis at around one week of age, with profuse watery diarrhoea, unresponsive to treatment, lasting two or more weeks and usually fatal.

Bovine cryptosporidiosis was first reported by Panciera *et al.* in 1971, in an 8 months old calf which had chronic diarrhoea of several weeks' duration, debilitation and progressive weight loss, curiously not unlike chronic diarrhoea in humans with immune defects. It was unusual because, in cattle, illness is seldom seen in animals older than several weeks. Meuten *et al.* (1974) described the first detailed histopathology of the gastrointestinal tract in a 2 weeks old diarrhoeic calf. They believed that *Cryptosporidium* was the pathogen responsible for the lesions and diarrhoea.

The most prominent lesions occur in the ileum. They include shortened, blunted, and often fused villi; the lining epithelium is flat or cuboidal; and the lamina propria is infiltrated with plasma cells, lymphocytes and occasional neutrophils and eosinophils (Fig. 12). Focal cryptitis can be seen in the small and large intestines. Colonic lesions are more focal with characteristically decreased and irregular mucosal height, decreased number of goblet cells, and mononuclear cell infiltration of the lamina propria. Cryptitis in the colon is characterized by desquamated epithelium and accumulation of polymorphs.

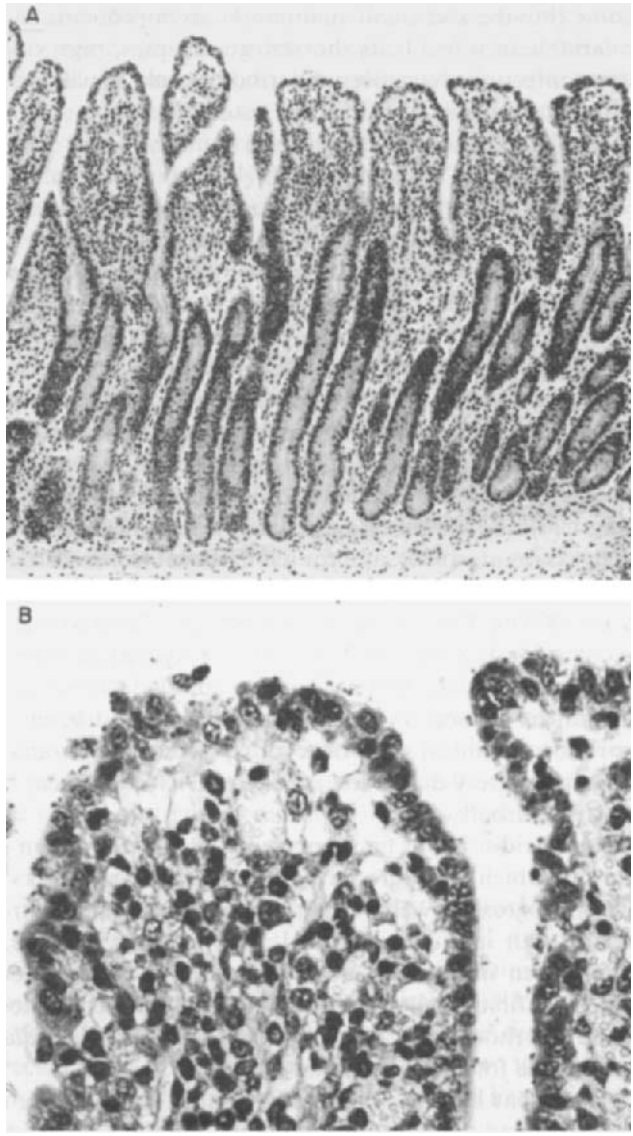



FIG. 12. Histological section from the mid ileum of a calf infected with *Cryptosporidium* of human origin. (A) Note the partial villous atrophy, crypt hyperplasia, increased cellular infiltration of the lamina propria, and stunted enterocytes (haematoxylin and eosin). (B) Higher magnification of an area from (A) showing numerous organisms embedded in the microvillous border of the flat, irregular and slightly eroded epithelial surface (haematoxylin and eosin staining).

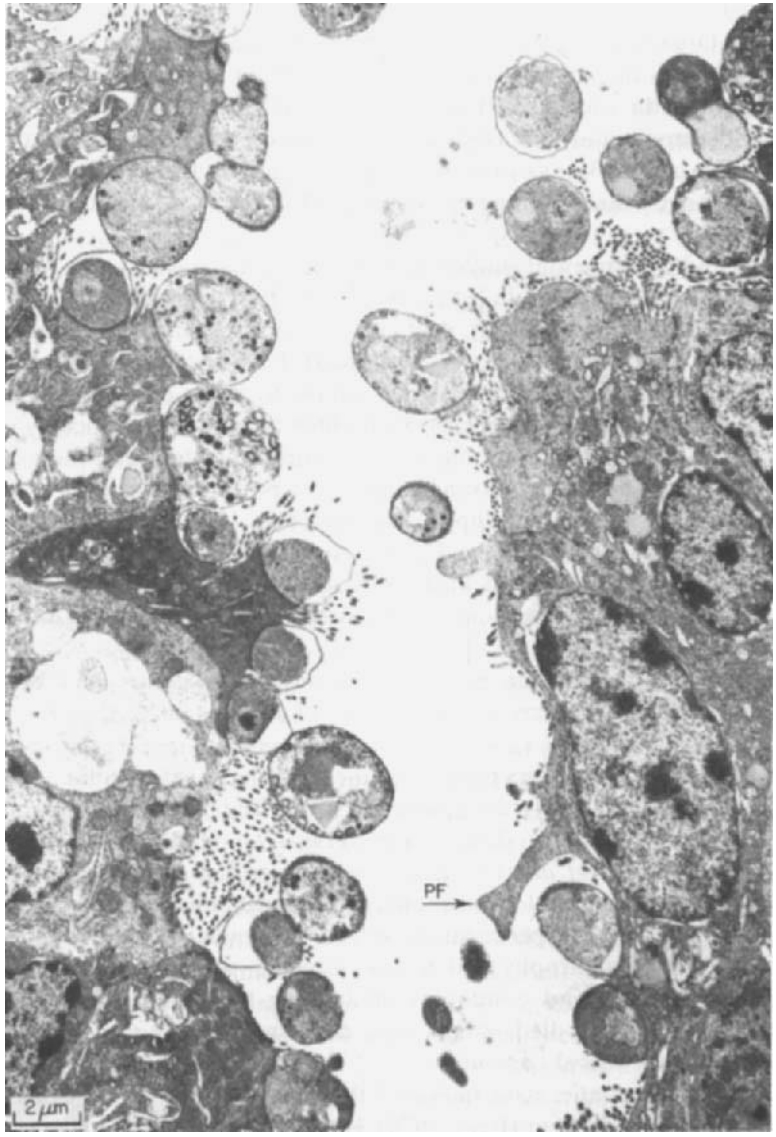



FIG. 13. Heavily infected site in the ileum of a calf showing two adjacent villous surfaces. One is extensively colonized; the other obviously has been, and its apical surface appears irregular and has lost the microvillous border, which would undoubtedly affect fluid and nutrient absorption. Note that trophozoites and macrogamonts are the predominant forms. The epithelium is fragmented, vacuolated, and often with extreme pedestal formation (PF).

The proximal small intestine is less severely affected and occasionally is spared. Organisms normally occupy the entire villus surface causing effacement of the microvillus border and often dissolution of the cell membrane at the site of attachment (Fig. 13). However, extensively infected cells in the small intestine of calves seldom survive after parasite maturation (Tzipori *et al.*, 1983a). Organisms isolated originally from calves have also been extensively studied in newborn lambs (Tzipori *et al.*, 1981c,e; Angus *et al.*, 1982c).

Field and experimental studies in small ruminants, including lambs reared both naturally (Angus *et al.*, 1982a; Anderson, 1982) and artificially (Tzipori *et al.*, 1981a; Anderson, 1982), deer calves (Tzipori *et al.*, 1981b; Orr *et al.*, 1985), and goat kids (Tzipori *et al.*, 1982d), have shown that clinical and pathological findings were essentially similar to those described in calves. The infection in lambs was extensive with high mortality in artificially reared animals, but was more variable under natural (suckled) conditions. In a study in Scotland, high mortality from cryptosporidiosis was reported in suckled lams, which developed diarrhoea 8 to 12 days after birth; 58 of 200 animals died within 2 to 3 days (Angus *et al.*, 1982a). However in Idaho, USA, diarrhoea in suckled lambs of a similar age lasted 4 days and the animals recovered without treatment after a mild illness (Anderson, 1982).

Mucosal legions of the magnitude described in calves and in other ruminants are seldom seen in other species of animals infected with *Cryptosporidium*. In mice and rats mucosal alterations are minor, despite often extensive infestation by the parasite of the epithelial surface in the lower gut (Sherwood *et al.*, 1982). In the guinea-pig subclinical enteritis, with moderate mucosal legions, has been described by Jervis *et al.* (1966) and Vetterling *et al.* (1971a). Angus *et al.* (1985), however, described a much more severe clinical illness and pathological changes in natural infections in guinea-pigs, and subsequently in experimentally infected animals. Within 5 days after inoculation villous atrophy and fusion occurred, with macrophages, other mononuclear cells, and eosinophils infiltrating the lamina propria of the ileum. Some animals died; others were depressed and anorectic with diarrhoea or watery caecal contents.

Experimental studies have indicated that cryptosporidiosis in cats caused only subclinical infections (Iseki, 1979). However, more recently Bennet *et al.* (1985) and Poonchia and Pippin (1982) observed that infection in cats, aged four months to five years, was associated with lack of appetite, weight loss and persistent diarrhoea. Asymptomatic cats can act as a source for human infection (Bennet *et al.*, 1985).

Naturally occurring cryptosporidiosis has been reported in dogs (Wilson *et al.*, 1983; Fukushima and Helman, 1984), but the nature of the infection remains unclear as the cases described were complicated by other factors

including other infections. The role of cryptosporidiosis in foals is also doubtful. Clinically obvious infection was reported in five immunodeficient foals (Snyder *et al.*, 1978), and limited serological observations suggest the infection may be widespread (Tzipori and Campbell, 1981). However, the clinical outcome of infections in immunologically normal foals is unknown. Reinmeyer *et al.* (1984) failed to detect oocysts in the faeces of 14 scouring foals aged 7–22 days. Over a 4 year period, I also failed to detect *Cryptosporidium* infection in 52 foals with diarrhoea (Tzipori, 1985a). Furthermore, in transmission experiments of bovine *Cryptosporidium* to newborn, colostrum-fed or colostrum-deprived foals, only subclinical infections were induced. One report, however, described natural infection in two foals, but its significance was not clear (Gajadhar *et al.*, 1985).

Cryptosporidiosis is not a serious cause of enteritis in either neonatal or postweaning pigs (Tzipori, 1985a). The infection has been observed, on occasions, under natural conditions in older animals (Kennedy *et al.*, 1978; Links, 1982; Tzipori, 1985a), though its role remains uncertain. Yet experimental inoculation of piglets, suckled (Tzipori *et al.*, 1981d) or artificially reared (Moon and Bemrick, 1981; Tzipori *et al.*, 1982e), can cause moderate to severe diarrhoea, extensive mucosal changes, and death.

An interesting case of cryptosporidiosis was reported in the kidney of a black-throated finch (Gardiner and Imes, 1984). Organisms were seen in the lumen of the tubules and it was assumed that the parasite had gained access to the kidney via the ureter from the cloaca. This was the first report of urinary tract infection. Reports of diarrhoea and deaths due to cryptosporidosis in wild species, including red deer calves (Orr *et al.*, 1985) and other artiodactyls (Van Winkle, 1985), both small ruminants, have been published. The disease occurred in very young animals that were either held in enclosures or artificially reared.

Upper respiratory tract infection with *Cryptosporidium* has been reported in turkeys (Hoerr *et al.*, 1978) and peacocks (Mason *et al.*, 1981), and has been observed in experimentally infected chicks (unpublished data). It is not clear in what circumstances predilection for a particular site occurs, as infection of the gastrointestinal tract has also been reported in turkeys (Slavin, 1955; Hoerr *et al.*, 1978). Infection of the respiratory tract in turkeys was associated with clinical signs of varying severity. Histological changes in the trachea included thickened mucosa due to infiltration of the lamina propria with histiocytes, lymphocytes and heterocytes, and flattening of the epithelium. Organisms were also observed in the nasal conjunctiva and sinus mucosa of infected peacocks which experienced severe depression, gurgling respiration, coughing and sneezing with a serous ocular discharge (Mason *et al.*, 1981). Outbreaks of respiratory infections, with 30% morbidity and 20% mortality, have been reported in broiler chickens and turkeys (Hoerr *et al.*, 1978; Dhillon *et al.*, 1981; Glisson *et al.*, 1984).

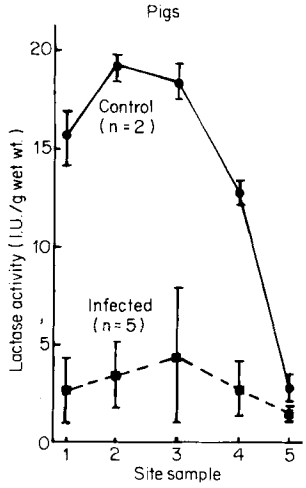



FIG. 14. Graph showing membrane-bound lactase activity (I.U. = μmol minute$^{-1}$ g$^{-1}$ wet weight) measured in the microvillous border of the small intestine of five piglets infected with *Cryptosporidium* of human origin, and in two control animals (means ± standard errors of the means), from the duodenum (site 1) to the terminal ileum (site 5).

### B. PATHOGENIC MECHANISMS

There are marked variations between different hosts in their responses to gastrointestinal infections with *Cryptosporidium*. Natural or experimental infection of young calves, lambs, goat kids and piglets is associated with generalized infection of the gastrointestinal tract, with moderate to severe mucosal changes as described earlier. Diarrhoea is caused by brush border maldigestion due to a loss of membrane-bound digestive enzymes in the upper small intestine (Fig. 14) and reduced absorptive capacity in the ileum (Fig. 12), which is attributed to marked villous atrophy and fusion reducing the overall surface area. In addition, the apical surfaces of absorptive cells are altered due to loss of microvilli and other membrane changes (Fig. 13), and severely damaged epithelial cells are replaced by functionally immature cells. These factors all reduce the efficiency of absorption of fluids and nutrients. The large bowel in these animals is not fully functional for the first few weeks after birth, therefore infection of this organ, at least initially, may not play a significant role in the pathophysiology of diarrhoea. Furthermore, the mucosal changes in the large bowel caused by cryptosporidiosis are focal and not extensive.

Susceptibility to cryptosporidiosis of domestic animals varies with age.

Lambs become resistant to experimental infection by about 15–20 days of age (Fig. 15) (Tzipori *et al.*, 1981c), and piglets by 12–15 days (Tzipori *et al.*, 1982e). Experiments conducted in specific-pathogen-free (SPF) animals show that this resistance is unrelated to the specific immune status of the animal. This innate resistance, which develops with age, is presumably associated with the ability of the host to prevent or reduce the infection in the proximal small intestine, and thus reduce the effect of maldigestion. In addition, the absorptive capacity of the colon improves with age to compensate for the reduced absorptive capacity of the ileum. Mucosal damage resulting from

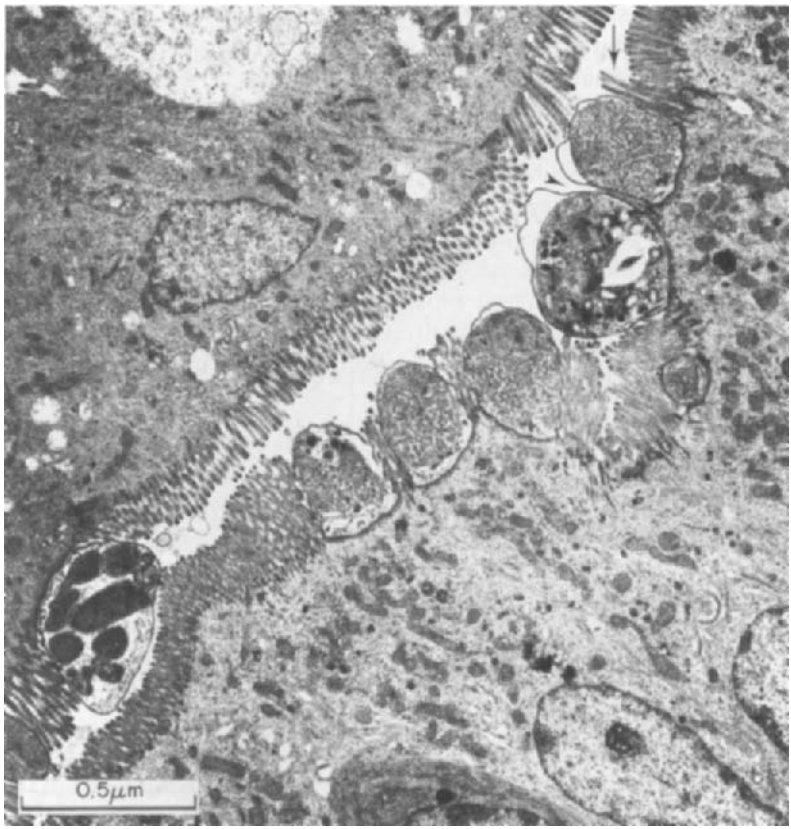



FIG. 15. Ileal section from an infected lamb showing early colonization of the surface, with as yet intact epithelium and microvillous border. Note, however, the marked elongation of microvilli adjacent to parasites (arrow). It is interesting that elongation is also apparent in the cell opposite the parasite (arrowhead); this may result from stimulation related to the ability of the parasite to induce the formation of a host-derived envelope.

severe inflammatory reactions may be induced by a number of mechanisms, including hypersensitivity reactions to parasite antigens or metabolites. Our observations suggest that (a) the mucosa of an older animal may be less reactive; (b) the infection in older animals is largely restricted to the ileum; and (c) development of the large bowel can compensate for an injury to the small intestine. These factors apparently limit the effect of infection with *Cryptosporidium* in older animals, in addition to specific immunity resulting from repeated exposure with increasing age. In this context it is worth mentioning the role of the large intestine in domestic animals. It is a highly complex organ which has a significant role in fermentation and absorption of fluids, electrolytes and nutrients. It can compensate for abnormalities associated with injury and reduced absorption in the ileum much more, it seems, than the human large bowel.

*Cryptosporidium* can cause severe disease in newborn piglets (Fig. 16) but, because of good lactogenic immunity resulting from the high prevalence of infection in the population (Tzipori *et al.*, 1981d; Tzipori and Campbell, 1981), piglets are protected against the infection up to the age of 12–15 days, after which they usually become inherently resistant (Fig. 17) (Tzipori *et al.*, 1982e). Rodents and chicks can be experimentally infected with heterologous "strains" of *Cryptosporidium* during the first 2–3 weeks of life only. Furthermore, they remain clinically normal throughout the course of the infection. The sites most commonly infected are the lower small intestine,

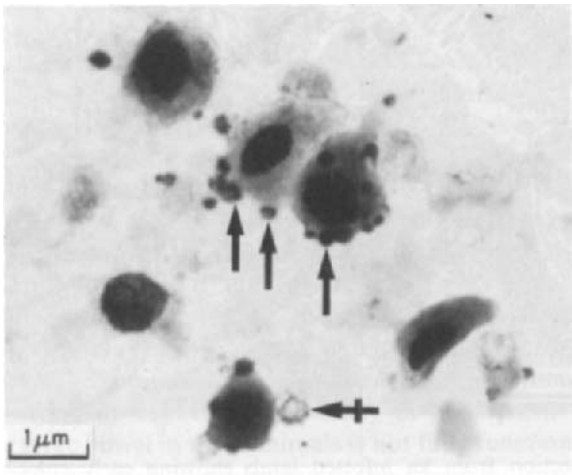



FIG. 16. Faecal smears from a piglet experimentally infected with *Cryptosporidium* isolated from humans. Overwhelming infection can lead to shedding of intact enterocytes with endogenous stages (arrows) or oocysts (crossed arrow), still attached to the microvillus border (Giemsa).

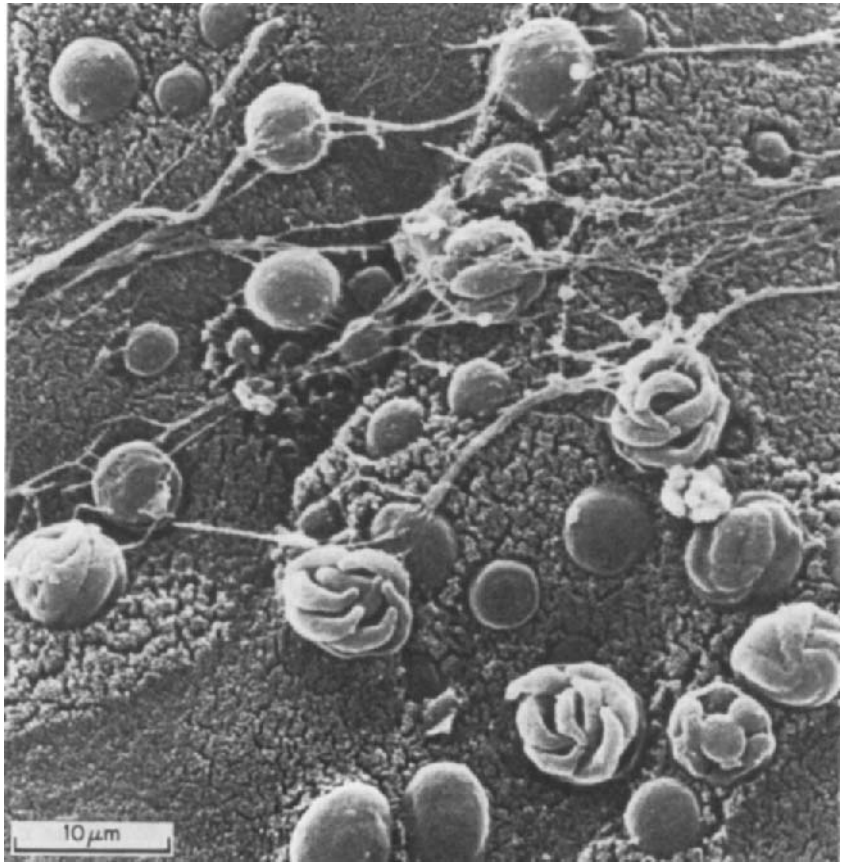



FIG. 17. Scanning electron micrograph of villous surface of the ileum of a piglet infected with *Cryptosporidium* isolated from calves. Note deeply embedded trophozoites and schizonts, most with ruptured parasitophorous envelopes, each containing eight merozoites. The microvillous border appears to be intact.

caecum and colon, apparently with little or no mucosal change (Sherwood *et al.*, 1982). This contrasts with extensive tissue reaction seen in calves and other small ruminants. Apparently, in susceptible animals, diarrhoea is a consequence of heavy infestation with extensive tissue damage of the proximal small intestine. In mice the proximal small intestine, and hence digestion, is less affected and, with minimal mucosal reaction in the distal small intestine, the capacity for absorption is only marginally impaired. Both these factors may explain the lack of diarrhoea in mice and possibly in other rodents. Lack of profound tissue reaction to infection may indicate that the

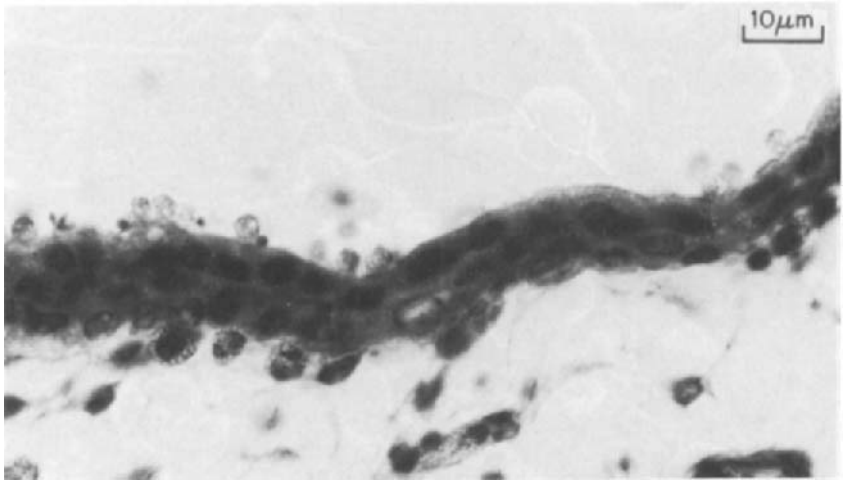



FIG. 18. Section of chorioallantoic membrane from a 16-day-old chick embryo infected with *Cryptosporidium* of bovine origin via the allantoic route 5 days earlier. Note endogenous forms on the endoderm cell surface (haematoxylin and eosin).

gut of the mouse is less sensitive to parasite products. In older piglets and lambs lacking specific antibodies, infection mimics the situation in the mouse, being sparse in the proximal small intestine with less mucosal reaction.

## VI. LABORATORY INVESTIGATION

### A. DIAGNOSTIC PROCEDURES

Diagnosis at present is based largely on identification of *Cryptosporidium* oocysts in faecal smears. Many techniques have been described by various investigators to improve the speed of diagnosis by devising methods for scanning at low magnification. Some staining techniques were said to improve the sensitivity of identification, particularly when only a few, damaged oocysts were present; others claimed higher specificity.

Among the most widely used are the modified acid-fast stains with or without initial concentration by flotation in a high-density sugar solution (Sheather's) (Ma and Soave, 1983; Garcia *et al.*, 1983). Other techniques said to be rapid and specific include a modified Ziehl–Nielsen method, staining with auramine phenol or Giemsa, and a dimethyl sulphoxide modified acid-

fast stain. Some investigators recommend the use of combinations of staining methods: e.g. auramine phenol and carbol fuchsin for scanning, and modified Ziehl–Nielsen for confirmation (Casemore *et al.*, 1986). Unlike serological tests, in which sensitivity may vary because of detection of different antigens, examination of stained faecal smears, whichever method is used, depends for success on the number of intact oocysts present.

A direct immunofluorescence (IF) test has recently been described for the detection of oocysts in the faeces utilizing specific monoclonal antibody directed against an oocyst wall antigen determinant (Sterling and Arrowood, 1986). Oocysts fluoresce brightly, and the authors suggested that the method may be useful for epidemiological studies in which unconcentrated faeces or other specimens can be screened rapidly. Another advantage was that it could detect poorly preserved or excysted oocysts which normally do not take up stain. Indirect IF was also used to confirm the presence of oocysts in tracheal aspirates from a child with suspected upper respiratory cryptosporidiosis (Harari *et al.*, 1986). IF applied directly to faeces has the inherent problem of lack of specificity; fluorescing particles of 3–6 μm can be extremely difficult to identify conclusively as oocysts, no matter how many negative or positive control specimens are included with each test. Enzyme-linked immunosorbent assays (ELISA), using antibodies raised against endogenous forms which may be shed in faeces, as well as against oocysts, may be more sensitive and specific and may simplify diagnosis.

Serological tests that measure specific circulating antibodies have been described. These include indirect IF (Tzipori and Campbell, 1981; Campbell and Current, 1983) and, more recently, ELISA (Ungar *et al.*, 1986). The indirect IF uses heavily infected intestinal segments from SPF animals as a source of antigen (Tzipori and Campbell, 1981). It has been used in preliminary studies to establish the presence of circulating specific antibodies in 10 species of domestic and human animals. The test was sufficiently sensitive to detect specific antibody in 80–100% of sera tested, a level which has been shown, over the past 2 years, to be a realistic target. In the ELISA test described by Ungar *et al.* (1986), oocysts which were purified from faeces of experimentally infected calves, and sonicated to liberate the sporozoites, were reacted with human sera and labelled anti-human IgG and IgM. The test for IgG was assessed to have a positive predictive value, during illness, of 93%, and a negative predictive value of 97%. Levels of specific antibody after clinical cryptosporidiosis in immunocompetent humans followed the normal pattern: an initial rise of IgM, which declined within several weeks after the infection, and the subsequent appearance of IgG, which was delayed but which lasted several months. Specific IgG was undetectable by this test within a year after infection. The test detected antibodies in only 3 of 60 randomly tested individuals with no history of exposure to infection. There is

no evidence of antigenic cross-reactivity between *Cryptosporidium* and other intestinal parasites, including coccidia (Campbell and Current, 1983; Ungar *et al.*, 1986). Undoubtedly, as more sensitive serological tests using a wider range and higher concentration of antigens are developed, the true prevalence of infection in various animal and human populations will become apparent and quantitation will permit a distinction to be made between recent, primary and secondary infections. Endogenous forms will probably provide a better source of antigen for immunodiagnostic tests than oocysts, particularly oocysts which have been sonicated, kept frozen, or fixed in formalin, all of which processes destroy sporozoites.

Hyperimmune serum, raised against oocysts in rabbits, recognized oocysts in cell culture (Fig. 19) or in the small intestine of piglets (Fig. 20), but not other endogenous stages, at high dilutions in a peroxidase–antiperoxidase (PAP) test. Conversely, hyperimmune serum raised in gnotobiotic piglets which had received, after recovery from diarrhoea, repeated injections of infected gut scrapings, recognized endogenous forms but not oocysts (Fig. 20). This was illustrated in studies in cell culture, in which infected cells fluoresced, during the first 3 days, only when reacted with serum raised in piglets, while from the third day onwards fluorescence occurred only with serum raised in rabbits (Waldman and Tzipori, unpublished data). The reason could be that oocysts with double walls have antigenic determinant(s) on their "extra" wall, not shared with other endogenous forms, and that fixed oocysts that have lost their sporozoites have little else in common with

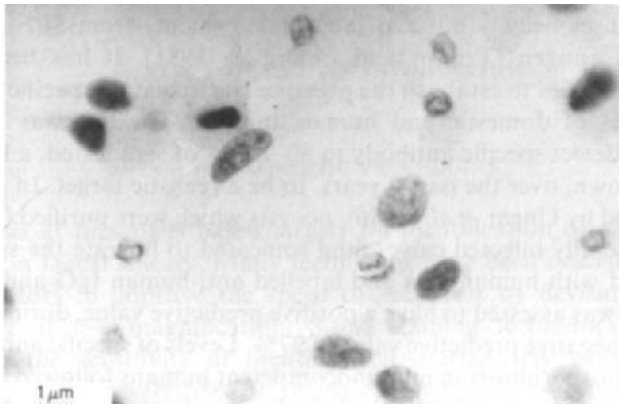



FIG. 19. Monolayer of embryonic mouse lung cells grown *in vitro* and stained with peroxidase–antiperoxidase, labelled with antibody raised against oocysts in rabbits. The cells, which appear morphologically intact, were infected with *Cryptosporidium* 5 days earlier. Only oocysts are visible at this stage.

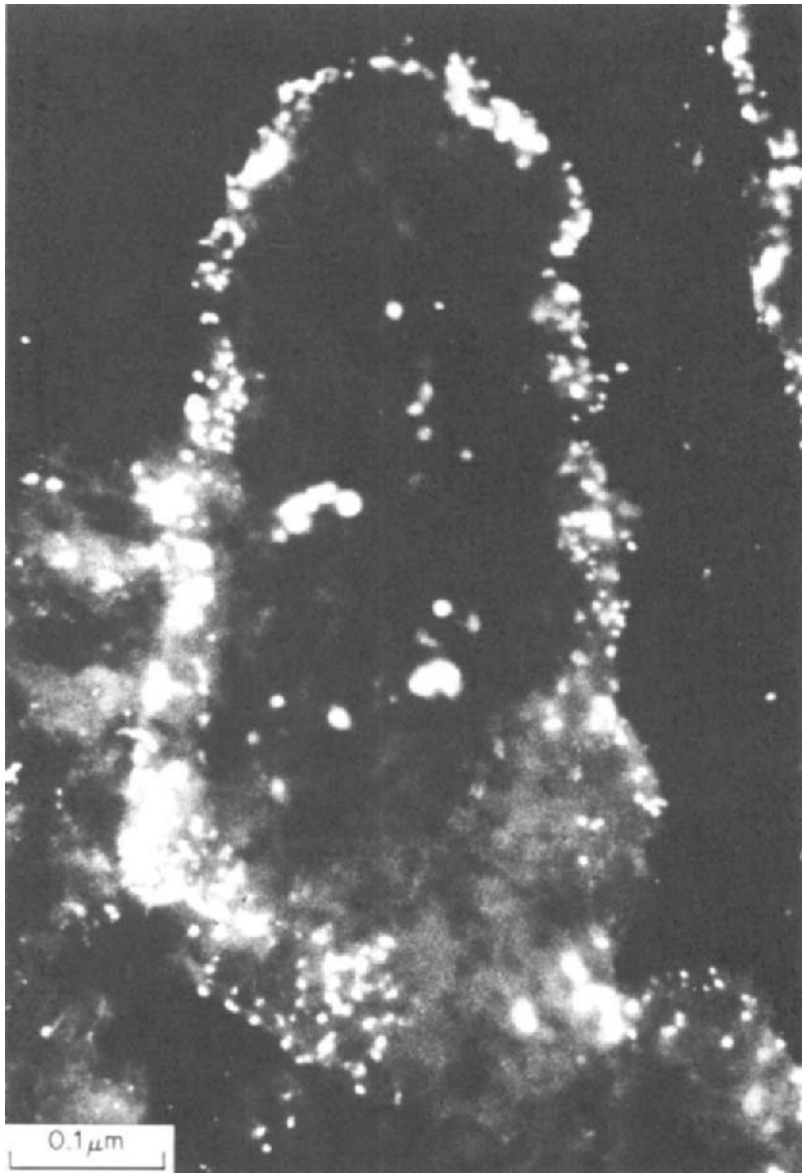



FIG. 20. Ileal mucosa of a gnotobiotic calf infected with *Cryptosporidium* of human origin and stained by indirect immunofluorescence using specific antibody produced against mostly endogenous forms of a calf isolate.

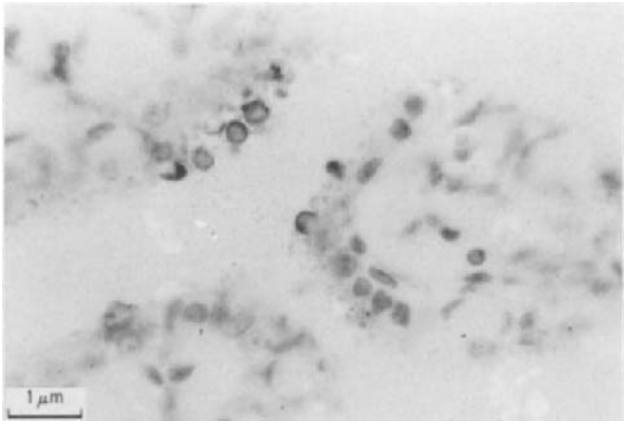



FIG. 21. Section of ileum from a piglet infected with *Cryptosporidium* of human origin and stained with peroxidase–antiperoxidase (PAP), using hyperimmune rabbit serum raised against Percoll-purified oocysts from a calf. Only the outer coat of oocysts is stained at a dilution of 1:2500; other unstained forms can be seen occupying the microvillous border. At dilutions up to 1:300, other forms also take up the stain (Waldman and Tzipori, unpublished data).

other forms. It is further proposed that these oocysts remain in the body only briefly and hence induce a poor immunogenic response compared with that induced by other forms. This would explain the poor reaction of the pig serum with oocysts. As mentioned earlier (Section III), oocysts are seen infrequently on infected mucous membranes. They are mostly within parasitophorous vacuoles until discharged in the faeces. Merozoites have been observed deep within the cytoplasm of M cells overlying Peyer's patches, but not in absorptive cells adjacent to macrophages, which indicates that these antigenic forms (merozoites) are sampled by intestinal lymphoid cells (Marcial and Madara, 1986). This lack of cross reaction between oocyst wall and other endogenous forms may explain the discrepancy between our preliminary study, which demonstrated a high frequency of circulating antibody against endogenous forms of *Cryptosporidium* in various populations—86% in randomly selected humans (Tzipori and Campbell, 1981), and that of Ungar *et al.* (1986), in which only 3 of 60 individuals were found to have antibody against *Cryptosporidium* oocyst wall.

### B. PURIFICATION AND CONCENTRATION OF OOCYSTS

Studies on the biology, pathogenesis, immunity and future control of infection have been aided by the discovery that *Cryptosporidium* isolated

from calves and humans, the most readily available sources of organisms, can be propagated in laboratory animals (Tzipori *et al.*, 1980a). The disease can be studied in gnotobiotic lambs (Tzipori *et al.*, 1981c; Angus *et al.*, 1982c), piglets (Tzipori *et al.*, 1981d, 1982e; Moon and Bemrick, 1981), and calves (Tzipori *et al.*, 1983a; Heine *et al.*, 1984c). The initial difficulty in obtaining bacteria-free oocysts was overcome as more information became available about the resistance of oocysts to various disinfectants including ethanol (Campbell *et al.*, 1982). Faecal suspensions containing oocysts can be decontaminated with 60% ethanol (Tzipori *et al.*, 1982e, 1983a), antibiotics (Snodgrass *et al.*, 1984) or 3.2% peracetic acid (Heine *et al.*, 1984c). While such decontamination procedures were adequate for pathogenesis studies in animals, for propagation in cell culture (Current and Haynes, 1984) or chick embryos (Current and Long, 1983) more elaborate purification and concentration procedures are required. Oocysts can be concentrated by ordinary centrifugation at 500*g*. It is more difficult when oocysts free from faecal debris and other organisms are required for cell culture or serology. Methods of purification described so far include flotation followed by passage of oocysts through Whatman CF-11 and DE52 cellulose columns, providing a yield of $5 \times 10^6$ intact oocysts (Sterling and Arrowood, 1986); separation by sucrose density gradient centrifugation and passage through glass bead columns, in which most of the oocysts were located in the 30% sucrose layer, though no final yield was given (Heyman *et al.*, 1986); and separation by Percoll discontinuous density gradient centrifugation (Waldman *et al.*, 1986). The last method involved a two-step procedure in which oocysts were first concentrated by sedimentation in a mixture of ether and phosphate buffered saline (PBS) and then separated on a discontinuous Percoll density gradient, without loss of infectivity of oocysts as determined by inoculation of piglets. I found the ether–PBS sedimentation to be superior to Sheather sugar flotation as the initial concentration step, largely because of the ability of the ether to extract lipids, which results in dispersion of the oocysts in the aqueous phase. This method provided a simple and efficient way of obtaining highly concentrated suspensions of $10^5$–$10^7$ oocysts per ml, free of faeces and intestinal contents and largely bacteria-free, as required for laboratory studies. In addition, Percoll solutions have low osmolality and cell toxicity, which is advantageous if oocysts are required for cell culture studies.

### C. EXCYSTATION OF OOCYSTS

After purification, excystation of oocysts was achieved by incubation at 37°C for one hour in a mixture of trypsin and bile salts (Current and Haynes, 1984). Recently, however, Fayer and Leek (1984) have shown that the

requirements for excystation of *Cryptosporidium* oocysts differ from those of *Eimeria*, *Isospora*, *Sarcosystis* and other related coccidia. Sporozoites of *Cryptosporidium* can be liberated from oocysts suspended in water, saline or other salt solutions without the need for incubation under reducing conditions or the use of digestive enzymes. However, excystation is greatly enhanced by incubation at 37°C and pH 7.6. Fayer and Leek (1984) considered that this, together with the fact that *Cryptosporidium* oocysts are sporulated when released, enable the recycling by autoinfection, without the need for an exogenous stage of development, required by other coccidia. Excystation is inhibited if oocysts are incubated in saliva, and they remain intact for several months when stored at 5°C in water, which may prove to be a simple method for preservation. Incubation in a $CO_2$ atmosphere has no advantage over incubation in air (Fayer and Leek, 1984; Reduker and Speer, 1985).

### D. PROPAGATION IN CELL CULTURE

*Cryptosporidium* can complete its life-cycle from sporozoites to infective oocysts in various primary and continuous cell lines, as well as in experimental animals. It has been propagated in human foetal lung (HFL), primary chicken (PCK) or porcine kidney (PC-10) cells (Current and Haynes, 1984), human intestinal cells (Soave *et al.*, 1985), human embryonic kidney (Reduker and Speer, 1985), and in several cell lines such as Vero MA-104, PK-1, PK-15, MDCK and NCTC (mouse lung) cells (Waldman and Tzipori, unpublished data). The development of the parasite in cell culture is similar to that observed in animals. However, whereas in mice the number of endogenous forms continued to increase after 4 days, in cell culture they remained the same or decreased (Current and Haynes, 1984). This was interpreted as indicating the lack of an autoinfective stage in cell culture, because of the absence of the thin-walled oocysts seen in animals. Most of the thick-walled oocysts apparently remain within parasitophorous vacuoles and are not released into the medium. Studies in human embryonic 407 intestinal cells (Soave *et al.*, 1985) suggest a greater degree of release of infectious forms into the supernatant medium. These forms, which were infective to fresh intestinal cells, consisted mainly (90%) of oocysts. *Cryptosporidium* causes no apparent morphological or functional damage to infected cells *in vitro* (Fig. 21).

Propagation in cell culture from sporozoites to infective oocysts is a characteristic of *Cryptosporidium* not shared by other coccidia (except *Eimeria tenella*). Growth *in vitro* provides a very useful tool for the study of various aspects of the life cycle and the action of chemicals and drugs.

However it may prove to be of most value in studying, in a "neutral" host, speciation of the parasite and characterization of the subtle biological variations noted among *Cryptosporidium* strains. Propagation in cell culture unfortunately has not yet replaced the need for maintaining the parasite in animals. It is clear that the best source of oocysts is either experimentally infected calves, or chronically infected patients shedding large numbers in their stools. The reason is that *Cryptosporidium* undergoes only one developmental cycle in cell culture. The infection in cell culture (Fig. 19), chick embryos (Fig. 18) and, to some extent, mice, does not generate as many oocysts as does that in calves and other small ruminants (Figs 12, 13).

## VII. Treatment and Control

Prevention or treatment of cryptosporidiosis in humans and calves will undoubtedly be of benefit in some instances. Although the infection in humans and animals is usually self-limiting, effective treatment would be useful for the occasional outbreaks causing high morbidity and mortality in neonatal ruminants, and the occasional occurrence of severe clinical illness lasting several weeks in humans. Neither the incidence and severity of the disease in humans, nor the extent of economic losses in other animals, however, warrants the development of a vaccine, although, with improvements of methods of purification and cell culture propagation, this should be technically feasible. Nevertheless, cryptosporidiosis is a major problem in immunologically compromised humans.

Chemotherapy of the infection in humans and animals has been uniformly unsuccessful (Casemore *et al.*, 1985b; Anonymous, 1984a). A variety of antimicrobial agents has been tested in calves (Moon *et al.*, 1982b), mice (Tzipori *et al.*, 1982b), and piglets (Moon *et al.*, 1982a; Tzipori, 1985a) without success. In addition to various antiprotozoal, anthelmintic, and antimalarial drugs tested, several others (including anti-*Mycoplasma*, anti-*Treponema*, anti-viral and anti-tuberculosis agents, antihistamines, the "wonder" drug Avermectin, and many others) were ineffective in arresting the infection in experimentally infected piglets (Tzipori, 1985a); some modification of the infection was observed only with lectin. Casemore *et al.* (1985b), who summarized the literature concerning treatment of cryptosporidiosis in 60 immunocompromised patients, reported that treatment with spiramycin resulted in the recovery of four patients, and four others were said to have shown some benefit. The therapeutic value of spiramycin remains doubtful; at best it may benefit a few individuals (Portnoy *et al.*, 1984). In most cases, however, it has not been effective, nor did it prevent infection in cell culture (C. Chapman, personal communication).

At present, the testing of chemotherapeutic agents is largely empirical and has proved to be futile. In the future, as more information on the biology of the parasite becomes available, particularly from tissue culture studies, the development of "tailored" drugs directed against specific targets may be more effective.

Remission of cryptosporidiosis in a child with congenital agammaglobulinaemia, following treatment with hyperimmune bovine colostrum, was reported recently (Tzipori *et al.*, 1986). The child developed persistent vomiting and watery diarrhoea at the age of 3 years, and oocysts were detected repeatedly in his stools. Three weeks after admission to hospital he began treatment with 200 ml of bovine hyperimmune colostrum mixed with 800 ml of Digestalac®, given daily by nasogastric tube for 12 days followed by oral intake for a further 4 days. His vomiting, diarrhoea and the shedding of oocysts in stools ceased within a week of beginning treatment, and he has been free of infection for more than 6 months (unpublished data). The hyperimmune colostrum was prepared by immunizing two pregnant dairy cows, one with Percoll-purified whole oocysts (Waldman *et al.*, 1986) and one with sporozoites excysted, by treatment with trypsin and bile salts, from Percoll-purified oocysts. The cows were given an intramuscular injection of parasite antigen mixed with complete Freund's adjuvant, followed by an intramammary infusion 2 weeks later using incomplete adjuvant. A mixture of colostra from the 2 cows was used for therapy. It is impossible to be certain that the treatment with hyperimmune cow colostrum was responsible for elimination of the infection and the remission of illness, in this one case. However, in previously reported cases of cryptosporidiosis in individuals with hypogammaglobulinaemia (Lasser *et al.*, 1979; Sloper *et al.*, 1982), the infection persisted for several years. It remains to be seen whether this form of treatment will be effective in patients with T-lymphocyte dysfunction.

## VIII. Summary and Conclusions

In this review I have examined the vast literature which has accumulated on *Cryptosporidium*, particularly in the past 3 years, in an attempt to highlight areas in which progress has been made in relation to the organism and the disease, and to indicate areas in which knowledge is still lacking.

Since 1982, a global effort by scientists and clinicians has been directed towards determining the nature of the disease in humans and the relative contribution of cryptosporidiosis to gastroenteritis. From published data, the incidence of diarrhoea is 1–5% in most developed countries, and 4–7% in less developed countries, when measured throughout the year and in all age groups. The frequency of cryptosporidiosis is highest in children aged

between 6 months and 3 years, and in particular locations (e.g., day-care centres) and at particular times of the year. Although susceptibility to infection is life-long, one suspects that the lower prevalence among older children and adults is due to immunity acquired from frequent exposure. Other important factors contributing to higher prevalence are the season—it is more frequent in a wet, warm climate—association with travel to particular destinations, poor hygiene, intimate contact with certain animals, and congregation of large numbers of young previously unexposed children in day-care centres. The association between cryptosporidiosis and giardiasis presumably results from the existence of a common source of infection.

The immune status of the host appears to be a major determinant of whether the infection is self-limiting or persistent. It is clear that both branches of the immune system are required for complete recovery, since T-lymphocyte dysfunction or hypogammaglobulinaemia can both lead to persistent illness. Chronic diarrhoea and malabsorption attributed to cryptosporidiosis also occur in the absence of evidence of immune defect. The importance of respiratory tract infection in humans, other than in the terminal stages of chronic illness, requires investigation.

The infection has now been identified in all classes of vertebrates; it has been observed in all domestic animals including pets, and a wide range of wildlife including birds. Cryptosporidiosis seems to cause diarrhoea in young ruminants, less frequently in pets. In birds the parasite has been observed in the gastrointestinal tract, without ill effect, and in the respiratory tract, in which clinical symptoms of variable severity have been described.

The mucosal response of the gastrointestinal tract to infection appears to vary among mammals and may be the key to the variable clinical manifestations observed. In humans the major effect is hypersecretion induced by the parasite in the proximal small intestine, with minimal mucosal changes. It is suggested that enterotoxic or cytotoxic substances released by the parasite may be responsible. In young ruminants there is strong evidence of severe mucosal injury causing brush border damage leading to maldigestion in the proximal small intestine, in addition to reduced capacity for absorption. It is suggested that hypersensitivity or "allergic" reaction of the mucosa to parasite antigen may be responsible. Presumably it is unique to these young animals: examination of cells cultured *in vitro*, epithelium from experimentally infected rodents and human biopsy specimens reveals that *Cryptosporidium* causes little morphological damage to infected cells. The infection in rodents is largely subclinical, presumably because the proximal small intestine is not involved, as it is in humans, and the mucosal reaction is minimal, unlike that in ruminants.

Detailed studies on the life-cycle and the ultrastructure have provided a better understanding of the biology of the parasite and highlighted its unique

characteristics. Of these, the existence of sporulated, thin-walled oocysts and their independence of reducing conditions for excystation, is of major importance and may explain the occurrence of autoinfection and persistent infection. The possible existence of more than one species of *Cryptosporidium*, and of different strains remains to be determined, and cross-transmission studies between different classes of vertebrates should help to clarify this issue. However, while there may be more than one species, it is quite clear that the same, or closely related and interchangeable, species perpetuate infection among humans and their domestic animals.

Diagnostic procedures are still based on detection of oocysts in faecal smears, which can be confirmed by immunofluorescence. The development of serological tests is now feasible, as methods for concentration and purification of parasite antigen and propagation in cell culture are available.

Treatment of cryptosporidiosis has been disappointing so far, despite concerted efforts by medical and veterinary scientists. Spiramycin is the only drug which has been claimed to be of some benefit in the treatment of a few patients with persistent diarrhoea. The use of hyperimmune cow colostrum for treatment should be further explored.

## Acknowledgements

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# Parasites and Complement

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## I. Introduction

From the perspective of the parasitologist interested in the pathology and pathophysiology of parasite infections, one of the most exciting recent advances in immunology and biochemistry has been the emergence of a comprehensive account of the pathways that lead to the inflammatory response in mammals. As the complement cascade is a major contributor to acute and chronic inflammation, the isolation and characterization of the various complement proteins and their inhibitors has been particularly satisfying and revealing. This review focuses on the complement system, including the two main pathways to activation, the membrane attack sequence and the control mechanisms which operate under normal homeostatic conditions. The possibility of positive regulation of the host immune response by certain complement fragments, a new and rapidly growing

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research area, has also been addressed. After briefly defining the complement cascade, I will examine the effects which various parasites have had on these proteins. I hope that those who read this review will appreciate the almost limitless possibilities for parasite interaction with complement, in ways that may be beneficial or detrimental to the parasite's well-being.

Inflammation represents the sum of the host responses to injury (Ryan and Majno, 1977). The cardinal signs of increased blood flow to the affected area and the development of swelling and pain result largely from the biologic activities of the various systems of inflammation. These systems include the proteins of the complement cascade; the Hageman-factor dependent pathways to coagulation, kinin generation and fibrinolysis; the chemical mediators of mast cells/basophils; the products of the metabolism of arachidonic acid and exceeding active lipids such as platelet activating factor (PAF, AGEPC) (Leid and Potter, 1985). Activation and secretion of these mediators of inflammation lead to changes in the permeability of blood vessels, the tone of smooth muscle in blood vessel walls and the movement and secretory functions of cells that are recruited to participate from local or circulating pools.

Many physiologic processes involve enzymatic activation of plasma proteins that circulate as inactive precursors or zymogens and are activated by limited and specific proteolytic cleavage. Cleavage may be singular and give rise to one biologically active product, or cleavage may result in sequential multiple fragments, giving rise to several biologically active proteins and peptides. In the latter case, two such extremely important and critical pathways or cascades are the complement cascade and the Hageman-factor dependent pathways to coagulation, kinin generation and fibrinolysis. The reader is encouraged to examine several other reviews for analysis of the Hageman-factor dependent pathways (Leid and Williams, 1979; Sundsmo and Fair, 1983; Leid and Potter, 1985).

## ABBREVIATIONS USED

ATS = anti-thymocyte serum; CVF = cobra venom factor; EAC = erythrocytes coated with antibody and complement; IBS = immune bovine serum; ICS = immune canine serum; IGpS = immune guinea pig serum; IHS = immune human serum; IMS = immune mouse serum; IRS = immune rat serum; NBS = normal bovine serum; NCS = normal canine serum; NES = normal equine serum; NGpS = normal guinea pig serum; NHamS = normal hamster serum; NHS = normal human serum; NJS = normal jird serum; NMS = normal mouse serum; NRbS = normal rabbit serum; NRS = normal rat serum; PBC = peripheral blood cells; PEC = peritoneal exudate cells.

## II. The Complement Cascade

Isolation and characterization of the human complement proteins have proceeded quite rapidly over the past 10–15 years (Fearon and Austen, 1980; Muller-Eberhard and Schreiber, 1980; Hugli, 1981; Brown *et al.*, 1983; Hugli and Morgan, 1984). We currently understand much about the molecular interactions of the 20 or more proteins of the human and guinea-pig systems than similar reactions in the rabbit and rat. Little is known at present about the complement cascade in many of our domestic animal species. This latter defect is particularly critical to our veterinary parasitology colleagues because of the importance of parasitism to domestic animal health and well-being. In future years more information will become available on complement activation in domestic animal species, permitting us to better interpret the interactions between parasites of domestic animal importance and their respective host complement proteins.

The complement cascade can be activated by either the classical or alternative pathways, both of which have a common terminal portion designated as the membrane attack components, or MAC (Muller-Eberhard, 1986). The classical proteins are identified by the capital letter C, followed by a number from one to nine. Subunits are indicated by lower-case letters and the active form of each component is shown by a bar over the letter or number (that is, $C\overline{42}$). Proteins unique to the alternative pathway are identified by the capital letters B, D, P, I and H. Fragments of these proteins are identified by lower-case letters (that is Bb).

## III. Classical Pathway

The classical pathway is activated by the binding of C1q to the Fc portion of either IgM or IgG antibody molecules in immune complexes. This specific interaction results in C1r binding and activation, which in turn converts C1s to its active form, $C\overline{1s}$. This activation of C1s can also occur via proteolytic cleavage of native C1s by plasmin or trypsin (Leid and Potter, 1985; Perlmutter and Colton, 1986). $C\overline{1s}$, alone or as the trimolecular complex ($C1\overline{qrs}$), cleaves C4 and C2 to form the active enzyme $C\overline{42}$. One of the minor cleavage fragments of C4 activation is C4a. The $C\overline{42}$ enzyme cleaves a major plasma protein, C3, into the large fragment, C3b, and a small peptide, C3a. The C3b binds to the $C\overline{42}$ enzyme and alters its specificity such that C5 is now cleaved into a major fragment, C5b, and a small peptide, C5a. The C5b binds to the $C\overline{423}$ enzyme and initiates the nonenzymatic and noncovalent assembly of the five components of the membrane attack sequence (C5b6789).

The peptides C3a, C4a and C5a are very closely related, as deduced from

their primary structures, and are referred to collectively as anaphylatoxins (Hugli, 1981). These peptides have many potent biologic activities that contribute to the development of acute inflammation. The three anaphylatoxins increase vascular permeability, contract smooth muscle and release mediators from mast cells and basophils. C5a and C5a minus its C-terminal arginine, $C5a_{des-arg}$, are potent chemotactic molecules and are probably responsible for much of the neutrophil chemotactic activity in plasma (Hugli and Morgan, 1984). C5a can also induce the chemotactically responding cells to metabolize arachidonic acid, giving rise to phlogistic metabolites or to molecules with pronounced down-regulatory effects. C3b, which is the major cleavage fragment of C3, promotes the adherence and/or subsequent phagocytosis of particles to cells bearing the C3b/C4b immune adherence or complement 1 (CR1) receptor. The bound C3b is cleaved by the C3b/C4b inactivator or I into a fluid phase fragment, C3c, and a bound C3d-g fragment. The C3d-g fragment is cleaved further, with the release of the peptide into the fluid phase. C3d and C3d-g have specific receptors on the cells, termed CR2 and CR3 complement receptors, respectively (Ross, 1982; Fearon and Wong, 1984).

The C3a and C3b molecules can also be generated by proteolytic cleavage of native C3 with the enzymes plasmin and trypsin. This activation scheme leads to the generation of important and potent biologic activities from complement proteins without sequential activation, and permits anaphylatoxin generation through activation of the Hageman-factor dependent systems. These latter anaphylatoxins possess many of the same biologic properties expressed by the peptides generated from alternative or classical pathway activation.

## IV. Alternative Pathway

The alternative pathway (C3, B, D and P) is activated by particles or surfaces that permit uncontrolled expression and amplification of alternative pathway enzymes (Fearon and Austen, 1980; Muller-Eberhard and Schreiber, 1980). Under normal conditions, the alternative pathway C3 converting enzyme or convertase, C3bBb, is constantly formed by binding of B to C3, the initial cleavage of C3B to C3Bb by D, followed by generation of C3b fragments from the C3 by the $\overline{C3Bb}$. Subsequent C3bBb formation results from binding of B to the C3b generated by $\overline{C3Bb}$, and formation of the relatively poor C3 convertase, $\overline{C3bB}$. D cleavage of the bound B occurs rapidly, resulting in the very active alternative pathway convertase, $\overline{C3bBb}$. The alternative pathway C3 convertase is quickly inactivated by either intrinsic decay of the sequential action of the C3b/C4b inactivator (I) and $\beta_1$-H (H) (Fearon and Austen,

1980; Muller-Eberhard and Schreiber, 1980). Properdin, or P, stabilizes the $\overline{C3bBb}$ enzyme and slows down its endogenous decay. A particle that is an alternative pathway activator permits the $\overline{C3bBb}$ enzyme to be formed on its surface but does not permit either I or H ready access to the active enzyme. Lack of access for the control proteins allows amplification of C3 cleavage and the generation of increased substrate around which the membrane attack complex can assemble.

Alternative pathway activation therefore, is quite distinct from classical pathway activation. Alternative pathway activation is generally considered to be antibody-independent, although antibody can enhance activation (Ratnoff *et al.*, 1983). Most importantly, activation represents escape from host control mechanisms rather than activation of zymogens, as is the rule for classical pathway activation.

Recent work has shown that the anaphylatoxins or the fragments of C3 and C5 can have marked host immunoregulatory function (Egwang and Befus, 1984; Hugli and Morgan, 1984). Anaphylatoxin regulation of the immune responses will be a particularly important area of research in the coming years. Parasites which can inhibit or interfere with the control proteins I and H of the alternative pathway, C4 binding protein (C4Bp) and C1 inhibitor (C1inh) of the classical pathway, would lead to marked effects on the host and parasite. The alternative pathway of complement seems to be a critical part of the first line of the host response to microbiologic and parasitologic invasion (Fearon and Austen, 1980; Brown *et al.*, 1983; Joiner *et al.*, 1984). The parasitic, bacterial and viral challenges with which domestic animals must cope on a daily basis are extensive. The maximal host protective potential of the complement cascade may be attained under intensive Darwinian selection pressure, as experienced by cattle, sheep, goats and horses in paddocks and on pastures every day. Agents which generate the anaphylatoxins, C3a, C4a and C5a, either through direct proteolysis or by presenting a surface for uncontrolled activation, would have serious consequences for the host.

## V. Parasites and Complement

I have elected to review the literature from 1980 until the present, as several reviews (Leid and Williams, 1979; Santoro *et al.*, 1979) covered the subject in depth prior to 1980. Moreover, I have limited the papers covered to those in which complement effects were clearly documented. I have focused on the quantitation of specific complement components during the course of various parasitic diseases *in vivo* or after interaction of animal sera with the parasite *in vitro*. In recent years, detection and quantitation of specific

complement components in an array of disease states has increased markedly. The vast majority of the literature which I have included has focused almost exclusively on the detrimental effects of complement activation upon parasite survival. Few reports have attempted to elucidate conditions in which parasites may use the complement system to their advantage, and Befus and Bienenstock (1982) have noted the lack of studies for interactions at the mucosal surface. A previous review (Leid, 1982) noted that Greenblatt *et al.* (1979) had pointed out the advantage of the complement system for the successful transformation from cercaria to schistosomulum of *Schistosoma mansoni*. Such lines of investigation in parasite immunology and biochemistry need to be pursued, and should form the basis for another review in several years.

For ease of reading and understanding, the review is divided into five major sections; (A) Protozoa, (B) Trematoda, (C) Cestoda, (D) Nematoda and (E) Arthropoda.

## A. PROTOZOA

Investigations on protozoan parasites and their interaction with the complement system have been very prolific in the past 7 years. The availability of methods to quantitate human complement components has permitted basic studies on parasites of human importance. In particular, quantitation of specific complement components has allowed a more detailed picture of the host–parasite interaction to emerge.

### 1. *Trypanosomiasis*

Rickman and Cox (1980), investigating *Trypanosoma brucei rhodesiense* infections, found that $CH_{50}$ titers plummeted during the first week to 10 days after infection, while the immunoconglutinin titers increased, apparently inversely related to the drop in hemolytic titers. Lindsley *et al.* (1981) investigated this infection in rabbits and found a significant increase in $CH_{50}$ levels during the first week of infection, a decrease to normal by the end of the second week, and a further decrease to one-half of normal levels by the end of the fourth week. Furthermore, rabbit C3 was present in six of 11 immune complexes detected by the C1q solid phase assay, and in all of 11 immune complexes precipitated by polyethylene glycol. Shirazi *et al.* (1980) followed the levels of C3 during the course of *T. brucei* infections in mice that were either untreated, irradiated and reconstituted, T-cell deprived, or T-cell deprived and CVF treated. In the first three groups, C3 levels rose to two to three times normal from the first to third weeks and then returned to normal.

The fourth group had < 10% of normal C3 levels during the first to third weeks, with C3 levels returning to normal thereafter. Greenwood and Whittle (1980) noted that both the alternative (AP) and the classical (CP) pathways were activated in sleeping sickness, with concomitant decreased levels of C3, C4 and factor B. Macaskill *et al.* (1980), using C3- and C5-deficient mice, showed that the clearance of *T. brucei* was normal with or without the utilization of hyperimmune sera. Lambert *et al.* (1981) showed a significant decrease in 67% of the samples from 36 patients with trypanosomiasis. C3 levels were quantitated in the serum and CSF from these patients; the mean C3 values were 62.9% of normal. This decrease suggests that complement activation occurred both peripherally and centrally within the CSF. Devine *et al.* (1986) have shown that bloodstream forms of *T. brucei gambiense* activated the alternative pathway in NHS, and they detected deposition of both C3 and factor B on the parasite's surface. Both C3 and B binding was inhibited by EDTA but not by EGTA. More importantly, C5 and C9 deposition was not observed, nor was the assembly of the membrane attack complex detected in the fluid phase around the parasite. No IgG was detected on the parasite membrane as well, and the binding of C3 was enhanced by factor B, D and $Mg^{2+}$ ions. These results would suggest that even though the C3 convertase could be assembled on the parasite surface, the enzyme was rapidly inactivated by I and H, although this remains to be proven. More studies need to follow the approach of these investigators in the exploration of the molecular events surrounding complement activation and inhibition at the parasite membrane/host interface.

Scharfstein *et al.* (1982) observed no change in C3 levels in mice infected with *T. cruzi* until day 9 PI (post infection) when C3 levels were 94% of normal. Levels dropped to 35% below normal values on day 11 PI and returned to normal concentrations by day 13 PI. No changes were observed in C4 levels other than a slight increase on day 13 PI (19%). Dalmasso and Jarvinen (1980) investigated *T. cruzi* infections in C5-deficient mice and C4-deficient guinea pigs. In both cases there was little or no difference between the deficient animals and their normal counterparts, with respect to levels of parasitemia or time to peak parasitemia. Rodriguez *et al.* (1981) compared *T. cruzi* infections in rats which were anti-mu treated to suppress antibody formation to untreated rats. Rat C4 levels did not change in anti-mu treated rats, while in normal rats C4 levels decreased to about 65% of normal. However, $AP_{50}$ levels diminished in both groups of rats, with a peak reduction to 50% of normal by 6 weeks PI. Cunningham *et al.* (1981) noted that $CH_{50}$ levels decreased to < 10% of normal at 35–45 days PI, and returned to 90% of normal by day 65 PI. These investigators also detected a soluble factor released from trypanomastigotes which decomplemented human and guinea pig sera within minutes, reaching maximum depressions

30 minutes after the start of incubation. This factor was heat stable and insensitive to trypsin digestion, suggesting a polysaccharide. This factor also resulted in the decomplementation of normal animals *in vivo*. Kierszenbaum *et al.* (1981) investigated *T. cruzi* infections in birds. Trypanomastigotes were lysed in agammaglobulinemic chicken sera and normal chicken sera. The lytic titers were the same or nearly so in both serum sources. This lytic activity was inhibited if the sera was heated, or treated with EDTA or CVF prior to incubation with the parasites. EGTA-treated chicken sera were as lytic as normal untreated sera, while decomplemented chicken sera were markedly impaired in lytic activity. Normal or agammaglobulinemic chicks cleared an IV parasite load within 7 minutes, but the complement depleted chicks required 1740 minutes or longer to clear the parasites. No host immunoglobulin was detectable on the parasite surface. Molina *et al.* (1984) reported the deposition of C3 on the endocardium, interstitium, vascular walls and myocardial fiber surfaces of *T. cruzi* infected humans.

Banks (1980), working with *T. congolense* infections in cattle, showed $^{51}Cr$ release from bovine erythrocytes in the presence of trypanosomes and immune bovine sera. Heat-inactivated IBS or NBS were without effect. Tizard *et al.* (1980) investigated immunoconglutinin and C3 levels in *T. congolense* infected cattle. These investigators found that immunoconglutinin levels were normal or only slightly elevated, but that concurrent trypanosome infection inhibited any immunoconglutinin rises due to *Brucella abortus* vaccinations. C3 levels in trypanosome-infected cattle were significantly depressed around day 20 PI (67% of normal). Rurangirwa *et al.* (1980) followed both *T. congolense* with *T. vivax* infections in cattle, and reported that C3 levels began to drop by 10 days PI and remained low until day 37 PI, when the animals were cured of the infections by the drug Berenil. The depressed C3 levels rose slowly to normal. $CH_{50}$ titers paralleled the C3 antigenic quantification, going from 7 $\log_2$ units per ml to 2.5 $\log_2$ units per ml over the 37 days of infection. Ferrante and Allison (1983) showed that a mutant of *T. congolense* lacking a surface variant specific glycoprotein (VSG), a procyclic form of *T. congolense* and a *T. brucei brucei* procyclic form without a VSG were all alternative pathway activators. The coated procyclic form of *T. b. brucei* was not an alternative pathway activator. Lysis of the parasites did not occur in normal human serum (NHS) heated at 56°C for 30 minutes nor in NHS made 10 mM in EDTA. Lysis did occur in NHS made 10mM in EGTA and in C2-deficient sera. Malu and Tabel (1986) noted a marked depression of C3, factor B and $CH_{50}$ titers after *T. congolense* infections in cattle. Following drug treatment, C3 recovered very slowly. while factor B and $CH_{50}$ returned to normal levels quickly. Tabel (1982) showed that homogenates of *T. congolense* activated the bovine alternative pathway with the cleavage of factor B shown by electrophoretic conversion.

Ferrante (1984) observed that *T. musculi* was lysed in normal human, cattle, sheep, rabbit, guinea pig and rat sera, but was not lysed in normal mouse sera. Treatment of lytic positive sera with EDTA or heating abolished lysis, while EGTA ± $Mg^{2+}$ ions resulted in normal lysis. C2-deficient sera lysed the parasites. Incubations of trypanosomes with NHS resulted in the electrophoretic conversion of C3. Addition of normal mouse serum (NMS) to NHS or normal bovine serum (NBS) inhibited lysis of the parasites, while addition of NMS to normal rat serum (NRS) still resulted in parasite lysis. C6-deficient normal rabbit serum (NRbS) did not lyse the trypanosomes. Wechsler and Kongshavn (1985) found that cobra venom factor (CVF) treatment of mice depleted circulating C3 levels but did not interfere with parasite replication; nor did CVF treatment inhibit parasite elimination. Albright and Albright (1985), using *T. lewisi* infections in mice, showed that there was no difference in natural resistance mechanisms between C5-deficient or C5-sufficient mice. Treatment of recipient mice with CVF prolonged the subsequent survival times of the parasite. CVF and silica pretreatment of mice prior to *T. lewisi* challenges resulted in the death of the mice. No evidence of C3 deposition on the surface of the parasite was obtained.

2. *Leishmaniasis*

Agu *et al.* (1981a,b) showed that for *Leishmania donovani* infections in hamsters a > 1 SD of the mean elevation in C3 levels was observed from the third to ninth week of infection. Factor B was also increased > 1 SD from the third week of the infection until the end of the experiment (106 days). C3 was also detected in the urine of some infected animals in the ninth and tenth weeks of the infections. Moreover, C3 deposition was detected in the kidney glomeruli during the fifth week of the infection. Franke *et al.* (1985) showed that promastigotes of *L. donovani* obtained from log phase cultures were always killed in NHS, while organisms from stationary cultures were either resistant to lysis by NHS (*L. braziliensis panamensis*), or slightly resistant (*L. donovani*), or not resistant (*L. major*). *L. b. panamensis* was killed in NHS, but not in EDTA or heat-treated sera. EGTA pretreatment of NHS allowed killing to be observed. However, no C3 deposition on the parasite was detected by immunofluorescence. Mosser *et al.* (1986) observed that promastigotes of *L. major*, *L. donovani*, *L. mexicana mexicanis*, *L. m. amazonensis* and *L. braziliensis guyanenis* were lysed in NHS or normal guinea pig serum (NGpS). C8-deficient sera did not result in lysis of any of the parasites. *L. major*, *L. m. mexicanis* and *L. braziliensis* lysed in Mg-EGTA treated sera or in C4-deficient NGpS. A small amount of C4 was noted to be consumed, however. *L. donovani* activated the classical pathway and $Ca^{2+}$ ions were

required. $^{125}$I-C3 was bound to the promastigotes of *L. major* and C3 was detected on the parasite surface by immunofluorescence using monoclonal antibodies. *L. major* promastigotes consumed 32% of the C4 available, while *L. donovani* promastigotes consumed 90% of the available C4. The classical pathway activator, the antibody-sensitized SRBC, consumed only 29% of the C4. Pearson and Steigbigel (1980) also showed that promastigotes of *L. donovani* were killed by NHS. $Mg^{2+}$-EGTA treatment of NHS blocked killing and C2-deficient sera did not kill the parasites. However, if exogenous C2 were added to the incubation mixtures the parasites were destroyed. Heat treatment (56°C for 30 minutes) inhibited lysis by NHS. Mosser *et al.* (1985) determined that amastigotes of *L. major*, *L. mexicana mexicanis*, *L. m. amazonensis* and *L. donovani* were killed in NHS. The killing of amastigotes ranged from 5% to 85%, with *L. donovani* the most resistant to NHS, *L. m. mexicanis* the next most resistant, while all the other strains were susceptible. *L. major* bound $6.8 \times 10^4$ C3 molecules per amastigote, *L. donovani* $4.1 \times 10^4$, *L. m. mexicanis* $6.2 \times 10^4$, *L. m. amazonensis* $4.2 \times 10^4$ and *L. major* $6.5 \times 10^4$. Kager *et al.* (1982) studied kala azar in 12 human patients and found C1q levels to be elevated five- to seven-fold, while C1s levels remained normal to slightly depressed. C1q levels decreased after drug treatment, but C3, C4 and C9 levels were highly variable, with no clear pattern of either decrease or increase. Haldar *et al.* (1981) came to similar conclusions, but in their case only C3 levels were quantitated and this complement protein did not significantly differ from control values. An earlier study (Ghose *et al.*, 1980), which involved 49 kala azar patients and 47 controls, showed a significant decrease in C3 levels with 0.90 mg $ml^{-1}$ of C3 in the patients and 1.47 mg $ml^{-1}$ in the controls. The C3 decrease appeared to be independent of either immunoglobulin or specific antibody titers. Hoover *et al.* (1985) confirmed that amastigotes were killed by the alternative pathway. NHS was cytotoxic at $\geqslant 1$ in 20 dilutions. Treatment of NHS with EDTA or heat (56°C for 30 minutes) rendered the NHS noncytotoxic. Treatment of NHS with $Mg^{2+}$-EGTA or the use of C2-deficient sera resulted in the death of the amastigotes. Immunoglobulin from sera of patients with kala azar enhanced killing by the alternative pathway by three- to five-fold. Ridley and Ridley (1984) investigated cutaneous leishmaniasis and found that the complement proteins C3, C1q and C3d were present in high levels extracellularly, following necrosis of the skin lesions. The presence in these lesions of C4 was minimal.

### 3. *Giardiasis*

Hill *et al.* (1984) investigated the killing of *Giarda lamblia* trophozoites by NHS. A dose-dependent killing was observed, with cytotoxicity varying from

8 to 76%. Heat treatment (56°C for 30 minutes) of NHS inhibited killing, as did pretreatment of NHS with EDTA or $Mg^{2+}$-EGTA. C2-deficient sera did not kill, and C3 was detected on the parasite surface by immunofluorescence. Trophozoites that were killed or were treated with trypsin or neuraminidase activated the AP, consumed B and had C3 deposited on their surfaces.

4. *Amoebiasis*

Ghadirian and Meerovitch (1982) treated recipient hamsters with CVF followed by challenge with *Entamoeba histolytica*. These animals had a marked increase in pathology, as evidenced by reduced survival time, increased loss of body weight, larger liver abscesses and amoebic metastatic foci. Reed *et al.* (1983) showed that five nonpathogenic strains of *E. histolytica* were susceptible to lysis by NHS, while nine of 11 pathogenic strains were resistant to lysis. Moreover, five of these resistant strains were isolated from liver abscesses. Meri *et al.* (1985) showed that incubation of amoeba with NHS resulted in consumption of C3, C4 and factor B. Treatment of NHS with EDTA blocked consumption of these three components, while treatment of NHS with $Mg^{2+}$-EGTA resulted in C3 and factor B consumption only. Calderon and Schreiber (1985) showed that C3b was deposited on the trophozoites, and that this deposition did not depend upon specific antibody. Heat treatment decreased C3b binding by five-fold ($65.6 \times 10^6$ molecules per parasite vs $13.5 \times 10^6$ molecules per parasite). The kinetics of C3b binding was very rapid, plateauing within 10 minutes. EGTA-treated NHS mediated lysis, but this lysis was slower and reached a lower peak (60% of untreated NHS).

Reilly *et al.* (1983) reported no difference in $CH_{50}$ titers between *Naegleria fowleri* infected and control mice. However, treatment of recipient mice prior to parasite challenge with CVF to deplete complement resulted in increased death rates, and these deaths occurred sooner. This was true for two challenge doses of amoeba given ($1 \times 10^5$ and $5 \times 10^4$), but not for a third challenge dose of $1 \times 10^4$ parasites.

5. *Cryptosporidiosis*

Stemmerman *et al.* (1980) reported on one patient with a *Cryptosporidium* infection and found a low normal level of C4, a normal level for C3, a low $CH_{50}$ titer and a low level of C3 activator or factor B.

6. *Malaria*

Ade-Serrano *et al.* (1981) investigated *Plasmodium falciparum* infections in

42 children with gametocytemia, 49 children without gametocytemia and 50 children who did not have malaria. The children with gametocytemia had 72% of normal C3 levels, 149% of C4 and 65% of C3b. The children without gametocytemia had 50% of normal C3 levels, 45% of C4 and 92% of C3b. Ward *et al.* (1981) showed that CVF-treated rats that were challenged with *P. berghei* had twice the number of infected rbc's. Rbc infectivity by *P. falciparum* parasites was not impaired using C2-, C3-, C4- or C5-deficient sera. The malarial parasites appeared not to utilize the complement system for invading rbc's. Abdalla and Weatherall (1982) investigated the presence of complement components on rbc's by the direct antiglobulin test (DAT). In 134 Gambian children, of which 52 were positive by DAT, 25 had C3 only on their rbc's, while 13 had both IgC and C3. C4 was rarely present on the rbc. Gupta *et al.* (1982) showed that all malarial patients had significantly decreased C3 and $CH_{50}$ titers. Vergani *et al.* (1983) showed that malaria patients had markedly elevated levels of C3d present, indicating widespread complement activation. The four malaria patients studied had from 14.5 to 37 C3d units per ml when the upper limit of normal was 6 units per ml. Stanley *et al.* (1984) studied the surface of *P. falciparum* infected erythrocytes. Trophozoite-infected rbc's activated the CP when using IHS. Significant quantities of C3b accumulated on infected rbc's during development of the parasite to the trophozoite stage. As the trophozoites developed in infected rbc's, the rbc's became an alternative pathway activator with up to 15000 C3b molecules deposited per rbc. In this case, the AP was augmented by the presence of specific antibody, and in the presence of specific antibody up to 36000 C3b molecules per rbc were detected. These amounts of C3b deposition did not lead to rbc lysis, however. Drouin *et al.* (1985) investigated a patient with both a *P. falciparum* infection and a hemolytic anemia. C3 levels were within normal ranges and no C3b, C3c or C4 was detected on the rbcs. Only IgG was detectable on the rbc surface, suggesting this was a noncomplement-mediated hemolytic anemia. Chakrabarty *et al.* (1985) investigated the possibility of chloroquine as a complement activator. Chloroquine interaction with NHS led to C3 and factor B cleavage, cleavages which did not require $Ca^{2+}$ or $Mg^{2+}$ ions. However, individual complement components did not alter significantly in normal human volunteers given the drug. In four patients with *P. falciparum* infections who were given the drug, marked decreases in C3 and factor B were observed. C4, C1q and properdin levels were normal or slightly elevated. These facts suggest that widespread complement activation can occur after antiparasitic drug therapy, and must be considered in treatment of the patient. Phanuphak *et al.* (1985) observed depressed C3 levels in 183 patients, while C1q, C4 and factor B levels were slightly elevated. There appeared to be no activation of the alternative pathway in an acute malarial crisis.

Salmon *et al.* (1986) examined the possible role of neutrophils in *P. falciparum* killing. Chemiluminescence observed after interaction of neutrophils and infected rbc's was positively correlated with the level of parasitemia ($r = 0.94$). In the presence of immune human serum (IHS), neutrophil chemiluminescence increased greater than three-fold. This increase was not observed in EGTA treated IHS or in IHS treated at 56°C for 30 minutes. Heating at 50°C for 15 minutes to inactivate factor B resulted in a slight reduction in chemiluminescence. Fandeur *et al.* (1984) investigated *P. falciparum* infections in Saimiri monkeys. They found that fresh NHS plus monkey antibodies to the parasite increased the number of parasites in human rbc's. However, fresh NHS plus monkey antibodies to the parasite had no effect on the number of parasites in the monkey rbc's.

Ganguly *et al.* (1980) quantitated C3 levels in *P. vivax* infected patients. C3 levels in all malarial cases including acute cases were similar to that observed in the control patients.

Contreras *et al.* (1980) investigated *P. berghei* infections in mice, and found a decrease in C3 levels over the course of an infection in four of five of the strains examined, the only exception being the CBA mouse. The four mouse strains which had decreased C3 levels had $<20\%$ of normal C3 detectable between the 9–12 days PI. This depression appeared to be correlated to the increase in circulating immune complex levels. Five of six nude mice had normal levels of C3 throughout the course of the infection, while their euthymic controls had 56.9% of normal levels. Pappas *et al.* (1981) noticed a decreased clearance of antibody-sensitized rbc's in *P. berghei* infected mice at 14 and 21 days PI. However, when these same cells were sensitized with complement *in vitro* and then given to recipient animals, the clearance was normal. Unsensitized rbc's or rbc's sensitized with complement alone were cleared in the same time period in both infected and noninfected mice. Finley *et al.* (1982) showed that although nude mice infected with *P. berghei* had lower levels of circulating immune complexes and total IgG, anemia developed more slowly in the nude mice, with a smaller decrease in the C3 levels (75% of normal for nu/nu versus 25 % of normal for the euthymic controls). However, Pakasa *et al.* (1985) showed the presence of C3 antigen in the glomeruli of *P. berghei* infected mice.

Cox *et al.* (1983) investigated *P. chaubaudi* infections in rats and found that $CH_{50}$ titers were markedly depressed between days 7–9 PI. The immunoconglutinin titers were elevated during the same time period. DeGraves and Cox (1983) showed that $CH_{50}$ titers went from 256 to 28 on day 10 PI and were almost back to normal on day 35 PI. Immunoconglutinin levels were a mirror image, with a peak titer of 640 on day 8 PI and a return to normal titers of 0 on day 35 PI. Delvinquier *et al.* (1984) showed the deposition of C3 in the glomeruli of *P. chaubaudi* infected mice. This deposition started about

day 7 PI and carried through 86 days PI, with the peak of C3 deposition occurring between days 22–29 PI.

7. *Babesiosis*

Jack and Ward (1980a,b) have investigated the role of complement in assisting the invasion of the host rbc by *Babesia rodhaini*. These investigators found that C5-, C6- or C8-deficient sera did not inhibit infection of rbc's, but C3-deficient sera did. C2-deficient sera inhibited rbc infections slightly. NHS to which EDTA was added strongly inhibited rbc invasion by the parasites. *B. rodhaini* parasites consumed complement components present in NHS in a dose-dependent manner, and there was an electrophoretic conversion of both C3 and factor B in the presence of EGTA, but not EDTA. The parasites were found to bind radiolabelled C3, C5 and properdin. The use of trypan blue inhibited rbc infections through inhibition of the C3b receptor. Jack and Ward (1980b) also found that cobra venom factor (CVF) pretreatment of rats markedly suppressed the subsequent parasitemias in animals later challenged with *B. rodhaini*, but normal parasitemia levels were again evident with the return of C3 levels to normal. Rats given trypan blue also had reduced parasitemias. Trypan blue blocked the uptake of radiolabelled C3 by *B. rodhaini* and suramin pretreatment enhanced infectivity, presumably through inhibition of I or the C3b inactivator. The infected rbc's were Coombs positive within 2 days PI. Ward and Jack (1981) also showed that the parasite itself possesses a C3b receptor.

Goodger *et al.* (1981a,b) examined *B. bovis* infections in cattle. They detected C3 in all circulating immune complexes precipitated by polyethylene glycol. Circulating C3 levels remained normal until day 5 PI, then entered a period of decline, reaching 68% of normal levels at day 13 PI and 46% of normal levels at day 19 PI. Conglutinin levels remained normal until day 5 PI (titer of 4076), then began a decline which bottomed out at day 9 PI (titer of 128), and by day 19 PI were back to normal levels. Splenectomized cattle showed an eight-fold decrease in conglutinin levels. Immunoconglutinin was detected in all plasma samples obtained 18 days PI. Benach *et al.* (1982) investigated acute cases of babesiosis in humans and found decreased levels of C3 and C4 in the acute phase sera. In addition, the $CH_{50}$ titers in these patients were depressed. An increase in C1q binding appeared to be unrelated to the polyclonal hypergammaglobulinemia observed in the same animals.

8. *Theileriosis*

Maxie *et al.* (1982) investigated *Theileria parva* and *T. lawrencei* infections in

cattle. These investigators found no significant changes in the circulating $CH_{50}$ titers in either infection. Shitakha *et al.* (1983) found that, in *T. parva* infections of cattle, the $CH_{50}$ titers remained at 100 units per ml in animals which survived the infections, while in animals which died, the levels were $<40$ units per ml. In recovered animals the C3 levels plateaued at 60% of normal, while in animals which died in $<3$ weeks the levels were 50% of normal, and in animals which died in $>3$ weeks the C3 levels plummeted to $<25\%$ of normal. This latter group indicates a continuing consumption of C3, a depressed synthesis of the complement protein, or both.

### 9. *Toxoplasmosis*

Reid *et al.* (1982) investigated *Toxoplasma gondii* infections in sheep and lambs. These workers found that C3 levels were reduced about 10% as a result of the *T. gondii* infection. Whether such a depression is biologically significant remains to be answered.

### 10. *Sarcocystosis*

Mahaffey *et al.* (1986) investigated *Sarcocystis cruzi* infections in cattle. These investigators used a direct antiglobulin test for the detection of C3 on the rbc. These tests were uniformly negative in all cattle tested. However, one of the animals tested had a slightly positive Coombs test during the third and fourth weeks of infection.

## B. TREMATODA

Although the vast majority of the investigations on complement interactions with trematodes have focused on schistosomiasis, other important trematode infections are beginning to be studied. The results of the work on schistosomes has led not only to a detailed account of the parasite and its host–parasite relationship, but also to basic findings concerning the immunopathology of complement proteins and their roles in other diseases.

### 1. *Opisthorchiasis*

Sirisinha *et al.* (1986) studied complement-mediated killing of *Opisthorchis viverrini*. These investigators found that NHS lysed the adult parasites, while sera heated at 56°C for 30 minutes did not. NHS treatment with 1 mM EDTA inhibited killing, but treatment with 1 mM EGTA did not. NHS, which had been heated at 50°C for 15 minutes, did not kill the parasite, but

factor B reconstitution of such sera did restore the helminthicidal activity. NHS serum treated with zymosan either did not kill the parasites or there was a dramatic reduction in the killing observed. Absorption of NHS with an anti-C4 antisera did not reduce killing, while absorption with an anti-factor B antisera did reduce the killing capacity of the NHS. The use of NRS, NRbS, NGpS, NHamS and IHamS in the assay led to parasite death.

2. *Fascioliasis*

Duffus and Franks (1980) showed that bovine antibodies in the presence of bovine complement did not result in damage or death of the newly excysted juvenile flukes (NEJ) of *Fasciola hepatica*. Davies and Goose (1981) investigated *F. hepatica* infections in rats and found no evidence of C3 deposition on the NEJ either after incubations in rat sera or after injection of the NEJ into the peritoneal cavity of recipient rats. Montgomery *et al.* (1986) observed that extracts of adult worms of *F. hepatica* can activate both the classical and alternative pathways of bovine and human complement. There was no evidence of complement activation when NEJ were incubated in IBS.

3. *Schistosomiasis*

Samuelson *et al.* (1980) showed that schistosomula of *Schistosoma mansoni* bound rat C3, but this binding did not result in capping or patching of the bound ligand. Heat-inactivated serum showed no binding of C3 ($< 5\%$). The binding of C3 was time-dependent, reaching a maximum within 1–2 hours. A reduction in binding over time was observed, as 50% of the label was removed by 3 hours after initiation of the incubation. This *in vitro* incubation mimicked the *in vivo* situation, as 100% binding was observed at 3 hours, 9% at 48 hours and 5% at 96 hours. Capron *et al.* (1981) showed that heat-inactivated NRS or IRS was half as effective at killing schistosomula when obtained up to 10 weeks PI. Treatment of 8 weeks IRS with 10 or 100 mM EDTA or EGTA did not decrease the helminthocidal activity of the sera.

Dean (1983) reviewed acquired resistance in schistosome-infected mice. Pepys *et al.* (1980) detected an elevation of C3 which occurred around day 44 PI in *S. mansoni* infected mice. C3 levels peaked at twice the control values and remained there until the infection was terminated. T-cell deficient mice did not give evidence of this rise, while mice that were thymectomized only and not given anti-thymocyte serum (ATS) for immunosuppression still had the same C3 pattern of elevation. Tarleton and Kemp (1981) showed the presence of C3 receptors on adult worms of *S. mansoni*. Van Marck *et al.* (1981) investigated the possibility of C3 deposition in the glomeruli of mice infected with *S. mansoni*. C3 was detected from the seventh to thirty-third

week PI and the C3 deposited appeared to parallel the increased deposition of IgG. Bickle and Ford (1982) showed a marked decrease in cell adherence to schistosomula of *S. mansoni* after skin penetration, either *in vitro* or *in vivo*, when compared to mechanically prepared schistosomula. Schistosomula obtained from the lungs of infected mice were relatively resistant to both complement and eosinophil-mediated killing, although mechanically derived schistosomula were still sensitive to lysis. Even in the mechanically derived schistosomula, surface activation and consumption of complement decreased markedly with time. Sher *et al.* (1982) showed that C5-deficient and CVF-treated mice, which were vaccinated and challenged 4 weeks later with *S. mansoni*, were equally resistant to challenge compared to untreated controls with respect to worm burdens. Santoro *et al.* (1982) on the other hand, found CVF treatment of mice led to an approximate doubling of the worm burdens. Injections of C3 2 hours prior to infection restored the resistance in the CVF treated mice to levels observed in untreated mice. C1q by itself was without effect, while C1q and C3 were effective. These same investigators confirmed the work of Sher and colleagues (1982), showing no difference in worm burdens between C5-deficient and C5-sufficient animals. Ruppel *et al.* (1982) gave further confirmation of the lack of difference in worm burdens in these latter two mouse strains.

Anwar *et al.* (1980) showed that the mast cell derived ECF-A tetrapeptides enhanced complement dependent eosinophil-mediated schistosomula killing by an increase of C3b receptors on the eosinophil. De Simone *et al.* (1986) found that eosinophils obtained from patients with filariasis, trichinosis, schistosomiasis or hookworm infections became markedly cytotoxic when incubated with zymogen-activated serum. This cytotoxicity was mediated through oxygen-derived products of the eosinophil. Young and Podesta (1984) investigated choline uptake and incorporation in *S. mansoni* adults and found that, in part, the process of this lipid's incorporation was complement-dependent. Choline incorporation increased whether or not the host was a permissive one and this stimulation of incorporation did not occur through increased uptake. The increase appeared to be mediated through the classical pathway, as NHS heated at 50°C to destroy the factor B did not impair the incorporation, but NHS heated to 56°C had little to no activity. NHS heated at 56°C, but given fresh C3 and C4, was almost as active as untreated NHS. This is the first evidence that parasites can use the complement system to their advantage in incorporating biochemical nutrients.

Fatima *et al.* (1984) investigated complement-mediated killing of *S. mansoni* schistosomula *in vitro* and *in vivo*. Rat $IgG_{a+b}$ antibodies plus complement were lethal both *in vitro* and *in vivo*. The killing required antibody as the complement was not lethal on its own. Carvalho-Castro *et al.* (1984) found in patients with schistosomiasis complicated by renal involve-

ment, approximately one-third the C3 levels of those patients without renal involvement (60mg $dl^{-1}$ vs 165mg $dl^{-1}$, respectively). Schistosomiasis patients with secondary *Salmonella* infections and with or without renal involvement had even lower C3 levels (32mg $dl^{-1}$ vs 74mg $dl^{-1}$, respectively). Santoro *et al.* (1980a) showed the binding of C1q to schistosomula of *S. mansoni* via a C1q receptor on the parasite surface or through IgG already bound. Binding to IgG already present on the parasite surface led to classical pathway activation, while direct binding to the C1q receptor did not lead to complement activation. C1q binding peaked at around 3 hours and declined rapidly thereafter, until by 24 hours C1q binding was down to background (10%). De Brito *et al.* (1983) detected C3 deposition in liver granulomas from 3–10 month *S. mansoni* infected mice. The C3 deposition appeared to peak around months 3–4 PI. Santoro *et al.* (1980b) showed that 86% of schistosomiasis patients had increased C3d levels, which indicates widespread complement activation. Moreover, there appeared to be a direct correlation between egg count and C3d levels. Patients with > 100 *S. mansoni* eggs per gram of stool had significantly raised C3d levels, while patients with 3–100 eggs per gram of stool had less C3d. Ouaissi *et al.* (1981) showed that schistosomula incubated in agammaglobulinemic sera resulted in no complement consumption, while, if IgG was added, C1, C4 and C2 were consumed (70%, 70% and 60%, respectively). This consumption was dependent upon first, the binding of IgG to the parasite surface and second, the binding of C1q to the immunoglobulin. Galvao-Castro *et al.* (1981) attempted to correlate circulating immune complex levels and complement breakdown products with the severity of the disease in *S. mansoni* infected human patients. C1q levels were slightly increased, as were levels of C5 and B, but there was no correlation between these changes in individual complement components and the severity of the disease. C3 levels were slightly decreased but again were not correlated to the severity of the disease. However, C4 decreases did correlate with an increased severity of the disease, as did increases in C3d levels. Linder and Huldt (1983) detected C1q, C4, C3 and $\beta_1$-H on the surface of adult *S. mansoni*. No factor B was detected on the parasite surface. Dias da Silva and Kazatchkine (1980) showed that newly transformed skin or mechanically transformed schistisomula activated the AP. The skin-prepared schistosomula consumed 22% of factor B and 25% of C3, while mechanically derived schistosomula consumed 25% of factor B and 27% of C3. Consumption was linear over 45 minutes and lung schistosomula did not activate the alternative pathway even if they were treated with the enzymes, neuraminidase, heparinase, trypsin or chrondroitinase ABC. Marikovsky *et al.* (1986) confirmed that schistosomula lost sensitivity to complement-mediated killing within hours after *in vitro* culture. An increase in viability was observed, falling from 40% dead within minutes to 20% dead at 2 hours to 10% dead at 6 hours. C3

deposition on the parasite was noted to occur within 1–2 hours, and the parasite membranes appeared to be the source for the complement consumption. Van Egmond *et al.* (1981) showed a dose-dependent consumption of C4 and factor B by soluble egg antigens and extracts of adult worms.

Robinson and Lewert (1983) detected the presence of C3 in cryoprecipitates obtained from 8 week *S. japonicum* infected rabbits. Pun *et al.* (1984) showed that humans infected with *S. japonicum* had markedly depressed levels of C3 and C4 (32 mg $dl^{-1}$ and 8 mg $dl^{-1}$, respectively). The normal levels were 86–184 mg $dl^{-1}$ for C3 and 24–80 mg $dl^{-1}$ for C4. After 2 weeks of methylprednisolone therapy, these depressed values returned to normal limits.

## C. CESTODA

Cestode infections have not been as thoroughly studied as other host–parasite systems, but this lack of breadth has not hampered the depth in which the few parasites have been investigated. In particular, the taeniid parasites have served as a model for investigations on mechanisms whereby parasites evade the destructive properties of the host complement cascade. Hammerberg and Williams (1978a,b) were the first to show that a polysaccharide secreted by the parasite was able to consume complement away from the surface of the parasite. This dramatic and illuminating finding has led to similar and more detailed studies on many other parasites.

### 1. *Mesocestodiasis*

Toye *et al.* (1984) showed that tetrathyridia of *Mesocestoides corti* activated the complement system. C3 cleavage was detected by electrophoretic conversion of C3. This conversion was blocked by EDTA and not by EGTA. C3 was also detected on the surface of the tetrathyridia. However, this deposition of C3 did not decrease the parasites' subsequent survival in recipient mice. These investigators used NGpS as the complement source, and the guinea pig C3 may not have been as effective in generating the alternative pathway convertase in the mouse, when compared to the guinea pig.

### 2. *Hymenolipidiasis*

Furukawa *et al.* (1984) investigated *Hymenolepis nana* infections in mice. They found that C3 levels did not change after immunization and challenge of the recipient mice. Moreover, mice given CVF prior to immunization or challenge were still completely resistant to the infection. Bogh *et al.* (1986) investigated complement-mediated lysis of newly excysted *H. nana*, *H.*

*diminuta*, *H. microstoma* and *H. citelli*. Newly excysted *H. diminuta* lysed in all animal sera tested (13 species). There was no lysis in NRS treated with 8 mM EDTA, NRS heated at 56°C for 30 minutes, CVF-treated NRS, nor in C5-deficient NMS or C6-deficient NRbS. Lysis of the larvae did occur in NRS treated at 50°C for 30 minutes, C2-deficient NGpS or $Mg^{2+}$-EGTA treated NRS. There was no lysis of *H. microstoma* in C6-deficient NRbS. *H. nana* and *H. citelli* were lysed in all animal sera tested (eight and six different sera, respectively). *H. microstoma* was not lysed by sera from its hosts, rats, mice and hamsters, but was lysed in all other animal sera tested. This finding again emphasizes the differences which can occur between investigations on a normal host–parasite relationship, and investigations of a parasitic infection in an abnormal host or host components.

Christensen *et al.* (1986) investigated the effects of NRS on excysted stages of different ages of the parasite *H. diminuta*. Newly excysted and 2-day-old parasites were lysed completely in NRS, while 4 day to 10-day-old parasites were destrobilated, with survival of the scolex and neck regions. Older parasites were also destrobilated, but the tails had portions lysed.

### 3. *Cysticercosis/Hydatidosis*

Letonja and Hammerberg (1983) observed the deposition of rat C3 on metacestodes of *Taenia taeniaeformis*, with a particular heavy deposition around the scolex. Using NRS, this C3 deposition was limited to the scolex alone, while with IRS, C3 was deposited on both the scolex and bladder. Attachment of host leukocytes depended upon labile serum factors which were inactivated by heat treatment of NRS at 56°C for 30 minutes. Suquet *et al.* (1984) isolated a proteinase inhibitor from metacestodes of *T. taeniaeformis* which subsequently was found to markedly down-regulate many host immunologic functions, including the complement cascade. Leid *et al.* (1987a,b) showed that C5a-mediated neutrophil chemotaxis and aggregation can be inhibited by a proteinase inhibitor, taeniaestatin. This inhibition of two neutrophil functions may be occurring through direct binding and inhibition of C5a. Moreover, Leid, Suquet, Blanchard and Tanigoshi (unpublished observations) have shown that taeniaestatin can inhibit factor D of the alternative pathway, a trypsin-like enzyme. The precise mechanism of this inhibition remains to be elucidated, but taeniaestatin is the first parasite-derived proteinase inhibitor of the complement system to be described, and is one of a few inhibitors known to affect D.

Machnicka and Grzybowski (1986) detected and quantitated the presence of hemolytic complement within the cysts of *T. saginata*. The circulating $CH_{50}$ titers in infected cattle ranged from 20 to 128, while titers within the parasite bladders from these same animals ranged from 14 to 36.

Correa *et al.* (1985) studied 12 patients with neurocysticercosis caused by

*T. solium* infections and found that 6/12 had C3b deposited on the surface of the cysticerci. In three patients with parasites in the CNS parenchyma no C3b was detected.

Perrigone *et al.* (1980) investigated cyst fluid from *Echinococcus granulosus* cysts for the cyst fluid's effects on C3, C4, C1q, C1s and factor B. C4, C1q and C1s were not activated upon incubation with cyst fluid, while C3 and factor B were cleaved. This C3 activation was not inhibited in the presence of $Mg^{2+}$-EGTA but was inhibited by heating the sera at 50°C to destroy factor B. Cicardi *et al.* (1985) have noted an acquired C1 inhibitor deficiency in a patient infected with *E. granulosus*. This patient also presented with angioedema. Leid and Williams (1979) noted that bottle jaw caused by *Haemonchus contortus* may well reflect perturbations in C1 inhibitor. More investigations such as this one with *E. granulosus* need to be done.

Vuitton *et al.* (1984) studied 12 patients with *E. multilocularis* infections that were treated with flubendazole. These 12 patients had elevated levels of C3 and C4 when compared to noninfected humans. The levels of C4 returned to normal within 12 months after drug treatment, while the C3 levels remained elevated. The $CH_{50}$ titers followed the C3 levels and remained elevated after drug therapy. Ali-Kahn and Siboo (1981) have detected C3 on the surface of the cyst germinal layer of *E. multilocularis*, but not on the laminated layer.

## D. NEMATODA

Nematode infections have also served as a particularly fruitful group of host–parasite relationships for investigations on parasite–complement interactions. Many of these studies investigated the role of complement in leukocyte killing of the parasites. The number of infections investigated and the depth to which they have been studied is gratifying and bodes well for the future.

### 1. *Trichuriasis*

Preston *et al.* (1986) studied the surface properties of *Trichuris muris*. Using immunofluorescence, these investigators detected C3b on the surface of the parasites incubated in NRS. This binding of C3b was eliminated if the sera were heated or treated with EDTA, but was not impaired if the sera were treated with EGTA. IMS showed retention of murine C3b binding after heating but NMS sera did not.

### 2. *Trichinosis*

Kazura (1981) observed that specific antibodies and an intact complement system did not result in destruction of either adult *Trichinella spiralis* or

larvae. Moreover, eosinophils were not enhanced in their killing of the parasites by the presence of complement components. Jensen and Castro (1981) detected no endogenous chemotactic activity in muscle larvae, pre-adults and adult worms. Nor did the incubation of spleen cells from normal or immunized hosts result in chemotactic activity in the culture supernatants. However, when larvae, pre-adults and adults were incubated with NRS, chemotactic activity increased markedly over background levels. When larvae were incubated with IRS, chemotactic activity increased over that observed when larvae were incubated with NRS. When pre-adults or adults were incubated with IRS, chemotactic activity did not increase over that observed for the same incubations with NRS. These results suggest that cellular infiltrates around the larvae in sensitized animals may be a result of complement activation, either locally or systemically.

3. *Ancylostomiasis*

Klaver-Wesseling *et al.* (1982) followed the interaction of *Ancylostoma caninum* with canine sera and cells. They detected the presence of C3 on the parasites by immunofluorescence. This binding did not occur in the presence of 10 mM EDTA or with sera treated at 56°C for 30 minutes, but did occur in 10 mM EGTA-treated sera. Erythrocytes obtained from both normal and immune animals bound to the parasites in the presence of EGTA, but not in the presence of EDTA, nor if the NCS were heated. Erythrocytes bound in all instances if immune canine serum (ICS) was used, and this was enhanced by EGTA.

4. *Trichostrongylosis*

Stankiewicz *et al.* (1981) observed alternative pathway activation in lamb sera by *Trichostrongylus vitrinus* larvae. These investigators found that sheep leukocytes were adherent to larvae treated with fetal lamb sera. This adherence was abrogated by heating the sera (56°C for 30 minutes or 50°C for 30 minutes) and the cell adherence was restored, in the latter case, by the addition of exogenous factor B. Fetal lamb sera chelated with 10 mM EDTA or treated with zymosan before incubation with the larvae inhibited leukocyte adherence. However, fresh lamb serum made 10 mM in EGTA permitted the adherence of leukocytes to return to normal levels.

5. *Nematospiroidiasis*

Penttila *et al.* (1984) showed that heat-activated IMS was not effective at mediating eosinophil or neutrophil inhibition of infectivity for larvae of

*Nematospiroides dubius*. Moreover, neutrophils from normal mice were not active in killing infectious larvae using NMS, IMS or heat-inactivated IMS. Antibody plus complement and neutrophils was only marginally better at killing larvae than complement and neutrophils alone. However, eosinophils plus antibody and complement was much more effective than complement and eosinophils alone. Complement activation would seem a particularly effective activator of eosinophil-mediated killing mechanisms.

6. *Nippostrongylosis*

Mackenzie *et al.* (1980) studied the activation of complement by several parasites, including *Nippostrongylus brasiliensis*. These investigators found that infective larvae of *N. brasiliensis* were killed by complement and cells alone, and did not require the presence of specific antibodies. Egwang *et al.* (1984) showed that larvae of *N. brasiliensis* activated the alternative pathway in NRS. Normal peritoneal macrophages adhered and killed the L3 larvae in the presence of complement. Bronchoalveolar macrophages obtained from infected rats adhered and killed L3 larvae, while those obtained from normal animals did not do so. Heat-inactivated NRS did not mediate adherence and killing and when adherence occurred after incubation with untreated NRS alone, killing was not observed. CVF and zymosan treatment of NRS did not permit cell adherence to occur. Bronchoalveolar macrophages obtained 2 days PI adhered but did not kill. Egwang *et al.* (1985a) studied C3-dependent phagocytosis in *N. brasiliensis* infected rats. These investigators found increased phagocytosis by alveolar macrophages with increased time PI. Alveolar macrophages were obtained at 0, 2, 8 and 32 days PI, and of these cells 9%, 19%, 26% and 18% were actively phagocytic, respectively. The cells obtained at days 8 and 32 PI released 81 and 71% more β-glucuronidase than normal alveolar macrophages. Egwang *et al.* (1985b) showed that multinucleate giant cells obtained from *N. brasiliensis* infected rats had a pronounced decrease in binding of erythrocytes coated with antibody and complement (EAC), 7% as compared with 47%. Lamontagne *et al.* (1984) detected an increased level of circulating C3 in mice infected with *N. brasiliensis* when the parasites were in the intestine. These levels started to increase at day 10 PI, were twice normal around day 12 PI and returned to normal by day 15 PI.

7. *Ascariasis*

Fattah *et al.* (1986) studied the adherence of human blood eosinophils with *Toxocara canis* larvae. They found a pronounced eosinophil adherence with NHS-treated larvae, a feature which was abolished by heat or EDTA treatment of the sera. Adherence was reduced in $Mg^{2+}$-EGTA and zymosan-

treated NHS, but was not abolished. IHS gave a positive adherence reaction which also was reduced after heat treatment. Collectively, these results indicate that eosinophil adherence was both classical and alternative pathway dependent.

8. *Dipetalonemiasis*

Haque *et al.* (1982) detected C3 deposition on *Dipetalonema viteae* obtained from infected rats. NRS depleted of complement by heating at 50°C or 56°C, or by treatment with zymosan-inhibited leukocyte adherence. EGTA-treated NRS did not alter the adherence of host cells. Factor B consumption was evident, as was alternative pathway activation when larvae were incubated with NRS. However, C4 consumption was much lower than factor B utilization. Eosinophil and macrophage adherence was dependent upon an intact host complement system.

9. *Dirofilariasis*

El-Sadr *et al.* (1983) investigated *Dirofilaria immitis* infections in dogs. They detected C3 deposition on the cuticle of microfilariae (mf) when these larvae were incubated in occult sera or sera from infected dogs without circulating mf. C3 and IgM antibodies were involved in killing the mf. Hamilton *et al.* (1986) showed that both C3 and C1 levels declined after diethylcarbamazine (DEC) treatment, or after challenge with specific antigen. $CH_{50}$ levels also were reduced in both of these groups. Staniunas and Hammerberg (1982) have shown complement activation by living microfilariae of *D. immitis* or their *in vitro* secreted products. C3, C5 and properdin were detected on the parasites incubated in NHS by fluorescinated antibodies to the human proteins. Conversion of C3 was also detected after incubation of the mf in NHS, and diethylcarbamazine enhanced this conversion in the presence of living mf, but did not do so with the lyophilized mf. The mf secreted into tissue culture media a polysaccharide which was a potent complement consuming factor.

10. *Wuchereriasis*

Ottesen *et al.* (1982) observed decreased C3 levels in human patients infected with *Wuchereria bancrofti*. Interestingly, they also found elevated C3 levels in the control patients from the filarially endemic areas. Simonsen (1985) has shown the deposition of C3 on the microfilarial sheath of *W. bancrofti*. DEC treatment of infected human patients or addition of DEC to the *in vitro* incubations did not cause C3 to be deposited on the parasites.

11. *Brugiasis*

Barta *et al.* (1981) showed that moderate changes in C3 and $CH_{50}$ levels occurred in hamsters infected with *Brugia pahangi* for 4 to 5 months. Other complement components were relatively unchanged. Karavodin and Ash (1982) examined the effects of sera from jirds on *B. pahangi*. They found that heat inactivation of jird sera at 56°C for 30 minutes decreased PEC adherence. Moreover, the percentage of mf with adherent PEC went from 87% to 10% after heat inactivation, while the cytotoxicity dropped from 72% to 38%. Normal jird serum (NJS) restored these values to 47% and 56%, respectively. Johnson *et al.* (1981) detected the deposition of guinea pig C3 on the surface of *B. pahangi* by immunofluorescence. Chandrashekar *et al.* (1985, 1986) investigated the complement dependence of neutrophil adherence to L3 larvae of *B. pahangi*. These investigators found marked deposition of C3 on the larvae using NRS and with or without 2 mM EGTA. EDTA (2 mM) treatment, heat inactivation (56°C for 20 minutes or 50°C for 60 minutes) or zymosan treatment abolished C3 binding. Utilization of a reagent deficient in factor D did not result in C3 deposition, cytoxicity or neutrophil adherence. Both neutrophil adherence and cytotoxicity followed the course of C3 binding.

Fanning and Kazura (1983) found that C3 levels remained the same in *B. malayi* infections of mice, whether there was a long or short period of microfilaremia. Sim *et al.* (1983) showed that complement was not apparently involved in the antibody-dependent cell adherence reaction to this parasite. Piessens and Dias da Silva (1982) investigated the adherence of buffy coat leukocytes to microfilariae of *B. malayi*. These investigators found that the cell adherence depended upon an intact complement system, as heating NGpS at 56°C for 60 minutes or 50°C for 30 minutes inhibited adherence, as did the presence of EDTA. Treatment of NGpS with 5 mM EGTA restored the reaction to 75% of normal, and C3 cleavage was detected in the presence of $Mg^{2+}$-EGTA, but not in the presence of EDTA.

12. *Onchocerciasis*

Coley and Leid (1982) investigated complement activation by the equine parasite, *Onchocerca cervicalis*. They found that adult extracts of the parasite activated human, murine and equine complement by both the alternative and classical pathways. Hemolytic activity was reduced 96, 95 and 79% in these three sources of sera, respectively. Heat inactivation (56°C or 50°C) or calcium chelation by EDTA inhibited this consumption. Treatment of sera with $Mg^{2+}$-EGTA restored the hemolytic activity to 60% of normal. Camp and Leid (1983) showed the complement dependence of chemotactic factor

generation in this infection. These investigators found that incubation of sera with extracts of the adult worms gave rise to factors which induced equine neutrophil migration. Heat inactivation (50°C or 56°C for 30 minutes) or chelation with 10 mM EDTA inhibited the generation of this factor, while sera obtained after treatment with 10 mM EGTA were as active as untreated sera. No chemotactic activity was present in the extracts of mf or adults alone. Gel filtration chromatography of the chemotactic factor, when combined with other data described, argue for the generation of C5a or a C5a-like molecule.

Loos and Dierich (1980) studied three patients with *O. volvulus* infections, and showed that $CH_{50}$ were not detectable in the patients, while they quantitated a mean of 40 units per ml in the control patients. C1 and C4 levels were markedly depressed with C4 levels $< 1\%$ of normal. All other components were low, but not as low as that observed for C4. Incubation of the patients' sera with NHS resulted in the consumption of C4 and C2. C4, plus the patients' sera and exogenous C1 inhibitor, restored C4 activity. The C1 inhibitor levels in these patients were only 50% of normal antigenically, but most importantly, these antigenic levels of the complement protein had no functional activity. This was the first study to document that parasites could have a marked effect on the control proteins of the complement cascade. More investigations of this nature need to be carried out.

Greene *et al.* (1983) found that 7 out of 114 patient sera had decreased C3 levels, 15 out of 114 had decreased C4 levels, while no patients of 58 examined had any changes in factor B levels during DEC therapy. King *et al.* (1983) investigated chemotactic factors generated by microfilariae of *O. volvulus*. They found that mf (nodule- or skin-derived) incubated in NHS or NGpS resulted in no chemotactic generation. Mf incubated in heat-inactivated NHS or NGpS was negative as were mf incubated in heat-inactivated sera supplemented with C4-deficient NGpS. However, mf incubated in heat-inactivated IHS, and to which NHS or NGpS was added, showed an increase in chemotactic activity, two to two and one half fold over that seen in the uninfected controls. These results would argue strongly for antibody and complement dependent generation of chemotactic factors, most probably C5a or $C5a_{des\text{-}arg}$.

## 13. *Litomosoidiasis*

Zahner (1983) noted the deposition of C3 on the microfilariae of *Litomosoides carinii* and activation of the complement pathway.

### E. ARTHROPODA

Arthropod infections and interactions with mammalian complement systems have not been investigated to any extent, although many of these parasites are intimately associated with the vascular system through feeding and attachment mechanisms. It would seem that investigations focused on inhibitors of complement activation, or of individual complement component consumption, would be important and fruitful research areas.

#### 1. *Hypodermiasis*

Boulard and Bencharif (1984) observed that three proteinases derived from *Hypoderma lineata* gave a dose-dependent activation of the complement cascade. In normal bovine serum (NBS), proteinase A acted on the complement components C3–C9, while proteinase B acted on the components C1–C3 and proteinase C was without effect. In IBS, proteinase A and C were slightly active in classical pathway activation, and proteinase B was a strong classical pathway activator.

#### 2. *Sarcoptiasis*

Falk (1980) showed that C3 and C4 levels in 151 human patients infected with *Sarcoptes scabei* did not differ from normal controls over the course of a 9 month period. Salo *et al.* (1982) found C3 deposition in skin lesions of 13 of 18 patients. The latter authors suggested that local complement activation may be important in the pathogenesis of this disease.

## VI. CONCLUSIONS

Parasitology has moved from investigations in which some anti-parasitic activity in animal sera was observed to be heat labile, to quantitation of selected complement components in an array of parasitic diseases, and finally to initiation of work on the molecular basis of parasite interactions with complement proteins. More of the latter studies will come to the front in the next 5 to 10 years, and are to be encouraged. These studies will focus on how complement is detrimental to the parasite's survival. I would hope that more investigations may be initiated on how the parasite utilizes the complement system to its advantage, with the hope that abrogation of this latter event might either prevent or limit the socioeconomic impact of these devastating infections. As has been so wisely pointed out to me over and over by my veterinary parasitology colleagues, parasite survival in domesticated animals

is the rule and not the exception. Therefore, the vast majority of parasites have evolved very potent mechanisms for forestalling the detrimental effects of the complement pathway, and these mechanisms need to be detected, investigated and finally used to control the infections.

This concludes my review on observed interactions between the mammalian complement system and their parasites. Undoubtedly, some papers have not been cited because they were overlooked in my computer searches. For these predicted omissions, I extend my apologies.

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# Chemical Communication in Helminths

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## I. Introduction

Chemical communication is ubiquitous in the animal kingdom, and has been studied extensively in insects (Wilson, 1968; Shorey, 1977). Less information is available on invertebrates other than insects (Frings and Frings, 1968).

It was known by the end of the nineteenth century that substances emitted by one organism influenced the behavior of another. The French naturalist, Jean H. Fabre used crushed female moths to attract male conspecifics (Wilson, 1963). By the 1890s entomologists were aware of sexual dimorphism in moth antennae, and believed that the plumed antennae of the male had receptors for chemical stimuli from the female (Hecker and Butenandt, 1984).

Studies in the first half of the twentieth century on lepidopterans and hymenopterans suggested that females emitted chemical substances (pheromones) that attracted conspecific males (Wilson, 1968). Female insects or specific organs were extracted in organic solvents to obtain presumptive chemical attractants, which were purified by chromatographic procedures to obtain substances that attracted conspecific males. Relatively large numbers of insects were processed to obtain minimal amounts of attractant; two well known sex attractants are bombykol from the silkworm moth, and gyplure from the gypsy moth (Wilson, 1968).

The insect pheromone work of the 1950s influenced nematode chemoattraction studies of the 1960s. The first observation on sexual attraction in a nematode was that of Greet (1964) on the free-living nematode, *Panagrolaimus rigidus*. Frings and Frings (1968), in their review on animal communication in invertebrates other than insects, mentioned Greet's work and aggregation studies in nematomorphs and rotifers; no mention was made of chemical communication in flatworms. Shorey's (1977) review on pheromones in the animal kingdom discussed chemical communication studies on *Trichinella spiralis* by Bonner and Etges (1967), *Camallanus* sp. by Salm and Fried (1973) and *Nematospiroides dubius* by Marchant (1970).

Bonner and Etges' (1967) work on *T. spiralis* was a forerunner of other studies on plant and animal parasitic nematodes in the 1970s and 1980s (Bone, 1982b, 1986). Recently, Huettel (1986) mentioned some 30 species of free-living, phyto- and zooparasitic nematodes which exhibit pheromone-mediated behavior (Table 1).

The observations of Sogandares-Bernal (1966) on wandering pre-adults of *Paragonimus kellicotti* first suggested chemotactic factors in worm attraction of hermaphroditic digeneans. Fried and Roberts (1972) provided further evidence of chemical attraction in digeneans when they showed that free metacercariae and young adults of *Leucochloridiomorpha constantiae* were attracted to each other *in vitro* (Nollen, 1983; Fried, 1986).

Chemical communication between male and female schistosomes was

TABLE 1 *List of nematodes exhibiting pheromone-mediated attraction*

| Species | Habitat[a] | Attracted sex(es) | Reference(s) |
|---|---|---|---|
| *Ancylostoma caninum* | AP | M | Roche, 1966 |
| *Ascaris suum* | AP | M, F | Garcia-Rejon *et al.*, 1982 |
| *Aspiculuris tetraptera* | AP | M, F | Anya, 1976<br>Verdejo *et al.*, 1983 |
| *Camallanus* sp. | AP | M, F | Salm and Fried, 1973 |
| *Cephalobus persignis* | FL | M | Cheng and Samoiloff, 1971 |
| *Chiloplacus symmetricus* | FL | M | Ahmad and Jairajpuri, 1980 |
| *Cylindrocorpus curzii* | FL | M | Chin and Taylor, 1969 |
| *Cylindrocorpus longistoma* | FL | M | Chin and Taylor, 1969 |
| *Ditylenchus dipsaci* | PP | F | Windrich, 1973 |
| *Heterodera avenae* | PP | M | Green and Plumb, 1970 |
| *Heterodera carotae* | PP | M | Green and Plumb, 1970 |
| *Heterodera cruciferae* | PP | M | Green and Plumb, 1970 |
| *Heterodera glycines* | PP | M | Green and Plumb, 1970<br>Rende *et al.*, 1982 |
| *Heterodera goettingiana* | PP | M | Green and Plumb, 1970 |
| *Heterodera mexicana* | PP | M | Green and Plumb, 1970 |
| *Heterodera oryzicola* | PP | M | Jayaprakash and Rao, 1982 |
| *Heterodera pallida* | PP | M | Green and Plumb, 1970 |
| *Heterodera* (now *Globodera*) *rostochiensis* | PP | M | Green, 1966 |
| *Heterodera schachtii* | PP | M | Green, 1966 |
| *Heterodera tabacum* | PP | M | Green and Plumb, 1970 |
| *Heterodera trifolii* | PP | M | Green and Plumb, 1970 |
| *Hoplolaimus indica* | PP | M | Azmi and Jairajpuri, 1977 |
| *Nippostrongylus brasiliensis* | AP | M, F | Alphey, 1971<br>Bone *et al.*, 1977b<br>Roberts and Thorson, 1977a |
| *Panagrellus silusiae* | FL | M | Cheng and Samoiloff, 1971 |
| *Panagrellus redivivus* | FL | M | Balakanich and Samoiloff, 1974<br>Duggal, 1978 |
| *Panagrolaimus rigidus* | FL | M, F | Greet, 1964 |
| *Pelodera strongyloides* | FP | M, F | Stringfellow, 1974 |
| *Pelodera teres* | FL | M | Jones, 1966 |
| *Rhabditis pellio* | FP | M, F | Somers *et al.*, 1977 |
| *Rotylenchulus reniformis* | PP | M | Nakasono, 1977 |
| *Syphacia obvelata* | AP | M,F | Verdejo *et al.*, 1983 |
| *Trichinella spiralis* | AP | M,F | Bonner and Etges, 1967<br>Belosevic and Dick, 1980 |

[a]Habitat refers to that of the reproductive adults. AP = Animal parasite; FL = free-living; FP = facultative parasite; PP = plant parasite.

Modified after Bone and Shorey (1978) and Green (1980).

suggested in early work (Armstrong, 1965); however, it was demonstrated *in vitro* by Imperia *et al.* (1980). Also, see Eveland and Haseeb (1986) and Haseeb and Eveland (1986).

The purpose of this review is to examine chemical communication in pre-adult and adult helminths, i.e. how helminths communicate with each other

using chemical signals. Information on host location by helminths has been reviewed by Ulmer (1971), Saladin (1979) and Kemp and Devine (1982) and will be considered here mainly as it relates to worm-mediated chemical communication. Most of the literature on chemical communication in helminths concerns free-living and parasitic nematodes, and digenetic trematodes. Little information exists on Acanthocephala, Cestoda, Monogenea, Turbellaria, and Aschelminthes other than nematodes.

This review considers terminology used in chemical communication; *in vitro* and *in vivo* bioassays; specific structures involved in the production and release of pheromones; chemoreception; and analytical techniques used to study pheromones. We first consider helminths in general and then specifically those organisms which have been studied extensively.

## II. Terminology

Terminology in chemical communication is often confusing and consensus does not exist on many of the definitions. The following discussion considers the most frequently used terms and relates them to helminthology.

Prior to 1959, *ectohormone* was used to describe chemical substances released to the outside by an organism and received by another. The term was confusing since it implied the release to the outside of an internal secretion. The term *pheromone* was coined by Karlson and Butenandt (1959) and Karlson and Lüscher (1959) from the Greek "*pherein*" (to carry or to transfer) and "*hormon*" (to excite or to stimulate). It has had wide acceptance by chemical ecologists and, as used today, refers to a substance released by an organism and received by another, usually of the same species. This is the definition used frequently in helminthology. The original definition of Karlson and Butenandt (1959) implied species specificity, whereas that of Karlson and Lüscher (1959) did not and allowed for chemical communication between closely related species. The latter definition is more appropriate here, since helminthologists have described interspecific chemical communication both *in vitro* and *in vivo*: e.g. attraction of *Nippostrongylus brasiliensis* to other nematode species (Roberts and Thorson, 1977b); interspecific communication in hermaphroditic digeneans (Fried and Jacobs, 1980; Fried and Wilson, 1981a; Fried and Leiby, 1982); interspecific communication in schistosomes (Taylor, 1970; Southgate *et al.*, 1982; Eveland and Fried, 1987).

Wilson (1963) described primer and releaser pheromones from insects. Primers cause physiological changes without immediate accompanying behavioral responses, whereas releasers cause an immediate behavioral response to a chemical stimulus (Wilson and Bossert, 1963). Releaser and primer pheromones have been described from trematodes and nematodes (Bone, 1982a,b).

Whittaker (1970) used the term *allelochemic* to describe chemicals involved in interspecific interactions, i.e. mainly host recognition responses. Of several allelochemics described, only *allomones* and *kairomones* are considered here since they occur in nematodes (Huettel, 1986). Allomones produce a response favorable to the releaser, whereas kairomones produce a response favorable to the recipient. Allelochemic messages may be released by insect, plant or animal host of nematodes. Food-finding behavior of nematodes is probably influenced by host-released allelochemics (Huettel, 1986).

Law and Regnier (1971) used the term *semiochemicals* ("*semion*" = mark or signal) for substances that carry signals from one organism to another. Huettel (1986) reviewed the terminology in nematode chemical communication following the schema of Law and Regnier (1971). She urged for a unification of nematode terminology to relate the field to other behavioral disciplines. She considered semiochemicals as chemical signals released by one organism and received by another, and divided them into pheromones which elicit intraspecific responses, and allelochemics which elicit interspecific responses. She considered sex, epidietic and alarm pheromones; sex pheromones cause attractive or aggregative behavioral responses between or within sexes and have been well described from nematodes; epidietic pheromones are chemical substances that regulate population densities, e.g. population regulation in *Caenorhabditis elegans* as reported by Golden and Riddle (1982). The third category, alarm pheromones, has been described from insects but not nematodes.

The terms *taxis* and *kinesis* are used in the helminth literature to describe worm responses; if movement is directed it is considered a taxis, and if not, a kinesis. It is often difficult to determine if the helminth response is chemotactic or chemokinetic. See Fraenkel and Gunn (1940) for the classification of terms used to describe animal orientation.

## III. Chemoattraction Bioassays

### A. NEMATODA

#### 1. *In vitro*

Nematode pheromone bioassays are usually based on the movement of individuals in an aqueous or semiaqueous medium in response to a gradient (Bone and Shorey, 1978a). Different apparatuses have been designed to meet individual needs, e.g. Greet (1964) for *Panagrolaimus*; Bonner and Etges (1967) for *Trichinella*; Salm and Fried (1973) for *Camallanus*; Garcia-Rejon *et al.* (1982) for *Ascaris*.

Green (1980) used the term *arena* to describe nematode bioassays and noted that arenas vary in shape from narrow strips to circular plates. He suggested that a medium similar to the natural habitat should be used since it allows for "normal" movement of organisms. He also suggested that the source of attractant in the bioassay could be live worms, e.g. if immobile such as *Heterodera* can be placed directly in the medium, but if mobile such as most zooparasites, must be restrained in a part of the arena by permeable barriers. He further noted that worms could be removed from cultures and the media used as the source of attractant.

The bioassay is the basic tool for the detection and eventual quantification of nematode biological activity (Huettel, 1986). Numerous bioassays have been developed for rapid screening of pheromones, and some are based on copulatory behavior patterns (Huettel, 1986). Such patterns have been described for *H. glycines*, based on *in vitro* observations of root explant culture or on isolated crude pheromone (Huettel, 1986; Huettel and Rebois, 1986; Huettel and Jaffe, 1987). Most bioassays developed for nematode sex pheromones have used dose responses of males to determine the optimal number of females needed to establish a pheromone gradient; the establishment of threshold levels for response of males is also necessary to assure that complete behavioral activity is measured (Green and Plumb, 1970; Bone *et al.*, 1977b; Huettel *et al.*, 1982).

It took at least 2 hours for *N. brasiliensis* females to establish an attractive gradient in a bioassay (Bone *et al.*, 1977b). According to Huettel (1986), 12–24 hours were required for *Heterodera* to establish an attractive gradient. Some bioassays have used copulatory behavior, as in the free-living nematode *Radopholus* sp., and such behavior has also been used to distinguish closely related species (Huettel *et al.*, 1982). This is in contrast to most bioassays, which have used observations only of male movement in response to a pheromone source either established from entire females or from agar disks impreganated with the attractant.

Bioassays demonstrate that oversaturation with female pheromone reduces the response of males, whereas using too few females fails to establish a gradient that males can follow (Bone *et al.*, 1977b; Huettel and Rebois, 1986). Using a three-choice maze bioassay (Fig. 1) in studies on *Ascaris suum*, Garcia-Rejon *et al.* (1985a) observed that worms responding positively undulated rapidly through the medium towards the inoculation zone; movement was slower in worms that responded negatively. Garcia-Rejon *et al.* (1985b) also noted that the response to sex attractant in *A. suum* was affected by the number of pheromone-emitting worms and the pH of the medium. The optimal migration response of both sexes was obtained when three individuals were used as releasers. Males and females showed significant responses to sex pheromones at pH 7.4, but did not respond at pH 5.5 or 9.0.

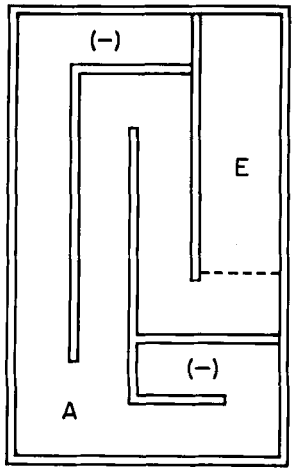


FIG. 1. The plastic choice-chamber (24.3 × 14.5 × 2.3 cm) used in studies on sexual attraction in *Ascaris suum*. The emitting worm is placed in the incubation zone (E, 14 × 4 cm) and the migrating worm is placed in the inoculation zone (A, 6.5 × 4 cm). The chamber provides three choices; two negative channels (−) and a positive channel leading to the zone of incubation. The chamber is filled with 300 ml of Tyrode's solution to a height of 1 cm and maintained at 34–37°C in the dark. The chamber is covered with a plastic sheet to restrain worms and to prevent evaporation of the medium. Redrawn after Garcia-Rejon *et al.* (1982), with the permission of Dr Verdejo Lucas, Division of Nematology, University of California, Davis, Calif. 95616.

2. *In vivo*

There are reports of mate location by zooparasitic helminths in both natural and experimental infections. *In vivo* studies do not eliminate host-mediated influences and are therefore more equivocal than *in vitro* observations. Oshima *et al.* (1962) observed 11 fertilized females but only a single male of *Ancylostoma caninum* along an 80 cm dog intestine. Roche (1966) placed *A. caninum* in the intestine of a dog and found that posteriorly placed males moved toward anteriorly located females, which suggested that female worms produced an attractant that moved with peristalsis. Similar results were reported by Gimenez and Roche (1972) for *N. brasiliensis* in the rat.

The influence of *N. brasiliensis* males and females on each other's dispersal in the intestine of rodents has also been studied (Brambell, 1965; Alphey, 1971; Roberts and Thorson, 1977a). Glassburg *et al.* (1981) surgically implanted males of *N. brasiliensis* posterior to females in the mouse intestine. An anterior movement of males demonstrated a dose-dependent response toward females within 6 hours. Glassburg *et al.* (1983) observed the behavior of single- and mixed-sex infections of *N. brasiliensis* in fed and fasted mice.

They noted differences in the behavior of male and female worms, and related the differences to intrinsic reproductive and nutritive needs of each sex.

## B. TREMATODA

### 1. *In vitro*

The bioassay for hermaphroditic digeneans observes worm pairing in petri dishes maintained at 35–41 °C. In this design, a 1% agar substratum which is overlaid with sterile Locke's solution is used, and allows worms to migrate at the agar/Locke's interface. In this nonbarrier design, worms show a maximal tendency to pair. However, this design does not exclude worm thigmotaxis, i.e. worm-to-worm contact pairing; worms are usually started 2cm from each other, and the tendency of one worm to attract the other is examined at various intervals up to 24 hours (Fried, 1986).

Worms in contact or within 5mm of each other are considered paired (Fried and Roberts, 1972). The digeneans often show noncontact pairing, i.e. worms come within 1–2mm of each other, do not contact, and then retreat to about 5mm from each other. Initial starting distance is a factor in subsequent pairing. *Echinostoma revolutum* started more than 8cm apart did not pair, whereas those started less than 8cm did. Other factors involved in *E. revolutum* pairing are temperature and the overlay medium. More pairing occurred at 39°C that at 35°C, and an overlay of sterile Locke's was better than the defined medium NCTC 135 (Fried and Pallone, 1984).

Attraction of a single worm to either a filter paper disk or a silica gel square containing worm homogenate or excretory–secretory (ES) products has also been examined (Fried and Gioscia, 1976; Fried and Robinson, 1981). A worm is placed equidistant between the experimentally impregnated disk or square and its control. Migration toward or contact with the experimental disk or square is considered as attraction.

In barrier designs, one worm is restrained, thus eliminating the possibility of worm thigmotaxis. Such designs have been used to study heterosexual and homosexual attraction in schistosomes maintained in polycarbonate linear chambers (Imperia *et al.*, 1980; Eveland and Haseeb, 1986). The barrier usually consists of a chimney made of dialysis tubing or a dialysis sac.

A bioassay system, with Earle's balanced salt solution (EBSS) containing 0.1% glucose and 0.5% lactalbumin hydrolysate, was developed by Imperia *et al.* (1980) to study chemoattraction in *Schistosoma mansoni*. The bioassay design was a modification of those used in studies of nematode behavior (Bonner and Etges, 1967; Salm and Fried, 1973). In the nonbarrier design,

polycarbonate chambers with 14 linear channels, each channel 3cm long, 1cm wide and 1.5cm high, were used. The channels were marked into 0.5cm zones. Each channel was filled to a height of 0.85cm with 1% agar. The agar was overlaid with EBSS. The chamber was maintained at 37°C and worms were placed into channels, 15mm apart. This distance allowed each worm to move either toward the other or 7.5mm in the other direction, toward the end of the channel. Worm movement was measured as distance traveled over time, and the percent attraction determined by the formula $15 - D/15 \times 100$, where $D$ is the actual distance between a worm couple at a particular point in time. Thus, by this formula, 0% attraction would indicate that worms remained at their original distance, and 100% attraction would indicate that all the worms were in contact (Eveland and Haseeb, 1986; Eveland and Fried, 1987).

The advantage of the nonbarrier design is that by allowing both worms to migrate, any chemoattractive effect can be cumulative and therefore maximized. However, some limitations of this design are: (1) it does not permit distinction between releasers or responders; and (2) it cannot rule out the possibility that pairing is accomplished by thigmotaxis or trial and error rather than by chemotaxis.

The barrier design permits such distinctions and it allows testing of ES products. In this design (Fig. 2), channels contained 0.6 cm diameter dialysis tube chimneys to restrain worms but allow the passage of ES products. A worm was placed at the zero (starting) point, equidistant between the chimneys. Worms which moved toward occupied chimneys were considered

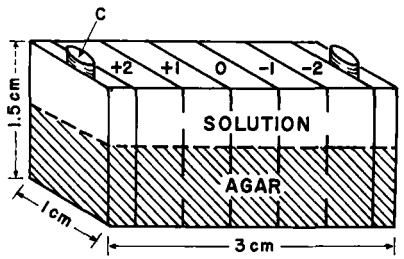


FIG. 2. One linear channel of a 14 × 3 × 1.5cm Plexiglass chamber used in studies on chemoattraction in adult schistosomes. The channel is filled to a height of 0.85cm with 1.0% phosphate buffered agar (pH7.0) which is overlaid with 1.5ml of EBSS. The dialysis tube chimney (C), 0.6cm in diameter and 1.0cm in length is placed in the agar at both ends of the channel. A worm is placed in the chimney adjacent to the + 2 zone, and another worm is placed in the 0 zone. Chambers are maintained at 37°C. Worm locations are recorded at various time intervals. Worms in + 2 and + 1 zones are considered attracted, whereas those in − 2 and − 1 zones are not. Worms remaining in the starting zone are neither attracted nor unattracted. Redrawn after Imperia *et al.* (1980), with the permission of the Editor of the *Journal of Parasitology*.

attracted, whereas those moving toward unoccupied chimneys were scored unattracted. Control experiments have been carried out with single worms placed equidistant between empty chimneys and, at all time points, most of the "migrating" worms remained at zero, and the remaining worms were distributed equally on both sides of the zero point. Thus, a 50% attraction would be expected if migration were random, and therefore only those responses greater than 50% are regarded as attraction (Eveland and Haseeb, 1986).

Extracts and ES products of schistosome adults have also been tested for chemoattractancy *in vitro*. Lipid extracts of worms and their ES products were prepared in various organic solvents. Agar plugs were impregnated with presumptive chemoattractants and placed in bioassay systems which were either of a linear or circular design (Childs *et al.*, 1986; Eveland and Haseeb, 1986).

In circular bioassay systems parasite orientation was determined by calculating the mean vector obtained from summing individual coordinates of parasite locations. A modified Rayleigh test was used to compare mean directions of vectors to the expected preferred direction towards the experimental agar plugs; the Rayleigh test was also used to test for significant directional preferences (clustering) which could have occurred independently of orientation towards a test compound (Childs *et al.*, 1986).

2. *In vivo*

Studies *in vivo* are based on examining the number and distribution of digeneans in natural or experimental infections. In these studies metacercarial cysts of *E. revolutum* were fed to experimental hosts (Fried and Weaver, 1969); free (unencysted) metacercariae of the brachylaimid, *Leucochloridiomorpha constantiae* were inoculated per cloaca into domestic chicks (Fried and Harris, 1971). At necropsy, observations were made on pairing, i.e. the tendency of two worms to come together, or clustering, i.e. aggregation of three or more worms in their normal sites (Fried and Roberts, 1972; Fried and Alenick, 1981; Fried and Robinson, 1981).

Observations on adult hermaphroditic digeneans, both in nature and in the laboratory, indicate that isolated worms are rare. Digeneans are usually paired or clustered in their natural host site. In 15 out of 16 cases, *Deretrema* sp. was found in pairs in the gall bladder of naturally infected flashlight fish, *Anamalosis katopteron*, and in only one case was a solitary fluke found (Burn, 1980).

In single-worm infections of various digeneans normal growth and development does not occur (Nollen, 1983). Fried (1962) found that the eyefluke, *Philophthalmus hegeneri*, when maintained singly in experimentally infected

domestic chicks, stopped growing after 20 days and failed to produce fertile eggs. Moseley and Nollen (1973) confirmed Fried's (1962) findings on *P. hegeneri*, and Nollen (1983) suggested that contact with another eyefluke of the same species is required for normal growth and sexual maturity of *P. hegeneri*. This may be due to mutual tactile stimulation by worms, or stimulation of growth by the presence of sperm in the seminal receptacle (Nollen, 1983).

Sogandares-Bernal (1966) observed that monometacercarial infections in the domestic cat with *Paragonimus kellicotti* never developed beyond the wandering pre-adult stage. When this host was later infected with other metacercariae, pre-adults paired and developed to mature worms in the lungs. Sogandares-Bernal postulated that chemotactic factors are involved in both worm attraction and sexual maturation in *P. kellicotti*. Worms do not find each other by chance alone, and factors other than "host mediated" are involved in worm chemoattraction.

Foreyt *et al.* (1977) studied the pairing tendency of *Fascioloides magna* in naturally infected white-tailed deer, *Odocoileus virginianus*. Pairing of worms was important for normal capsule formation in the liver and for worm maturation.

*In vivo* observations on schistosomes were made by Armstrong (1965), who examined the distribution of adult *S. mansoni* in unisexual infections by quick-freezing mice in liquid nitrogen and dissecting them after partial thawing. He found that 97% of the worms of both sexes were in portal veins, with their anterior ends directed toward the flow of blood. He observed homosexual pairing of males and such pairs showed a larger male harboring one to several immature males in its gynecophoral canal. He also studied the pairing behavior of X-irradiated schistosome males and females, and observed that deformed and sterile males paired less frequently with females than did normal males. He believed that worms find each other because they are attracted to the liver, and that tactile attraction is responsible for bringing conspecific members together.

There is some evidence that schistosomes release lipid *in vivo*. Cryostat sections of mouse mesenteric veins harboring adult worms revealed neutral lipid droplets in the part of the vessel wall that contracted males. Only parts of the male gynecophoral fold exposed to the female contained lipid, suggesting an influence of females on lipid secretion in males (Haseeb *et al.*, 1984).

### 3. *In ovo*

The use of the chick chorioallantois as a bioassay for trematode behavior was first suggested by Fried (1969). To eliminate host factors associated with

worm migration and establishment *in vivo*, pairing and clustering have been studied on the chorioallantois of domestic chick embryos maintained *in ovo* or *in vitro*; significant pairing of *L. constantiae* and *E. revolutum* have been reported from the chorioallantois (Fried and Roberts, 1972; Fried *et al.*, 1980a). Although these studies eliminate definitive host factors associated with the site, they do not eliminate chick embryonic or extraembryonic factors.

## IV. Chemical Nature of Helminth Pheromones

### A. NEMATODA

Greet (1964) showed that the sex pheromone of female *Panagrolaimus rigidus* was soluble in aqueous agar, and Balakanich and Samoiloff (1974) used ether–water extraction to demonstrate the water-solubility of the female sex pheromone of *Panagrellus redivivus*. Green (1967) reported that sex pheromones of *P. rigidus* were nonvolatile; Cheng and Samoiloff (1971) noted similar findings in *Panagrellus silusiae*. Treatment of *P. silusiae* pheromone with pronase decreased its activity, and column chromatographic analysis of the pheromone suggested both low and high molecular weight components. The male attractants of *P. redivivus* did not fractionate from water into ether, and two fractions were isolated on a Sephadex G-25 column (Green, 1980).

Stringfellow (1974) reported that hydroxyl ions emitted by females of *Pelodera strongyloides* attracted males. Bone and Shorey (1978a) suggested that although pH gradients stimulated some nematode behavior, premating attraction probably relied on more specific stimuli. Green (1980) doubted that simple inorganic ions were sex pheromones in *P. strongyloides*.

Green (1966) reported that pheromone from female *Heterodera* was labile in agar at 20°C, and little biological activity occurred one day after females were removed from the agar. Green (1967) reported that sex attractants of *H. schachtii* and *H. rostochiensis* did not fractionate from water into ether unless the aqueous layer was acid or alkaline. Active material was recovered from dried solutions or secretions by dissolution in water but not in acetone, methanol or ether. Greet *et al.* (1968) implicated a volatile component as a premating attractant in *H. rostochiensis* and *H. schachtii*. Aqueous components of *Heterodera* pheromone were not affected by UV, drying or moderate heat. Green and Plumb (1970) examined the female sex pheromones of ten *Heterodera* spp. and suggested the presence of at least six distinct attractive compounds. The dried pheromones were stable for one month at 5°C. Green (1970) noted that the activity of *H. schachtii* and *H. rostochiensis* phero-

mones was reduced by absorption on anionic exchange resins, Sephadex G. 10 and cellulose. Clarke *et al.* (1976) used paper chromatography to separate two active components of the secretions of *H. rostochiensis*. Green (1980) noted that nematode sex attractants are polar organic materials with several active components; pheromones of *H. schachtii* and *H. rostochiensis* are probably neutral or amphoteric and physically stable; these properties are characteristic of nematode sex attractants which operate in aquatic or semiaquatic environments, i.e. animal intestines, plant tissue and soil that are buffered against change.

The sex pheromone of the female pinworm, *Aspiculuris tetraptera*, was more heat labile than that of the male; boiling destroyed both male and female pheromones (Anya, 1976). *Nippostrongylus brasiliensis* females were attracted to male- or female-produced lipophilic substances which migrated with cholesterol and β-sitosterol on thin-layer chromatograms; authentic cholesterol or β-sitosterol was not attractive (Roberts and Thorson, 1977c). Two active compounds account for the pheromonal activity in incubates of *N. brasiliensis* females (Ward and Bone, 1983). Based on pronase activity, one compound is a peptide of about 600 $M_r$ as determined by gel filtration. The second compound is probably a hydrocarbon of 375 $M_r$ and its isolation by high-pressure liquid chromatography indicates that it is moderately polar and soluble in water and alcohols. The compound is synthesized rapidly, and analysis of its elements suggests the presence of one nitrogen; spectral analysis indicates that an amino acid accounts for about 50% of the molecular weight. Mycotoxin inhibitors of amino-acid transport in the host intestine yield a 72% increase in pheromone production by female *N. brasiliensis*, while amino-acid depletion in the host's diet causes a 34% reduction in pheromone production. Studies using radiolabelled amino acids may contribute to pheromone identification and allow insight into pheromone biosynthesis (Bone, 1986).

### B. TREMATODA

Lipophilic factors are involved in chemoattraction of trematodes (Childs *et al.*, 1986; Eveland and Haseeb, 1986; Fried, 1986; Gloer *et al.*, 1986; Haseeb and Eveland, 1986). Fried and Roberts (1972) demonstrated pairing of *Leucochloridiomorpha constantiae in vitro*, and Fried and Shapiro (1975) showed that *L. constantiae* metacercariae released lipids into the medium; the free sterol fraction was the major neutral lipid egested by this organism as determined by thin-layer chromatography (TLC). Chloroform–methanol extracts of ES products of *L. constantiae* metacercariae contained free sterols which attracted worms significantly *in vitro* (Fried and Gioscia, 1976). Berger

and Fried (1982), using densitometric-TLC, confirmed that free sterol was the major ES-lipophilic product of *L. constantiae* metacercariae; they reported that worms released an average of 3.2ng of free sterol per metacercariae per hour.

*Amblosoma suwaense* metacercariae paired and aggregated *in vitro*. Their major lipophilic ES product was sterol ester(s), and free sterol(s) was a minor fraction. When tested in the bioassay, *A. suwaense* metacercariae were attracted to sterol ester but not the free sterol fraction. Densitometric-TLC showed that *A. suwaense* metacercariae released a mean of 128 ng of sterol ester per metacercariae per hour compared to a mean release of free sterol of 49 ng per metacercariae per hour (Fried and Robinson, 1981; Berger and Fried, 1982).

*Echinostoma revolutum* adults were attracted to lipid but not aqueous fractions of ES products. Preparatively isolated sterol, triglyceride-free fatty acid, sterol ester and phospholipid fractions of chloroform-methanol extracts of *E. revolutum* were tested in the bioassay. The worms were attracted only to the sterol fraction, whereas the fraction containing phospholipid and other polar compounds acted as a chemorepellant. The use of authentic cholesterol as an attractant for *E. revolutum* in the bioassay was equivocal (Fried *et al.*, 1980b).

Analyses of free sterols in *E. revolutum* by gas–liquid chromatography (GLC) and TLC showed that the major free sterol is cholesterol (Barrett *et al.*, 1970; Fried and Boddorff, 1978). Chitwood *et al.* (1985) examined *E. revolutum* sterols using capilliary GLC-mass spectrometry (GLC–MS). They identified the relative percentage of ten different sterols, of which cholesterol was the most abundant (96.73%). They concluded that free sterol fractions prepared by TLC may contain additional components; positive identification of the lipophilic chemoattractants requires further purification and bioassay.

*S. mansoni* adults attract each other *in vitro*; ES products of worms of one sex attract worms of the opposite sex, and lipid fractions of worm ES products are attractive. Lipids released by adult males attract females *in vitro* and lipid release is modulated by the presence of other worms (Eveland and Haseeb, 1986; Haseeb and Eveland, 1986). Childs *et al.* (1986) reported that *S. mansoni* males were attracted to substance(s) in female extracts and ES products. Gloer *et al.* (1986) extracted *S. mansoni* females and their ES products with a series of solvents that provided fractions of varying polarity. *N*-Pentane- and ether-soluble fractions derived from ES products exhibited chemoattractancy comparable to that of whole-worm extracts. Analyses of the least polar fractions (*N*-pentane- and ether-soluble) by TLC and NMR spectroscopy suggested that some of the major components were steroids. The major steroid was cholesterol, but when authentic cholesterol was tested in the bioassay, it was unattractive to both sexes (Childs *et al.*, 1986).

## V. Structures Involved in the Production and Release of Pheromones

### A. NEMATODA

The vulva of *Nematospiroides dubius* females was implicated as a site of pheromone release, since conspecific males did not show bursal response to females whose vulva was smeared with silicone grease (Marchant, 1970). Croll and Wright (1976) were not able to demonstrate bursal flaring in *Nippostrongylus brasiliensis*. Dissolved cuticular material produced by *Panagrellus silusiae* females at ecdysis was thought to be a sexual attractant for males (Samoiloff, 1970). There was evidence of pheromone release from the body surface of female *Heterodera schachtii* and *H. rostochiensis*, suggesting that it was produced in the hypodermis or stored in the pseudocoel after production in an undetermined site (Green and Greet, 1972). Hydroxyurea-treated larvae of *P. silusiae* developed into females that did not attract males suggesting that the gonads were a likely source of pheromone production (Cheng and Samoiloff, 1972). Similar results were reported in *N. brasiliensis* adults in which gonadal development was inhibited by actidione (Bone and Shorey, 1978b).

Mid-region sections (containing vulva) of the female pinworm, *Aspiculuris tetraptera* attracted males, whereas those from the anterior or posterior regions did not, suggesting that the pulvillar secretory cells were a presumptive source of pheromone. Caudal gland secretions of males also attracted females (Anya, 1976). *Ascaris suum* males were attracted to the sex organs of females, and females were attracted to testes and male body fluid (Garcia-Rejon *et al.*, 1985a), implicating sex organs as the site of pheromone production in both sexes.

### B. TREMATODA

Most trematodes are small (< 1 cm in length) and it is difficult to isolate organs associated with production or release of pheromone. Lipophilic substances are involved in trematode chemoattraction (Eveland and Haseeb, 1986; Fried, 1986) and the source of lipids has not been unequivocally demonstrated, although lipid released from the tegument of *S. mansoni* males has been implicated as a presumptive pheromone in heterosexual schistosome chemoattraction (Haseeb *et al.*, 1984, 1985b; Haseeb and Eveland, 1986). In echinostomes and fasciolids, lipids accumulate in the excretory system and are released through the excretory pore (Burren *et al.*, 1967; Fried *et al.*, 1980b). In brachylaimids, epithelial cells of the intestinal ceca release

lipids into the lumen, and the lipids are egested through the mouth (Harris and Cheng, 1973). Lipids released from the excretory system in *E. revolutum* and from the alimentary tract in *L. constantiae* and *A. suwaense* are attractive (Section IV).

## VI. Receptors and Chemoreception

### A. Nematoda

Amphids are blind, pouch-like invaginations in the cuticle located in the head region of nematodes. One amphid is located on each side of the head, usually posterior to the cephalic setae, and they contain sensory receptors (Barnes, 1987). In the tail region of some nematodes is a pair of unicellular glands, phasmids, which open separately on either side of the tail. Phasmids are probably glandulosensory structures that function in chemoreception and are best developed in parasitic nematodes (Barnes, 1987).

Sensory neurons in the head of a nematode are grouped into sensilla. The neurons in each sensillum end in a channel enclosed within two non-neuronal cells, the sheath and socket cells. Neurons located in channels that penetrate the cuticle are considered to be chemoreceptors (Ward *et al.*, 1975). The ultrastructure of nematode sensory structures has been reviewed by McLaren (1976) and Wright (1983).

Ward (1973) suggested that chemotaxis in the nematode *Caenorhabditis elegans* requires comparison of chemical concentrations by the anterior sensory receptors. Using mutants, he showed that orientation of *C. elegans* depends on chemoreceptors in the head. The tips of spicules were believed to have pheromone receptors when Samoiloff *et al.* (1973) inhibited male to female attraction in *Panagrellus silusiae* by extirpating one spicule in males with a laser microbeam. The lack of oriented male response following irradiation of one spicule suggested that orientation to female pheromone required comparison of the chemical concentration at the receptors of both spicules. Such a chemoreceptive capacity seems reasonable, since the spicules of many nematodes are innervated, but it seems odd that chemoreceptors controlling anterior-directed orientation should be located in the spicules (Bone and Shorey, 1978a).

Wright (1983) noted similarities of nematode chemosensilla to vertebrate olfactory cells. Enzyme-mediated inhibition of chemotaxis in *C. elegans* was demonstrated by Jansson *et al.* (1984) who reported that mannosidase and sialidase caused 100% inhibition and trypsin caused 50% reduction of chemotactic behavior. They proposed a mechanism of chemoreception in

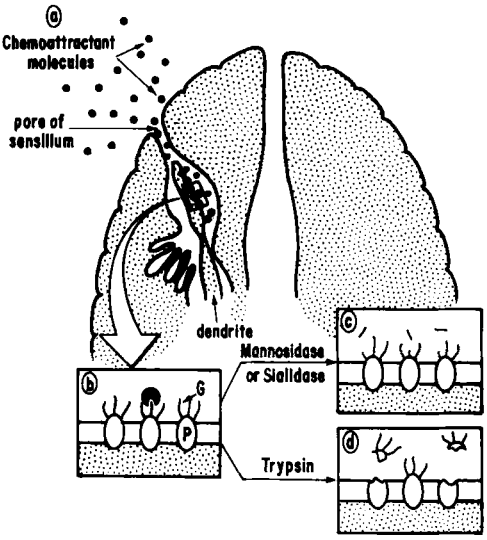


FIG. 3. Proposed mechanism of chemoreception in nematodes based on chemotaxis studies on *Caenorhabditis elegans* and *Panagrellus redivivus*. (a) Chemoattractant molecules diffuse through the pore of the sensillum located in the head area of the nematode. The attractant molecules are recognized by membrane receptors within the sensillum. (b) Schematic of glycoprotein membrane receptors. The protein segment (P) is depicted as passing through the membrane lipid bilayer. Glycans (G) are sites of recognition. A chemoattractant molecule is specifically recognized by a terminal sialyl or mannosyl moiety. (c) Treatment with mannosidase or sialidase alters the glycan residue, resulting in conformational changes and total loss in function of the receptors. (d) Proteolysis affects some, but not all, of the receptors, causing a partial blocking of chemotaxis. Redrawn after Jansson *et al.* (1984), with the permission of Academic Press.

nematodes that is initiated by the binding of chemoattractant to cephalic surface carbohydrates which are exudates of the amphids or other sensilla (Fig. 3). Lectin-mediated functional impairment and inhibition of chemotaxis was also demonstrated in *C. elegans* by Jeyaprakash *et al.* (1985). Mannose-containing receptors have been implicated in chemoreception of *Trichostrongylus colubriformis* by Bone and Bottjer (1985).

### B. TREMATODA

Studies on the reception of chemical stimuli in trematodes are sparse (Kemp and Devine, 1982). Scanning electron microscopy and light-level silver staining studies have shown that larval and adult digeneans have various

types of sensory papillae, and that uniciliate and multiciliate papillae probably play a role in chemoreception (Hoole and Mitchell, 1981; Fried and Fujino, 1984). Although electron microscopy has revealed structures in schistosome surfaces which may serve as sensory receptors, there is still no information on their function (Hockley, 1973; Haseeb *et al.*, 1985b).

Michaels (1969) postulated that the mating position in *S. mansoni* is determined by linear receptors on males and females. She used ribonucleases, deoxyribonucleases, and snake venoms to determine the nature of postulated receptors, but these attempts were unsuccessful. Results of her studies are difficult to interpret, since normal and abnormal mating positions of transected worms are not clearly defined.

Lectin-binding sites have been demonstrated on both schistosomula and adult schistosomes (Stein and Lumsden, 1973; Bennett and Seed, 1977; Murrell *et al.*, 1978). Several oligosaccharide residues, notably alpha-methyl-D-mannoside, D-galactose, D-mannose and/or D-glucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine and sialic acid, occur on the schistosome surface (Simpson and Smithers, 1980; Simpson and McLaren, 1982). In these studies, however, distinction between male and female worms was not made. Bennett and Seed (1977) believed that these lectin-binding carbohydrates occur as glycoproteins. Results of other studies did not support this notion (Murrell *et al.*, 1978). A study on the interaction of *Salmonella* and schistosomes suggests that at least the mannose-containing receptors are glycolipids (Melhem and LoVerde, 1984). Although these studies have provided information about lectin-binding receptors in schistosomes, there has been no attempt to determine the role of receptors in chemoattraction.

Host immunoglobulin binding to schistosomes is mediated by Fc receptors present on the worm surface. Receptors for C3 and C1q have been demonstrated on schistosomula (Kemp *et al.*, 1986). Benzodiazepine binding sites have beeen demonstrated on the tegument of *S. mansoni* adults (Bennett, 1980). At present, there is no evidence of involvement of these receptors in interactions between male and female worms.

## VII. Specific Organisms

### A. NEMATODA

#### 1. *Heterodera*

Considerable work has been done on pheromones of the cyst nematode *Heterodera* (of which one species investigated, *H. rostochiensis*, has since

been put in a new genus, *Globodera*). Females of *Heterodera* spp. produce sex pheromones that attract conspecific males; males are less attracted to females that are heated, treated with methyl bromide or extracted in acetone; the pheromones are nonvolatile (Section IV) and remain active after vacuum drying at 35°C or air drying at 45°C (Green, 1966).

Newly emerged males of *Heterodera* spp. do not respond to females, indicating that some aging is required for responsiveness; the reaction of males is dosage-dependent and requires an optimal concentration of pheromone for maximal response (Greet *et al.*, 1968). Numerous species of *Heterodera* have been described and Green and Plumb (1970) studied the specificity of *Heterodera* pheromones. Based upon the observed intra- and interspecific patterns of chemical communication they suggested that the genus *Heterodera* can be divided into three subgenera and ten species.

Rende *et al.* (1982) showed that *H. glycines* males were attracted to females in an *in vitro* bioassay. Responses were dependent on dosage and diffusion time. Males were most responsive three days after they emerged from the host plant, at which time production of the pheromone by females was also maximal. Jayaprakash and Rao (1982) studied the life history of *H. oryzicola* and noted that virgin females secreted a strong male attractant; the males migrated with them. Huettel and Rebois (1986) developed a bioassay for *H. glycines* that allowed for the use of behavioral characteristics to detect pheromone; a typical coiling or encirclement behavior of males in the presence of isolated females on agar plates was noted. *H. glycines* showed chemotaxis to various test substances without coiling behavior, suggesting that coiling is a specific behavior associated with sex pheromone (Huettel and Jaffe, 1987).

### 2. *Nippostrongylus brasiliensis*

Roberts and Thorson (1977a) studied attraction of *N. brasiliensis* adults *in vivo* and *in vitro*. Their *in vitro* studies showed that heterosexual and homosexual attraction occurred in the absence of host-mediated factors, and attraction to worm ES products indicated that chemical factors were involved. Roberts and Thorson (1977c) showed that *N. brasiliensis* females were attracted to the lipophilic ES products of both males and females (Section IV). Roberts and Thorson (1977b) also showed that *N. brasiliensis* adults could pair with nematodes of other species *in vitro*, indicating that nonspecific attractants were involved.

Bone and Shorey (1977b) described the interactive influences of male- and female-produced pheromones on attraction of *N. brasiliensis* males to females. Contrary to the observations of Roberts and Thorson (1977a), they found no evidence for homosexual attraction *in vitro*. Bone and Shorey

(1977b) noted that males were strongly attracted to a source of sex pheromone emitted from females. Bone *et al.* (1977a) showed that males were highly responsive to female pheromone after the final larval molt about 5 days after infection. The time of day did not influence either sex's production of or response to pheromone. Bone *et al.* (1977b) reported that 7-day-old male and female worms showed mutual heterosexual attraction *in vitro* and that a strong dosage dependency to the female pheromone was present in responding males. Males were significantly attracted to as few as three females, and females were less responsive to male pheromone dosage *in vitro*. Bone and Shorey (1977a) maintained males in an environment permeated with female pheromone. When males were removed from the environment, their subsequent ability to orient to female pheromone was significantly reduced for up to 2 hours. Bone and Shorey (1977a) suggested that this phenomenon could serve as the basis for a selective anthelmintic technique in which premating communication between males and females is disrupted. Bone *et al.* (1978) studied factors influencing the movement of males toward a female pheromone; they noted that single responding males showed a dosage-dependent movement toward a source of pheromone from females incubated or homogenized in Tyrode's solution.

Increase in female pheromone beyond an optimal concentration resulted in reduced male movement toward the pheromone source. Bone (1980) was able to activate male worms by *in vitro* exposure to female pheromone using a spot plate bioassay. Pheromone prepared as an incubate elicited an almost 80% male response within 10 minutes at a 15 females per hour dosage; the male response was dosage dependent. Bone (1982c) showed that the release of pheromone by females as a crude incubate was linear for the first 2 hours, but declined after that; pheromone release in solution increased with higher temperature up to 37°C. Gel filtration of female pheromone prepared as a crude incubate showed biologically active elutions at $K_{av}$ 0.64 and 1.0. Bone *et al.* (1979) used gel filtration chromatography on homogenates of males and females to demonstrate two fractions with pheromonal activity. One fraction with a molecular weight over 500 was found in aqueous extracts of both sexes, and was attractive to both sexes. A second region of activity was found only in females and attracted only males; a molecular weight under 400 was proposed for this region. Bone *et al.* (1980a) noted that the $K_{av}$ 0.64 fraction showed reduced activity for the male's locomotor response when obtained from female worms older than 10 days. Bone *et al.* (1980b) described partial purification of the aggregation pheromone, *nippolure*, from female worms. This polypeptide pheromone is degraded by pronase, while increased recovery is obtained after treatment with several protease inhibitors. The pheromone has a net negative charge based on an isoelectric point

of 7.3 and retention in DEAE cellulose. Ward and Bone (1983) described chromatographic isolation procedures on the $K_{av}$ 1.0 pheromone of female worms.

### 3. *Caenorhabditis elegans*

The two sexes of *C. elegans* are the male and the self-fertilizing hermaphroditic female (Albert *et al.*, 1981); to our knowledge there are no studies on sexual attraction between males and self-fertilizing hermaphrodites. However, numerous studies are available on the chemotaxis of *C. elegans* (Ward, 1978; Dusenbery, 1980). Ward (1973) designed a bioassay to show that *C. elegans* is attracted by four classes of chemical substances, i.e. cyclic nucleotides, cAMP and cGMP; anions, $Cl^-$, $Br^-$, $I^-$; cations, $Na^+$, $Li^+$, $K^+$, $Mg^+$; and alkaline pH values. Worms orient toward the gradient and the response is klinotactic. Dusenbery (1980) studied the behavioral responses of *C. elegans* to controlled chemical stimuli by holding the worm's tail in a suction pipette; behaviour was recorded by light sensors connected to a multichannel recorder; a type of behavior described as "the reversal bout" was observed, suggesting that klinokinesis with adaptation plays a role in the movement of *C. elegans* in a chemical gradient.

Huang *et al.* (1982) placed hydroxytryptophan (5-HTP) on the vulva of *C. elegans* to examine behavioral responses to this neurotransmitter; vulval contraction occurred, but there was little relationship between dosage and behavioral response.

The food supply and a pheromone influence both entry and exit from the dauer stage of *C. elegans*. The pheromone does not affect adult chemotaxis or egg-laying, but increases the frequency of dauer larva formation and inhibits recovery. The pheromone was described as fatty-acid-like and only partially characterized (Golden and Riddle, 1982).

## B. ASCHELMINTHES OTHER THAN NEMATODA

Females of the rotifer, *Asplanchna brightwelli*, did not respond to males, and the response of males to a female pheromone was not studied (Souchek and Scarfe, 1981). Gilbert (1976) reported that chemical recognition reduced cannibalism in the rotifer, *A. sieboldi*. Female rotifers in the genus *Brachionus* attract males with a heterocyclic compound of a low molecular weight. The pheromone was probably detected by receptors on the corona and it stimulated turning behavior of the male (Gilbert, 1963).

## C. TREMATODA

### 1. *Monogenea*

Although there is information on the influence of chemical substances from fish hosts on hatching and host-finding in larval monogeneans (Kearn, 1986), there is scant material on chemical communication in adults. S. Macdonald (1974. Ph.D. Thesis, University of East Anglia) found some evidence for clustering in the gill parasite, *Diclidophora merlangi*. She reported that when a juvenile and an adult worm occupied the same gill arch they were on neighboring lamellae in only 31% of the cases. When two or more adult worms occupied the same gill arch they were on neighboring lamellae in 74% of the times. Her findings suggest that adult worms migrate towards each other.

Although self-fertilization can occur in some monogeneans, others have adaptations to assure cross-fertilization (Smyth and Halton, 1983). A remarkable adaptation for cross-fertilization is in *Diplozoon paradoxum* which consists of two fused individuals, with fusion occurring during larval development. The mechanism of how *Diplozoon* larvae find each other is unknown (Smyth and Halton, 1983).

### 2. *Digenea*

(*a*) *Schistosoma.* Recent studies have demonstrated that adult *S. mansoni* attract each other *in vitro*, and the attraction is chemically mediated (Imperia *et al.*, 1980; Eveland *et al.*, 1982). Imperia *et al.* (1980) stated that adult males emit pheromone(s) which attract females. The *in vitro* studies by Eveland *et al.* (1983) and Shirazian and Schiller (1982) suggest worm-finding and pairing mechanisms which may operate *in vivo*.

Shaw *et al.* (1977) showed that acetone and ether extracts (presumably lipids) of *S. mansoni* males induced development of vitelline cells, with a corresponding increase in body length of females from unisexual infections. This study suggests that lipophilic factors from males induce developmental changes in females. The active factors may be lipids or, alternatively, lipids may serve as carriers or solvents. The pheromone of hard ticks has been shown to be dissolved in neutral lipid (Sonenshine *et al.*, 1981).

Histochemical and TLC studies clearly indicate that lipid release is affected by the presence of other worms. These observations are in accord with the findings of Eveland *et al.* (1983), who reported that worms of either sex do not show attraction to worm-pairs, two males or two females, although homosexual attraction to single worms occurred. They proposed that either the "window effect" (Kemp and Devine, 1982) or a "shutdown"

mechanism is involved in diminishing attraction. Demonstration of a dose–response relationship would help clarify this issue (Haseeb and Eveland, 1986).

(*b*) *Echinostoma revolutum.* Numerous *in vitro* studies have demonstrated a tendency of this organism to pair or cluster (Fried, 1986). Isolated organisms in the domestic chick, although capable of self-fertilization, do not grow as well as paired or clustered organisms and produce fewer viable eggs (Fried and Alenick, 1981). The tendency to pair *in vitro* was studied by Fried *et al.* (1980b) using a linear chamber. Whereas adults paired *in vitro*, chemically excysted metacercariae did not. Adults were attracted to isolated worms restrained in a dialysis sac, and were also attracted to lipid fractions of worm ES products. TLC of ES products showed that the free sterol fraction was attractive. Organisms were not unequivocally attracted to an authentic cholesterol standard; also GLC–MS analysis of the sterol fraction showed that it was complex and consisted of at least nine minor sterols in addition to cholesterol (Section IV).

Single *E. revolutum* adults migrated significantly toward dialysis sacs containing conspecific adults (Fried *et al.*, 1980b). Worms isolated in the sac were designated "releasers" and those outside, "migrators" (Belosevic and Dick, 1980; Fried and Wilson, 1981b). *E. revolutum* pheromonal dosage was examined in a barrier design bioassay where either two, four, or six releasers were isolated in a chimney barrier and a single migrator was allowed to move toward or away from the barrier; four and six releasers were better at attracting a single migrator than two releasers (Fried and Wilson, 1981b). Tracking of *E. revolutum* adults in an 0.8% agar-Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum was done; worms survived up to 48 hours in the medium and left distinct acetabular tracks in the agar; some worms showed directed movement toward each other, suggesting chemotaxis (Fried and Vates, 1984).

### D. ACANTHOCEPHALA

Crompton (1985) stated that there is no evidence to suggest that male or female acanthocephalans produce chemical attractants to assist in mate finding. Circumstantial evidence of chemical communication is good since male and female *Moniliformis moniliformis* ( = *M. dubius*), when surgically transplanted into rats, find each other and copulate, regardless of age and numbers (Crompton, 1974). There is little information on pairing in acanthocephalans and it is not known whether this phenomenon begins prior to sexual maturation (Crompton, 1970).

Bone (1976) considered heterosexual mating attraction in *M. moniliformis*

to be absent, and suggested that mating resulted from trial and error pairing through thigmotactic stimulation. This statement was based on controlled exposures of 20-day-old male and female worms *in vitro* in an apparatus designed to detect secretions either sex might have released into the medium. According to Bone (1982b) studies on reproductive communication in acanthocephalans are scarce, probably because of their minor economic and medical significance. However, sexual dimorphism is suggestive of the occurrence of chemically mediated interactions.

### E. TURBELLARIA

Few studies are available on pheromones in turbellarians (Bone, 1982a), and they are restricted to freshwater planarians of *Dugesia*. Reynierse and Gleason (1975) reported that when a single planarian was attracted to a conspecific aggregation, visual and chemical cues were involved. Perkins and Fried (1982) showed that fed *D. tigrina* and *D. dorotocephala* paired intraspecifically in the absence of light, indicating that visual cues are not essential for pairing; starved planaria did not pair significantly and fed planaria egested free sterols and free fatty acids into the medium; lipids were proposed as chemoattractants. The role of lipids in planarian behavior is not unusual, since Mason (1975) showed that free fatty acids are involved in food location in *D. nuemani*.

### F. CESTODA

Information on pheromones in cestodes is not available; Bone (1982a) suggested that pheromones are probably involved in behavior and sexual phenomena in monoecious and dioecious cestodes.

Observations on some cestodes suggest the presence of chemical communication. Intestinal location of *Hymenolepis diminuta* was determined by gradients of 5-hydroxytryptamine (Mettrick and Cho, 1981). Self-fertilization in *H. nana* for a number of generations yielded eggs and cysticercoids with reduced viabilty, and selfing beyond the fifth generation was lethal (Rogers and Ulmer, 1962).

Crowding factors in cestodes are probably pheromonal, i.e. worm-secreted substances that inhibit conspecifics. Insler and Roberts (1980) prepared a worm-conditioned saline (WCS) by incubating 10-day-old *H. diminuta* from crowded infections in balanced salt solution, and then tested the effects of the WCS on the incorporation of $^{3}$H-thymidine into the DNA of *H. diminuta* from uncrowded infections; thymidine incorporation was inhibited about 60% compared to controls.

## VIII. Site-Finding Behavior

### A. NEMATODA

Several investigations have demonstrated chemical attraction and oriented migration of phytoparasitic nematodes to diffusates of roots (Ulmer, 1971). Chemotactic factors are also involved in site finding of zooparasitic nematodes, e.g. attraction of *Ancylostoma caninum* juveniles to a low molecular weight component of dog serum (Vetter *et al.*, 1985), an adaptation that probably helps the parasite find a vein upon entry into the host (Huettel, 1986).

Some nematodes use kairomones, i.e. interspecific chemical substances that elicit a behavioral response favorable for the receiver (Huettel, 1986). *Bursaphelenchus xylophilis*, an entomophilic nematode, is attracted to β-myrcene, a terpene which is also attractive to insects. A related terpene, ipsenol, is the aggregation pheromone of *Ips proconfusus* (Coleoptera). Some entomophilic nematodes use host pheromones for site location (Huettel, 1986).

Passive or nonoriented behavioral phenomena have been noted in nematodes. Migration of *N. brasiliensis* larvae through the host's intestine and blood vessels is due in part to passive transport (Croll, 1976; Croll and Ma, 1978). Site selection by gastrointestinal helminths is influenced by physiological cues of host origin, the most important of which were bile secretions, diet, pH, $pCO_2$, $pO_2$ (Sukhdeo and Mettrick, 1983).

The behavioral repertoire of nematodes is complex, and at least 22 distinct reactions including site location have been described for nematodes (Croll and Sukhdeo, 1981). Both site finding and worm-mediated chemical signals should be considered an integral part of the behavioral repertoire of nematodes.

### B. TREMATODA

Considerable literature is available on how miracidia locate their snail hosts (Saladin, 1979), and how oncomiracidia find their fish hosts (Kearn, 1986). Relatively little information is available on how cercariae find their hosts, although lipids play a role in the penetration of schistosome cercariae into host skin (Zibulewsky *et al.*, 1982; Granzer and Haas, 1986). Precise information on how non-schistosome cercariae find their hosts is scant, although a recent study by Anderson and Fried (1987) suggested that renal secretions of snails are involved in cercarial penetration and location of site for encystation.

Little information is available on site finding of metacercariae and adult trematodes (Ulmer, 1971; Kemp and Devine, 1982). Danley (1973) reported that secretions from the chick Harderian gland attract the eyefluke, *P. megalurus*, and Fried and Bradford (1984) suggested that free sterols from the bursa of Fabricius are chemoattractants for *L. constantiae* metacercariae. Sukhdeo and Mettrick (1986) noted that extracts from the rat duodenum and liver affected the rate of locomotion and orientation of newly emerged juveniles of *F. hepatica*, but the mechanisms by which larvae orient are not clear.

Little is known about site selection in adult trematodes; fecal extracts are chemoattractants for paired adult schistosomes (Awwad and Bell, 1978) and adult schistosomes are attracted *in vitro* to indole derivatives (Schwabe and Kilejian, 1968). Unidentified portal serum factors stimulate the oviposition of *S. mansoni* maintained *in vitro* and may play a role in site-location of adult schistosomes *in vivo* (Wu and Wu, 1986). Single *E. revolutum* adults placed on the chick chorioallantoic membrane localize on the chorioallantois above the embryo. A second worm placed on the membrane paired with the first, but worm-pairs were located less frequently above the embryo than single worms. Worm-released chemoattractants probably mask factors associated with chick-embryo secretions that attract a single worm to the preferred site (Fried and Diaz, 1987).

## IX. Analytical Techniques Used in Studies of Chemical Communication

Modern analytical techniques have played an important role in the isolation and identification of pheromones in more than 300 insects (Leonhardt, 1985). There were 234 references on pheromones in 1976 compared with 661 in 1982, and many described methods of isolation and identification (Leonhardt, 1985).

The voluminous literature on microanalytical chemistry of insect pheromones has been summarized in two recent books edited by Hummel and Miller (1984) and by Acree and Soderlund (1985). A comprehensive earlier review was presented by Beroza (1975) on the extraction, isolation, and identification of insect pheromones; that review contains an excellent discussion of chromatographic and spectrometric procedures used in analysis of pheromones.

Studies on arthropods should provide useful models for work on helminths. Sonenshine (1985) described techniques for studies on pheromones of ticks, i.e. scanning and transmission electron microscopy of the foveal gland, X-ray microanalysis and gas–liquid chromatography (GLC) to ident-

ify the sex attractant, 2,6-dichlorophenol in *Dermacentor*. Hecker and Butenandt (1984) described the events that led to the chemical identification of the sex pheromone, bombykol, from the female silkworm moth, *Bombyx mori*. They also discussed the use of paper chromatography to identify samples of nitroazobenzene-carboxylic acid (NABA)—ester fractions of the pheromone; column chromatography for further purification of the fraction; and other chromatographic procedures for elucidation of structure of the NABA–ester of bombykol.

Relatively few studies are available on the microanalyses of pheromones in helminths. Roberts and Thorson (1977c) used TLC to isolate lipid pheromones from homogenates of *N. brasiliensis* females. Bone (1982c) used TLC, HPLC and gel filtration to show that pheromones from *N. brasiliensis* females contain water- and organic-soluble components.

Fried (1986) described sample preparation, and TLC procedures for the isolation and tentative identification of lipophilic pheromones in various hermaphroditic trematodes. The techniques included Folch extraction procedures, qualitative and densitometric TLC; argentation and preparative-TLC (Fried and Sherma, 1986). Because preparative TLC procedures may not exclude extraneous compounds from a given sample, Chitwood *et al.* (1985) used capillary GLC–MS to demonstrate numerous minor sterols in addition to cholesterol in a sterol fraction presumed to have pheromonal activity.

TLC was used to identify presumptive lipophilic pheromones in schistosomes (Haseeb and Eveland, 1986). TLC and GLC–MS were used by Gloer *et al.* (1986) to examine *S. mansoni* females and their ES products for presumptive lipophilic pheromones.

## X. Concluding Remarks and Future Prospects

Helminthologists are about 30 years behind entomologists in their studies on chemical communication. There is only partial characterization of two helminth pheromones, both from *N. brasiliensis* females; one is probably a hydrocarbon with a molecular weight of about 375 and the second a peptide of moderate polarity, soluble in water and alcohols, and with a molecular weight of about 600 (Bone, 1986). Less information is available on the characterization of trematode pheromones, which appear to be unidentified lipophilic substances, probably sterols (Eveland and Haseeb, 1986; Fried, 1986; Gloer *et al.*, 1986; Haseeb and Eveland, 1986; Section IV). It is not known if lipids are the pheromone(s) or carriers of pheromone(s) as in hard ticks (Sonenshine, 1985).

Details on the biosynthesis, storage, release and reception of helminth pheromones are meager, especially when compared to studies on lepidopter-

ans (Hecker and Butenandt, 1984) or acarines (Sonenshine, 1985). Knowledge of diffusion and transport of helminth pheromones in aqueous or semiaqueous environments is also meager (Bone and Shorey, 1978a; Green, 1980). Lipophilic substances are only sparingly soluble in aqueous environments (Haberland and Reynolds, 1973), and the mechanism of their transfer in helminths is not known. The fact that labelled cholesterol and its metabolites can be transferred between adult male and female *S. mansoni in vitro* (Haseeb *et al.*, 1985; Silveira *et al.*, 1986) attests to the transfer of lipids in aqueous or semiaqueous environments.

Transfer of volatile insect pheromones in various media has been considered by Shorey (1976) and Wilson (1968) and techniques are available for their collection, isolation, and bioassay (Gaston, 1985; Parliament, 1985) that are applicable in helminthology should the need arise. Although dosage-dependent studies on pheromones in nematodes are commonplace (Bone and Shorey, 1978a; Bone, 1982b), they have been used infrequently with trematodes (Fried and Wilson, 1981b; Bone, 1982a).

Studies of behavior are difficult to do *in vivo*, and there is a scarcity of *in vitro* cultivation studies with emphasis on worm behavior. No economically important trematode has yet been cultured to an ovigerous adult stage. Further studies that combine *in vitro* culture and pheromone behavior work should be promising. A recent study on newly emerged juveniles of *F. hepatica* has revealed the behavioral repertoire of young digeneans (Sukhdeo and Mettrick, 1986). Information on the behavioral analysis of nematodes surpasses what is known about trematodes, mainly because of the pioneering work of Croll and co-workers (Croll and Sukhdeo, 1981).

New parasite models should be sought that may be more suitable for pheromone studies. Schistosomes in the genera *Schistosomatium* and *Trichobilharzia* are dioecious and dimorphic, easy to maintain in the laboratory, and reach sexual maturity earlier than other schistosomes (Malek, 1977; Meuleman *et al.*, 1984). They may provide a better model for chemical communication studies than schistosomes which infect humans.

Hermaphroditic digeneans of economic significance, i.e. *Fasciola*, *Fascioloides* and *Paragonimus*, show a tendency to pair *in vivo* and could provide interesting material for *in vitro* studies. Some background literature on *in vivo* pairing of *Fascioloides* and *Paragonimus* is available (Sogandares-Bernal, 1966; Foreyt *et al.*, 1977).

There is a need for further purification and chemical characterization of helminth attractants. Excretory–secretory products from worms need to be collected and screened for potential pheromone activity, procedures which first may require partial purification and characterization. Such studies need joint efforts of analytical chemists and helminthologists.

New approaches to the control of helminthiases are needed. A knowledge

of pheromones that regulate sexual attraction in nematodes and dioecious trematodes or pairing and aggregation in hermaphroditic digeneans could lead to immunological or chemotherapeutic means to interfere with pheromonal communication. Preventing worms from mating would eliminate the pathology associated with egg production in such helminths as the schistosomes, and prevent the completion of the life-cycle for most helminths.

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# The Nature, Extent and Significance of Variation Within the Genus *Echinococcus*

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# I. Introduction

## A. Variation in parasites

The genetic heterogeneity of many parasite species has long been suspected, because of ill-defined differences in the epidemiology of a particular disease in different endemic areas and variation in clinical manifestations. The inability of traditional taxonomic criteria (i.e. morphology) to accurately detect and determine the extent of variation between and within species of parasites led to the application of more reliable biochemical/molecular criteria (see Section III, C). As a result, it has been possible not only to confirm the enormous genetic heterogeneity that exists within parasite species, but also to relate this heterogeneity to factors of epidemiological and clinical significance. This has been well demonstrated for the causative agents of a variety of important parasitic diseases such as trichinosis, schistosomiasis, African and South American trypanosomiasis, leishmaniasis, malaria and haemonchosis (reviewed in Thompson, in press). It is the purpose of the present review to examine the nature, extent and significance of variation, particularly below the species level, in the causative agent of hydatid disease, *Echinococcus*.

## B. Current status of hydatid disease

Hydatid disease is medically and economically one of the most important of the zoonoses. This importance shows little sign of diminishing, and in fact there is evidence that the causative agents of both unilocular (*E. granulosus*) and multivesicular (= alveolar) (*E. multilocularis*) forms of the disease are spreading and extending their ranges into areas previously considered to be free of infection (Matossian *et al.*, 1977; Anonymous, 1982; Rausch, 1986; Schwabe, 1986; Thompson and Allsopp, in press).

Medically, hydatid disease is significant because surgery remains the only effective cure for patients with active, viable hydatid cysts (Schantz, 1984), although even with surgical intervention, some authorities regard multivesicular hydatid disease to be incurable (Mosimann, 1980). There is still widespread optimism for the role of chemotherapy using the benzimidazole compounds mebendazole and albendazole. At this stage, however, chemotherapy is not widely advocated as an alternative to surgery in operable cases but shows promise post-operatively in preventing recurrences and as a means of impeding the progression of disease in inoperable cases (Eckert, 1986; Rausch *et al.*, 1986; Mansueto *et al.*, 1987).

On a global basis, *E. granulosus* must be the most important of the four

species. Geographically, it is the most widespread, with endemic foci on every inhabited continent (Matossian *et al.*, 1977; Rausch, 1986; Thompson and Allsopp, in press). Because of the adaptability of *E. granulosus* to a wide variety of domestic food animals, there is a direct relationship between the prevalence of unilocular hydatid disease and human activity. Man's role in perpetuating the life-cycle of *E. granulosus* through inappropriate husbandry practices is the most important factor for transmission (Thompson and Allsopp, in press). Sylvatic cycles play only a minor role, if any, as reservoirs of infection for domestic cycles. Fortunately, control programmes which rely on education backed up by legislation have demonstrated how well *E. granulosus* will respond to control (Gemmell and Lawson, 1986; Gemmell *et al.*, 1986a). The causative agent of multivesicular hydatid disease has a more restricted geographical distribution than *E. granulosus* (Table 1). Unfortunately, however, *E. multilocularis* poses a far greater problem to control because of its occurrence in sylvatic host assemblages.

The economic effects of hydatid disease arise from public health aspects and costs of hospitalization, costs associated with control programmes, losses from the condemnation of affected organs from livestock at slaughter, the consequences of import restrictions and effects on animal production and meat quality (Anonymous, 1982; Schwabe, 1986; Thompson and Allsopp, in press).

### C. BIOLOGY AND LIFE-CYCLE OF THE CAUSATIVE AGENT OF HYDATID DISEASE, *ECHINOCOCCUS*

There are four recognized species of *Echinococcus*; *E. granulosus*, *E. multilocularis*, *E. oligarthrus* and *E. vogeli* (see Section II A; Table 1). The following is meant as a brief overview of the biology and life-cycle of *Echinococcus* and summarizes the more detailed descriptions given in Schantz (1982), Rausch (1986), Thompson (1986) and Thompson and Allsopp (in press).

Members of the genus *Echinococcus* are cyclophyllidean cestodes belonging to the family Taeniidae. As such, they are characterized by an indirect two-host life-cycle involving carnivorous definitive and non-carnivorous intermediate mammalian hosts. Unlike *Taenia*, adult *Echinococcus* rarely exceed 7 mm in length and usually have fewer than five proglottids. The hermaphroditic adults live firmly attached to the mucosa of the small intestine of the definitive host. Sexual maturity is attained within 3–4 weeks, with egg production commencing as early as 28 days, depending on the species and strain. Shed gravid proglottids containing eggs are voided in the faeces of the definitive host, although eggs are undoubtedly released from the shed proglottid as it passes down the gut, so that free eggs are also liberated

in the faeces. Eggs voided in the faeces are thought to be fully embryonated and infective, although immature eggs may be released which require a period of maturation in the environment. The eggs can withstand a long sojourn in the environment due to the resistant nature of their outer keratinized covering (embryophore).

Ingestion of viable, embryonated eggs by a suitable intermediate host leads to the development of the cystic, metacestode (larval) stage. The eggs hatch in the stomach and small intestine releasing the embryo, or oncosphere. Once activated, the oncosphere rapidly penetrates the villous epithelium and reaches the lamina propria, assisted by hook movements and histolytic enzymes. Upon gaining access to a venule or lacteal, the oncosphere is passively transported to the liver or lungs, although a few may be transported further to the kidneys, spleen, muscles, brain or other sites. The organs affected vary in different species of intermediate host and also with the species or strain of *Echinococcus*. Once the oncosphere has reached its final location, it develops into the cystic, asexually proliferating metacestode. The type of cyst which develops varies markedly between the different species (Table 1). Cysts of all species characteristically comprize an inner germinal or nucleated layer which is supported externally by an acellular laminated layer. A host-produced fibrous adventitial layer surrounds the laminated layer in all species except *E. multilocularis*.

Hydatid cyst development varies between different species and strains of parasite and is also influenced by the age, sex, immune status, species and breed of host as well as the degree of infection. With *E. granulosus*, full development, i.e. production of protoscoleces, may take 2 years in naturally infected intermediate hosts such as sheep, but with *E. multilocularis* protoscoleces are produced within 2–4 months in its natural intermediate hosts, arvicolid rodents. Protoscoleces, which arise by a process of asexual budding (= polyembryony; Smyth, 1964), are not always produced. It is quite common, particularly in unsuitable intermediate hosts, for sterile cysts to develop in which protoscoleces have not been produced.

Asexual proliferation of the metacestode is entirely endogenous in *E. granulosus*. In contrast, both endogenous and exogenous proliferation occur in *E. multilocularis* due to the lack of any limiting host-tissue barrier, in the form of an adventitial layer. This permits larval *E. multilocularis* not only to spread by infiltration of surrounding host tissues, but also to produce distant metastatic foci as a result of the distribution of detached germinal cells via the lymph or blood. Some exogenous proliferation also occurs in larval *E. oligarthrus* and *E. vogeli*, but this is limited by the presence of an adventitia.

Definitive hosts become infected by ingesting protoscoleces from a hydatid cyst. Within a few hours, ingested protoscoleces descend between the villi of the small intestine, where they attach and proceed to develop to the adult worm.

## II. TAXONOMY

### A. SPECIES

Classification and nomenclature within the genus *Echinococcus* Rudolphi, 1801 has long been controversial. The life-cycle of *E. granulosus* (Batsch, 1786) was determined experimentally by von Siebold (1852). By 1855 two different types of hydatid cyst had been described, and there was much debate over whether one or two species of *Echinococcus* were the causative agents. The dualist view, that *E. granulosus* was responsible for cystic hydatidosis and another species, *E. multilocularis* Leuckart, 1863, for alveolar hydatidosis, was not universally accepted until Vogel (1957) completed the life-cycle of *E. multilocularis* in the laboratory.

Between 1910 and 1972, 14 other species of *Echinococcus* were described (Kumaratilake and Thompson, 1982a). Most of these were subsequently invalidated by Rausch (1953), Vogel (1957), Rausch and Nelson (1963) and Schantz *et al.* (1976), on the grounds that their morphological descriptions were either based on unreliable characters or fell within the limits of normal morphological variation of established species. Only *E. oligarthrus* (Diesing, 1863) and *E. vogeli* Rausch and Bernstein, 1972, have retained their status as valid species.

Thus, there are four currently accepted species in the genus *Echinococcus*. Their major distinguishing characteristics are summarized in Table 1.

### B. SUBSPECIES

Between 1954 and 1969, ten subspecies of *E. granulosus* and three subspecies of *E. multilocularis* were described (Kumaratilake and Thompson 1982a). Of the subspecies of *E. granulosus*, all except two were subsequently invalidated by Rausch (1967a) and Schantz *et al.* (1976) on the grounds that they were not shown to be segregated geographically or ecologically from the nominate subspecies, *E. granulosus granulosus* Verster, 1965, and therefore did not conform to the definition of a subspecies proposed by Mayr (1963). *E. granulosus canadensis* Webster and Cameron, 1961, described from wolves and cervids in northern North America, was considered by Rausch (1967a) to be geographically separated from *E. g. granulosus* and was not invalidated.

The three subspecies of *E. multilocularis*; *E. multilocularis multilocularis* Vogel, 1957, *E. multilocularis sibiricensis* (Rausch and Schiller, 1954) and *E. multilocularis kazakhensis* Shul'ts, 1961, were regarded as valid by Rausch (1967a). *Echinococcus m. multilocularis* and *E. m. sibiricensis* are geographically separated, being restricted to Europe and North America respectively.

TABLE 1 *Characteristics[a] of the four species currently recognized within the genus* Echinococcus

| | *E. granulosus* | *E. multilocularis* | *E. oligarthrus* | *E. vogeli* |
|---|---|---|---|---|
| Geographical distribution | Cosmopolitan | Central and northern Eurasia, northern North America | Central and South America | Central and South America |
| Host range | | | | |
| (i) *Definitive host* | Primarily dogs and other canids | Primarily foxes, also other canids and cats | Wild felids | Bush dog |
| (ii) *Intermediate host* | Primarily ungulates, also marsupials and primates, including humans | Primarily arvicolid rodents, also other small mammals and humans | Rodents; agoutis, paca, spiny rats | Primarily agoutis, also other rodents and humans |
| Metacestode | | | | |
| (i) *Nature of cyst* | Unilocular, endogenous proliferation, no infiltration or metastasis | Multivesicular, endogenous proliferation, infiltration and metastasis | Polycystic, endogenous and exogenous proliferation, no infiltration or metastasis | Polycystic, endogenous and exogenous proliferation, no infiltration or metastasis |
| (ii) *Location of cyst* | Visceral, primarily liver and lungs | Visceral, primarily liver | Peripheral, primarily muscles | Visceral, primarily liver |
| (iii) *Protoscoleces* | | | | |
| (a) *Mean length* (μm) *of large hooks (range)* | 25.9–35.0 (19.4–44.0) | 26.7–28.5 (25.0–29.7) | 30.5–33.4 (29.1–37.9) | 39.3–41.6 (38.2–45.6) |
| (b) *Mean length* (μm) *of small hooks (range)* | 22.6–27.8 (17.0–31.0) | 23.1–25.4 (21.8–27.0) | 25.4–27.3 (22.6–29.2) | 32.5–34.0 (30.4–36.9) |

| | | | | |
|---|---|---|---|---|
| Adult | | | | |
| (i) *Mean length* (μm) *of large hooks (range)* | 32.0–42.0 (25.0–49.0) | 31.0 (24.9–34.0) | 52.0 (43.0–60.0) | 53.0 (49.0–57.0) |
| (ii) *Mean length (μm)* of small hooks (range) | 22.6–27.8 (17.0–31.0) | 27.0 (20.4–31.0) | 39.0 (28.0–45.0) | 42.6 (30.0–47.0) |
| (iii) *Mean number of segments (range)* | 3 (2–7) | 5 (2–6) | 3 | 3 |
| (iv) *Total length of strobila* (mm) | 2.0–11.0 | 1.2–4.5 | 2.2–2.9 | 3.9–5.5 |
| (v) *Ratio of length of anterior part of strobila to length of gravid segment* | 1:0.86–1.30 | 1:0.31–0.80 | 1:0.96–1.10 | 1:1.90–3.0 |
| (vi) *Position of genital pore* | | | | |
| (a) *Mature segment* | Near (usually posterior) to middle | Anterior to middle | Anterior to middle | Posterior to middle |
| (b) *Gravid segment* | Posterior to middle | Anterior to middle | Near to middle | Posterior to middle |
| (vii) *Mean number of testes (range)* | 32–68 (25–80) | 18–26 (16–35) | 29 (15–46) | 56 (50–67) |
| (viii) *Form of uterus* | Lateral sacculations | Sac-like | Sac-like | Long, tubular and sac-like |

[a]Data from Kumaratilake and Thompson (1982a), Rausch (1986) and Thompson (1986).

*Echinococcus m. kazakhensis* was originally reported from sheep in Kazakhstan, USSR (Rausch, 1967a; Kumaratilake and Thompson, 1982a). Rausch (1967a) considered this subspecies to be geographically and ecologically isolated from *E. m. multilocularis*. However, more recent reports have thrown doubt on the occurrence of *E. multilocularis* in domestic ungulates, and questioned the existence of *E. m. kazakhensis* (Kumaratilake and Thompson, 1982a; Rausch, 1986; Eckert, in press).

### C. STRAINS

There is only one category below the rank of species in which the names of taxa are regulated by the International Code of Zoological Nomenclature; that of subspecies. The invalidated subspecies of *E. granulosus* were therefore not recognized taxonomically and this may have led to neglect of the inherent variability within the species (Thompson, 1986). Smyth and Smyth (1964) referred to populations which differed from the established species of *Echinococcus* as variants, races or strains. Rausch (1967a) suggested that different populations, including invalidated subspecies, should be designated as strains or forms. The term "strain" is now commonly accepted to refer to intraspecific variants of uncertain taxonomic status (Thompson, 1986). Recently, even subspecies which are regarded as taxonomically valid have been referred to as strains. For example, *E. g. canadensis* is also known as the northern form (Rausch, 1986) or sylvatic strain (Anonymous, 1982) of *E. granulosus*. *Echinococcus m. multilocularis* is referred to as the central European strain (Vogel, 1977) and *E. m. sibiricensis* as the St Lawrence Island strain (Rausch and Bernstein, 1972) of *E. multilocularis*.

There is now considerable evidence of strain variation within the recognized species of *Echinococcus*. This evidence will be documented (Section III, A), before we consider the concept of a "strain" more critically (Section III, B).

## III. Beyond Taxonomy: Variation Within Recognized Species

### A. EVIDENCE AND EXTENT OF VARIATION

#### 1. Echinococcus granulosus

(*a*) *Horse strain.* In 1963, Williams and Sweatman carried out comprehensive, comparative morphological studies on both gravid adult worms and protoscoleces of *E. granulosus* of horse and sheep origin. They demonstrated

significant morphological differences between parasites from the two hosts, particularly in rostellar hook morphology and the anatomy of the male reproductive system. These authors also carried out some limited cross-infection experiments in sheep and horses, which suggested that horses are refractory to infection with *E. granulosus* of sheep origin, whereas sheep may support limited development of the horse form. In light of these differences in morphology and apparent host specificity, Williams and Sweatman (1963) proposed the designation of a new subspecies, *E. g. equinus*, for the form of *E. granulosus* occurring in British horses. A few years later, the taxonomy of *Echinococcus* at the intraspecific level was reviewed by Rausch (1967a), who invalidated the taxon created by Williams and Sweatman (1963). The basis for dismissal of subspecific status was not the nature of the differences described by Williams and Sweatman but rather the definition of a subspecies. Rausch (1967a) argued that since subspecies are capable of interbreeding, there must be evidence of geographical or ecological isolation to prevent reproduction. Since the two populations of *E. granulosus* occur sympatrically in Great Britain and both utilize dogs as definitive hosts, Rausch (1967a) could not support Williams and Sweatman's proposal for a new subspecies. It has since been argued that definitions developed for cross-fertilizing dioecious organisms may be inapplicable for potentially self-fertilizing hermaphrodites such as cestodes (see Section IV, C, 1). However, the most unfortunate aspect of the taxonomic invalidation proposed by Rausch for the equine form of *E. granulosus*, and also numerous other subspecies of *Echinococcus*, was that it appeared that no credence was given to the characteristics described for individual variants when formal taxonomic status was originally proposed. Although this was not the intention of the taxonomic revisions it appears that, to many workers, the taxonomic arguments negated the fact that the described subspecies exhibited pronounced and distinct biological characteristics.

Fortunately, the situation changed dramatically in the early 1970s, by which time the incidence of equine hydatidosis in Great Britain had risen alarmingly, with prevalence rates of over 60% reported (Thompson and Smyth, 1975). J. D. Smyth stimulated renewed interest in intraspecific variants of *Echinococcus* by adopting an entirely different approach to the problem. Smyth had developed a reproducible procedure for the *in vitro* cultivation of *E. granulosus* of sheep origin from the protoscolex to the sexually mature adult (Smyth and Davies, 1974a). However, when protoscoleces from horse hydatid cysts were exposed to the same culture conditions, they repeatedly failed to segment or mature (Smyth and Davies, 1974b). The observation that different populations of *E. granulosus* may have quite distinct metabolic requirements reinforced the fact that although there may be difficulties associated with formal taxonomic "pigeon-holing" because of

traditional taxonomic concepts, intraspecific variation does occur. Smyth's observations complemented and extended those of Williams and Sweatman (1963) which were primarily based on morphological differences, and stimulated an intense period of research which has led to the demonstration of numerous differences between *E. granulosus* of horse and sheep origin (Table 2) and to their designation as different strains. The morphology, developmental biology, physiology, biochemistry, metabolism, molecular biology, host specificity and epidemiology of the horse strain have been studied in great detail and compared with that of other strains (Thompson and Smyth, 1975; Smyth, 1977, 1982a; Thompson, 1978, 1979, 1986, in press; Thompson and Kumaratilake, 1982; McManus and Simpson, 1985; Kumaratilake *et al.*, 1986a).

Recent comparative studies on *E. granulosus* of equine origin from England, Scotland, Ireland, Belgium, Switzerland, South Africa and New Zealand (Kumaratilake *et al.*, 1986a) have complemented and confirmed earlier studies on the parasite from Italy (Macchioni and Gallo, 1967), Lebanon and Syria (Dailey and Sweatman, 1965), and have demonstrated the uniformity and widespread geographical distribution of the horse strain of *E. granulosus*.

(*b*) *Cattle strain.* A similar situation to that involving *E. granulosus* from horses was recently reported for a bovine form of the parasite (Thompson

TABLE 2 *Demonstrated differences*[a] *between the horse and sheep strains of* Echinococcus granulosus

| | |
|---|---|
| Adult morphology | Hook dimensions and anatomy of reproductive system |
| Larval morphology | Hook dimensions |
| Intermediate host specificity | Horse strain not infective to sheep; sheep strain not infective to horses |
| Infectivity to humans | Horse strain probably not infective to humans |
| Developmental characteristics | Adult and larval stages shown to differ in their development *in vivo* and *in vitro* |
| Chemical composition | Differences in relative quantities of protein, RNA and lipid |
| Metabolism | Differences in energy metabolism |
| Protein separation | Protein and enzyme profiles shown to differ using electrophoresis and isoelectric focusing |
| DNA hybridization and restriction site analysis | |

[a]See Thompson and Smyth (1975), Anonymous (1982), Kumaratilake *et al.* (1986a), McManus and Bryant (1986), Thompson (1986, in press) and Eckert (in press) for further details.

*et al.*, 1984). In Switzerland, cattle commonly harbour hydatid cysts and are the most important intermediate host for perpetuating the parasite (Eckert, 1981). Sheep in Switzerland are rarely affected and horses harbour the equine strain (Kumaratilake *et al.*, 1986a). Cysts in Swiss cattle are usually fertile. This is in contrast to many other parts of the world where cattle, although commonly infected, serve no useful role in the life-cycle since cysts are usually sterile or degenerate; such infections are considered to result from non-bovine strains of *E. granulosus* (Thompson *et al.*, 1984).

The form of *E. granulosus* found in Swiss cattle has been shown to be quite different to that occurring in sheep and horses in Europe and elsewhere (Thompson *et al.*, 1984). It differs not only in its unique morphology but also biochemically, in its precocious development in dogs, and in its predilection site in the intermediate host, where cysts occur more frequently in lungs than liver. As with the horse strain, the form of *E. granulosus* described from cattle in Switzerland does not appear to be confined to that country. High fertility rates in cattle in South Africa, Belgium, Germany, Sri Lanka and India suggest forms of *E. granulosus* adapted to cattle (Dissanaike, 1957; Verster, 1962; De Rycke, 1968; Abraham *et al.*, 1980; Sanyal and Sinha, 1983; Thompson *et al.*, 1984; Hörchner *et al.*, 1986; Worbes, 1986). Of particular interest is the fact that the unusual adult morphology of *E. granulosus* from Swiss cattle has been shown to be virtually identical to that of *E. granulosus* of cattle origin from South Africa (Verster, 1965; Kumaratilake, 1982). This form was previously given both specific (*E. ortleppi*) and later subspecific (*E. g. ortleppi*) status (Lopez-Neyra and Soler Planas, 1943; Verster, 1965), which was subsequently invalidated (Rausch, 1967a).

(*c*) *Domestic sheep strain.* By far the most important and commonly infected intermediate host of *E. granulosus* is the domestic sheep. The majority of comparative studies have been undertaken on ovine material from Australia, New Zealand and England, and these suggest that the same strain of *E. granulosus* occurs in the three areas (Kumaratilake *et al.*, 1986a). Although most work on *E. granulosus* of sheep origin from Europe has been undertaken on material from England, recent comparative studies on isolates from Italy, Portugal, Turkey, Switzerland and Scotland using morphology, biochemistry and recombinant DNA techniques strongly suggest the uniformity of the sheep strain in Europe and adjacent areas (Kumaratilake *et al.*, 1986a; K. W. Yap, I. D. Pawlowski, R. C. A. Thompson and A. J. Lymbery, unpublished observations). In this respect, it is interesting that Saiz-Moreno (1984) recently concluded that the primary focus of hydatidosis in Australia and New Zealand originated from Spain. Studies on *E. granulosus* from the USA have also demonstrated its similarity to the parasite occurring in sheep in Europe and Australasia (Kumaratilake *et al.*, 1986a).

This is perhaps not surprising since separate introductions of *E. granulosus* into the USA from Europe and Australia are believed to have taken place (Rausch, 1967b; Crellin *et al.*, 1982).

(*d*) *Other domestic sheep strains?* There is evidence that *E. granulosus* of sheep origin from the Australian island state of Tasmania is a separate strain to that occurring on the mainland of Australia and elsewhere. Morphological, biochemical and developmental studies have shown it to be distinct, yet its origins are obscure (Kumaratilake *et al.*, 1983; Kumaratilake and Thompson, 1983, 1984a,b; Thompson and Kumaratilake, 1985). It is possible that the initial focus of *Echinococcus* infection in Tasmania had a different origin to that on the Australian mainland (e.g. South Africa), or that the founding population was small and, by chance, genetically different ("founder effect"; Mayr, 1940). Alternatively, different selection pressures in Tasmania, perhaps as a result of a diagnostic programme of arecoline treatment of the definitive host, may have led to adaptive genetic differentiation. As regular drug treatments have been implemented in Tasmania only within the past 20 years, this would require rapid evolutionary change. Recent studies in our laboratory have suggested that there are few significant differences at enzyme loci between *E. granulosus* from the mainland of Australia and Tasmania, but significant differences appear to exist in highly repeated sequences of genomic DNA (A. J. Lymbery, R. C. A. Thompson, K. W. Yap and I. D. Pawlowski, unpublished observations). One hypothesis which explains these results is that a few mutations over a short period of time in regulatory DNA have produced large adaptive changes in development rate, but have not affected structural gene loci. At this stage, our results are preliminary and this hypothesis has not been adequately tested.

Studies in Australia have suggested, firstly, that the same strain may occur in more than one intermediate host (e.g. the mainland domestic strain can infect domestic ungulates, macropod marsupials and humans) and, secondly, that a particular host species may be susceptible to more than one strain of *Echinococcus* (e.g. separate strains on the Australian mainland and Tasmania both utilize sheep as an intermediate host). Both situations also appear to occur in Kenya. Recent biochemical studies indicate that Kenyan sheep may harbour a distinct strain of *E. granulosus* (McManus, 1981), and evidence suggests that the same strain occurs in goats and humans, although further work is required to determine the status of the parasite in cattle and camels (Macpherson and McManus, 1982). The Kenyan sheep strain appears to be the major source of infection to humans (Macpherson, 1983), and it has been suggested that the high prevalence of infection in certain tribes may be because the strain to which humans are susceptible is particularly virulent (Thompson, 1979; French *et al.*, 1982). Humans play an active role as

intermediate host in the life-cycle of *E. granulosus* in Kenya, and human cysts are invariably fertile (Macpherson, 1983). The presence of unusual strains in East Africa had been suggested previously by Nelson and Rausch (1963), and more recently by Eugster (1978) who speculated that this might explain the prevalence of the parasite in the hot, arid areas of East Africa. Further work is required to determine whether the strain of *E. granulosus* in sheep in Kenya is the only strain present in the region and whether sheep are indeed the major intermediate host.

Few comparative studies have been undertaken on *E. granulosus* from sheep in other endemic areas. In this respect, there is a need to examine sheep isolates from the Mediterranean region, Middle East, China, South America and much of Asia.

(*e*) *Pig strain?* Although pigs are known to be susceptible to strains of *E. granulosus* normally perpetuated in sheep (Kumaratilake and Thompson, 1984a,b), extensive studies in eastern Europe (Bulgaria, Czechoslovakia, Hungary, Yugoslavia and Poland) and the Soviet Union have suggested that a pig strain of *E. granulosus* exists which is distinct from that occurring in other domestic animals (Slepnev, 1975; Kuznetsov *et al.*, 1975; Slais, 1980; Stoimenov and Kaloyanov, 1980; Voloschchuk, 1980; Wikerhauser *et al.*, 1986). In these areas, a dog–pig cycle may be the most important for perpetuating the parasite (Eckert, in press). This pig strain is considered to have a low infectivity to humans. For example, in parts of Poland, 31% of pigs and 11% of dogs on private farms are infected but only one case of cystic hydatid disease in humans has been diagnozed in local hospitals during 20 years (Pawlowski, 1985). *Echinococcus granulosus* of pig origin from Mississippi in the USA is also considered to be a "variant" of *E. granulosus*. This conclusion followed the results of host specificity experiments and observations on the morphology of larval and adult stages (Hutchison, 1970).

The possible existence of a distinct strain of *E. granulosus* adapted to pigs is interesting, since the classical description of *E. granulosus* given by Vogel (1957) was based on observations of adult worms of pig–dog origin (Kumaratilake, 1982). It would appear that this description may therefore only apply to the pig strain of *E. granulosus* (see Kumaratilake, 1982).

(*f*) *Buffalo strain?* Buffaloes are a common intermediate host for *E. granulosus*, particularly in Asia (e.g. Gill and Venkateswara, 1967; Islam, 1982). Infections are characterized by predominantly pulmonary localization and high cyst fertility (Schwabe, 1986). The only detailed comparative studies on *E. granulosus* of buffalo origin were carried out by Gill and Rao in 1967. Their studies on the morphology and development of the adult worms revealed differences which resulted in these authors not being able to assign

their buffalo/dog material to any described subspecies. They considered their material to be closest to *E. granulosus canadensis* (cervid strain, see i(i)). Interestingly, this form from cervids has morphological characteristics which correspond more closely to those of *E. granulosus* from Swiss cattle than any other form (Kumaratilake, 1982; Kumaratilake *et al.*, 1986a; L. M. Kumaratilake, R. C. A. Thompson and P. M. Schantz, unpublished observations). The most characteristic feature shared by these forms of *E. granulosus* is the occurrence of gravid worms with only two segments. Although further studies are required to characterize *E. granulosus* of buffalo origin, and in particular, to compare it to the strain of *E. granulosus* adapted to cattle, it is possible that the form of *E. granulosus* in buffaloes is the same as that in Swiss cattle.

(*g*) *Camel strain?* The camel is an important host of *E. granulosus* and is found commonly infected throughout much of Africa and the Middle East (see Schwabe, 1986). Cysts are usually fertile, often in contrast to other intermediate hosts in the same endemic area (e.g. Al-Yaman *et al.*, 1985). Although there has been much interest in the camel as an intermediate host of *E. granulosus*, particularly of its role as a reservoir of infection in humans and the possibility of the camel form being a different subspecies or strain (Pandey *et al.*, 1986), very little comparative work has been carried out to characterize isolates of *E. granulosus* from camels. Dailey and Sweatman (1965) found significant differences in the number and size of rostellar hooks of *E. granulosus* from camels compared to the parasite of sheep origin. However, these authors considered that the results could reflect host-induced morphological variation. Studies in Kenya have so far proved equivocal, with biochemical data suggesting distinctness for the camel form, whereas comparative *in vitro* studies suggest uniformity with forms from other intermediate hosts (McManus, 1981; Macpherson and McManus, 1982; Macpherson and Smyth, 1985).

(*h*) *Goat strain?* Very little specific information is available on isolates of *E. granulosus* of goat origin, apparently because goats are often not distinguished from sheep in reports (Rausch, 1986). Pandey (1972) could not assign isolates of *E. granulosus* of Indian goat–dog origin to any described subspecies and considered that it may be a "strain or mutant in the process of evolution". He noted a long prepatent period of 60–90 days. Prasad (1983) also emphasized the fact that goat material has a longer prepatent period than *E. granulosus* from other species of intermediate hosts. However, further work is required to confirm the distinctness of *E. granulosus* of goat origin.

(*i*) *Strains originating in wild host assemblages*

(i) *Cervids.* Perhaps the most important wild-animal cycle for *E. granulosus* is that in which cervids are the intermediate hosts. As discussed below (Section IV, D), this form of *E. granulosus* is considered to be most similar to the ancestor of strains of *E. granulosus* in domestic ungulates (Rausch, 1986). *Echinococcus granulosus* in cervids is primarily perpetuated by a predator–prey relationship involving wolves and large deer, such as elk (moose) and reindeer, in northern North America and Eurasia (Rausch, 1967a,b, 1986). However, domestic cycles involving dogs and domesticated reindeer operate in parts of Canada, Alaska, Siberia, Norway and Sweden (Rausch, 1986).

*Echinococcus granulosus* of cervid origin differs in many respects from other forms of *E. granulosus*. It does not readily infect domestic ungulates and, in contrast with domestic strains of *E. granulosus*, is virtually asymptomatic in humans (Cameron, 1960; Cameron and Webster, 1961; Wilson *et al.*, 1968). It also differs serologically from domestic strains of the parasite (Cameron, 1960), and exhibits characteristic differences in the type of infection produced in laboratory mice (Webster and Cameron, 1961). More recently, Russian workers have shown that *E. granulosus* of cervid origin is not infective to cattle (Safronov and Isakov, 1982, 1984), and develops rapidly in dogs (Mankhaeva and Shumilov, 1982). Recent research has also demonstrated the distinctness of *E. granulosus* from cervids on the basis of larval and adult morphology and isoelectric focusing of soluble proteins (L. M. Kumaratilake, R. C. A. Thompson and P. M. Schantz, unpublished observations). These studies have suggested that *E. granulosus* of cervid origin has affinities with cattle rather than sheep strains of *E. granulosus* (see Section IV, D). There is also evidence that more than one strain of *E. granulosus* occurs in cervids (L. M. Kumaratilake, R. C. A. Thompson and P. M. Schantz, unpublished observations), as initially proposed by Sweatman and Williams (1963) who recognized two subspecies (*E. g. canadensis* and *E. g. borealis*).

(ii) *Macropod marsupials and dingoes in Australia.* On the mainland of Australia, two distinct life-cycle patterns operate to maintain *E. granulosus* (Thompson and Kumaratilake, 1982). One involves sheep and farm dogs, with cattle and pigs as potential accidental intermediate hosts. The other involves macropod marsupials, mainly wallabies, and dingoes (*Canis familiaris dingo*) (Thompson and Kumaratilake, 1985). Knowledge of how these cycles interact is critical for the implementation of control programmes (Thompson, in press, and see Section V, A, below). There is evidence that the two life-cycle patterns reflect the existence of two strains on the Australian mainland. *Echinococcus granulosus* of sheep and

macropod origin are morphologically distinct, notable features in macropod isolates being the possession of twice the normal number of rostellar hooks, as well as the unique shape of adult hooks (Kumaratilake and Thompson, 1984a). This morphological variation is complemented by biochemical differences (Kumaratilake and Thompson, 1984b). Both strains develop well in dogs but, interestingly, the sylvatic (macropod) strain develops more rapidly in dingoes than the domestic (sheep) strain (Thompson and Kumaratilake, 1985). Differences in the development of the metacestode have also been found, and in particular it appears that the sylvatic strain is not infective to sheep (Thompson and Kumaratilake, 1985; Thompson, in press and unpublished observations).

The fact that the Australian sylvatic strain develops more rapidly in the dingo than the domestic dog supports the theory that it was introduced into Australia before the domestic strain, which arrived with European settlers in the late nineteenth century (Thompson and Kumaratilake, 1985). The sylvatic strain may have been introduced in the dingo with migrating aborigines from Asia many thousands of years ago, and subsequently became adapted to macropod marsupials. To test this theory, comparative studies of Australian and South-East Asian forms of *E. granulosus* are necessary, although today *E. granulosus* appears to be uncommon in South-East Asia.

(iii) *African wildlife.* Wild ungulates of several species have been found infected in Africa, where wild canids such as hunting dogs (*Lycaon pictus*), jackals (*Canis mesomelas* and *C. aureus*) and hyaenas (*Crocuta crocuta*) as well as domestic dogs, act as definitive hosts (Eugster, 1978; Nelson, 1982; Macpherson *et al.*, 1983; Rausch, 1986). Of most interest is the form of *E. granulosus* found in lions. From the results of surveys on felids in other parts of the world, and numerous attempted experimental infections of domestic cats (see Thompson, 1979), it was concluded that felids do not act as definitive hosts for *E. granulosus*. However, sylvatic cycles involving the lion and a number of intermediate host species have been reported from several African countries (reviewed by Nelson, 1982, 1983; Macpherson *et al.*, 1983; Craig, 1986). In South Africa, one of 15 wild cats (*Felis silvestris* = *F. libyca*) was also found infected with *E. granulosus* (Verster and Collins, 1966).

The African situation suggests the existence of a unique strain susceptible to wild felids, although it is not known whether this strain is also infective to domestic cats. The most significant evidence for the existence of a distinct strain in lions has been reported by Graber and Thal (1980) in the Central African Republic. They suggest that a sylvatic cycle between the lion and wart-hog occurs, and they have experimental evidence that the local parasite

is not only distinct morphologically, but also is not infective to dogs. Because of this, both Macpherson (1986) and Rausch (1986) have emphasized that the taxonomic status of this strain needs consideration, and suggest that specific status (*Echinococcus felidis* Ortlepp, 1937) may be applicable.

(iv) *Lagomorphs.* Until 1972, lagomorphs were not considered suitable intermediate hosts for *E. granulosus*, and previous reports to the contrary were shown to involve other taeniids (Gemmell, 1960; Kumaratilake and Thompson, 1982b). However, Schantz and Lord (1972) found 4 out of 71 European hares (*Lepus europaeus*) in the Province of Neuquén, Argentina, to be infected. More recently, Thakur and Eddi (1982) refer to the fact that there are many infections in hares in parts of Argentina, and stress the zoonotic significance of this cycle. However, further work is required to determine whether a true sylvatic cycle operates between hares and local foxes of the genus *Dusicyon*, or whether infection may originate from domestic dogs carrying *E. granulosus* of sheep origin. Nevertheless, the existence of a form of *E. granulosus* infective to lagomorphs is significant and warrants further investigation.

2. E. multilocularis, E. oligarthrus *and* E. vogeli

Of the other three species of *Echinococcus*, evidence for intraspecific variation has so far only been reported in *E. multilocularis*. *E. oligarthrus* and *E. vogeli* have restricted geographical distributions and host ranges (Rausch, 1986) which may account for any lack of reported variation within these species, although few comparative studies have been undertaken.

Similarly, there have been few comparative studies on isolates of *E. multilocularis* from different endemic areas. Investigations have been limited in their approach to only a few criteria and more comprehensive studies on a greater number of isolates are required. However, from the available data (Vogel, 1957; Rausch and Richards, 1971; Ohbayashi *et al.*, 1971; Thompson and Eckert, 1983; Kamiya *et al.*, 1985), there is some evidence of variation in morphology, pathogenicity, developmental characteristics and host specificity.

*Echinococcus multilocularis* is predominantly maintained in sylvatic life-cycles and has a more restricted intermediate host range than *E. granulosus*. The fact that *E. multilocularis* may occasionally overlap into synanthropic situations may, in the future, lead to the recognition of intraspecific variants. There is also the possibility that apparent differences in reproductive behaviour between *E. granulosus* and *E. multilocularis* (Kumaratilake *et al.*, 1986b) may account for more extensive intraspecific variation in the former (see Section IV, A).

## B. NOMENCLATURE

The identification of variant strains within the recognized species of *Echinococcus* has been very important to the epidemiology and control of hydatid disease. Unfortunately, because of its informal nature, the term "strain" lacks precision. It has rarely been defined explicitly and, from their implicit definitions, different authors appear to use the term in different ways.

The broadest definition is that of Thompson (1982; in press), who regarded strains as populations of a species which have been characterized by one or more criteria. Other authors are more restrictive. Eckert (in press) defined strains as local populations differing intrinsically in morphological and biological features. This implies, firstly, that strains should be geographically restricted, a point also made by Schantz (1984) and, secondly, that differences between strains should have a genetic basis. Smyth (1977, 1982a), McManus and Smyth (1979), McManus (1981), McManus and Macpherson (1984) and McManus *et al.* (1985) regarded strains as intraspecific variants restricted to different host species. Elsewhere, however, Smyth (1964) and McManus and Smyth (1986) implied that different host species are not essential and are only one of the biological characteristics by which strains may differ.

These differences in the use of the term "strain" have led to confusion. For example, the FAO/UNEP/WHO Guidelines for Echinococcosis/Hydatidosis Surveillance, Prevention and Control state that morphological features "should not be considered as the sole criteria for the discrimination between strains", yet also recommend that "minor morphological variants within the genus should be designated as strains until their biological status has been clarified" (Anonymous, 1982; pp. 18–20). Confusion could be avoided if the term is defined explicitly where it is used. We believe that the recognition of strains should depend upon differences in characters of practical significance. By this we mean anything which is, or may be, relevant to the epidemiology and control of hydatid disease, such as development rate, geographical distribution, host range, infectivity, pathogenesis and susceptibility to chemotherapy (see Section V). To avoid arbitrary and misleading designations of strains, the differences in these characters should have a genetic basis and be sufficiently marked so as to be statistically detectable. **An ideal definition for a strain of *Echinococcus* would be a group of individuals which differs statistically from other groups of the same species in one or more genetically determined characters of actual or potential significance to the epidemiology and control of hydatid disease.** However, such a definition is not operational because it is very difficult to prove a substantial genetic component to variation in characters of practical significance. These characters are often complex, their mode of inheritance is not usually known, and their expression may be greatly influenced by environmental factors.

A possible solution to this problem lies in the recognition that restricted gene flow will lead to differentiation between populations and, perhaps, to their subsequent designation as variants or strains (Bryant and Flockhart, 1986). The demonstration that two populations have restricted gene flow will therefore provide the best predictor of a genetic basis to any differences between them in characters of practical significance. Restricted gene flow between populations may sometimes be surmised from their geographical or ecological isolation, but is more reliably indicated by differences in equilibrium gene frequencies, subject to the assumptions and errors inherent in such measurements (Slatkin, 1985; Richardson *et al.*, 1986). Therefore, the recognition of strains will usually depend not only upon differences in characters of practical significance but also upon differences in characters whose genetic basis can be established. The value of different characters for this purpose is discussed below (Section III, C). We should emphasize that it is not sufficient to show differences in these characters between just a few individuals; samples from putative strains should be large enough so that differences in the frequencies of genes coding for the characters can be statistically detected.

**Operationally, then, we define a strain of *Echinococcus* as a group of individuals which differs statistically from other groups of the same species in gene frequencies, and in one or more characters of actual or potential significance to the epidemiology and control of hydatid disease.** The major advantage of this definition is its applicability. In our view, differences in gene frequencies will provide measurable and reliable evidence of restricted gene flow between strains. We stress that restricted gene flow is used as an indication of a genetic basis to differences in characters of practical significance. Therefore, by our definition, the term "strain" is used as a practical descriptor; it denotes neither an evolutionary unit nor a taxonomic category. A number of corollaries follow logically from the definition.

(1) A strain may include one or more populations. For example, the domestic sheep strain of *E. granulosus* consists of populations infecting sheep in a number of different countries around the world. Gene exchange between some of these populations is undoubtedly restricted and they may differ in gene frequencies, but until they are shown to also differ in characters of practical significance we would not regard them as different strains.

(2) Described subspecies may or may not be regarded as strains. For example, the European and North American populations of *E. multilocularis* have been described as separate subspecies (*E. m. multilocularis* and *E. m. sibiricensis*, respectively), because they differ morphologically and are geographically separated. However, as yet there is no convincing evidence of differences between them in characters of practical

significance. Until such evidence is found we would not describe them as different strains.

(3) Host specificity is not a necessary characteristic of strains. For example, in Australia, the mainland domestic strain of *E. granulosus* can infect a number of different intermediate hosts, while different strains on the mainland and Tasmania may both utilize the same intermediate host (Section III A 1 (d)).

### C. DIFFERENTIAL CRITERIA OF VALUE

Table 3 lists those criteria which have been used to characterize strains in *Echinococcus* and other parasitic organisms. Although we believe that strains should differ in ecological, physiological or behavioral characters of significance to the epidemiology and control of hydatid disease, such characters cannot usually be related directly to the genome. For this reason, they are often referred to as extrinsic (Lumsden, 1974; Chance, 1979; Peters, 1981; Thompson, in press). Extrinsic criteria may often provide an excellent "field" indication of variation, but they need to be substantiated by criteria which more closely reflect genomic differences. These criteria are usually regarded as intrinsic. Of the intrinsic criteria listed in Table 3, only DNA sequencing provides a direct measure of genotypic differences. The other characters, like extrinsic criteria, may be affected by both genotype and environment; morphological, immunological and biochemical variation, in particular, may reflect regulatory changes to a particular host or ecological situation, rather than fixed genetic differences. The intrinsic criteria in Table 3 have been arranged in descending order of the likelihood of a substantial environmental component to their variation.

With varying degrees of confidence, intrinsic criteria may be used to measure differences in gene frequencies, and thus restricted gene flow, between putative strains. Morphology has been the most widely used criterion in studies on the characterization of *Echinococcus* populations (reviewed in Kumaratilake and Thompson, 1982a). This is because morphology was, and is, universally accepted for taxonomic determination and, until recently, taxonomy was the dominant motivation for undertaking comparative studies on *Echinococcus*. However, there has been much disagreement over the value of morphology in characterizing species and intraspecific variants of *Echinococcus* (e.g. Rausch, 1953; Vogel, 1957; Sweatman and Williams, 1963; Rausch and Nelson, 1963; Verster, 1965; Kumaratilake and Thompson, 1982a). A number of factors have led to this disagreement (Rausch, 1953, 1967a; Thompson, 1979). Often, too few characters or specimens were examined, or different methods of fixation produced

TABLE 3 *Criteria[a] used for strain differentiation and characterization in parasites*

**Extrinsic criteria**

1. *Ecology, epidemiology*
   - Geographical distribution[b]
   - Range of hosts[b]
   - Host specificity[b]
   - Vector distribution
   - Influence of environment on parasitic and non-parasitic stages

2. *Physiology, behaviour (in vivo, in vitro)*
   - Development rate[b]
   - Reproductive biology[b]
   - Growth *in vitro*[b]
   - Infectivity/pathogenesis/virulence[b]
   - Susceptibility to chemical agents

**Intrinsic criteria**

1. *Morphology*
   - Gross
   - Ultrastructural

2. *Immunology*
   - Immunological response[b]
   - Immunodiagnosis[b]
   - Serotyping[b] – serial dilution, gel diffusion, immunoelectrophoresis, microcomplement fixation, radioimmunoassay, etc.

3. *Biochemistry*
   - Metabolism[b]
   - Lectin binding
   - Total protein analysis[b] – electrophoresis, two-dimensional electrophoresis, isoelectric focusing
   - Isoenzyme analysis[b] – electrophoresis, isoelectric focusing
   - Amino-acid sequencing

4. *Karyotype*
   - Chromosome number-microscopy, pulsed-field gradient gel electrophoresis
   - Chromosome structure

5. *DNA* (chromosomal, mitochondrial, kinetoplast)
   - Structure/size/density
   - Base composition[b]
   - DNA–DNA hybridization[b]
   - Restriction site analysis[b]
   - Base sequencing

[a]Information from Lumsden (1974), Chance (1979), Peters (1981) and Thompson (in press).
[b]Used for strain differentiation in *Echinococcus*.

spurious differences between specimens. More importantly, no studies have been specifically designed to determine the range of variation of commonly used morphological characters, or to identify covariation between different characters and hence eliminate character redundancies (see Thorpe, 1976). Also, there have been no attempts to measure the heritability of morphological characters, although there is evidence that at least some characters are significantly affected by the host. For example, Schantz *et al.* (1976) demonstrated that protoscoleces of *E. granulosus* from the same intermediate host gave rise to morphologically distinct adults in experimentally infected dogs and foxes.

In general, therefore, without an analysis of the genetic and environmental components of variation, morphological differences may not provide reliable estimates of differences in gene frequencies between populations of *Echinococcus*. However, if morphological differences are validated by results obtained with other criteria, they may provide effective diagnostic markers for identifying strains. This has been demonstrated with the horse strain of *E. granulosus*, which possesses distinct morphological features that have been substantiated by a number of other differential criteria. Consequently, it has been possible to determine the geographical distribution of this strain using morphology alone (Kumaratilake *et al.*, 1986a).

Immunological, biochemical, chromosomal and DNA criteria are less likely to be influenced by environmental factors than morphology, and hence are more likely to provide reliable measures of genetic differences between strains. Such criteria do not, as yet, have wide acceptance taxonomically, but have proved invaluable as epidemiological indicators of variation throughout all parasite groups (Peters, 1981; Thompson, 1982, in press; Godfrey, 1984; Gibson and Miles, 1985; Rollinson *et al.*, 1986; Simpson, 1986). We would, however, sound a note of caution about drawing sweeping conclusions from data obtained with modern biochemical or molecular techniques. On their own, such techniques can be used to identify genetically independent populations when applied to samples of appropriate size. This suggests that the populations may be following separate evolutionary paths and warrant more detailed study, but should not form a basis for the recognition of different strains. In our view, strains should also differ in characters of practical significance. From this perspective, the most useful picture of strain variation in *Echinococcus* or any other parasite, will be obtained when as many criteria as possible have been applied, preferably simultaneously, to the populations under study.

### D. FUNCTIONAL SIGNIFICANCE OF STRAIN VARIATION

The use of biochemical and DNA criteria promises not only to enable a more valid and discriminatory approach to the identification and characterization of strains, but also to provide a better understanding of the functional nature and significance of strain variation. It may be possible to link variation in characters such as development rate, host specificity, infectivity, susceptibility to chemotherapy and metabolism, to changes in, or regulated by, the genome. As a consequence, we may understand the basis of differences between strains and the advantages such differences confer on the parasite. In some cases, this may appear obvious since, for example, different strategies

of metabolism are associated with different hosts (Bryant and Flockhart, 1986), and thus seem essential for parasite survival; but why are such changes necessary? In other cases, there may be no apparent advantages of subtle shifts in metabolism exhibited by different strains (McManus and Bryant, 1986).

One thing is clear. An understanding of the molecular/biochemical basis of strain variation will not only provide us with a better picture of the evolutionary potential of strains of *Echinococcus*, but will allow a more rational approach to the development of chemotherapeutic agents.

## IV. Intraspecific Variation and Speciation

### A. Models of Strain Variation

The extensive intraspecific variation of *Echinococcus* has been explained by two conflicting models, one attributable to J. D. Smyth and the other to R. L. Rausch.

#### 1. *Smyth's model*

Smyth and Smyth (1964) proposed that species of *Echinococcus* possess two characteristics which favour the production of variants. Firstly, adults are self-fertilizing hermaphrodites, so that any mutation arising in an adult is likely to be transmitted in both heterozygous and homozygous form to its offspring. Secondly, larvae reproduce clonally in the intermediate host, so that a large number of genetically identical individuals may arise from a single mutant offspring. Under this scheme, mutations are likely to be expressed even when recessive and, if they are not selected against in the intermediate host, are likely to increase in number. The clone of mutant individuals which arises may be recognized as a new strain, if sufficiently different from the original population.

Since it was first proposed, this model has been widely invoked to account for strain variation in *Echinococcus* (e.g. McManus and Smyth, 1979, 1986; Anonymous, 1982; Thompson, 1979, 1986; Bryant and Flockhart, 1986). Kumaratilake and Thompson (1982a) and Kumaratilake *et al.* (1986b) suggested that the model is valid for *E. granulosus*, but not for *E. multilocularis*, which may have different reproductive behavior and less extensive strain variation.

### 2. *Rausch's model*

Rausch (1967a,b, 1986) distinguished two forms of *Echinococcus granulosus*; a northern form (or cervid strain), which is found in wolves and reindeer and has a holarctic distribution, and a European form, which occurs in dogs and domestic ungulates and has a cosmopolitan distribution. According to Rausch (1973, 1985) the northern form of *E. granulosus*, the European and North American subspecies of *E. multilocularis* and possibly the other two species of *Echinococcus* are morphologically and biologically uniform over wide geographical ranges. This is attributable to extensive gene flow between conspecific populations, mediated by cross-fertilization.

Cross-fertilization is also presumed to occur in the European form of *E. granulosus* and the extensive strain variation which it exhibits is attributed to its association with domestic hosts (Rausch, 1985). Practices of animal husbandry may have erected geographical and ecological barriers between populations associated with different domestic intermediate hosts and subjected these populations to different selection pressures. Because of their store of genetic variation, maintained by cross-fertilization, these populations responded rapidly to selection, diverged genetically and are recognized as different strains (Rausch, 1985).

## B. DISTINGUISHING BETWEEN THE MODELS

These two models differ widely in their explanation of intraspecific variation in *Echinococcus*. Their fundamental point of departure is the mode of fertilization of adult worms. Smyth's model regards self-fertilization as the norm, at least in *E. granulosus*, whereas Rausch's model assumes that all species of *Echinococcus* are predominantly cross-fertilizing. While neither model may be correct in its extreme form, by testing the contrasting predictions they make about mode of fertilization, we should understand more about the origins of strain variation in *Echinococcus*.

### 1. *Direct observational data*

Direct observational evidence of the predominant mode of fertilization in internal parasites is difficult to obtain. Three methods of sperm transfer have been described in cestodes: self-insemination (within a single proglottid or between proglottids of the same strobila); cross-insemination; and hypodermic impregnation—random insertion of the cirrus through the tegument (Williams and McVicar, 1968; Smyth, 1982b; Nollen, 1983). Cross-insemination has never been observed in *Echinococcus*. Smyth and Smyth (1969)

reported self-insemination in 10% of a large (unspecified) number of whole mounts of *E. granulosus* of Australian sheep–dog origin. Self-insemination was also reported by Kumaratilake (1982) for *E. granulosus* of Australian sheep–dog origin and by R. C. A. Thompson (1975, Ph.D. Thesis, London University) for *E. granulosus* of British horse–dog origin (Fig. 1). Smyth (1982b) suggested that a cuticular membrane, sometimes found over the genital pore in mature proglottids of *E. granulosus*, may function to prevent cross-insemination. Kumaratilake *et al.* (1986b) observed self-insemination in 12 of 13 specimen of *E. oligarthrus* collected from a naturally infected ocelot from Colombia, and in 2 out of over 300 specimens of *E. multilocularis* of European origin collected from experimentally infected dogs.

Observations of self-insemination, but not cross-insemination, in species of *Echinococcus* do not provide conclusive evidence that self-fertilization is the predominant breeding system in nature. Kumaratilake *et al.* (1986b) point out that lack of evidence for cross-insemination in *Echinococcus* may be due to technical difficulties in obtaining slides of two small cestodes in the

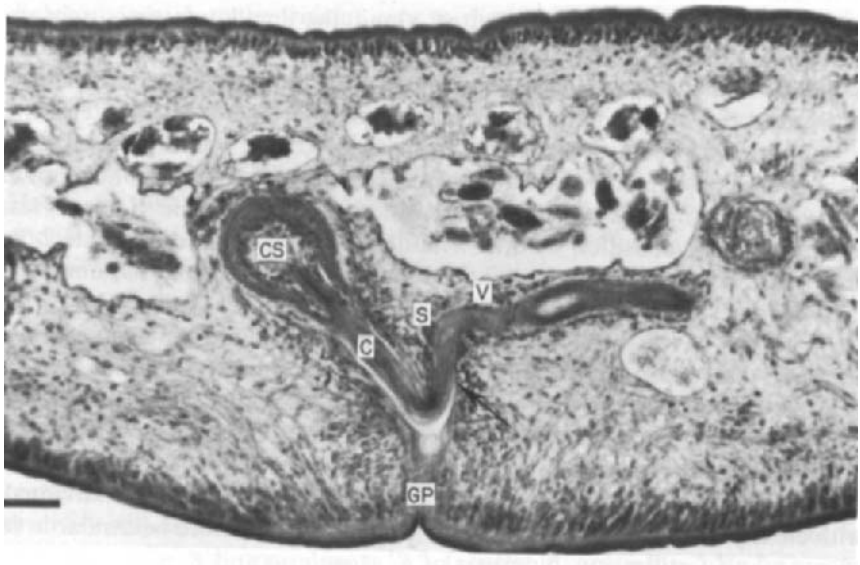


FIG. 1. Self-insemination in *Echinococcus granulosus* (horse strain). Section stained with haematoxylin and eosin through a sexually mature proglottid showing cirrus (C) deeply inserted (arrow) into the vagina (V). CS = cirrus sac; GP = genital pore; S = muscular sphincter. Scale bar = 40 μm.

act of copulation. Furthermore, fertilization may not result from observed self-insemination. Thompson *et al.* (1982) observed self-copulation in *Mesocestoides corti in vitro*, but found no evidence of fertilization. Rausch (1985) suggested that both self-insemination and hypodermic impregnation may be responses to a stressful situation, rather than natural processes of fertilization. He concluded that observations on the insertion of the cirrus by cestodes removed from their host may be of little value in the interpretation of reproductive behaviour.

## 2. *Behavioral and ecological characteristics*

In the absence of conclusive direct evidence on the breeding system of *Echinococcus*, Rausch (1985) argued that ecological and behavioral attributes provide indirect evidence for cross-fertilization. Adults of both *E. granulosus* and *E. multilocularis* have a restricted distribution in the intestine of the final host, *E. granulosus* attaching anteriorly and *E. multilocularis* posteriorly (Vogel, 1957; Thompson and Eckert, 1983; Kamiya *et al.*, 1985; Zhuravets, 1985). Sweatman and Williams (1963), Macpherson *et al.* (1985) and Gemmell *et al.* (1986b) found that, in dogs with natural or experimental infections of *E. granulosus*, adult worms have a restricted distribution in light infections but are evenly distributed along the small intestine in heavy infections. Rausch (1985) also stated that adults of both *E. granulosus* and *E. multilocularis* have a more restricted distribution at low densities, and interpreted this as an adaptation to ensure cross-fertilization. An alternative explanation is provided by competition theory, which predicts increased niche breadth at high densities due to intraspecific competition (O'Connor *et al.*, 1975). If competition for space or nutrients in the intestine of dogs is less intense at lower densities, then distributions should be more restricted. To distinguish between these possibilities requires a detailed analysis, similar to that carried out by Rohde (1979) and Rohde and Hobbs (1986) on ectoparasites of fish. Such an analysis has not been done for *Echinococcus*, although Gemmell *et al.* (1986b) found no correlation between density and size of *E. granulosus* in the intestine of dogs, suggesting that competition between adult worms may not be important in this species.

Smyth (1969) claimed that the ecology and behaviour of cestodes make it likely that cross-fertilization, when it occurs, will be between individuals with closely related or identical genotypes and will therefore be similar in its effects to self-fertilization. Numbers of *E. granulosus* and *E. multilocularis* in naturally infected definitive hosts show great variation (Rausch and Schiller, 1956; Rausch and Richards, 1971; Macpherson *et al.*, 1985; Gemmell and Lawson, 1986), but there are no data on genetic relationships between worms. Ecological information which bears on this question is confusing. It

is not known whether the definitive host normally acquires infection from one or several intermediate hosts. Intermediate hosts of both *E. granulosus* and *E. multilocularis* often contain a number of cysts (Rausch and Schiller, 1956; Gemmell and Lawson, 1986), but there are no data to indicate whether these are genetically different. Even if all the adult *Echinococcus* in the intestine of the definitive host are derived from a single cyst, Rausch (1985) has questioned the assumption that they will be genetically identical. This is based on studies by Schiller (1973), who found that in *Taenia crassiceps*, where asexual reproduction occurs by budding from the bladder wall of a cysticercus, some cysticerci produced multiple scoleces with different numbers of suckers. Schiller (1973) concluded that the various germinative loci in the bladder wall of the same cysticercus are not always genetically homogeneous. However, from the data presented, an alternative interpretation, that differences in the scoleces were environmentally induced, cannot be ruled out.

### 3. *Genetic structure*

Direct observation and inferences from behaviour and ecology have not provided the means for determining the breeding system of species of *Echinococcus*. A more fruitful way to distinguish between self- and cross-fertilization may be to examine their consequences in terms of genetic structure. A number of theoretical and empirical studies have found that the mode of reproduction and breeding system of a species are important determinants of the pattern of genetic variation within and between populations (Fryxell, 1957; Ellstrand and Levin, 1980; Saura, 1983; Loveless and Hamrick, 1984). The models of intraspecific variation proposed by Smyth and Rausch lead to very different predictions about patterns of genetic variation in *Echinococcus*.

(*a*) *Predictions from Smyth's model.* According to Smyth's model, *Echinococcus granulosus* is predominantly self-fertilizing and any cross-fertilization which occurs in either *E. granulosus* or other species of *Echinococcus* is likely to be between closely related individuals. Inbreeding will lead to an excess of homozygous genotypes, and exclusive self-fertilization to complete homozygosity within the species (Allard *et al.*, 1968). Genetic diversity will exist between strains, because different strains will be fixed for alternative alleles at one or more loci. Genetic diversity may also be present within strains, if mutant alleles do not produce phenotypic effects great enough to be recognized as strain differences. Any genetic diversity within strains will be largely distributed between self-fertilizing or inbreeding family groups (Wright, 1951), leading to non-random associations of alleles (Allard,

1975). Clonal reproduction in the intermediate host should lead to concentrations of genetically identical individuals in one geographical area and therefore to spatial structuring of genetically diverse family groups (Levin and Kerster, 1971). This effect will be enhanced by competition between different clones (Parker, 1979).

(*b*) *Predictions from Rausch's model.* Rausch's model regards cross-fertilization between genetically different individuals as the normal breeding system in all species of *Echinococcus*. Cross-fertilizing species usually maintain between 4 and 15% heterozygous loci per individual (Ayala, 1982) and in the absence of migration, mutation and selection, the number of heterozygous individuals at any one locus within a population will be in accord with Hardy–Weinberg equilibrium proportions. Genetic differences between populations will exist only where there are substantial barriers to gene flow (Lewontin, 1974; Slatkin, 1985), such as between European and North American populations of *E. multilocularis*, or between populations of the European form of *E. granulosus* in segregated host cycles. Within such populations, cross-fertilization in combination with clonal reproduction will prevent local differentiation and the spatial structuring of genetic diversity (Levin and Kerster, 1971).

(*c*) *Testing the predictions.* These predictions need to be tested before the models can be evaluated. As yet, there have been no comprehensive empirical studies on the genetic structure of any species of *Echinococcus*. However, some data are available from studies of morphological, biochemical and molecular differences between strains.

Although a large number of morphological differences have been documented between strains of *E. granulosus*, different isolates of the same strain, whether from the same or different countries, appear to be morphologically uniform (Sweatman and Williams, 1963; Kumaratilake and Thompson, 1984a; Kumaratilake *et al.*, 1986a). Similarly, consistent morphological differences have been found between European and North American populations of *E. multilocularis*, but there is little variation between different isolates from Europe (Vogel, 1957). Rausch *et al.* (1978) and Rausch *et al.* (1984) reported non-significant morphological variation between different isolates of *E. oligarthrus* and of *E. vogeli* in South America.

Rausch (1985) interpreted the morphological uniformity of different species and strains of *Echinococcus* as evidence of cross-fertilization. However, this conclusion may not be warranted. Little is known of the mode of inheritance of morphological characters used in studies on *Echinococcus*, but they usually show continuous variability and are likely to be affected by the segregation of many genes (Falconer, 1960). Although the original theoret-

ical models of Fisher (1918) and Wright (1921) concluded that self-fertilization increased the genetic variance of polygenic characters compared to random cross-fertilization, more comprehensive models, considering the effects of linkage, mutation and selection, suggest that the system of mating has no influence on the amount of genetic variance of polygenic characters at equilibrium (Lande, 1977; Clegg and Epperson, 1985). Therefore, the morphological uniformity within strains of *Echinococcus* tells us little about the predominant mode of fertilization.

Protein and DNA analyses provide a more direct measure of genetic variation than morphology (Section III C), and a number of biochemical and molecular studies have provided data on variation and between strains of *E. granulosus*. Kumaratilake and Thompson (1984b) found differences between the mainland domestic, Tasmanian domestic and sylvatic strains in Australia, in the banding patterns of soluble proteins following isoelectric focusing. Different isolates of each strain had identical banding patterns. Other studies have measured isoenzyme variation following electrophoresis or isoelectric focusing. Le Riche and Sewell (1978), McManus and Smyth (1979), McManus and Simpson (1985) and Harrison *et al.* (1986) found differences in one to seven enzymes between, but not within, horse and sheep strains in Europe. Le Riche *et al.* (1982) examined one enzyme and found differences between isolates from sheep and cattle in Argentina, but no variation between isolates from each host. Macpherson and McManus (1982), using two enzymes, found no variation between different isolates from humans and sheep or between different isolates from camels in Kenya, although all camel isolates differed from all human and sheep isolates. Limited variation was found for one enzyme between different isolates from cattle. McManus and Simpson (1985) compared restriction fragment banding patterns following digestion of genomic DNA with two restriction endonucleases and hybridization to a heterologous rRNA gene probe. They found no differences between eight isolates of the UK horse strain, but differences between UK horse and sheep strains.

These results have been interpreted as evidence that each strain of *Echinococcus granulosus* exists as a separate, monomorphic clone, as predicted by Smyth's model of strain variation (McManus and Smyth, 1979; Macpherson and McManus, 1982; McManus and Simpson, 1985; Bryant and Flockhart, 1986). However, no study has yet attempted a detailed analysis of the genome of each isolate, using a range of enzymes or DNA probes, or been designed to obtain comprehensive samples of either the population of isolates from each locality or the range of localities in which a particular strain is found. In our laboratory, we have recently applied isoenzyme electrophoresis to 23 isolates of *E. granulosus* collected from domestic intermediate hosts at six localities throughout mainland Australia.

Although all isolates conformed morphologically to the domestic sheep strain, polymorphism was detected at 4 out of 20 enzyme loci. There were no significant multilocus associations and no evidence of geographical structuring of genetic variation. At one locality, levels of heterozygosity at one locus suggested that at least some cross-fertilization was occurring (A. J. Lymbery and R. C. A. Thompson, unpublished observations). Although any conclusive interpretation of genetic structure would be premature, these data suggest that both cross-fertilization and self-fertilization occur in populations of *E. granulosus* in Australia. This approach offers the most hope of determining the natural breeding system of species of *Echinococcus*, and therefore of evaluationg the different models of strain variation.

## C. SPECIATION

### 1. *Species concepts*

The continuing controversy over the taxonomic status of variant populations of *Echinococcus* is due partly to disagreement over appropriate criteria for distinguishing different species and subspecies, but also to failure to apply a consistent species concept. A clear view of what constitutes a species is essential for constructing formal taxonomic groupings and understanding modes of speciation.

(*a*) *Species concepts derived from Rausch's model of strain variation.* Rausch (1967a) and Schantz *et al.* (1976) invalidated three previously described species of *Echinococcus* and a number of subspecies of *E. granulosus*, by applying the biological species concept. According to this concept, species are groups of interbreeding natural populations that are reproductively isolated from other such groups, while subspecies are taxonomically different populations of a species, prevented from interbreeding by geographical separation or exclusive host relationships (Mayr, 1963, 1970). The use of the biological species concept follows logically from Rausch's model of intraspecific variation in *Echinococcus*, which regards cross-fertilization as the normal breeding system.

Application of the biological species concept to cestodes in general, and *Echinococcus* in particular, has been criticized as inappropriate for potentially self-fertilizing hermaphroditic organisms (Stunkard, 1957; Beveridge, 1974; Kumaratilake and Thompson, 1982a). The biological species concept cannot be applied to groups which reproduce strictly asexually or by exclusive self-fertilization (Mayr, 1963). However, even occasional cross-fertilization appears to integrate normally selfing plants into discrete species

units (Grant, 1957). Theoretical studies show that very low levels of gene flow may prevent genetic differentiation between populations (Lewontin, 1974; Slatkin, 1985). It is likely, then, that self-fertilization would need to be almost absolute in *Echinococcus* before the biological species concept could be regarded as inapplicable.

(*b*) *Species concepts derived from Smyth's model of strain variation.* Smyth's model of strain variation in *Echinococcus* assumes either exclusive or predominant self-fertilization. In line with this, Smyth has disregarded the biological species concept and proposed that *E. granulosus* and *E. multilocularis* occupy opposite ends of a continuum of variation. Present data are inadequate to recognize the complete continuum, and forms incorporating characteristics of both species have themselves been variously designated as strains, subspecies or species (Smyth, 1964, 1977; Smyth and Smyth, 1964). This view does not derive from any recognized species concept; species, subspecies and strains are regarded as arbitrary categories. Although such a taxonomic situation may be applicable to micro-organisms (Hutchinson, 1968), most higher organisms appear to fall into discrete taxonomic groups, even in the complete absence of cross-fertilizing sexual reproduction. These groups are regarded as either the remnants of biological species derived from sexually reproducing ancestors (White, 1978), as ecologically similar organisms occupying a limited number of niches (Hutchinson, 1968), or as slowly evolving genotypes derived from common ancestors (Wiley, 1981). A phenetic species concept (Sokal and Crovello, 1970), which delimits species solely on the basis of their shared characteristics, is usually applied to these groups of asexual or self-fertilizing organisms. Alternative concepts, such as ecological species (Van Valen, 1976) and evolutionary species (Wiley, 1978, 1981) are available. These have often been criticized for lacking operationality, but in some cases appear to have provided useful and productive groupings of asexual organisms (e.g. Stoddart, 1985).

(*c*) *The application of species concepts.* For *Echinococcus*, further data on genetic structure and breeding systems are vital to realistic decisions on the taxonomic validity of variant populations. An appropriate species concept must be applied and data relevant to that concept collected. If *Echinococcus* are found to be exclusively self-fertilizing, then a phenetic species concept may be considered appropriate. The designation of specific or subspecific status will then depend upon the extent of overall genetic or phenotypic differences between groups. For example, described morphological differences between the horse and sheep strains of *E. granulosus* may be large enough for them to warrant description as subspecies, but probably not as species (Kumaratilake *et al.*, 1986a). Alternatively, if *Echinococcus* are found

to reproduce by cross-fertilization, then a biological species concept will be relevant. Decisions about species and subspecies will then depend on opportunities for, and the extent of, interbreeding between groups. For example, if the horse and sheep strains of *E. granulosus* maintain genetic differences in strict sympatry, they may be considered as separate species. In this respect, Kumaratilake *et al.* (1986a) suggested that the pronounced anatomical differences between *E. granulosus* of horse and sheep origin may act as a physical barrier to interbreeding. Rausch (in Schantz, 1982) has speculated that some populations of *E. granulosus* may be reproductively isolated and represent sibling species. It is perhaps ironic that if the horse strain had initially been described and accepted as a distinct species, rather than a subspecies, then it is unlikely that comprehensive characterization studies (described in Section III, A, 1(a)) would have been undertaken, since such studies were undoubtedly stimulated by the uncertainty and controversy of taxonomic status.

### 2. *Modes of speciation*

Different species concepts channel our thinking about possible modes of speciation. Organisms which reproduce asexually, or by exclusive self-fertilization, may speciate either allopatrically or sympatrically. Smyth's model of strain variation in *Echinococcus* envisages the instantaneous appearance of a variant clone (Smyth and Smyth, 1964). This clone is necessarily reproductively isolated from its ancestor, whether or not they are geographically separated. Subsequent adaptation or genetic drift may lead to the accumulation of sufficient genetic differences to constitute a new species.

If cross-fertilization is the normal breeding system in *Echinococcus*, as proposed by Rausch (1985), then the question of speciation is more complex. Various models have been proposed for the acquisition of reproductive isolation (or of different specific mate recognition systems; Paterson, 1981) in populations of cross-fertilizing organisms. The most widely accepted mode of speciation is allopatric, in which two populations diverge while geographically separated (Mayr, 1963). Recently, many authors have argued that sympatric speciation is common in parasitic organisms (Bush, 1975; White, 1978; Price, 1980). This is presumed to occur through host switching and subsequent divergence of populations which are ecologically, but not geographically, separated. There are, however, both theoretical and empirical objections to sympatric speciation, and its importance as an evolutionary process is uncertain (Futuyma and Mayer, 1980; Paterson, 1981). Rausch (1967a) recognized that gene flow between populations of cross-fertilizing *Echinococcus* in sympatric host species could only be prevented by rigidly exclusive predator–prey relationships. Given the apparent rarity of

such relationships amongst the hosts of currently recognized species of *Echinococcus* (Rausch, 1986), allopatric speciation is likely to have been predominant in the evolution of the genus.

### D. PHYLOGENY

There has been little work on the phylogenetic relationships between species and strains of *Echinococcus*. Rausch (1985) suggested that marked differences in the structure of the larval stage between the four recognized species of *Echinococcus* are indicative of a long period of separation (since at least the early Pleistocene). The genus is assumed to be monophyletic, with *E. granulosus* and *E. multilocularis* diverging in Eurasia as a result of ecological separation (in different host cycles), and *E. oligarthrus* and *E. vogeli* evolving in South America, having dispersed from Eurasia in their mammalian hosts (Rausch, 1973).

Rausch (1967a,b; 1986) and Schantz (1982, 1984) have attempted to infer the phylogeny of intraspecific variants of *E. granulosus* and *E. multilocularis* from host relationships and biogeographical data. The ancestral form of *E. granulosus* is considered to have infected wolves and cervids (principally reindeer and elk) in the northern parts of Eurasia and North America. Further south in Europe, the same form infected other intermediate hosts, including the wild ancestors of cattle, sheep and pigs. The present northern form of *E. granulosus* has retained the ancestral life-cycle, although its European distribution is now more restricted. The European form was derived from the ancestral form via the domestication of wolves and wild ungulates in Europe. This led to ecological, and probably also geographical, separation of populations of *E. granulosus* with sylvatic and domestic life-cycles. The present cosmopolitan distribution of the European form is considered to be a result of the dispersal of humans and their domestic animals through the eighteenth and nineteenth centuries.

In contrast to the marked changes in host relationships and distribution of *E. granulosus*, *E. multilocularis* is considered to have largely retained its ancestral life-cycle and geographical range. This involved foxes and rodents through central and northern Eurasia and northern North America. The Eurasian and North American populations were presumably separated with the flooding of the Bering land bridge, 13000–14000 years ago (Cox and Moore, 1980). Disjunctions also occurred in Europe, principally between populations in western Europe and the Soviet Union, but are not reflected in morphological differentiation.

While this scenario for the evolution of intraspecific variants of *E. granulosus* and *E. multilocularis* appears logically sound, there is little

evidence to support it. Schantz (1984) noted that cysts of *E. granulosus* are typically unilocular in cervids, but multilobular or chambered in domestic ungulates such as sheep. This is taken as evidence that cervids are the natural (ancestral) intermediate host and the parasite is less well adapted to other species of hosts. However, R. C. A. Thompson (1975, Ph.D. Thesis, University of London) and Schwabe (1986) argued that asymmetrical and multilobular cysts arise from external influences on development and are correlated with organ location rather than host species. Other predictions which appear to follow from the scenario proposed by Rausch and Schantz have not been tested. The separation of northern and European forms of *E. granulosus* and Eurasian and North American subspecies of *E. multilocularis* are considered to be vicariance events, and should therefore be paralleled by relationships in other parasite taxa inhabiting the same hosts. These data may be available from the literature, but, to our knowledge, they have not been analysed. Rausch (1967a) implied that the ancestral form of *E. granulosus* infected not only cervids, but also the ancestors of domestic ungulates. The phylogeny of strains of *E. granulosus* should therefore be congruent with the sequence of domestication of hosts, but not with the phylogeny of hosts. Other patterns would contradict this evolutionary scenario. For example, non-congruence between the phylogeny of strains and the sequence of domestication of hosts would imply that domestication did not produce ecological and geographical barriers to gene flow between parasite populations. Congruence between the phylogeny of strains and the phylogeny of hosts would imply that strains are host-specific and differentiated with the speciation of their hosts. To test these predictions, information is required on the phylogeny of hosts, the sequence of domestication of hosts and the phylogeny of strains. The first two are available; what is now needed is an assessment of the phylogeny of strains of *E. granulosus* independent of considerations about host relationships and biogeography.

Kumaratilake (1982) attempted a non-quantitative phenetic grouping of the four recognized species and a number of strains of *E. granulosus*, based on measurements of a range of morphological characters. Her analysis, shown in Fig. 2, suggests that all species share some characteristics, although *E. vogeli* and *E. oligarthrus* are morphologically intermediate between *E. granulosus* and *E. multilocularis*. Whereas the sheep and horse strains of *E. granulosus* are morphologically similar, the cattle strain is quite distinct and shares a number of similarities with *E. vogeli* and *E. oligarthrus*. Further studies indicate that the cattle strain is similar to *E. granulosus* from cervids in North America, particularly with respect to adult development and strobilar morphology (L. M. Kumaratilake, R. C. A. Thompson and P. M. Schantz, unpublished data). While these data suggest affinities between the northern form and cattle strain, and between the sheep and horse strain, of

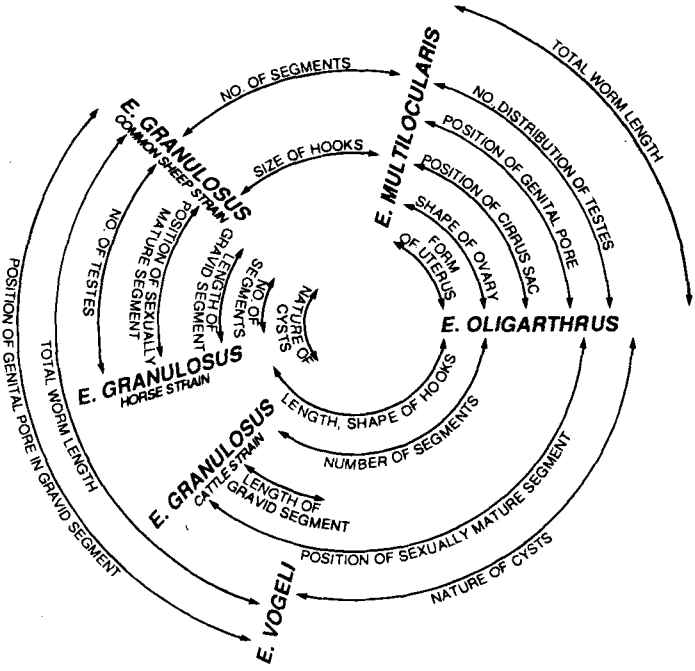


FIG. 2. Morphological similarities between species and strains of *Echinococcus*, based on measurements of 37 characters. Redrawn from Kumaratilake (1982).

*E. granulosus*, they should be interpreted cautiously. The groupings are purely phenetic, based on overall similarity rather than ancestral relationships. A necessary preliminary to estimates of phylogeny is a detailed character analysis, addressing not only the problems of heritability and covariation between morphological characters, but also the evolutionary polarity of character states. This has not yet been done. A wide range of morphological characters are available; whether their range of variation is sufficient to enable the determination of ancestral and derived states can only be answered by attempting the analysis. In addition, biochemical and molecular techniques, which are now being applied to *Echinococcus*, may provide other characters of value in phylogenetic studies.

## V. PRACTICAL SIGNIFICANCE OF STRAIN VARIATION

The practical significance of variation within the genus *Echinococcus* must relate to how this variation may influence our knowledge of the epidemiology

of hydatid disease and measures that may be taken to diagnose, treat and control the disease. As such, it is useful to consider this under the following headings.

## A. DYNAMICS OF TRANSMISSION AND CONTROL

In any endemic area, the prerequisite to implementing control programmes is a sound knowledge of the dynamics of transmission. In the past, such a requirement would have appeared straightforward, as long as the species of *Echinococcus* could be identified. This is not the case today, particularly in areas where *E. granulosus* is the causative agent. This is because of the extensive intraspecific variation that has been demonstrated and which is reflected in numerous factors of epidemiological significance.

Perhaps the most obvious factor of epidemiological importance is host specificity. It assumes most significance where there is the possibility of interaction between sylvatic and domestic cycles, as illustrated by the situation on the mainland of Australia (see Section III, A, 1, i(ii)). Control strategies depend on knowing whether the form of *E. granulosus* which is primarily maintained in a sylvatic cycle (wallaby–dingo) is infective to domestic intermediate hosts. If the sylvatic form proved to be infective to sheep, effective control would be impossible without eradication of the entire dingo population! Fortunately, present results suggest that control may be a realistic proposition, since two distinct strains of *E. granulosus* appear to occur on the Australian mainland, one of which is primarily perpetuated in the sylvatic cycle and does not develop well in sheep (Thompson and Kumaratilake, 1985; Thompson, in press and unpublished observations). Thus, control efforts can safely focus on the sheep–dog cycle without the worry of the wallaby–dingo cycle "spilling over" into the domestic animal environment. The possibility of differences in host specificity also assumes importance in endemic areas where there are numerous intermediate hosts (as in Kenya, see Sections III, A, 1(d) and (i), (iii)). The existence of numerous susceptible intermediate hosts in an endemic area, in all of which the parasite produces fertile cysts, may suggest either (1) a distinct strain with an unusually broad host range or (2) the presence of more than one strain, since in other endemic areas strains are adapted to particular species of intermediate host and do not develop as well in other animals. In England, the realization that sheep and horse forms of *E. granulosus* may exhibit specificity for their respective intermediate hosts was a major impetus in elucidating their life-cycle patterns and strain characteristics (see Section III, A(a)).

There is also the possibility that certain strains may not be infective to

humans. For example, current epidemiological information indicates that horse and pig strains of *E. granulosus* in Europe may not infect man, whereas the converse is proven for sheep and cattle strains (Thompson and Smyth, 1976; Thompson, 1977, in press; Pawlowski, 1985; Baldock *et al.*, 1985). In Australia, it has recently been demonstrated that humans are susceptible to both domestic and sylvatic strains of *E. granulosus* (Thompson *et al.*, 1987).

Apart from host specificity there are other manifestations of intraspecific variation which may affect transmission. The most important is that of developmental pattern. The recent demonstration that strains of *E. granulosus* and *E. multilocularis* may vary in their prepatent period (Kumaratilake *et al.*, 1983; Thompson and Eckert, 1983; Thompson *et al.*, 1984; Hörchner *et al.*, 1986) must be taken into account when considering control programmes involving regular drug treatment of definitive hosts. Such programmes aim to break the cycle by maintaining the definitive host population free of egg-producing worms. Thus, drug treatment is given regularly at a period designed to remove worms prior to patency. Variation in the timing of egg production between strains means that generalizations as to the timing of drug treatment should not be made.

Other factors that may influence transmission include the resistance properties of eggs and the development of the metacestode. Variation in such characteristics is suspected and future studies will hopefully elucidate the situation further. Evidence from Switzerland indicates that development of hydatid cysts may vary between strains of *E. granulosus*. The cattle strain usually produces fertile cysts in Swiss cattle, whereas other strains usually develop poorly, giving rise to sterile or degenerate cysts (Thompson *et al.*, 1984; and see Section III, A, 1(b)). The nature of hydatid infection in Swiss cattle is also unusual in that cysts occur more frequently in the lungs than in the liver, with small fertile cysts often developing deep within the substance of the lungs. As a consequence, cysts may not always be detected at meat inspection, giving rise to the unusual situation that dogs in urban areas are more at risk of contracting infection with *E. granulosus* than dogs in rural areas, due to the likelihood of eating contaminated offal bought at butchers' shops (Eckert, 1981).

### B. PATHOGENESIS AND CLINICAL COURSE

There is evidence that strains of *Echinococcus* may vary in their pathogenesis in intermediate hosts and that the clinical effects of infection with different strains may therefore differ. Strains of *E. granulosus* in Kenya and Libya are thought to exist which may be particularly virulent in humans, whereas in

North America the indigenous sylvatic strain causes a benign, often symptomless condition in humans (French *et al.*, 1982; Gebreel *et al.*, 1983; Wilson *et al.*, 1968). Unfortunately, there are no data from controlled animal experiments to corroborate speculation that strains differ in virulence. Indeed, the clinical effects, particularly of unilocular hydatid infection, in animals are poorly understood.

### C. IMMUNODIAGNOSIS AND VACCINATION

The serological response of the intermediate host is influenced not only by the species but also by the strain of *Echinococcus* (Cameron, 1960; Huldt *et al.*, 1973; Gottstein *et al.*, 1983, 1986, 1987; Leikina, 1984; Lightowlers *et al.*, 1984; Pezzella *et al.*, 1984; Rickard and Lightowlers, 1986). Consequently, such antigenic heterogeneity will have to be considered in the development and choice of immunodiagnostic procedures in different endemic areas, particularly with respect to the choice of reference antigens employed. As emphasized recently (Gottstein *et al.*, 1986), much work must be carried out to find antigen fractions for immunodiagnosis that are not affected by strain differences. Similarly, consideration must be given to the choice of antigens used in vaccine development, since a vaccine against infection with one strain may not elicit a protective response against a different strain.

### D. CHEMOTHERAPY

Considerable attention is being given to the development of effective chemotherapeutic agents against the metacestode of *Echinococcus*. At the present time, surgery remains the only effective curative treatment in the absence of a reproducibly effective metacestocide. Numerous clinical trials have been undertaken for both *E. granulosus* and *E. multilocularis* using several benzimidazole compounds. Results obtained have been variable and, as a consequence, views on the role of drug therapy are controversial. There is some agreement that chemotherapy has value with inoperable cases by impeding parasite proliferation and also post-operatively as a means of inhibiting recurrent cysts (Eckert, 1986; Rausch *et al.*, 1986).

An important factor that may contribute to the variability of success in clinical trials is the existence of different strains. A number of workers have suggested that strains may differ in their response to particular chemotherapeutic agents (Saimot *et al.*, 1981; Schantz *et al.*, 1982; Kammerer and Schantz, 1984). Such a possibility is supported by the extensive differences in physiology, biochemistry and metabolism between strains of *Echinococcus* (see McManus and Bryant, 1986; Bryant and Flockhart, 1986).

## VI. Conclusions

We are only now beginning to appreciate the extent of intraspecific variation in the genus *Echinococcus*. A comparison of currently recognized strains with previously described, and subsequently invalidated, species and subspecies demonstrates that at least some of this variation has been recognized by a number of workers in the past. The acceptance of such variation was undoubtedly hampered by rigid application of traditional taxonomic concepts, and by the lack of adequate differential criteria to characterize variants below the species level.

Today, the situation has changed dramatically. The discriminatory power of biochemical and molecular techniques, and their growing acceptance in taxonomic determinations, have provided the tools necessary for recognition and characterization of variants within the recognized species of *Echinococcus*. In addition, a more flexible taxonomic approach has arisen from the philosophical debates which have dominated the theory of classification over the past three decades. The application of current taxonomic procedures to the classification of a group of organisms should provide us with a great deal of implicit information about genetic relationships within the group. In *Echinococcus*, this will ensure an important role for the explanatory and predictive value of taxonomic determinations in studies on the epidemiology and control of hydatid disease.

A prerequisite to this approach is the collection of data on genetic variation within and between populations of *Echinococcus*. Without these data, no decisions can be made about breeding systems, extent of gene flow, appropriate species or modes of speciation. Biochemical and molecular techniques have provided the means of obtaining the data; what are now needed are studies designed to use these techniques to answer fundamental genetic questions.

Despite increasing access to highly discriminatory differential criteria and a more flexible taxonomic approach, problems of nomenclature are likely to remain. Many genetically distinct populations within the recognized species of *Echinococcus* cannot be adequately allocated to a formal taxonomic category. In itself, this is neither unique nor surprising. Genetic variation within and between populations is the rule for most species of animals and plants. This variation is well accepted and we agree with Mayr (1963) that for free-living organisms there is no need for informal taxonomic categories between the levels of population and subspecies. However, in parasitic organisms the recognition of variation may be of great medical and agricultural significance and it is for this reason that the term "strain" has been widely used to describe intraspecific variants in *Echinococcus* and other parasites. For the term to be of value, we believe that there must be some uniformity in

its use, and this belief underlies our attempts at creating a workable definition. We expect, and welcome, criticisms of this definition. However, we hope that it will generate discussion which may lead to an acceptable rationalization of the current confusing usage.

The influence of intraspecific variation in *Echinococcus* on the epidemiology and control of hydatid disease is now quite clear. It is essential that strains are indentified and characterized in each endemic area if effective control programmes are to be instituted. Future research should aim to develop even more reliable and practically applicable differential criteria for this purpose. Biochemical and molecular dissection of the parasite in a quest towards this end may also unravel the functional nature of strain variation, and provide direction for the development of chemotherapeutic agents.

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# Index

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