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PARASITOLOGY

VOLUME 22

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PREFACE

This volume of Advances in Parasitology continues the policy established by Ben Dawes and developed by Professor Lumsden, of attempting to review any aspect of parasitology in which significant developments are being, or have recently been, made. We feel that the traditional division of parasitology into protozoology and helminthology is becoming increasingly artificial. As more emphasis is laid on the subject's ecological aspects—including inter-relationships between parasites and their hosts—and on the cell biology of the parasites themselves, the common principles resulting from a shared life-style and a common eukaryotic nature, are becoming more evident. We hope to include in future volumes, papers dealing with general principles of parasitism, not subdivided on the basis of uni- or multicellularity and not necessarily restricted even to eukaryotic organisms.

Meanwhile, in the present volume, the traditional division is maintained, though our first criterion—topicality and significance—is, we believe, fully met by all the included papers. Perhaps the most controversial contribution is that by Evans and Ellis, questioning views which have been held more-or-less uncritically since the work of Muriel Robertson early this century.

1983

J. R. BAKER
R. MULLER

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CONTENTS

Recent Observations on the Behaviour of Certain Trypanosomes within their Insect Hosts

D. A. EVANS AND D. S. ELLIS

Cell-mediated Killing of Protozoa

KAREEN J. I. THORNE AND JENEFER M. BLACKWELL

Sexual Development of Malarial Parasites

R. E. SINDEN

The Genetic Basis of Diversity in Malaria Parasites

D. WALLIKER

Hydatidosis and Cysticercosis: The Dynamics of Transmission

J. RUTH LAWSON AND M. A. GEMMELL

Lipid Metabolism in Parasitic Helminths

G. J. FRAYHA AND J. D. SMYTH

Recent Observations on the Behaviour of Certain Trypanosomes within their Insect Hosts

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I. Introduction

Research work carried out over the past 10 to 15 years has given new insight into many of the problems associated with African trypanosomiasis. The discoveries in the laboratory, for example, of antigenic variation, of switches in metabolic pathways and of variations in the isoenzyme patterns of differing trypanosome populations have produced a clearer understanding of some of the complex interrelationships between hosts, parasites and vectors. In the field, however, many of the early problems remain.

As long ago as 1921 Duke noted that it was sometimes impossible to find trypanosomes in the salivary glands of tsetse flies caught in an area where transmission of trypanosomiasis was occurring. Buxton (1955) reviewed the problem, and the difficulty generally of correlating the extent of the disease found with the apparent degree of tsetse involvement. While Molyneux and his colleagues (1979) have shown how the behaviour of infected flies would tend to maximize transmission, there still may be other factors involved, and the behaviour of the trypanosomes described here may have some bearing on these and other problems long recognized in sleeping sickness and related trypanosomiases.

A. The traditional view of the developmental pathways of African trypanosomes within the tsetse fly

The parasites responsible for these diseases are those of the *Trypanosoma brucei* group; the generally accepted developmental pathways during that part of their life cycle which is within their vector, the tsetse fly (*Glossina* spp.), have been described by Buxton (1955, pp. 607–609) as follows.

> "If a tsetse takes blood containing trypanosomes (setting aside the possibility of direct transmission) the organisms may disappear, either while the meal of blood is being digested, or during the digestion of a subsequent meal: or they may survive in the crop, living there for many days but failing to establish themselves elsewhere (*T. rhodesiense* in *G. palpalis*, Duke and Mellanby, 1936); or they may establish themselves first in the midgut, later in the salivary glands and so complete the cyclical development. In the last case, the fly becomes capable of transmitting the infection to other mammals. The cycle is only completed in a very small proportion of flies, under normal circumstances (...).
>
> "In the event of the trypanosomes establishing themselves, they pass with the blood into the midgut, and at first lie inside the tubular peritrophic membrane which separates them from the epithelium of the midgut (...). After about 4 days they may be found in the ectoperitrophic space, i.e. outside the membrane, between it and the epithelium (Yorke,

Murgatroyd and Hawking, 1933). It appears highly probable that they have reached this position by passing down the alimentary canal inside the peritrophic membrane, as far as its free posterior end, which is in the hindgut; from that point they probably pass from within the membrane to the ectoperitrophic space, in which they migrate forward along the midgut to the proventriculus. . . .

"Having passed right forward in the ectoperitrophic space, the parasites find themselves in the annular space with midgut epithelium on one side and the base of the peritrophic membrane, in the proventriculus, on the other (...). It is probable that they escape by passing through the base of the peritrophic membrane at the point where it is being actively produced and is still fluid."

Thus, according to this pathway, the trypanosomes pass through the wall of the peritrophic membrane only to enter the endoperitrophic space, not to leave it. Having re-entered the endoperitrophic space, Buxton suggests, "the trypanosomes . . . might pass forward through the foregut and food canal to the distal extremity of the hypopharynx: from that position it is held that the organisms pass up inside the hypopharynx from tip to base, and so by way of the ducts to the salivary glands. It cannot be claimed that every stage of this cycle has been observed, indeed it would be a matter of extreme difficulty to obtain direct evidence of some parts of it."

Figure 1 shows the whole of this complex pathway diagrammatically. Figure 1(a) is usually stated to be based on the various observations and published work of Bruce and Robertson in the 20 years before the First World War. But their version, as will be seen from Fig. 1(b), did not involve the presence of the peritrophic membrane or a very clear journey to reach the open end of the hypopharynx that the parasites would have to travel to reach the salivary glands.

B. MAJOR CONTRIBUTIONS SUPPORTING THE TRADITIONAL VIEWS

It is necessary to examine here how the accepted developmental pathways (as quoted above, from Buxton, 1955) were built up, not only to establish how this was achieved historically, but also to relate the recent observations reported here to those of the original workers.

The first demonstration of the association between trypanosomes, thought to be *T. congolense* (Hoare, 1972), and tsetse flies (*G. morsitans*) was by Bruce (1895, 1897) who showed that tsetse could be used to transmit "live viruses" among horses. Later, Brault (1898) suggested that this could be the method of spread of human sleeping sickness. In 1903 Bruce and Nabarro reported from Uganda that wild-caught tsetse were able to transmit trypanosomiasis to monkeys. In the same year, from Zululand, Bruce noted that the

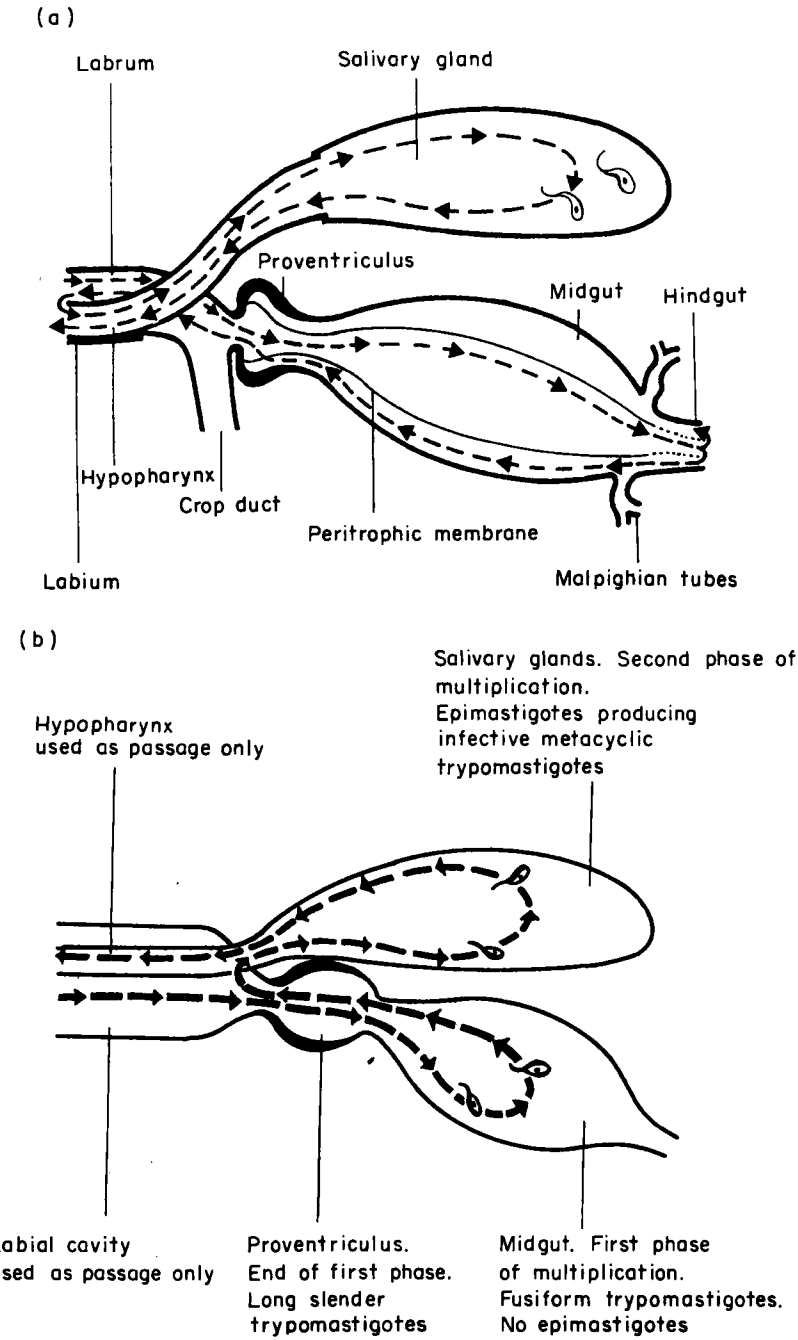

FIG. 1. Diagrams illustrating the pathway followed by *Trypanosoma brucei* within *Glossina* spp.: (a) as outlined by Buxton (1955: based on diagram by Davies, 1967); (b) adapted from diagram by Wenyon (1926).

trypanosomes found in tsetse guts were not infective to mammals (Bruce, 1903), yet the disease could be passed by flies (*G. palpalis*) from sleeping sickness patients to monkeys for up to 48 hours after the flies fed on these patients (Bruce *et al.*, 1903). Some form of development within the fly therefore seemed possible, and Kleine (1909) showed that a cycle did exist in his laboratory-reared *G. palpalis* flies, taking 20 days, this being the time between an infective feed and the moment when the flies first became capable of transmitting *T. brucei*. Bruce *et al.* (1909) reported that Kleine's tsetse remained infective for at least 50 days after infective feeds, and confirmed Kleine's findings using *T. gambiense*, then the only recognized cause of human sleeping sickness. They also reported completion of the cycle in tsetse salivary glands with the production of infective metacyclic forms. When the salivary glands were packed with trypanosomes, none was found in the "proboscis, proventriculus, thoracic gut, crop, hindgut and Malpighian tubules" but they were seen in the midgut from the fourteenth to the twenty-third day. Bruce noted also the low rate of fly transmission: 1 % of *G. morsitans* with *T. brucei* and 0.2 % of *T. gambiense* with *G. palpalis*. Two years later (Bruce *et al.*, 1911c), he had raised the percentage to 8 in some experiments with *T. gambiense*.

In 1911 Bruce *et al.* (1911a, b) summarized their findings as follows.

(a) *T. gambiense* multiplied in the gut of about one in every 20 *G. palpalis* which had fed on infected animals.
(b) Flies became infective, on average, 34 days after their first feed.
(c) Flies may remain infective for 75 days.

Later in the same report (Bruce *et al.*, 1911a) they added the following conclusions.

(a) In the course of development of *T. gambiense* in *G. palpalis* the proboscis did not become involved as in the case of some other species.
(b) A few days after the infective feed the trypanosomes disappeared from the great majority of the flies, but in a small percentage this initial disappearance was followed by renewed development.
(c) After a very short time the flies which had fed on an infective animal became incapable of conveying the infection by their bites, and this non-infectivity lasted for 28 days; when renewed or late infectivity developed.
(d) A fly in which this renewed or late infectivity occurred could remain infective for at least 96 days.
(e) Invasion of the salivary glands occurred at the same time as this renewal of infectivity, and without this invasion of the salivary glands there could be no infectivity.
(f) The type of trypanosome found in the salivary glands when the fly became infective was similar to the short stumpy form found in the

vertebrate's blood, and it was believed that this reversion to blood type was *sine qua non* in the infective process.

They also noted that, while other trypanosome forms were found in the salivary glands, the only place within the fly where the blood-like type was found was in these glands.

Throughout much of this period confusion had been caused by the added presence of *T. grayi* in the guts of wild-caught tsetse. Although this trypanosome develops its metacyclic forms in the hindgut—the "posterior station"—and infects reptiles by contamination with tsetse faeces, after initial ingestion by the fly it first develops in the midgut in the same way as do members of the *T. brucei* group. It was this similarity that caused the confusion among the earlier workers. The life-cycle of *T. grayi* was finally elucidated by Hoare (1931a, b).

It was recognized generally by the workers already cited that human sleeping sickness in the field was caused by what is now named *T. brucei gambiense* Dutton, 1902, spread by *G. palpalis*, though in many animal experiments *T. brucei brucei* had been used, with *G. morsitans* as its vector. Stephens and Fantham (1910) described a different agent of human sleeping sickness, which they called *T. rhodesiense*, now named *T. brucei rhodesiense* Stephens and Fantham, 1910, whose vector was *G. morsitans* and whose characteristics resembled the animal parasite *T. b. brucei* Plimmer and Bradford, 1899. The disease in man was much more acute than that caused by *T. b. gambiense*, and with a different pathology. However, the stages in the fly of these two agents appeared to be identical (Lloyd and Johnson, 1924).

Observations on the establishment of a trypanosome infection in the tsetse fly contained in the first paragraph of the quotation from Buxton (1955) above are derived from Robertson (1912a and 1913). She also noted (1913) that multiplication of trypanosomes occurred soon after ingestion in the mid- and hindguts. After 48 hours she found that the predominant form of the parasite resembled the stumpy form found in the blood of the vertebrate host. By 10 days these forms started to be replaced by longer, thinner forms which moved progressively up the gut towards the proventriculus. After 3 weeks these forms began to invade the salivary glands "from the hypopharynx". Her evidence for this last point rested on finding trypanosomes within the duct but not yet within the gland (Robertson, 1913).

Robertson (1912c) found no intracellular stage or attachment of the parasites to the gut wall, nor did she mention the peritrophic membrane, though this structure had already been reported by Stuhlmann (1907). She observed no sexual phase, but felt that there might well be one, probably in the salivary glands. However, in the second week of some gut infections she did note (Robertson, 1912c) the presence of multinucleate forms and speculated on their function. She dismissed the "male" form reported by Taute (1911) as irrelevant.

C. ROLE OF THE PERITROPHIC MEMBRANE

Thus the main characteristics of the fly infection were established before the First World War, although the final point, namely that salivary gland infections could not occur without previous gut infection, was not made until 1921 by Duke. An important anomaly was the lack of recognition of the role played by the peritrophic membrane. Southgate (1965) drew attention to this curious gap in the story. Robertson (1912b) believed that the absence of trypanosomes from the anterior part of the midgut for 7–12 days after the infected blood-meal was due to their being carried down the gut by the blood of later meals. She envisaged an ebb and flow of multiplying trypanosomes up and down the gut. Observations on the tsetse gut (Southgate, 1965) showed that new blood extends rapidly down to the junction with the hindgut and, before this, there may be forcible evacuation of the products of previous meals: clearly the midgut is an unstable region for the establishment of a large colony of parasites if there is not some method of attachment or special sequestration mechanism available.

The first mention of the peritrophic membrane in relation to development of trypanosomes in tsetse was by Johnson and Lloyd (1929) and Lloyd (1930), who noted that trypanosomes (*T. congolense*) could be found developing in the ectoperitrophic space. Wigglesworth (1929) described the origin of the membrane from an annular pad of proventricular epithelium, "pressed" out from the hardening secretions of those cells. He found it composed mainly of chitin and permeable to digestive enzymes, haemoglobin and haematin, although acting as a "filter" for intact erythrocytes.

Hoare (1931a) showed that the peritrophic membrane was a continuous tube down to the midgut/hindgut junction and first coined the phrases "intra" and "extra" peritrophic spaces, here referred to as "endo" and "ecto" peritrophic spaces. He believed the rectal "spines" disrupted the membrane's posterior end so that its contents could be liberated in the gut lumen.

Taylor (1932) described *T. gambiense* developing in *G. tachinoides* in the ectoperitrophic space. In 1933 Yorke and co-workers showed that within a few days of an infected blood-meal, trypanosomes could no longer be found in the endoperitrophic space—only in the ectoperitrophic space. The trypanosomes then moved up to the proventriculus, later reaching to within its lumen. These workers also believed that the trypanosomes attained the ectoperitrophic space via the torn posterior end of the peritrophic membrane, where it had been ruptured by the rectal "spines" or "teeth".

In 1957 Gordon first demonstrated the passage of trypanosomes across the peritrophic membrane at the upper end of the proventriculus. He cut serial sections of tsetse infected with *T. congolense* and found great numbers in the ectoperitrophic space, but only a few of the same morphological type within the lumen, which he assumed to have just passed through into

the endoperitrophic space. A year later Fairbairn (1958) published photographs of trypanosomes in the proventriculus, where the peritrophic membrane was still "soft", in the act of penetration.

D. OBSERVATIONS CONFLICTING WITH THE TRADITIONAL PATHWAYS

These, then, were the last points completing the cycle quoted from Buxton and shown in Fig. 1a. This complex pathway, involving as it does trypanosomes doubling back on several occasions through different environments within the fly, itself presents problems.

It is probable that its sojourn in the crop gives the trypanosome time to change its metabolism from one involving the use of glucose (in the vertebrate hosts' blood) to its new energy source of proline from the tsetse fly (Harmsen, 1973). The parasite, now a midgut trypomastigote form, enters the peritrophic membrane sac via the proventriculus. Hoare (1931a) and Willett (1966) have clearly demonstrated the peritrophic membrane initially to be a long intact sac that is ruptured or opened only when it reaches the "spines" of the rectum after the fly's first feed (although its proventricular end, as it is being produced from the annular ring of cells (Wigglesworth, 1929), might be soft enough to allow penetration by the parasites). Subsequently the hind end of the membrane remains open throughout the life of the fly. Thus the accepted route would take the trypanosomes into the hindgut itself, taking 3 or 4 days to reach there (Willett, 1966).

Bursell and Berridge (1962) have shown that while the pH of the tsetse midgut contents is around 7·2 (with low osmolarity), the hindgut osmolarity is very high, with a low pH of around 5·8. Thus a trypanosome would have to suffer very dramatic changes in its environment during any circumnavigation of the end of the peritrophic membrane, which it would be unlikely to survive.

This part of the accepted developmental pathway has thus several difficulties. It has been suggested (Wigglesworth, 1929) that the peritrophic membrane could be "inconsistent in the middle section" of the midgut, thus allowing trypanosomes direct access to the ectoperitrophic space: however, Willett (1966) found that, even after a very large first blood-meal, the peritrophic membrane remained intact throughout its length until it finally engaged with the rectal "spines" several days later; this was confirmed by Southgate (1965). Freeman (1970, 1973) suggested that trypanosomes might pass out of the endoperitrophic space via the soft forming peritrophic membrane at the upper end of the proventriculus in the first hour following an infected feed. This represented a reversal of the accepted view of the passage through this area of membrane, which proposed that only mature midgut forms re-entered the endoperitrophic space at this point on their way to the salivary glands.

The final route taken by *T. brucei* group trypanosomes to the salivary glands has been questioned over the last 20 years. Foster (1963, 1964) was the first to report the presence of trypanosomes of unknown species in the haemocoele of tsetse flies. Webber (1963) established that, once inoculated into the tsetse haemocoele, *T. rhodesiense* could survive and multiply. Later Otieno *et al.* (1976) performed similar experiments and reported that tsetse flies, so infected, could themselves transmit the infection. They also described multinucleate (cyst) forms in the haemocoeles of their infected flies (see below). Mshelbwala (1972) found that 15% of 262 tsetse flies, belonging to three different species, which had trypanosomes in their gut or salivary glands, also had trypanosomes in their haemocoelomic fluid; those from about half of the flies infected mice. The forms he found were similar to either midgut or salivary gland types (these latter presumably were the ones capable of infecting his mice). The suggestion was that these haemocoele parasites had come either through the gut wall (those non-infective to mice) or from the salivary glands (those that were infective to mice). Since then L. Jenni (personal communication) has regularly found examples of *T. brucei* in the haemocoeles of some of his experimentally maintained and infected tsetse at the Swiss Tropical Institute.

Thus it is clear that there were some serious difficulties in the accepted developmental pathways and some hints that perhaps other routes, which might be involved, should be investigated.

II. Methods used to Re-examine the Traditional Views

The methods we used to study the developmental cycles of trypanosomes within the tsetse fly may be summarized as follows (fuller details are given in our papers cited in the list of references on pp. 38–42).

1. *G. morsitans morsitans* were infected with cultured procyclic (= midgut) forms of three *T. brucei* stocks (see Table 1) from different sources in Africa (Ellis, 1978), which had been added to artificial blood meals (Evans, 1979) using the membrane feeding techniques of Mews *et al.* (1977).

2. The infected flies were dissected in glutaraldehyde fixative at suitable intervals and the guts and salivary glands removed and processed for electron microscopic (EM) examination.

3. These methods ensured suitably high infection rates in the flies used for EM examination, which was the only way to establish the exact location and state of the trypanosomes at various times during the developmental cycle within the fly.

TABLE 1

Details of experimental infections of Glossina m. morsitans *with* Trypanosoma brucei

Stock	180 TS1	180 TS1	180 TS1	LUMP 1026	STIB 488T
No. of guts processed for electron microscopy	21	46	13	9	12
Infective forms added to tsetse blood meal	Cultured forms		Long thin blood forms	Cultured forms	
No. of infected feeds	1	3	1	1	1
Average time (days) to:					
entrapment in folds of peritrophic membrane	9	8	—	10	6
entry into peritrophic membrane	10–12	9–10	—	12–13	7–9
entry into ectoperitrophic space	12–14	10–12	19	12–15	9–10
exit from ectoperitrophic space into midgut cells	20	20	24	24	16
earliest transmission via salivary glands	28	28	30–34	—	—

4. Parallel studies, using flies infected by feeding on trypanosome-infected laboratory rodents, were undertaken to insure that our use of cultured procyclic forms for tsetse infection was valid and that we were not introducing artifactual developmental pathways. Infection of the flies from rodents always led to lower numbers of trypanosomes in the fly, which were far more difficult to locate with the electron microscope.

5. Much of the above work was repeated using two additional *T. brucei* stocks, and one stock of *T. congolense*.

III. OBSERVATIONS

The methods involving infection with cultured procyclic trypanosomes enabled 60% of all flies examined 20 days after an infected blood-meal to have established midgut infections with all three trypanosome stocks; though over the following 2 weeks over half of these infections were lost. Those infections that did persist led to salivary gland infections and subsequent transmission of infection to laboratory mice, either by the bite of the infected tsetse flies or by intraperitoneal injection of macerated salivary glands.

A. PENETRATION OF THE PERITROPHIC MEMBRANE BY *Trypanosoma brucei*

The three different trypanosome stocks used all appeared to follow the same behaviour within the tsetse gut lumen, though the timings at

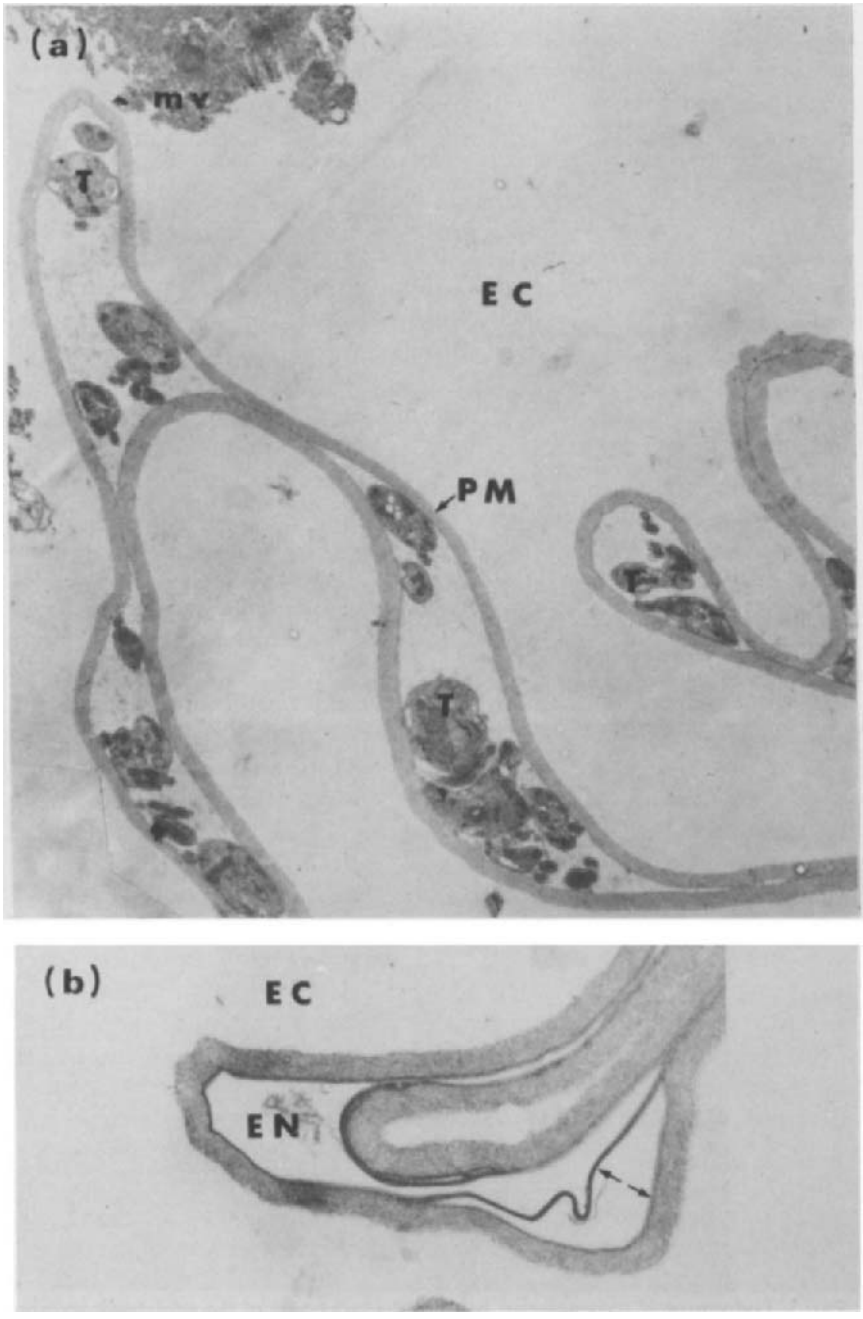

FIG. 2. Penetration of the peritrophic membrane by *Trypanosoma brucei*. (a) Trypanosomes (some marked T) of stock 180 TS1 trapped within the folds formed in the peritrophic membrane (PM) as the blood meal within it is digested. The microvilli of the tsetse midgut cells are marked mv; EC, ectoperitrophic space. Magnification ×3600. (b) The peritrophic membrane, separating the endoperitrophic (EN) and ectoperitrophic (EC) spaces, is splitting into two layers. Magnification ×16 000.

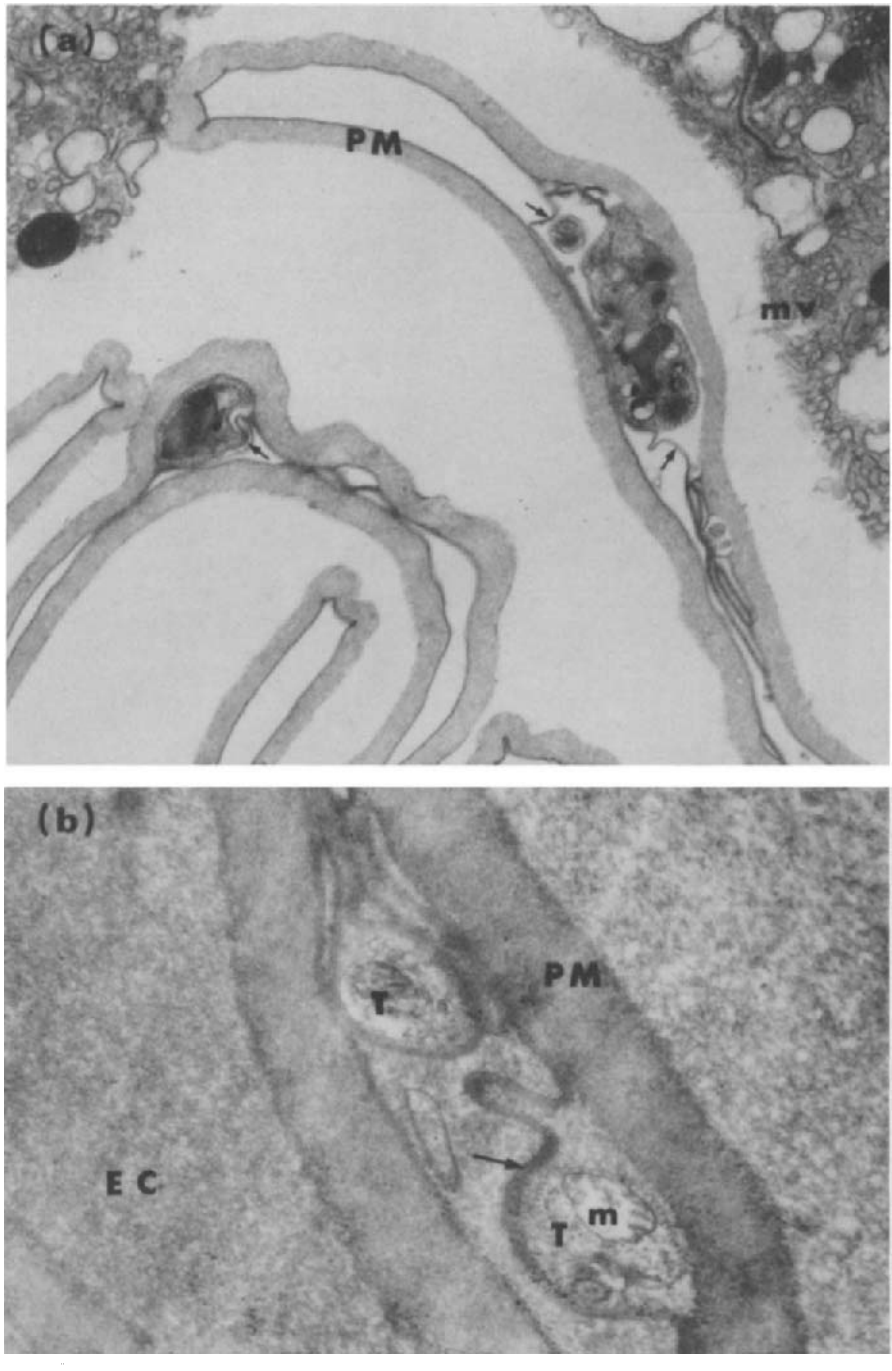

FIG. 3. Lodgement of trypanosomes between the two layers of the peritrophic membrane. (a) Trypanosomes (348 TA) lying between the two layers of the peritrophic membrane (PM). The inner layer is arrowed; mv, microvilli. Magnification ×12 600. (b) Trypanosomes (180 TS1) (T) 2 weeks after an infected meal. The inner osmiophilic layer of the peritrophic membrane (PM) is arrowed, and the trypanosome mitochondrion (m) is marked; EC, ectoperitrophic space. Magnification ×23 000. Reprinted by permission from *Nature*, Vol. 267, No. 5614, pp. 834–835.

which different stages were reached varied (see Table 1). As the volume of the blood meal decreased, the peritrophic membrane became thrown into folds and the trypanosomes were seen lying as if trapped in pockets (Fig. 2a). This same behaviour was repeated following later infected blood meals. At the apex of many of these pockets, or sharp folds, the inner osmiophilic layer of the peritrophic membrane was crumpled and separated from the wider external component (Fig. 2b). This occurred most frequently when the inner layer was found to be on the inside of a fold. Separation of these layers occurred usually in association with a trypanosome. Although this separation could be seen in a single thin section without accompanying trypanosomes, adjacent serial sections usually disclosed a trypanosome in contact with a more distant area of the separated membrane.

The presence of a large bacterial load in the tsetse gut lumen at this stage retarded or prevented these changes, which took 6–10 days (see Table 1) in the healthy tsetse gut. Infections with yeasts were as effective in preventing the establishment of trypanosome infections as were bacteria.

The largest concentrations of parasites at this stage, following a single infected meal, were found in the anterior midguts of tsetses. No parasite was found near the proventriculus. Those seen in the posterior part of the midgut or hindgut were either dead or appeared morphologically disorganized. With multiple infected feeds, the spread of the trypanosomes was more extensive along the gut, possibly as a result of temporary displacements following the arrival of later blood meals.

The passage across the peritrophic membrane took a further 2 days to complete and occurred over the central two-thirds of the anterior midgut. The process appeared to be in four stages: (a) the penetration of the inner osmiophilic layer where this was separated from the outer layer; (b) lodgement between the two layers (Figs 3a, b); (c) penetration into the thicker outer layer (Figs 4, 5); (d) exit from this outer layer (Fig. 6) into the ectoperitrophic space. Dividing trypanosomes were sometimes seen within the membrane. In the week following penetration, the ectoperitrophic space in this central area of the anterior midgut was packed with organisms (Fig. 7).

Trypanosomes were not found within the peritrophic membrane in the upper proventriculus region at any stage, nor were they to be found lower down within the membrane between 70 hours and 6–10 days (see Table 1) following an infected feed; at 10 days the majority of trypanosomes had already left the endoperitrophic space and thereafter the lower portions of the gut were no longer monitored.

B. PENETRATION OF TSETSE MIDGUT CELLS

Between 16 and 24 days trypanosomes were found to be leaving the ectoperitrophic space and penetrating the midgut cells (Figs 8a, 8b). The

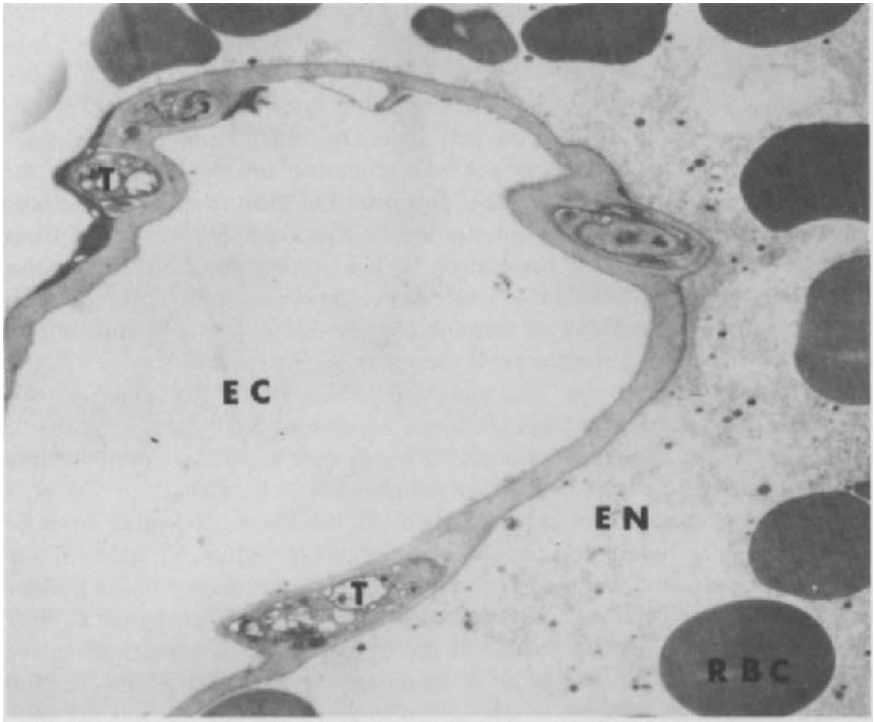

FIG. 4. Four trypanosomes (*T. congolense*) (T) lying within the outer layer of the peritrophic membrane. Note the presence of a later blood meal (RBC, red blood cell) in the endoperitrophic space (EN); EC, ectoperitrophic space. Magnification ×3200.

penetrations occurred flagella-first (Fig. 9a), the brush border membrane being invaginated (Figs 8a, 8b, 9b). The trypanosomes then left this invaginated pocket and came to lie free within the midgut cell cytoplasm, no longer enclosed by any membrane or vacuole (Fig. 10a). Eventually the trypanosomes reached the basement membrane of the midgut cells (Fig. 10b), lying outside the membranes of the gut cells. Trypanosomes were found trapped in folds of the gut in the haemocoelomic space (Fig. 11), following the dissecting out of the midgut under fixatives. All five *T. brucei* stocks examined followed this pathway, while *T. congolense* penetrated the peritrophic membrane in the same area and in the same way as shown in Figs 4 and 6 (Evans *et al.*, 1979).

As an alternative to giving single or triple infected feeds, five flies were fed an initial infected (stock 180 TS1) meal and, after further uninfected blood feeds, were given a second similarly infected meal 18 days after the first one. On examination on the thirtieth day (i.e. 12 days after the second infected feed), the ectoperitrophic space contained trypanosomes, but the gut cells and peritrophic membrane both had some trypanosomes within them. Thus the

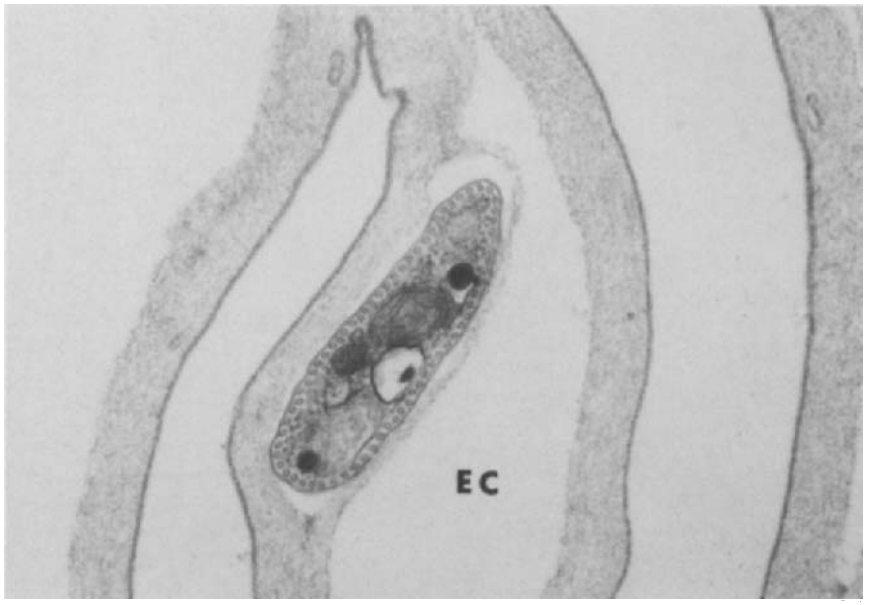

FIG. 5. A trypanosome (348 TA) about to break out of the outer layers of the peritrophic membrane into the ectoperitrophic space (EC). Magnification ×42 000.

last of this second infective wave was just leaving the peritrophic membrane on the twelfth day, showing that the trypanosomes in a second infected feed appeared to have taken 48 hours less in their journey into the midgut cells than those of the initial feed (see Table 1). Thus it was possible to follow successive waves of invasion of the gut cells by trypanosomes added at suitable intervals to the blood meals during the life of the tsetse fly (Fig. 11).

C. ORGAN CULTURE PREPARATIONS

In addition to following development within the living fly, trypanosome-infected tsetse organ cultures were prepared to study special parts of the cycle (Evans and Ellis, 1978).

1. *Tsetse gut explants*

Whole guts, dissected from uninfected tsetse flies, were incubated at 27°C in either RPMI tissue culture medium supplemented with 20% (v/v) foetal calf serum, or Cunningham's medium (Cunningham, 1977). Cultured procyclic forms of *T. rhodesiense* (stock 180 TS1) were added to the media and guts examined over 7 days. By the fifth day trypanosomes had found their way into the midgut and were multiplying. By the sixth day the parasites had

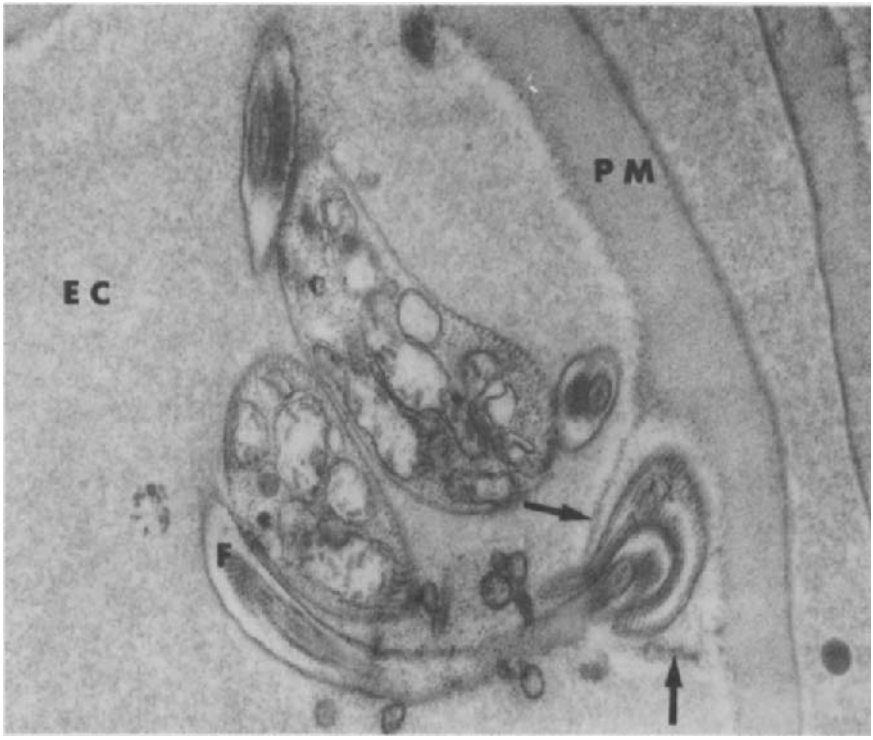

FIG. 6. A trypanosome (*T. congolense*) breaking free of the peritrophic membrane (PM), flagellum (F) foremost, into the ectoperitrophic space (EC). The remnants of the PM are arrowed. Magnification ×20 000. Reproduced by permission from *Journal of Protozoology*, Vol. 26, p. 559.

entered the epithelial gut cells and some had come to lie outside the gut cells against their basement membrane (Fig. 12). On the seventh day "cyst forms" were found within the gut cells and in the medium.

2. *Heads plus salivary glands*

Whole tsetse heads with salivary glands still attached were placed in the same culture media as (1). Similar cultured trypanosomes were added and the whole was placed in an incubator at 27°C for 60 days, in sealed 0·5 ml plastic containers. When examined, the salivary glands had not been invaded but the culture medium was full of dividing trypanosomes and large multi-nucleated giant forms, containing many sets of organelles, often within the mouthparts of the tsetse head.

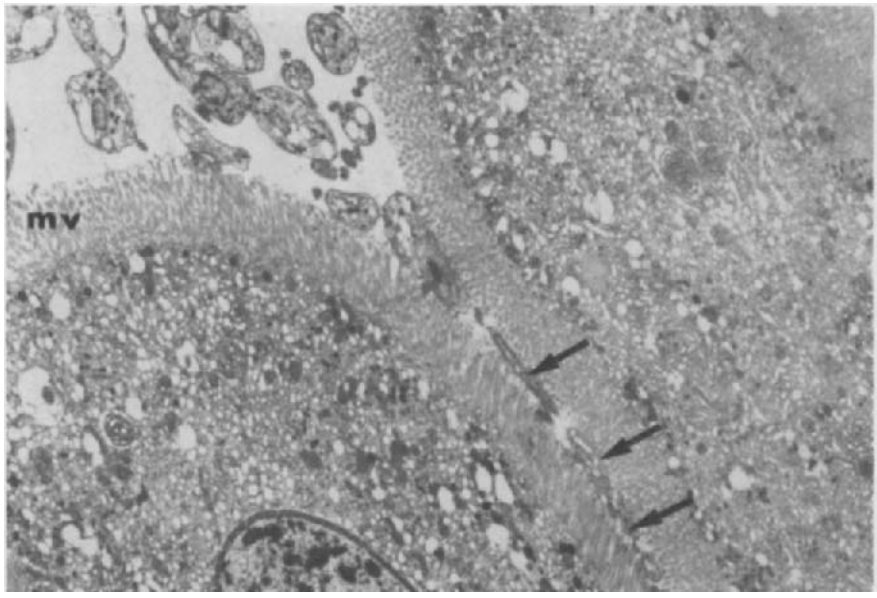

FIG. 7. Trypanosomes (180 TS1) in the ectoperitrophic space and within the narrow clefts (arrowed) between midgut cells, 3 weeks after an infected blood meal; mv, microvilli. Magnification × 3600.

D. GIANT FORMS

Giant forms of trypanosome such as those described above were also found during cultivation of the procyclic forms used to infect tsetse. After 4 days growth in culture medium both 180 TS1 and STIB 488T stocks produced a number of the "giant" or "cyst" forms referred to earlier, which made up about 10% of the general population. These forms were often more than 100 μm in diameter (Figs 13a, 13c) and contained large numbers of the various organelles found in normal trypanosomes, such as nuclei (Figs 13c, 14b), flagella (Figs 13c, 14a), mitochondria (Fig. 14a) and kinetoplasts (Fig. 15a). At the same time as these "cyst forms" appeared, trypanosomes were seen apparently attached to each other (Fig. 13b) in an unusual way.

Many of the large vacuoles (Figs 13a, 14a) found in these giant forms were lined with subpellicular microtubules. From their walls small trypanosome-like organisms appeared to bud (Figs 13a, 16b), containing all the organelles that are normally present in a trypanosome. Several of the giant trypanosomes, whose large vacuoles had opened to the outside, were noted apparently liberating a number of small trypanosome-like organisms

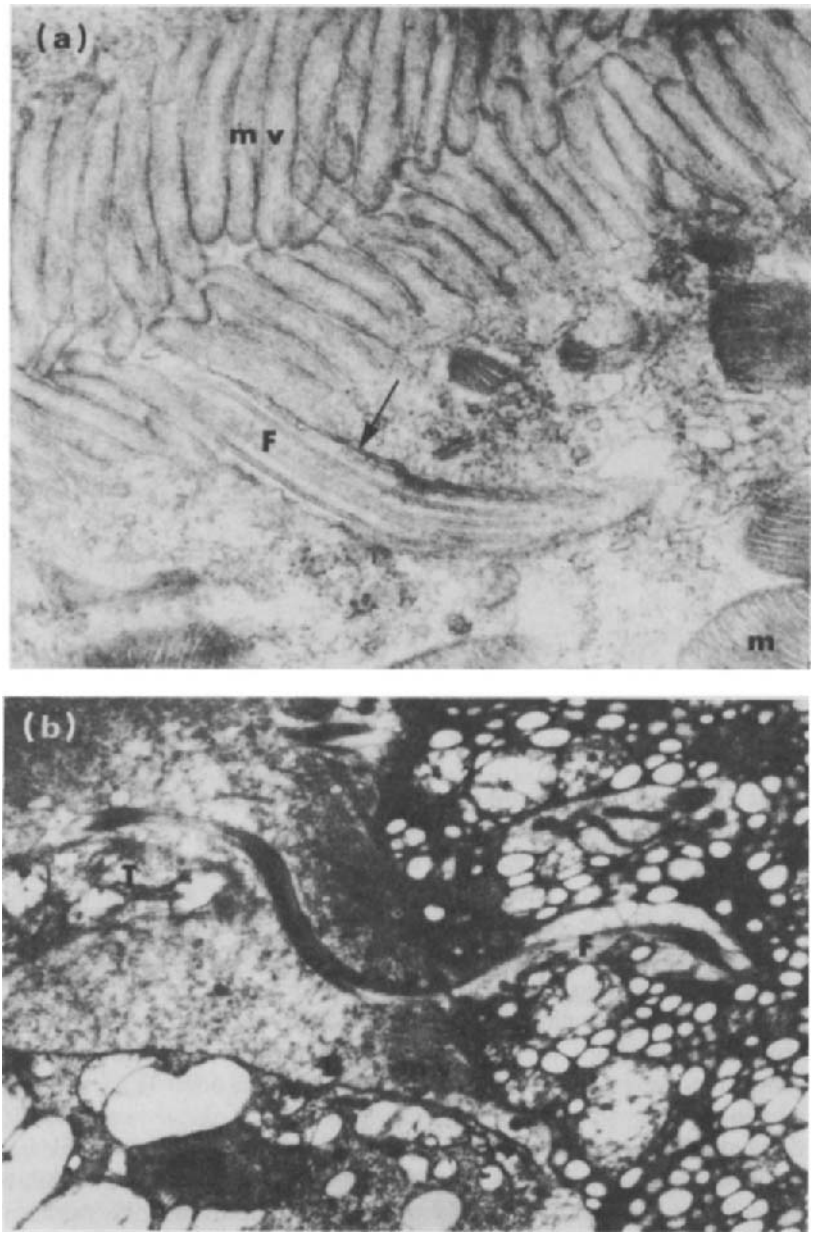

FIG. 8. (a) The flagellum (F) of a trypanosome (180 TS1) invaginating the plasmalemma between the microvilli (mv) of a midgut cell in a cleft as in Fig. 7. The cell membrane and flagellar membrane lie parallel to each other (arrow); m, gut cell mitochondrion. Magnification ×45 000. (b) A thick section showing the whole flagellum (F) of a trypanosome (180 TS1) (T) invaginating the midgut cell membrane; mv, microvilli. Magnification × 7600.

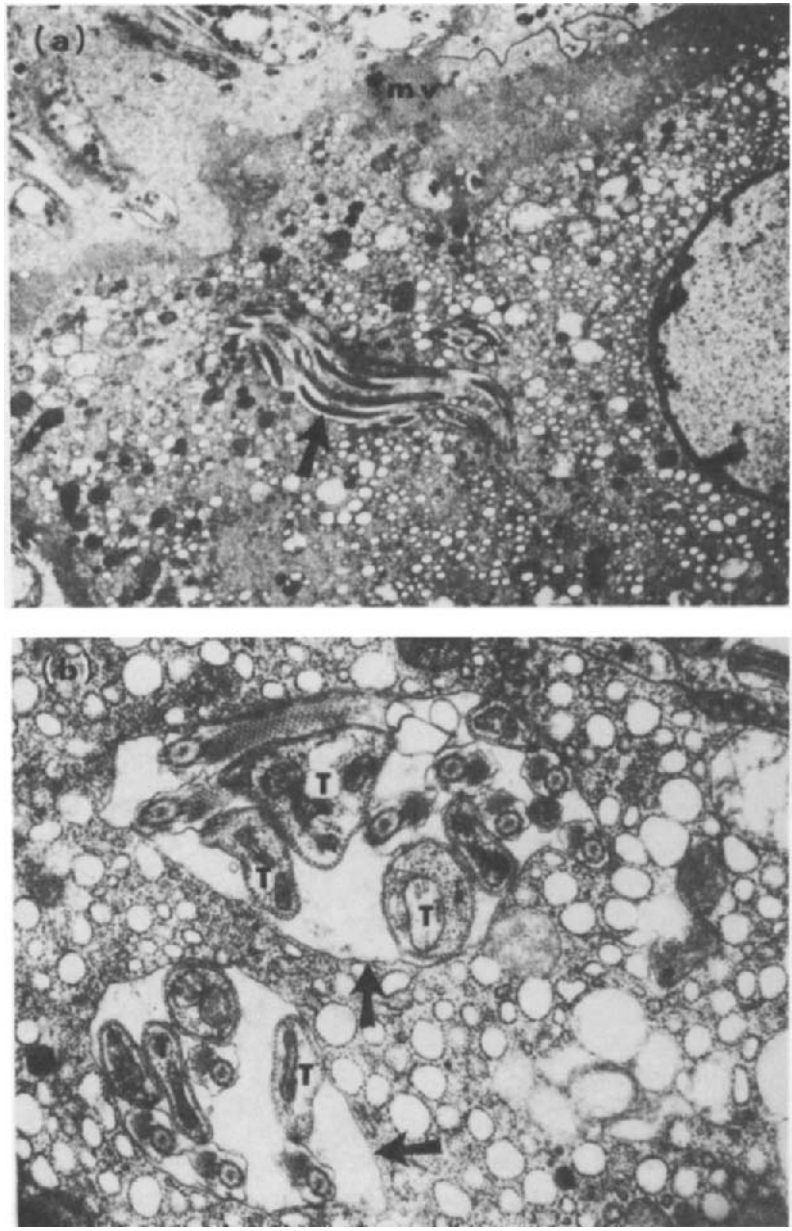

FIG. 9. (a) The flagella (arrowed) of several trypanosomes (180 TS1) entering a tsetse midgut cell, 3 weeks after an infected blood meal; mv, microvilli. Magnification ×4500. (b) A section, cut perpendicular to that of (a), from the same fly with many trypanosomes (T) within two invaginated spaces in the midgut cells. The walls of these spaces are arrowed. Magnification ×20 700. Reprinted by permission from *Nature*, Vol. 258, No. 5532, pp. 231–233.

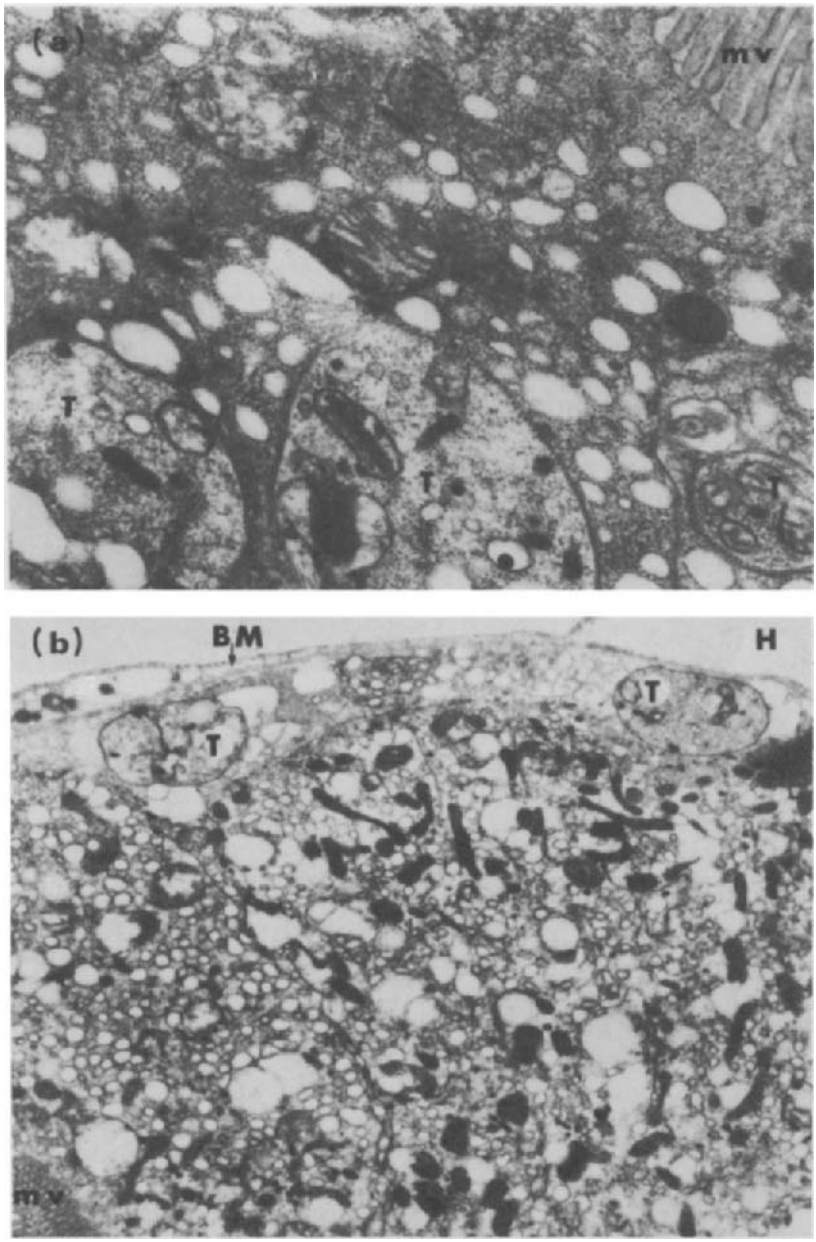

FIG. 10. (a) Three trypanosomes (180 TS1) (T) lying within the cytoplasm of the midgut cells, but no longer surrounded by invaginated plasmalemma, 21 days after an infected blood meal; mv, microvilli. Magnification ×20 700. (b) Two trypanosomes (T) (same specimen as in a) lying outside the midgut cells and beneath the basement membrane (BM). H marks the haemocoele; mv, microvilli. Magnification ×5400. Reproduced by permission from *Transactions of the Royal Society of Tropical Medicine and Hygiene*, Vol. 72, p. 653.

FIG. 11. Section of tsetse gut after weekly infected blood meals (180 TS1). At 31 days trypanosomes (T) are seen in the haemocoele (H). Parasites from the third infected feed are seen in the ectoperitrophic space (arrowed). Red blood cells of the last meal are still enclosed within the peritrophic membrane. Magnification ×2000.

(Figs 15a, 15b, 16a). A similar process involving giant trypanosomes was found to occur after 2 months of the organ culture experiments.

Similar giant forms were found within gut cells of tsetse flies during the passage of trypanosomes across these cells towards the haemocoele membrane (Fig. 17a). They were most frequently seen near the basement membrane. The number of organelles and the membrane arrangements appear to indicate the formation of a number of future individual trypanosomes (Figs 17b, 17c).

E. TRYPANOSOMES IN TSETSE SALIVARY GLANDS AND THE BEHAVIOUR OF *Trypanosoma rangeli*

Trypomastigotes were also found in the parenchyma cells of infected tsetse salivary glands (Fig. 18a). This happened most frequently using

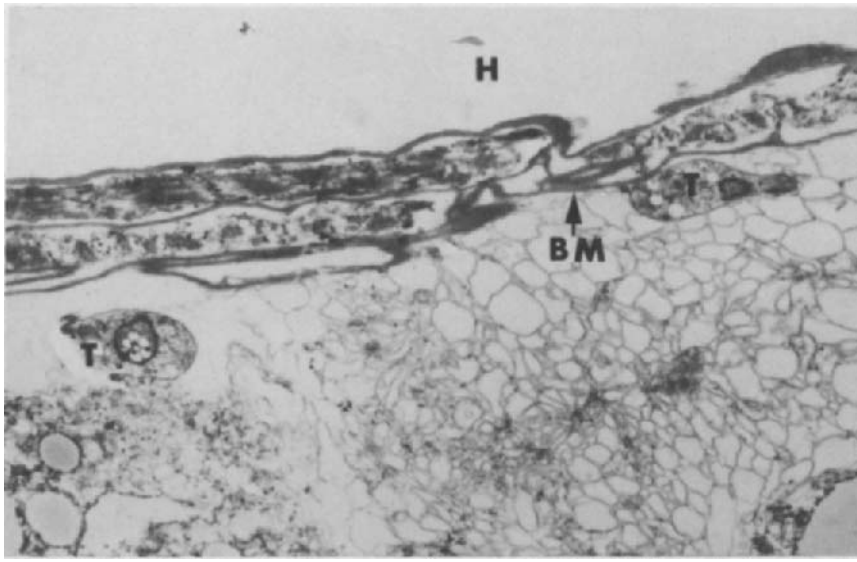

FIG. 12. Two trypanosomes (180 TS1) (T) are lying outside the midgut cells, but beneath the basement membrane (BM) in a whole gut organ culture preparation 6 days after infection with cultured procyclic forms (cf Fig. 10b). Magnification ×3200. Reproduced by permission from *Transactions of the Royal Society of Tropical Medicine and Hygiene*, Vol. 72, p. 653.

TREU 667 which is a rapidly developing stock of *T. b. brucei* that produces a high rate of haemocoele infections. We have not as yet been able to detect the passage of trypanosomes across the outer membranes of the tsetse salivary glands.

Study of the mechanism of salivary gland penetration by the South American trypanosome *T. rangeli* (Ellis *et al.*, 1980) revealed that these trypanosomes penetrate the membranes of the salivary glands of the reduviid bug *Rhodnius prolixus* flagellum foremost (Figs 18b, 18c), the method used by *T. brucei* group trypanosomes for their passage into midgut cells. *Trypanosoma rangeli* also produces giant forms in its insect host, but the only place we have found these has been in the outer membranes of the bug's salivary glands (Fig. 19), and not in the gut cells.

IV. DISCUSSION

These observations show that penetration of the fully developed peritrophic membrane can occur in the midgut region and that this penetration is outward into the ectoperitrophic space. It is related, in the model used,

to the concentration of parasites within the lumen, i.e. it occurs in those areas of the midgut that contain the highest concentration of trypanosomes, and its frequency tails off on either side of these areas of maximum parasite density. This distribution of trypanosomes corresponds exactly to that first noted by Yorke *et al.* (1933), in the tsetse gut lumen during 2–4 weeks following infection. This correlation supports the results obtained using our model by relating them directly to field studies and natural infections.

Looking through the photographs published by earlier workers, it seems clear that trypanosomes can get enmeshed in the soft, newly formed peritrophic membrane in the anterior proventricular area and penetration can occur, possibly in both directions. But it has yet to be shown that the few parasites demonstrated by these workers within this part of the membrane could provide the numbers required (whichever way they travel) for a satisfactory infection of either the ectoperitrophic space (penetrating outwards) or the salivary glands (passing back into the lumen).

It cannot be claimed here that the penetration of the peritrophic membrane observed in the midgut region is the only, or necessarily always the main, pathway to the ectoperitrophic space (Ellis and Evans, 1977a, b), but it clearly is so in this model, using *T. brucei* trypanosomes and *G. morsitans.*

The mechanics of penetration require a minimum of two trypanosome enzymes. Firstly a protease to allow passage across a chitin-containing layer of the peritrophic membrane. Lehane (1976 and personal communication) suggested that a protease would be effective because the peritrophic membrane chitin is "suspended" in protein and the removal of this latter matrix would result in localized disintegration of this layer of the membrane (Figs 4–6). The same enzyme would also assist the trypanosomes in their entry into the gut cells. The second obligatory enzyme would presumably have to be a lipase to attack the inner lipid-containing layer of the peritrophic membrane. A lipase would be necessary too for the trypanosomes to cross the cell membranes of the tsetse gut cells. Enzymes of both these types have been demonstrated in blood forms of trypanosomes and are discussed by Ormerod (1979).

A. INTRACELLULAR TRYPANOSOMES AND NEW DEVELOPMENTAL PATHWAYS

By using the electron microscope it was possible to establish conclusively that the different stocks of *T. brucei* group trypanosomes all penetrated into midgut cells of living tsetse (as they did in gut organ culture preparations).

Historically the first record of intracellular development of a flagellate was made in 1911 by Minchin and Thomson, and was of *T. lewisi* which they found within the midgut cells of the rat flea. They also found a form of multiplication within vacuoles which they called "schizogony-like" and which is discussed later in relation to "cyst forms". Later, Rodhain (1942a, b)

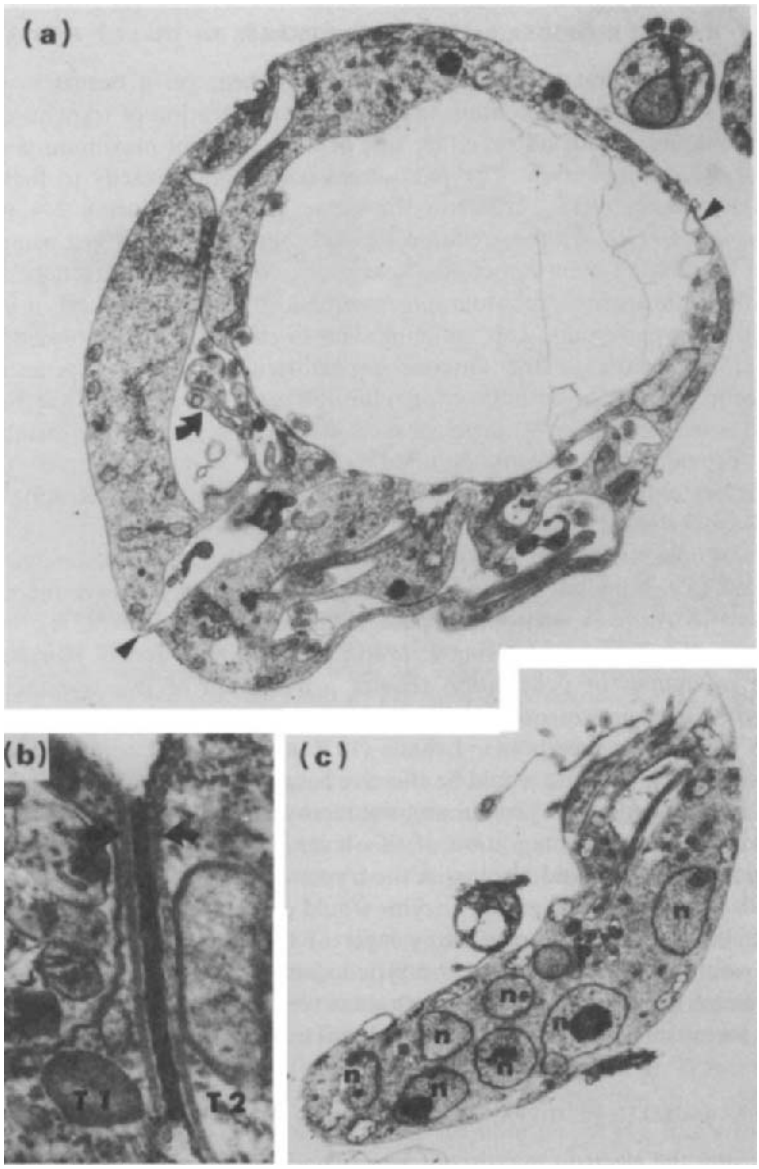

FIG. 13. Cultures of 180 TS1. (Figs. 13, 14, 15, 16 and 17 are reproduced by permission from *Folia Parasitologica*, Vol. 29, pp. 5–11.) (a) Giant form similar in shape to that of *T. conorhini* described by Deane and Milder (1972). Areas of suggested junctions between "parents" are arrowed. Curved arrow marks budding forms. The large vacuole is not lined with subpellicular tubules, in contrast to the many smaller vacuoles. Magnification ×7000. (b) A form of junction between two trypanosomes (T1 and T2) whose subpellicular tubules are nearly perpendicular to each other; an unlikely arrangement during a division process. Magnification ×61 200 (c) A multinucleate form in a 5-day culture. Seven nuclei (n) are seen in the section. A normal-sized organism lies above. Magnification ×4400.

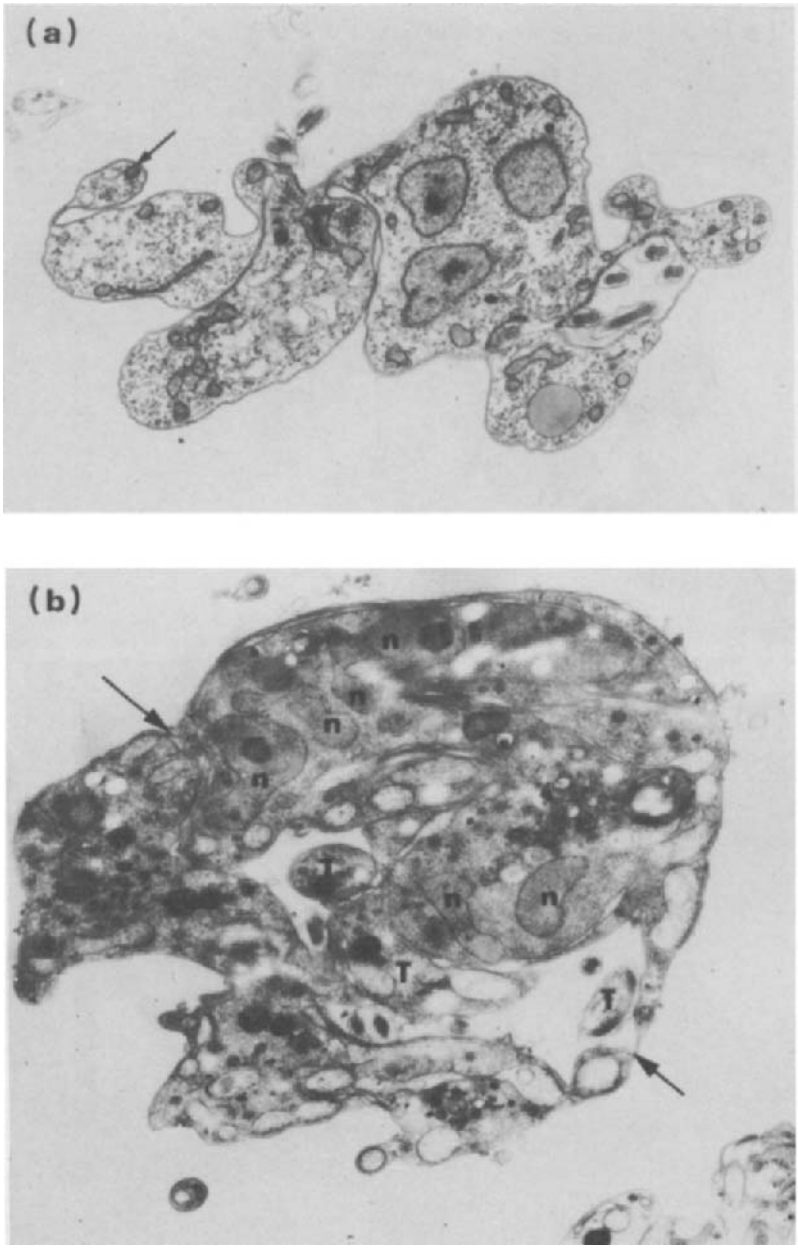

FIG. 14. Cultures of 180 TS1. (a) A giant form from an organ culture of tsetse head and salivary gland. Arrow marks a budding form. Magnification $\times 7000$. (b) A multinucleate form apparently made up of two individuals, whose areas of attachment are arrowed, containing three small forms (T); n, nuclei. Magnification $\times 7000$.

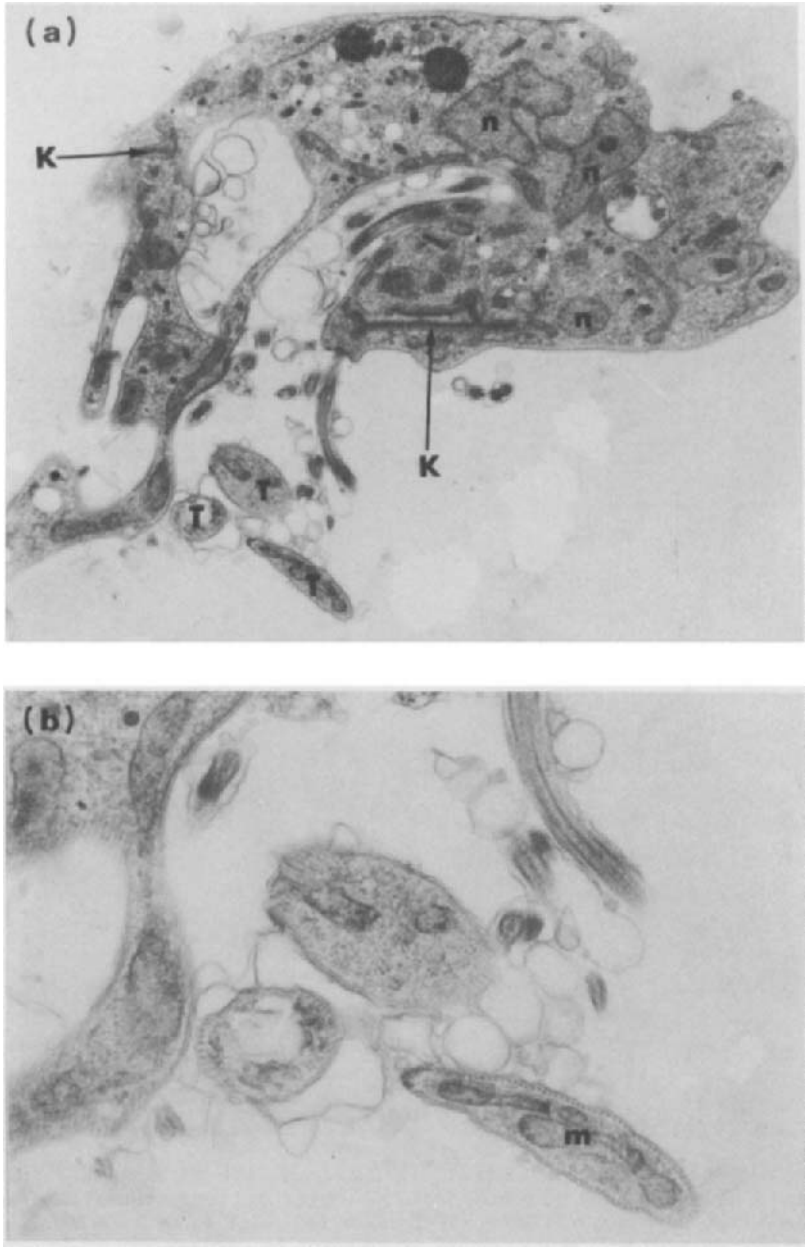

FIG. 15. Cultures of 180 TS1. (a) A giant form containing many organelles (kinetoplasts, K and nuclei, n) and apparently releasing new forms (T). Magnification ×7200. (b) An enlargement of the new forms shown in (a) showing their normal structure with mitochondria (m). Magnification ×18 000.

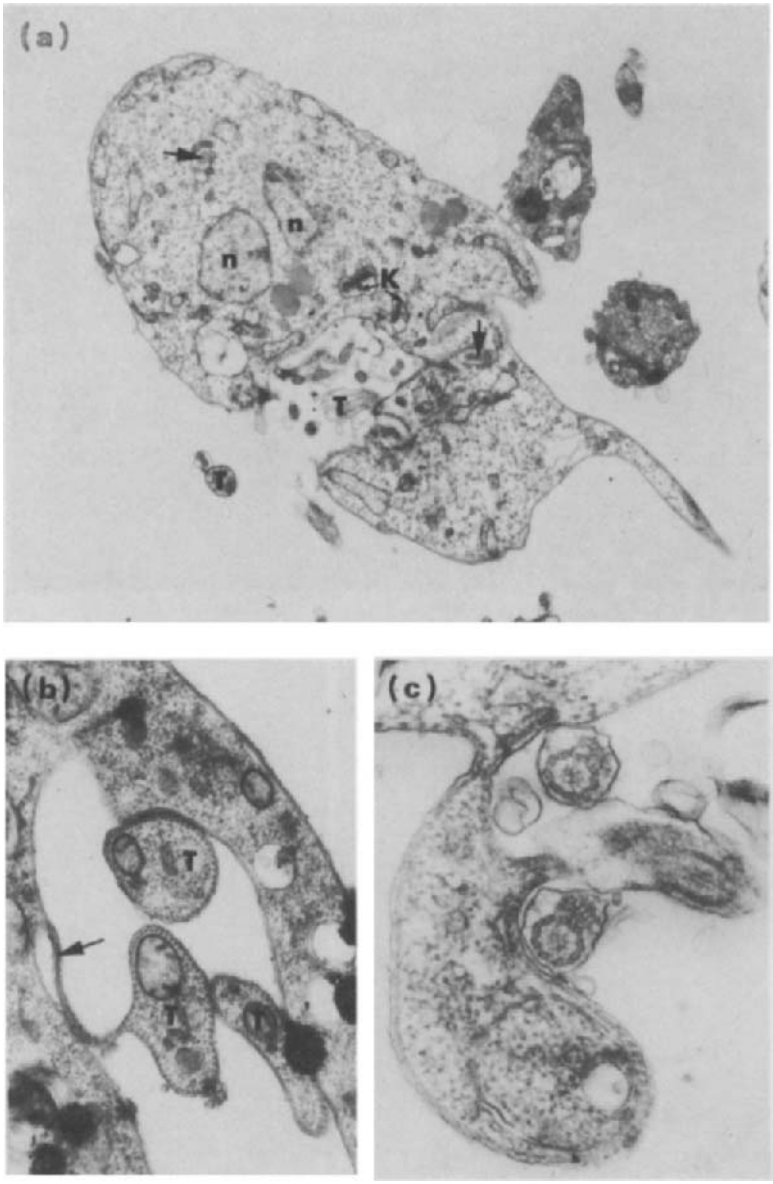

FIG. 16. Cultures of 180 TS1. (a) A giant form apparently liberating small organisms (T) to the exterior following the rupture of the large vacuole. Further flagella not associated with this vacuole are arrowed; K, kinetoplasts; n, nuclei. Magnification ×6500. (b) Three organisms (T) budding into a vacuole (lined with subpellicular microtubules) in a giant form. The attachment of one is arrowed. Magnification ×19 500 (c) Part of a giant form in a tsetse gut organ culture, showing a new form budding from the exterior. Magnification ×37 400.

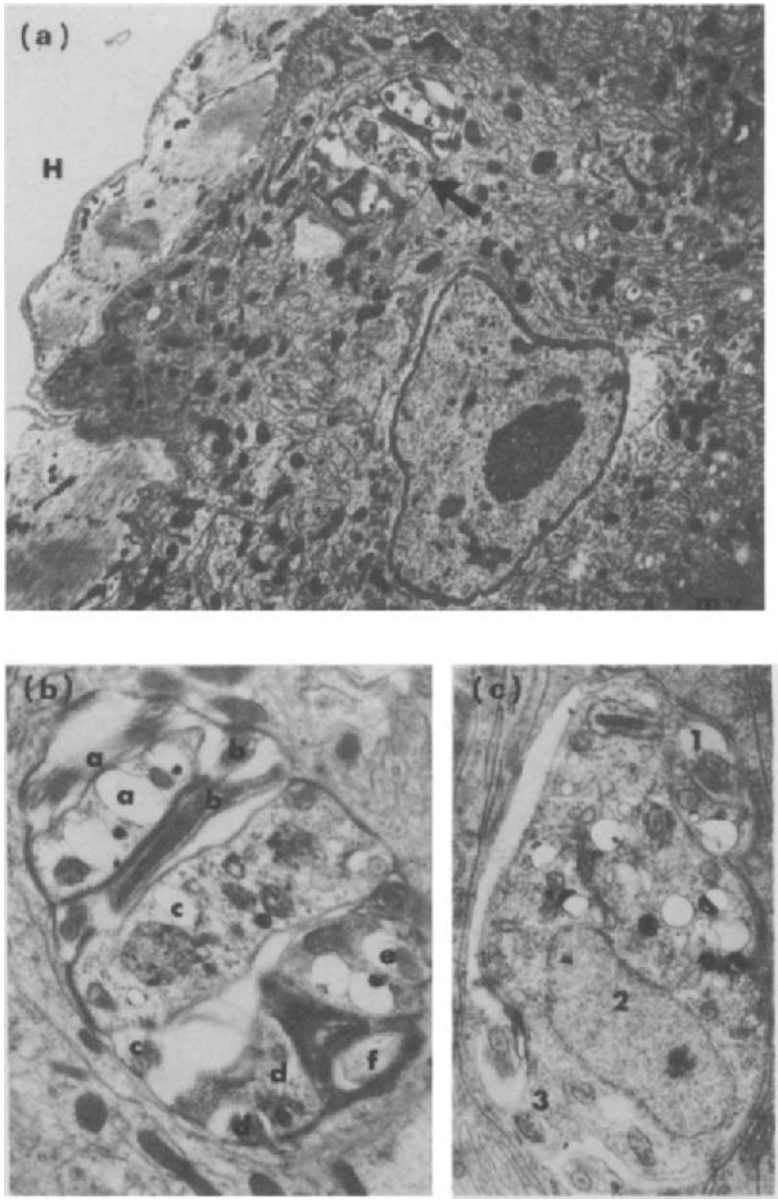

FIG. 17. (a) A giant form of 180 TS1 (arrowed) lying within the cytoplasm of a tsetse midgut cell; H, haemocoele; mv, microvilli. Magnification ×3400. (b) An enlargement of (a) with perhaps up to five individuals (a–f) within the giant form. Magnification ×19 100. (c) A similar giant form to that shown in (b) from a tsetse midgut cell, containing three possible individuals (1–3). Magnification ×20 400.

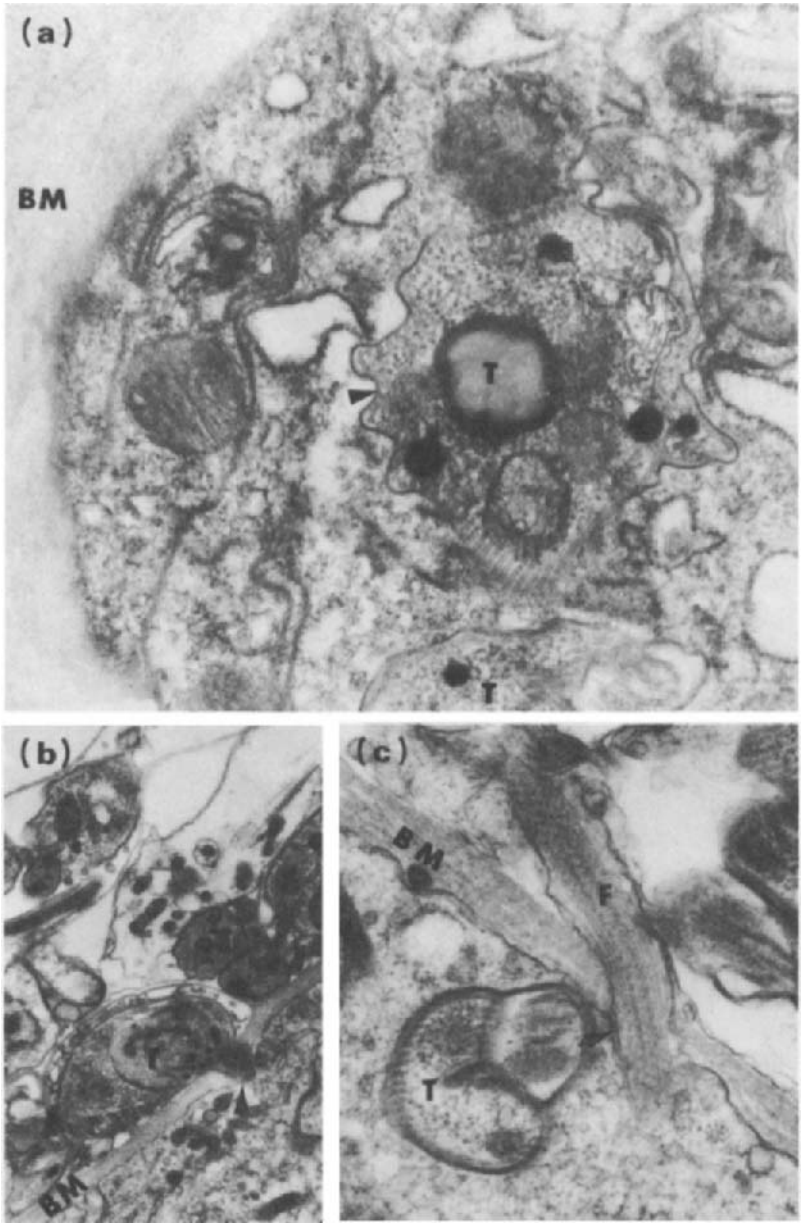

FIG. 18. (a) Two trypanosomes (TREU 667) (T) lying free within the cytoplasm of a tsetse salivary gland cell, below the basement membrane (BM). The trypanosome pellicle (arrowed) is no longer surrounded by any cytoplasmic membrane. Magnification ×32 000. (b) *T. rangeli* (T) penetrating the basement membrane (BM) of the salivary gland of *Rhodnius prolixus*, flagellum foremost (arrowed), to enter the gland cell. Magnification ×9000. (c) An enlargement of the process shown in (b). Note the invagination of the gland cell plasmalemma (arrowed). Another trypanosome (T) is already within the gland cell. BM, basement membrane; F, flagellum. Magnification ×37 800. (b) and (c) are reproduced by permission from *Zeitschrift für Parasitenkunde*, Vol. 62, pp. 70–72.

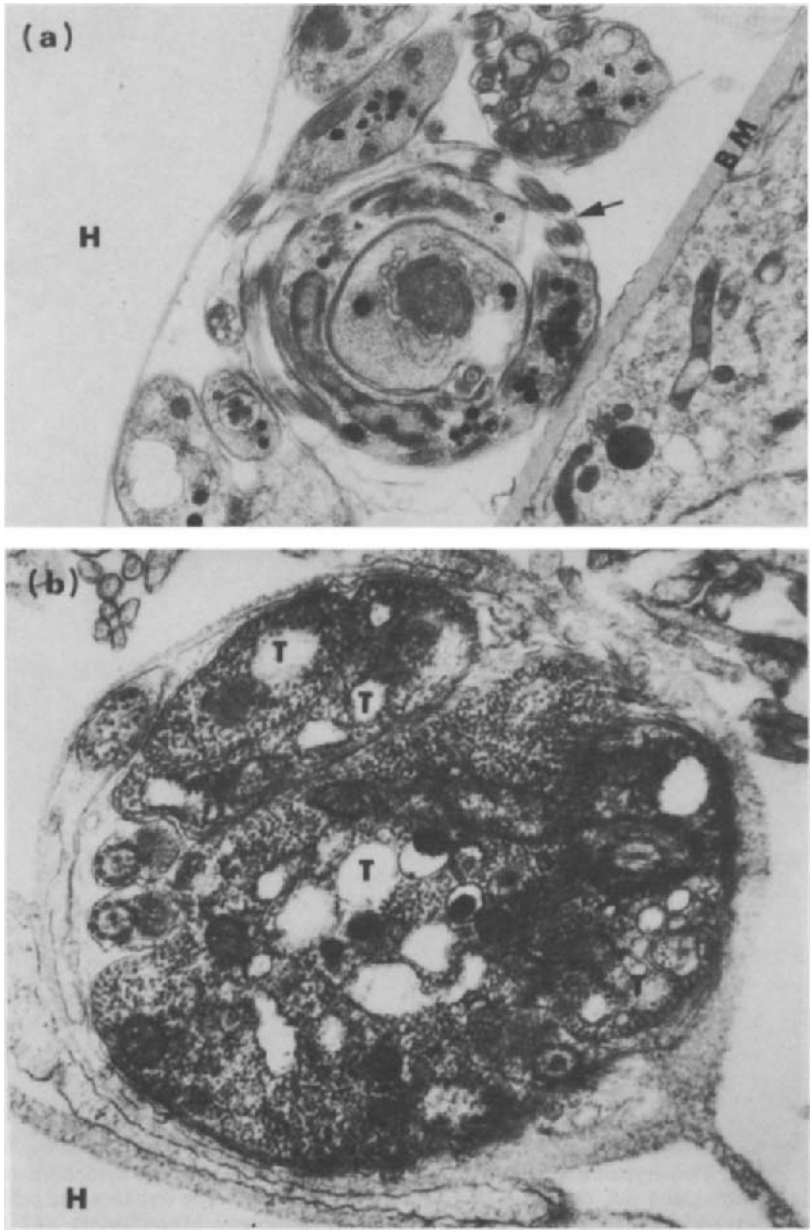

FIG. 19. (a) Two giant forms of *T. rangeli* lying in the outer structures of a salivary gland of *R. prolixus*. The outer membrane of the large one is arrowed. BM, basement membrane. Magnification ×11 340. (b) A similar giant form to that shown in (a) within the basement membranes of the bug's salivary gland. The individual organisms are marked T (cf Fig. 17b). Magnification ×37 800. Reproduced by permission from *Zeitschrift für Parasitenkunde*, Vol. 62, pp. 70–72.

published work on further intracellular forms. More recent observations have been made on leishmanias found in insect gut cells by Lelijveld (1966) and Killick-Kendrick *et al.* (1977). But until our initial observations (Evans and Ellis, 1975), no sleeping sickness trypanosome had been reported within the cells of either mammalian or insect host.

In their vertebrate host, *T. brucei* group trypanosomes are now known to exist in tissue spaces which they must have entered from capillaries, either through or between the endothelial cells (Ellis, 1978). However, earlier work on these tissue forms of the trypanosomes of stock 180 TS1 in the brains of rats (D. Ellis, unpublished observations) showed that they may be found within the connective tissue cells of the meninges, with no surrounding vacuolar membrane. The penetration of these cells is presumably an active process, there being no evidence of phagocytic activity in the types of cells within which they were found.

Figures 8–9 seem to indicate a similar form of active penetration, whereby the flagellum of the trypanosome appears to invaginate the brush-border membrane of the tsetse gut epithelial cell progressively until the whole flagellate is within the cell, but surrounded by the stretched membrane forming a "vacuole". Later the trypanosomes leave this "vacuole" to lie freely within the cytoplasm (Fig. 10a). It is assumed that the parasites' exit through the outer cell membrane is also achieved "flagellum first", though this part of the process has yet to be observed in the electron microscope.

Within the cytoplasm of the gut cells some trypanosomes appear vacuolated, though the proportion of those so affected does not seem greater than that seen in the ectoperitrophic space earlier in the cycle (Fig. 7). Others appear to be involved in multiple division (Fig. 7).

The strongest evidence for active penetration is provided by the organ-culture experiments. In this system the infecting trypanosomes find their way into the lumen of the inactive gut organ cultures, penetrating first the peritrophic membrane and then the gut cells. The micrographs in Figs 10b and 12, of trypanosomes reaching identical positions beneath the gut cells' basement membrane, the first within the living tsetse fly and the second as a result of contact with gut organ cultures, are strikingly similar and surely indicate an active process.

This last experiment, using organ cultures, could not identify any trypanosomes that continued their journey through the outer gut membranes, since those trypanosomes achieving this would only be returned to the pool of parasites in the medium outside the gut. But we believe that this experiment does settle one important point. When the presence of trypanosomes within the gut cells was first demonstrated (Evans and Ellis, 1975), it was suggested that perhaps these parasites might have somehow fortuitously been trapped by the peristaltic waves of the gut or by the great changes in the shape of epithelial cells during ingestion and digestion of a large blood-meal.

But with identical appearances and locations of trypanosomes occurring whether the gut is active or not, it seems clear that the penetration and passage across these cells is a process controlled by the trypanosomes themselves and not the result of chance entrapment, or other phenomena related to activities of the gut cells.

The path from the epithelial gut cells to the salivary glands has yet to be elucidated. Figure 11 shows trypanosomes outside the gut in the haemocoelomic cavity, but it is very difficult to devise experiments to prove that they got there as a result of direct penetration from the gut lumen. There is also the possibility that the parasites may travel up (or down) within the haemocoelomic membrane but outside the gut epithelial cells, to some suitable point of exit; or possibly remain within the membrane until near the salivary gland's external membrane (which connects with the gut-haemocoele membrane) and so reach the gland without necessarily becoming free within the haemocoele cavity.

Trypanosomes can be found in the tsetse salivary gland cells (Fig. 18a), but, unless penetration from the haemocoele can be observed, it is still possible that they entered these cells from the gland lumen. However, it is worth noting that the trypanosomes seen within the gland cells were trypomastigotes (without obvious surface coats) and not epimastigotes which are the forms lining these gland cells on their inner (lumen) sides.

Many protozoa, the malaria parasites being perhaps the most significant example, which are transmitted via salivary secretions use haemocoele fluid as a vehicle to reach the insect's salivary glands during their development within the vector. This route has been established to date for only one species of trypanosome, *T. rangeli* (Hoare, 1972; D'Alessandro, 1976). The parasites' principal arthropod vector is the bug *Rhodnius prolixus*, into the intestine of which the trypanosome passes with the blood meal obtained from its human host. From the bug's intestine the trypanosomes find their way into the haemocoele and phagocytic haemocytes within 72 days; from the haemocoele the parasites reach the salivary glands, there developing 15 days later (D'Alessandro, 1972) into the metacyclic trypomastigote forms which are responsible for transmission to further mammalian hosts.

We showed recently that *T. rangeli* produced salivary gland infections in *R. prolixus* within 7 days of its ingestion, and this enabled us to follow the process of invasion of these glands from the haemocoele (Figs 18b, 18c), which appeared to involve the formation of giant forms (Figs 19a, 19b) (Ellis *et al.*, 1980).

B. GIANT FORMS AND THEIR POSSIBLE ROLES

Mention has been made several times of "cyst" or "giant" forms of African trypanosomes found in liquid culture, in infected tsetse organ

culture, in the midgut cells of infected flies, as well as in the salivary glands of *R. prolixus* infected with *T. rangeli*. This term "cyst form" is used here to include and describe those morphological types of trypanosome which have in the past been referred to as "multinucleate forms" (Robertson, 1912c), "giant forms" (see above), "formes kystiques" (Jadin and LeRay, 1969) and "cyst-like bodies" (Deane and Milder, 1966, 1972; Ellis and Evans, 1977c). For a few years early in this century there was considerable interest shown in these giant forms and in the possibility of some process of sexual reproduction in which they might play a part. Novy (1906), in assessing the roles of the various forms of trypanosomes that had been described, discussed the "male" and "female" forms and referred to multinucleate parasites seen within the tsetse fly. Ottolenghi's (1910) dramatic illustrations of multinucleate forms, which he inferred were macrogametes, were followed a year later by "schizogony-like" forms of *T. lewisi* demonstrated by Minchin and Thomson (1911) within insect cells. Walker (1912) reported similar forms in the spleens of animals infected with *T. evansi*. Robertson's (1912c) observations have already been referred to.

From that time until quite recently (Deane and Milder, 1966) interest in multinucleate or cyst forms appeared to have evaporated, though Ormerod (1979) has shown that Italian workers still continued studies in this field. He suggested that lack of publications in the English language journals was the result of the great influence still exerted by the original (largely British) workers on trypanosomiasis who had abandoned their earlier speculations on these problems. However, Muniz (1927) did report (in a French journal) giant cyst forms in 15-day-old cultures of *T. cruzi*, many of them multinucleated, inside some of which he observed flagellar movements.

Recently interest in these cyst forms has revived: Deane and Milder, firstly following examination with the light microscope (Deane and Milder, 1966) and later using the electron microscope (Deane and Milder, 1972), published details of "cyst-like" bodies found in their cultures of the stercorarian trypanosome *T. conorhini*. Many of their electron micrographs closely resembled that in Fig. 13a. Jadin and LeRay (1969) reported similar findings in cultures of *T. b. brucei* and *T. b. gambiense*. In 1976 Otieno *et al.* found giant forms within the haemocoeles of tsetse flies infected with blood forms of *T. b. brucei*.

Deane and Milder suggested three possible explanations for the "cyst" forms, which they believed contained living (and, under phase contrast, moving) trypanosomes: (a) the organisms making up the rosettes in cultures of epimastigotes, which were normally ingesting debris, could perhaps ingest whole live trypanosomes, i.e. a form of cannibalism; (b) several trypanosomes had somehow penetrated into a large dying trypanosome; or (c) a complex, probably sexual, process was occurring.

By 1972 Deane and Milder had come to prefer the last possibility, and suggested the following sequence: two epimastigotes fused around their flagellar pockets; the DNA-containing organelles (nuclei and kinetoplasts, the latter with their conjoined mitochondria) underwent repeated division and were perhaps extruded into the large vacuole made up of the fused flagellar pockets of the "parents"; and the walls of this vacuole became thin and eventually complete new epimastigotes were liberated to live independently outside the bodies of the "parents", which now disintegrated. These authors did not regard this form of reproduction, presumably involving genetic interchange, as necessarily a common occurrence of *T. conorhini*, stressing that sexual activity is often incurred only under certain specific conditions, such as changes in the food supply, changes of environment (including hosts), or the influence of hormonal changes.

Figure 13b illustrates an unusual association between trypanosomes which was seen regularly in cultures of all the trypanosome stocks used in this work and also in the organ culture preparations. The highly osmiophilic material shown in this micrograph, possibly lipoprotein, is characteristic of these areas of association.

When trypanosomes divide by binary fission, the division is approximately longitudinal and the subpellicular tubules at areas newly separated from each other are roughly parallel to each other. Thus at whatever angle the sections of such areas are cut by the microtome, the tubules seen in each individual run roughly in the same direction. However, in Fig. 13b the subpellicular tubules in the two individual trypanosomes are running roughly at right angles to each other. This micrograph may therefore not represent part of the division process of a single trypanosome, and could be part of a fusion process between two mature organisms. The unusual osmiophilic material between the two apposed surfaces is clearly seen.

Deane and Milder (1972) concentrated their attention on areas looking very similar to those arrowed in Figs 13a and 14b as being the points of fusion between two trypanosomes. They suggested that this fusion occurred only at the edges of apposed flagellar pockets, and expressed the belief that these arrowed points were the old edges of such pockets. According to their hypothesis, these fused pockets enlarged to produce the large vacuoles found in cyst forms.

Figures 13a and 15a illustrate these vacuoles, many of the larger ones being lined with subpellicular tubules arranged with the same sort of spacing intervals as in normal trypanosomes. In vertebrate, culture and vector forms of *T. brucei* group trypanosomes, the normal flagellar pocket membrane is associated with only four subpellicular tubules and these are grouped together, leaving the rest of the pocket membrane unlined (D. S. Ellis and W. E. Ormerod, unpublished observation). These four microtubules are a continuation of the four found in association with the special area of endoplasmic

reticulum lying directly beneath the flagellum (Taylor and Godfrey, 1969). They can be traced from inside the pocket near the kinetoplast to the anterior extremity of the trypanosome body. A large vacuole created by the fusion of the two apposed flagellar pockets could therefore have, initially, only these four tubules from each trypanosome flagellar pocket to line it. It seems more probable, therefore, that any fusion giving rise to a large tubule-lined vacuole, would occur at areas already lined with regular subpellicular tubules, which are known to proliferate extensively during binary fission, and not at the flagellar pockets. The vacuoles are found both lined and unlined by subpellicular tubules. Serial sections show them to be true vacuoles, and not invaginations of the parasite's pellicle.

In general, this complex process does not seem to be related to any of the recognized multiple division processes (e.g. rosette formation) which are known to occur in some, but not in *T. brucei* group, trypanosomes. If the cyst forms do produce new individuals they would appear to do so by a process of "budding off" into the vacuoles described. All the normal trypanosome's organelles can be found in these forms, and "budding" may also occur directly from the trypanosome body to the outside (Fig. 16c), possibly without the creation of the special vacuole. Figures 15 and 16 appear to show the final liberation of the new individuals from the "parent".

Within the tsetse fly, cyst forms were never found within the lumen of the gut, nor beneath the basement membrane of the gut epithelium; they were seen only during the passage of trypanosomes through these gut cells (Fig. 17a). These observations suggest that such forms arise only within the gut cells and then either die off, or give rise to new individuals which then continue their journey as normal uninucleate trypanosomes. Because no dead cyst form was ever found in the gut cells, it is possible that this last explanation is the correct one.

To sum up, there is some evidence that an unusual association between individual African trypanosomes can be seen, most commonly in liquid culture and organ culture preparations. If fusion between sleeping sickness trypanosomes does occur it is probably not limited to areas surrounding the flagellar pocket. If the giant forms do result from such a fusion, the large tubule-lined vacuoles seen are not formed from enlarged flagellar pockets alone, since many of the cyst forms contain several such vacuoles. Our work suggests that the interpretation of Deane and Milder (1972) of such vacuole formation does not apply to *T. brucei* trypanosomes. The creation of new individuals, if it occurs, is by budding into these vacuoles and the new forms may then be liberated to the outside by rupture of the vacuoles. Cyst forms, apparently behaving in a similar fashion, were also observed in tsetse gut cells during the passage of trypanosomes across them following penetration from the ectoperitrophic space.

When culturing *T. rangeli*, we found that although some giant and multi-organelle forms were seen, they were far less frequent and also smaller than those found in the various *T. brucei* group trypanosomes studied. It is possible that giant forms generally are a response to unfavourable conditions and our *T. rangeli* cultures were not followed for sufficient time for their regular appearance. The giant forms in the bug's salivary gland outer structures (Fig. 19) were usually without the large cysts seen in salivarian trypanosome cultures, but were similar to the forms found in tsetse midgut cells (Figs 17b, 17c). They appeared there to represent a multiple division mechanism rather than a process involving budding into a vacuole. However, the cyst forms of unidentified trypanosomes in the gut cells of wild-caught reduviid bugs (Ellis *et al.*, 1982) did have large vacuoles containing many flagella, and appeared very similar to some of the *T. brucei* group giant forms (Fig. 14a) produced in culture.

The significance of all these giant forms is difficult to assess without any evidence of some genetic interchange or genetic shuffling. If one suggests that these forms are not dead-end, aberrant malformations of a disorganized binary fission process, then the possibility of their being a product of some "sexual" process must be considered. To date, no direct evidence has been produced to indicate genetic interchange between (genetically) marked individuals or populations, though there is some interesting evidence of genetic exchange among trypanosomes in Africa (Gibson *et al.*, 1980), derived from isoenzyme pattern analysis of widely scattered isolates collected from all over that continent. Also using isoenzyme studies of various African stocks, Tait (1980) has recently produced further evidence of random mating and recombination among trypanosomes. As this indirect evidence of sexual activity accumulates, it is possible that some of the processes described here may eventually be found to be involved.

Finally we do not claim that the new developmental pathways outlined here for African sleeping sickness trypanosomes within the tsetse fly, and summarized in Fig. 20, are the only ones used, nor that giant forms necessarily represent a sexual phase in many different trypanosomes' life cycles. Further work in many areas will be needed to discover the answers. However, we do feel that the evidence for these pathways is clear enough to justify further investigation of the possible consequences. If, for example, trypanosomes have to cross the tsetse haemocoele to reach the salivary glands, then the environment of this cavity may be crucial to transmission.

Croft *et al.* (1982) have found a trypanosomicide in haemocoele fluid, and the presence of this may well explain why, in general, up to 60% of a fly population may develop a midgut infection, but this dwindles so that perhaps less than 5% get salivary gland infections. The most rapidly developing stocks (e.g. TREU 667) produce the heaviest haemocoele infections, and the

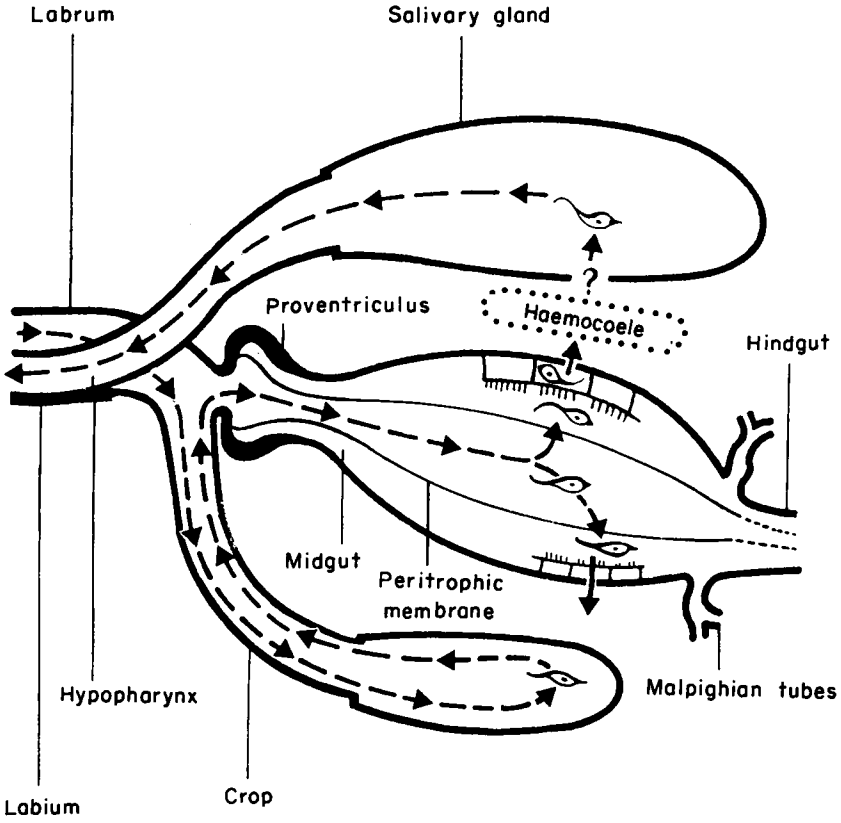

FIG. 20. Diagram illustrating proposed alternative developmental pathway of *Trypanosoma brucei* in *Glossina* (compare Fig. 1, p. 4).

highest number of gland infections. It is possible that the haemocoele, for these stocks, may act as a reservoir for later gland infections, and that the assessment in the field of gland infections only may not be the most accurate measurement of the potential of a fly population to transmit trypanosomiasis.

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Note added in proof

Work (to be published) after the completion of this chapter has shown that additional isolates of *T. brucei* group organisms behave similarly in *Glossina palpalis*, using bloodstream trypanosomes fed to the flies in a membrane-feeding system as outlined on p. 9 for *G. morsitans*. The parasites penetrated the peritrophic membrane and passed through the tsetse gut cells in the same areas and in the same fashion as reported for *G. morsitans*. In these later experiments, giant forms were seen associated with the outer structures of the salivary glands. Within the gland itself, trypomastigotes were found in the surrounding muscle layers, and inside the parenchymatous cells.

Meanwhile, it has been suggested (East *et al.*, 1983) that the trypanosomicidal factor reported by Croft *et al.* (1982) from tsetse haemocoele fluid (see p. 36) would rule out any possibility of an alternative pathway to the salivary glands such as the one we propose. Similar factors are well known in different fluids encountered by various trypanosomes during their life cycles, such as serum of vertebrate hosts and haemolymph of reduviid bugs, and have presumably evolved to establish and maintain suitable host/parasite balances. Instead of ruling out the presence of trypanosomes in their neighbourhood, such factors could be seen as implying the historical presence of the parasites.

A trypanosomicidal factor in tsetse haemolymph, if occurring under natural conditions, would be a factor contributing to the low infection rates which are observed for *T. brucei* group organisms in tsetse flies. The absence (or limited specificity) of such a factor might play a part in the occasional overwhelming haemocoele infections with *T. brucei* observed by L. Jenni (personal communication).

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Cell-mediated Killing of Protozoa

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I. Introduction

In recent years interest in the survival and growth of protozoan parasites in the vertebrate host has developed. Much of the earlier immunological work stressed the role of the humoral response and antibody-mediated lysis of protozoa, particularly for parasites which spent all or part of their life cycle in the bloodstream or extracellular fluid. More recent studies have demonstrated the importance of cell-mediated killing mechanisms, especially for the protozoan parasites that enter and multiply inside cells. This review attempts to summarize what is currently known about both extracellular and intracellular cell-mediated killing of protozoa. In so doing we will examine: (1) the means by which intracellular protozoa gain entry into host cells; (2) the conditions under which *in vivo* and *in vitro* survival and multiplication as opposed to stasis and cidal activity have been demonstrated for the various genera; (3) the known mechanisms for cell-mediated killing; (4) the means by which parasites evade even the most potent of these killing mechanisms; and (5) the genetic control of the host's resistance to infection. To maintain continuity throughout, parasites are grouped under classes according to the newly revised classification of the Protozoa by N. D. Levine and coauthors (1980), although this does not necessarily group them according either to their preferred host cell or to the particular mechanism by which they are killed.

II. Parasite Entry into Host Cells

Except for the microsporidia which directly penetrate the host cell membrane (Trager, 1974), the entry of protozoa into host cells occurs in two main stages. First the parasite becomes attached to the host cell via some form of receptor/ligand binding. This may involve Fc receptors demonstrable by preloading receptor sites by pretreatment of the host cell with, for example, anti-macrophage immunoglobulin G (IgG), the Fc portion of the IgG or specific monoclonal anti-Fc antibodies, or C3b receptors which are sensitive to trypan blue and trypsin but not chymotrypsin. Fc and complement receptors are especially important for the uptake of protozoa opsonized with specific antisera (Takayanagi and Nakatake, 1977; Stevens and Moulton, 1978; Cook, 1981), although some protozoa directly activate complement via the classical (Pearson and Steigbigel, 1980) or alternative (Nogueira *et al.*, 1975) pathway which, if it does not lead to direct lysis of the parasite, may mediate attachment to the host cell (Ward and Jack, 1980; Bray and Alexander, in press). Cytophilic antibody may bind to the host cell via the Fc receptor thus establishing an Fab site for parasite attachment (Herman, 1980; Bray and Alexander, in press). Alternatively, one of a number of glycoprotein (trypsin

sensitive) or glycolipid (trypsin resistant) receptors, which bind via their sugar moieties to lectin-like ligands, may be involved. This may be demonstrated either by competitive binding of the lectin-like ligand with saccharides such D-mannose, *N*-acetyl-D-glucosamine or D-glucose or by attacking the glycoprotein or glycolipid receptor with specific glycosidases like α-mannosidase, α-*N*-acetyl-glucosaminidase or β-glucosidase. There is also evidence for receptor/ligand binding via sialic acid residues which can be inhibited by pretreatment with neuraminidase. Table 1 includes a summary of the receptor/ligand combinations involved in attachment of protozoa to host cells. In some cases the glycoprotein or glycolipid receptor has been demonstrated on the host cell and the lectin-like ligand on the parasite (Bray and Alexander, in press) while in other situations the reverse is true (Chang, 1981b).

Following receptor/ligand binding, the parasite becomes enclosed in a vacuole with a lining membrane derived from the host cell. Griffin and coworkers (1975, 1976) proposed that the ingestion of particles, presumably including protozoa (Silverstein, 1977), by phagocytic cells results from the sequential and circumferential interaction of the receptors on the phagocyte's surface with the ligands on the particle's surface—the so-called 'zipper hypothesis'. The particle-bound ligands form a template that guides the phagocyte membrane. Hence, capping of surface ligands on the particle leads to reduced phagocytosis as the template is lost from most of the particle surface (see Section V, p. 108). The simple presence of the receptor for the ligand is not, however, the sole determinant of whether the ligand-coated particle is ingested. This depends on the physiological state of the host cell and may involve transmembrane potential changes (Miles *et al.*, 1981) and the degree of hydrophobicity of the particle relative to the host cell membrane (Wilkinson, 1976; Van Oss, 1978) as the initiating signals for the phagocytic process. The motor mechanism for ingestion is provided by the microfilaments (actin and myosin) which interact under the area of receptor/ligand contact. Cytochalasins B and D reversibly inhibit the action of the microfilaments and many workers have interpreted cytochalasin inhibition of parasite entry as indicative of phagocytic uptake by the host cell (see Table 1). Silverstein (1977) has suggested, however, that with parasite entry into non-phagocytic cells the force necessary to move the host cell membrane around the parasite may be generated by the contractile elements of the parasite. In a recent review of host cell invasion by coccidian and malarial parasites (class Sporozoea), Sinden (1981) has proposed that, following receptor/ligand attachment, invasion occurs by a cytochalasin-inhibitable active locomotion of the parasite into the host cell by means of a microfilament-based capping reaction. While it is generally accepted that the entry of *Plasmodium* into erythrocytes, for example, involves such an active process on the part of the

TABLE 1

Attachment and entry of protozoa into host cells[a]

Parasite: Species	Strain	Stage	Host cell: Cell-type	Source	Parasite inhibition	Attachment: Host cell inhibition	Proposed receptor	Entry: Cytochalasin sensitivity	Suggested mode of entry	Reference
A. Zoomastigophorea										
1. *Trypanosoma*										
T. cruzi	Y	Epimastigote	Macrophage	Resident P				+/−	Phagocytosis and active entry	Alexander (1975)
T. cruzi	Y; PF; Tulahuén	Epimastigote	Macrophage	Resident P; thiog PE		α-Mϕ −ve Tryp +ve Ch-tryp +ve	Not Fc or C3b	+	Phagocytosis	Nogueira and Cohn (1976)
T. cruzi	Y; PF; Tulahuén	Trypomastigote (metacyclic)	Macrophage	Resident P; thiog PE		α-Mϕ −ve Tryp +ve Ch-tryp +ve	Not Fc or C3b	+	Phagocytosis	
T. cruzi	Y; PF; Tulahuén	Epimastigote	HeLa; L and fibroblast	Cell lines and calf embryo					No entry	
T. cruzi	Y; PF; Tulahuén	Trypomastigote (metacyclic)	HeLa; L and fibroblast	Cell lines and calf embryo					? Active entry	
T. cruzi	Y; CL	Epimastigote	Macrophage	Resident P		α-Mϕ +ve Tryp +ve		+	Phagocytosis	Kipnis *et al.* (1979)
T. cruzi	Y; CL	Trypomastigote (bloodstream)	Macrophage	Resident P		α-Mϕ −ve Tryp −ve	Not Fc or C3b	−	Active entry	
T. cruzi (opsonized)	Y; CL	Trypomastigote (bloodstream)	Macrophage	Resident P				−	Active entry	
T. cruzi	EP	Trypomastigote (metacyclic)	Fibroblast; muscle	Cell lines; chick embryo		Con A +ve WGA +ve Ricin I +ve PHA +ve Tryp +ve		+	Phagocytosis and ? active entry	Henriquez *et al.* (1981)

Species	Parasite Strain[a]	Stage	Host cell Cell-type	Host cell Source	Attachment Parasite inhibtion	Attachment Host cell inhibition	Proposed receptor	Entry Cytochalasin sensitivity	Entry Suggested mode of entry	Reference
2. *Leishmania*										
L. donovani	1S	Promastigote	Macrophage	Resident P				+	Phagocytosis	Chang (1979)
L. donovani		Promastigote	Macrophage	Hanks PE				+	Phagocytosis	Ardehali *et al.* (1979)
L. donovani	1S	Promastigote	Macrophage	Resident P	Neur +ve α-Mann +ve α-*N*-Glu +ve β-Gluc +ve Tryp +/−	Sial +ve D-Mann +ve D-Gluc +ve Tryp +/−	GP/GL on parasite; *lectin on* Mϕ			Chang (1981c)
L. donovani	1S	Amastigote	Macrophage	Resident P			Not Fc or C3b		Phagocytosis	Chang and Dwyer (1978)
L. t. major	ex LUM[P] 344	Promastigote	Macrophage	Resident P	Glut −ve	4°C +ve Glut +ve	Membrane fluidity	+	Phagocytosis	Zenian *et al.* (1979)
L. t. major	ex LUM[P] 344	Promastigote	Macrophage	Resident P		Azide +ve 2-Deoxy-Glucose +ve	Glucose/ mannose		Phagocytosis (iodoacetamide inhibited)	Zenian (1981)
L. m. mexicana		Promastigote	Macrophage	Resident P				+	Phagocytosis	Alexander (1975)
L. m. mexicana		Promastigote	Macrophage	Resident P	*N*-Glu +ve Tryp −ve Glut −ve	WGA +ve Tryp +ve Glut +ve	GP on Mϕ; lectin on parasite			Bray and Alexander (iin press)
L. m. amazonensis		Amastigote	Macrophage	Resident P		4°C −ve Azide −ve			Phagocytosis (iodoacetamide inhibited)	Benoliel *et al.* (1980)
B. Sporozoea										
1. *Toxoplasma*										
T. gondii			Macrophage	Resident P				+	Phagocytosis	Ryning and Remington (1978)

[a] Abbreviations: α-Mϕ, antimacrophage serum; Glut, glutaraldehyde; P, peritoneal; PE, peritoneal exudate; Sial, sialic acid; Thiog, thioglycollate. *Enzymes:* α-Mann, α-mannosidase; α-*N*-Glu, α-*N*-acetyl glucosaminidase; β-Gluc, β-glucosidase; Ch-tryp, chymotrypsin; Neur, neuraminidase; Tryp, trypsin. *Lectins:* Con A, concanavalin A; PHA, phytohaemagglutinin; WGA, wheat germ agglutinin. *Receptors:* GL, glycolipid; GP glycoprotein. *Saccharides: D*-Gluc, *D*-glucose; *D*-Mann, *D*-mannose; *N*-Gluc, *N*-acetyl glucosamine.

parasite (Aikawa *et al.*, 1978), the process of parasite entry into phagocytic cells is less clear. Chow and Kreier (1972) observed very little entry of *P. berghei* into normal macrophages unless immune serum was present, whereas a combination of immune serum and immune macrophages resulted in effective immune phagocytosis. On the other hand, Danforth and coworkers (1980) demonstrated active entry of *P. berghei* and *P. knowlesi* sporozoites into normal resident macrophages in the presence of normal serum. Some controversy also exists as to whether *Toxoplasma gondii* enters host cells by phagocytosis (Jones *et al.*, 1972; Ryning and Remington, 1978) or by active penetration (Aikawa *et al.*, 1977; Tanabe *et al.*, 1978). Many experiments utilizing the inhibitory effect of cytochalasin, which is time and dose dependent, do not allow the investigator to distinguish successfully between effects on the host cell versus the parasite. Hence it is still unclear whether epimastigotes and trypomastigotes of *Trypanosoma cruzi* (see Table 1) enter host cells solely by phagocytosis or whether some active entry may occur. For *Leishmania* species more decisive evidence for the role of phagocytic uptake has been provided by the use of iodoacetamide, an irreversible inhibitor of glycolysis, when independent pretreatments of host cell and parasite are possible (Benoliel *et al.*, 1980; Zenian, 1981).

III. Intracellular Fate of Different Protozoal Species

A. Zoomastigophorea

1. *Trypanosoma*

(*a*) *Salivarian trypanosomes.* As early as 1908 Mesnil and Brimont described the cell-mediated killing of *T. brucei in vivo* (see Table 2 for summary of cell-mediated killing of *Trypanosoma* species). They observed, by microscopy of both fresh and stained material, that parasites from the blood stream of an infected mouse were rapidly phagocytosed and destroyed when injected, together with immune serum from goats or dogs, into the peritoneal cavity of an uninfected mouse. Both macrophages and neutrophils were active, with macrophages phagocytosing up to eight trypanosomes each. Destruction was complete in 1 hour. Extracellular trypanosomes were unaffected, as judged by motility and appearance. Immune serum was a necessary requirement and was equally active if used to pre-coat the trypanosomes or if present during the reaction. These authors were, however, unable to demonstrate phagocytosis of antibody-coated trypanosomes *in vitro*. Serum obtained from the mouse used as the source of trypanosomes had no opsonizing activity, presumably owing to antigenic variation of the *T. brucei*. The repeated peaks of parasitaemia in man, which are now known to be caused by the

emergence of antigenic variants (Cross, 1978), were observed by Ross and Thomson (1911) and were shown to be followed a few days later by peaks in the number of leucocytes, mainly monocytes, in the blood.

Sites of cell-mediated killing of *T. brucei* were investigated by Goodwin (1971). Chambers were inserted into rabbits' ears and 1 week later the animals were infected by subcutaneous inoculation with 6 million trypanosomes from mouse blood. The chambers were later removed and processed for light and electron microscopy. After about 3 weeks the venules began to disintegrate and leucocytes first adhered to the damaged endothelium and then migrated into the extravascular tissue. Adherent cells obstructed blood flow and the vessels disintegrated further with more migration of leucocytes into tissue spaces. The leucocytes involved were macrophages and not neutrophils, but since the chambers were left for 3 weeks, an early neutrophil response would have been missed. Trypanosomes were trapped alive by the macrophages and then destroyed. Cattle infected with *T. congolense* also have macrophages in their blood vessel walls and these too were reported to contain trypanosome debris (Fiennes, 1946). Goats infected by tsetse flies with *T. brucei* or *T. congolense* developed local skin reactions at the site of infection within 10 days. These were characterized initially by an infiltration of neutrophils, followed later by mononuclear cells. Only mononuclear cells and not neutrophils were seen after *T. vivax* infection (Emery and Moloo, 1980, 1981).

Cell-mediated killing of salivarian trypanosomes *in vitro* has now been demonstrated in several different systems. *Trypanosoma gambiense* from infected Wistar rats were freed of host blood components by chromatography on DEAE-cellulose by the method of Lanham and Godfrey (1970). Their attachment to, and phagocytosis by, rat macrophages, isolated from peritoneal exudates, was studied by light microscopy of Giemsa-stained preparations (Takayanagi *et al.*, 1974). Attachment was rapid and firm and was absolutely dependent on specific antibody. Phagocytosis took place within 2 hours. Complement had no effect on attachment or phagocytosis. There was no difference between macrophages from normal and immune rats.

Lumsden and Herbert (1967) observed that, in the presence of specific antiserum, *T. brucei* TREU-164 from infected mouse blood rapidly adhered to, and was taken up by, monolayers of mouse peritoneal macrophages on glass coverslips. Neither adherence nor phagocytosis occurred in the absence of antiserum. A more detailed analysis, by electron microscopy, of the phagocytosis of *T. brucei* (strain 110) by mouse peritoneal macrophages confirmed that immunoglobulin is necessary for attachment and ingestion (Stevens and Moulton, 1978). In addition, Stevens and Moulton (1978) showed (i) that complement enhanced antibody-dependent phagocytosis and was followed by destruction of the parasite, (ii) that antibody and complement

TABLE 2

Cell-mediated killing of Trypanosoma *species*[a]

Organism			Effector cell				Incubation		Killing		Reference
Species	Strain	Stage	Species	Cell-type	Source	Treatment	E:T	Time	Assessment		
T. brucei	NT, NZ	Trypomastigote	Mouse	Macrophage	Resident P	+Ab	*In vivo*	1 h	LM	++	Mesnil and Brimont (1908)
T. brucei	NT, NZ	Trypomastigote	Mouse	Neutrophil	Resident P	+Ab	*In vivo*	1 h	LM	++	Mesnil and Brimont (1908)
T. brucei	S42	Trypomastigote	Rabbit	Macrophage	Blood		*In vivo*	21 d	LM + EM	++	Goodwin (1971)
T. brucei	S42	Trypomastigote	Human	Neutrophil	Blood		5:1 + Ab	1 h	[^{3}H]Ur	++	D. Paton and D. Franks, unpublished observations
T. brucei	S42	Trypomastigote	Human	Monocyte	Blood		5:1 + Ab	1 h	[^{3}H]Ur	++	D. Paton and D. Franks, unpublished observations
T. brucei	110	Trypomastigote	Inf deermouse	Macrophage	Resident P	Inc 1–2 h	5:1 + Ab	1 h	EM	++	Stevens and Moulton (1978)
T. congolense			Cattle	Macrophage	Blood		*In vivo*		LM	++	Fiennes (1946)
T. gambiense	Wellcome type 0	Trypomastigote	Rat	Macrophage	Resident P	Inc 1 h	50:1 + Ab	1·5 h	LM	++	Takayanagi *et al.* (1974)
T. theileri		Epimastigote	Cattle	Neutrophil	Mammary exudate		20:1 + Ab	< 1 h	[^{3}H]Ur + EM	++	Townsend and Duffus (1982)
T. theileri		Epimastigote	Cattle	Neutrophil	Blood		20:1 + Ab	< 1 h	[^{3}H]Ur	++	Townsend and Duffus (1982)
T. theileri		Epimastigote	Cattle	Eosinophil	Mammary exudate		20:1 + Ab	4 h	[^{3}H]Ur + EM	++	Townsend and Duffus (1982)
T. theileri		Epimastigote	Cattle	Macrophage	Mammary exudate		20:1 + Ab	5 h	[^{3}H]Ur + EM	++	Townsend and Duffus (1982)
T. theileri		Epimastigote	Cattle	Monocyte	Blood		20:1 + Ab	5 h	[^{3}H]Ur + EM	++	
T. lewisi		Trypomastigote	Rat	Macrophage	Resident P		*In vivo*	4 h	LM	++	Laveran and Mesnil (1901)
T. lewisi		Trypomastigote	Rat	Neutrophil	Resident P		*In vivo*	4 h	LM	++	Laveran and Mesnil (1901)
T. lewisi		Trypomastigote	Wistar rat	Macrophage	Resident P		1:1 + Ab	2 h	LM	++	Lange and Lysenko (1960)
T. lewisi		Trypomastigote	Wistar rat	Macrophage	Resident P		+ Ab	5 h	^{32}P LM + EM	45%	Ferrante and Jenkin (1979)
T. lewisi		Trypomastigote	Wistar rat	Macrophage	Resident P			5 h	^{32}P LM + EM	12%	Ferrante and Jenkin (1979)
T. lewisi		Trypomastigote	Inf Wistar rat	Macrophage	Resident P			5 h	^{32}P LM + EM	12%	Ferrante and Jenkin (1979)
T. lewisi		Trypomastigote	Mouse LAC	Macrophage	Resident P		+Ab	5 h	^{32}P LM + EM	++	Ferrante and Jenkin (1979)
T. dionisii	P2 + P3	Epimastigote	Mouse	Macrophage	Resident P			5 h	LM + EM	+	Liston and Baker (1978)
T. dionisii	P2 + P3	Trypomastigote	Mouse	Macrophage	Resident P			5 h	LM + EM	+	Liston and Baker (1978)
T. dionisii	P3	Epimastigote	Human	Neutrophil	Blood		20:1 + Ab	1 h	^{99m}Tc + EM	60%	Thorne *et al.* (1979)
T. dionisii	P3	Epimastigote	Human	Neutrophil	Blood		20:1	1 h	^{99m}Tc + EM	20%	Thorne *et al.* (1979)
T. dionisii	P3	Epimastigote	Human	Monocyte	Blood		20:1 + Ab	2 h	^{99m}Tc + EM	20%	Thorne *et al.* (1979)
T. dionisii	P3	Epimastigote	Human	Monocyte	Blood		20:1	2 h	^{99m}Tc + EM	20%	Thorne *et al.* (1979)
T. dionisii	P2	Trypomastigote	Human	Neutrophil	Blood		20:1 + Ab	5 h	[^{3}H]Ur + EM	35%	Thorne *et al.* (1981)
T. dionisii	P2	Trypomastigote	Human	Neutrophil	Blood		20:1	5 h	[^{3}H]Ur + EM	20%	Thorne *et al.* (1981)
T. dionisii	P2	Trypomastigote	Human	Monocyte	Blood		10:1 + Ab	5 h	[^{3}H]Ur + EM	20%	Thorne *et al.* (1981)
T. dionisii	P2	Trypomastigote	Human	Monocyte	Blood		10:1	5 h	[^{3}H]Ur + EM	10%	Thorne *et al.* (1981)
T. cruzi	Ernestina	Epimastigote	Mouse BALB/cAnN	Macrophage	Resident P		1:1	4–30 h	LM	++	Dvorak and Schmunis (1972)
T. cruzi	Ernestina	Trypomastigote	Mouse BALB/cAnN	Macrophage	Resident P		1:1	1–4 h	LM	++	Dvorak and Schmunis (1972)
T. cruzi	Y	Epimastigote	Mouse A/Sn	Lymphocyte	Spleen		6:1	18 h	Loss of motility	1%	Abrahamson and Da Silva (1977)
T. cruzi	Y	Epimastigote	Mouse A/Sn	Lymphocyte	Spleen		6:1 + Ab	18 h	Loss of motility	33%	Abrahamson and Da Silva (1977)
T. cruzi	Y	Epimastigote	Mouse A/Sn	Lymphocyte	Spleen		20:1	18 h	Loss of motility	23%	Abrahamson and Da Silva (1977)
T. cruzi	Y	Epimastigote	Mouse A/Sn	Lymphocyte	Spleen		20:1 + Ab	18 h	Loss of motility	62%	Abrahamson and Da Silva (1977)

Organism			Effector cell				Incubation		Killing Assessment		Reference
Species	Strain	Stage	Species	Cell-type	Source	Treatment	E : T	Time			
T. cruzi	Y	Epimastigote	Rat	Splenocyte	Spleen		50 : 1	5 h	[^{3}H]Ur	10%	Sanderson *et al.* (1978)
T. cruzi	Y	Epimastigote	Rat	Splenocyte	Spleen		50 : 1 + Ab	5 h	[^{3}H]Ur	30%	
T. cruzi	Y	Epimastigote	Rat	Eosinophil	Resident P		8 : 1	4 h	[^{3}H]Ur	0%	Lopez *et al.* (1978)
T. cruzi	Y	Epimastigote	Rat	Eosinophil	Resident P		8 : 1 + Ab	4 h	[^{3}H]Ur	30%	
T. cruzi	Y	Epimastigote	Rat	Neutrophil	Dextran PE		8 : 1	4 h	[^{3}H]Ur	15%	
T. cruzi	Y	Epimastigote	Rat	Neutrophil	Dextran PE		8 : 1 + Ab	4 h	[^{3}H]Ur	35%	
T. cruzi	Y	Epimastigote	Human	Neutrophil	Blood		15 : 1	4 h	[^{3}H]Ur	23%	Madeira *et al.* (1979)
T. cruzi	Y	Epimastigote	Human	Neutrophil	Blood		15 : 1 + Ab	4 h	[^{3}H]Ur	60%	
T. cruzi	Tulahuén	Epimastigote	Human	Neutrophil	Blood		4 : 1	3 h	[^{3}H]Ur	23%	Olabuenaga *et al.* (1979)
T. cruzi	Tulahuén	Epimastigote	Human	Neutrophil	Blood		4 : 1 + Ab	3 h	[^{3}H]Ur	60%	
T. cruzi	Tulahuén	Epimastigote	Mouse B10; B10.D2	Macrophage	Resident P		1 : 10	24 h	LM + EM	–	Tanowitz *et al.* (1975)
T. cruzi	Tulahuén	Epimastigote	Mouse B10; B10.D2	Macrophage	Resident P		1 : 1	24 h	LM + EM	++	
T. cruzi	Tulahuén	Epimastigote	BCG mouse B10; B10.D2	Macrophage	Peritoneal		1 : 100	24 h	LM + EM	–	
T. cruzi	Tulahuén	Epimastigote	BCG mouse B10; B10.D2	Macrophage	Peritoneal		1 : 10	24 h	LM + EM	++	
T. cruzi	Y; PF; Tulahuén	Epimastigote	Mouse Swiss; C3H/He; C57BL	Macrophage	Resident P		1 : 1	24 h	LM + EM	++	Nogueira and Cohn (1976)
T. cruzi	Y; PF; Tulahuén	Epimastigote	Mouse Swiss; C3H/He; C57BL	Macrophage	Thiog PE		1 : 1	24 h	LM + EM	++	
T. cruzi	7	Epimastigote	Mouse	Macrophage	Resident P				LM	+	Behbehani (1971a)
T. cruzi	Y	Amastigote	Mouse Swiss	Macrophage	Resident P		1 : 10	2 h	EM	60%	Carvalho *et al.* (1981b)
T. cruzi	H 510	Epimastigote	BCG mouse C3H/StCr1	Macrophage	Resident P		1 : 1	48 h	LM	+	Hoff (1975)
T. cruzi	H 510	Trypomastigote	BCG mouse C3H/StCr1	Macrophage	Resident P		1 : 1	48 h	LM	+	
T. cruzi	H 510	Epimastigote	*T. cruzi* mouse C3H/StCr1	Macrophage	Resident P		1 : 1	48 h	LM	+	
T. cruzi	H 510	Trypomastigote	*T. cruzi* mouse C3H/StCr1	Macrophage	Resident P		1 : 1	48 h	LM	+	
T. cruzi	F	Trypomastigote (bloodstream)	Hamster	Macrophage	Resident P		1 : 1 + Ab?	2 h	LM	++	Milder *et al.* (1977)
T. cruzi	Tulahuén	Trypomastigote (bloodstream)	Human	Neutrophil	Blood		7 : 1	5 h	LM	3%	Kierszenbaum (1979)
T. cruzi	Tulahuén	Trypomastigote (bloodstream)	Human	Neutrophil	Blood		7 : 1 + Ab	5 h	LM	45%	
T. cruzi	Tulahuén	Trypomastigote (bloodstream)	Human	Monocyte	Blood		6 : 1	5 h	LM	0%	
T. cruzi	Tulahuén	Trypomastigote (bloodstream)	Human	Monocyte	Blood		6 : 1 + Ab	5 h	LM	40%	
T. cruzi	Tulahuén	Trypomastigote (bloodstream)	Human	Eosinophil	Blood		3 : 1	5 h	LM	0%	
T. cruzi	Tulahuén	Trypomastigote (bloodstream)	Human	Eosinophil	Blood		3 : 1 + Ab	5 h	LM	32%	

TABLE 2 (*continued*)

Organism			Effector cell				Incubation		Killing Assessment		Reference
Species	Strain	Stage	Species	Cell-type	Source	Treatment	E:T	Time			
T. cruzi	Tulahuén	Trypomastigote	Human	Macrophage	Blood	LK		72 h	LM	+	Williams and Remington (1977)
T. cruzi	Tulahuén	Trypomastigote	Mouse	Macrophage	Resident P		*In vivo*	4 h	LM	+	Kierszenbaum *et al.* (1974)
T. cruzi	Tulahuén	Trypomastigote	Inf mouse C3H	Macrophage	Tissue		*In vivo*		LM	+	Taliaferro and Pizzi (1955)
T. cruzi	Y; PF; Tulahuén	Trypomastigote	Mouse Swiss C3H/HeJ; C57BL	Macrophage	Thiog PE		1:1	24 h	LM	−	Nogueira and Cohn (1976)
T. cruzi	Y; Tulahuén	Trypomastigote	Inf mouse	Macrophage	*T. cruzi* Ag PE		1:1	24 h	LM	++	Nogueira *et al.* (1977a, b)
T. cruzi	Y; Tulahuén	Trypomastigote	BCG mouse	Macrophage	*M. tuberculosis* Ag PE		1:1	24 h	LM	+	
T. cruzi	Y	Trypomastigote	Mouse	Macrophage	Resident P	LK	1:2	72 h	LM	++	Nogueira and Cohn (1978)
T. cruzi	Y	Trypomastigote (bloodstream)	Mouse	Macrophage	Resident P	LK	1:2	72 h	LM	−	Nogueira *et al.* (1980)
T. cruzi	Y	Trypomastigote (bloodstream)	Mouse	Macrophage	Resident P	LK	1:2 + Ab	72 h	LM	++	
T. cruzi	Y	Trypomastigote (bloodstream)	Mouse DBA	Macrophage	Resident P		1:5 + Ab	24 h	LM	−	Alcantara and Brener (1978)
T. cruzi	CL	Trypomastigote (bloodstream)	Mouse DBA	Macrophage	Resident P		1:5 + Ab	24 h	LM	+	
T. cruzi	Y + CL	Trypomastigote (bloodstream)	Mouse C3H/He	Macrophage	Thiog PE		40:1	18 h	Motility infectivity	50%	Okabe *et al.* (1980)
T. cruzi	Y + CL	Trypomastigote (bloodstream)	Mouse C3H/He	Macrophage	Thiog PE		40:1 + Ab	18 h	Motility infectivity	50%	
T. cruzi	Y + CL	Trypomastigote (bloodstream)	Irr mouse C3H/He	Macrophage	Thiog PE		40:1	18 h	Motility infectivity	0%	
T. cruzi	Y + CL	Trypomastigote (bloodstream)	Irr mouse C3H/He	Macrophage	Thiog PE		40:1 + Ab	18 h	Motility infectivity	50%	
T. cruzi	Tulahuén	Trypomastigote (bloodstream)	Mouse	Macrophage	Spleen		2:1 + Ab	5 h	Motility	30%	Kierszenbaum and Hayes (1980)
T. cruzi	Tulahuén	Trypomastigote (bloodstream)	Mouse	Neutrophil	Spleen		1·5:1 + Ab	5 h	Motility	40%	
T. cruzi	Y	Trypomastigote (bloodstream)	Inf mouse *S. mansoni*	Neutrophil	Proteosepeptone PE		20:1 + Ab	16 h	Motility infectivity	76%	Kipnis *et al.* (1981)
T. cruzi	Y	Trypomastigote (bloodstream)	Inf mouse *S. mansoni*	Eosinophil	Proteosepeptone PE		20:1 + Ab	16 h	Motility infectivity	73%	

[a] Abbreviations: Ab, antibody; Ag, antigen; EM, electron microscopy; E:T, effector: target cell ratio; Inc, incubated; Inf, infected; Irr, irradiated; LK, non-specific lymphokine; LM, light microscopy; PE, peritoneal exudate; Thiog, thioglycollate.

alone, without macrophages, did not damage *T. brucei*, and (iii) that only activated macrophages from trypanosome-infected mice were active.

The loss of radioactive material from *T. brucei* prelabelled with [^{3}H]uridine was used by D. Paton and D. Franks (personal communication) to demonstrate that leucocytes from human peripheral blood are active against trypomastigotes obtained from feeder layers of irradiated African green monkey kidney cells. Isotope release was detected within 1 hour of adding leucocytes to *T. brucei* trypomastigotes, was induced more rapidly by neutrophils than by monocytes and was greatly enhanced by a specific antiserum.

While B lymphocytes and secreted antibody, especially of the M class (Takayanagi and Enriquez, 1973), are important in controlling infections with salivarian trypanosomes (Campbell *et al.*, 1977), there appears to be no obligatory role for T lymphocytes in the short term. Campbell and coworkers (1978) showed that the resistance of mice to re-infection with *T. rhodesiense* was relatively independent of thymic lymphocyte function since it could be induced in athymic nude mice.

(*b*) *Stercorarian trypanosomes.* Cell-mediated killing *in vivo* of the rat parasite *T. lewisi* was described in 1901 by Laveran and Mesnil. They introduced the blood stream form of *T. lewisi* into the peritoneal cavity of rats and took samples 4 hours later. Examination by light microscopy revealed that both peritoneal macrophages and neutrophils rapidly phagocytosed and digested the trypomastigotes.

Incubation of *T. lewisi* with rat peritoneal cells for 2 hours *in vitro* similarly showed that macrophages have a strong phagocytic activity towards these protozoa (Lange and Lysenko, 1960). Lange and Lysenko (1960) only rarely saw neutrophils phagocytosing *T. lewisi*, but it is possible that 2 hours was too long an incubation time for neutrophil activity to be still detectable. Antisera to both *T. lewisi* and *T. cruzi* enhanced the rate of phagocytosis.

Ferrante and Jenkin (1979) used the rate of ^{32}P release from *T. lewisi*, labelled by pre-incubation for 24 hours with [^{32}P]orthophosphate, to measure cell-mediated lysis by rat and mouse peritoneal macrophages. They showed that macrophage-mediated lysis is greatly enhanced by antiserum and that macrophages from immune animals are no more active than macrophages from normal animals. Antiserum alone does not induce lysis unless complement is also present (Ferrante and Jenkin, 1978).

Arguments over the importance of cell-mediated killing of *T. lewisi in vivo* were resolved by Patton (1972). He treated mice with 0·5 mg of dexamethasone daily to depress the reticulendothelial system. When *T. lewisi* was injected into these animals they developed a massive parasitaemia. This parasitaemia could be controlled if glycogen-induced peritoneal exudate cells from normal rats, together with hyperimmune sera, were injected into the peritoneum of

the dexamethasone-treated animals, but not if cells or antisera were used alone. After injection of cells and antisera the trypanosomes were retained in the peritoneal cavity where they agglutinated and were phagocytosed.

Evidence for cell-mediated killing *in vivo* of *T. musculi*, a trypanosome which infects mice, was obtained by Targett and Viens (1975). They obtained non-adherent peritoneal cells, presumably lymphocytes, from mice which had recovered from infection with *T. musculi* and injected them into infected mice. Elimination of the parasite from the infected mice was accelerated. They hypothesized that the immune lymphocytes were activating the macrophages of the infected animal to kill the *T. musculi*.

Trypanosoma dionisii is a stercorarian trypanosome isolated from bats. Evidence from light and electron microscopy showed that epimastigotes and trypomastigotes of *T. dionisii* are phagocytosed by mouse peritoneal macrophages and that some are subsequently digested (Liston and Baker, 1978).

Quantitative assay of cell-mediated damage to *T. dionisii* was made by pre-labelling epimastigotes with ^{99m}Tc-pertechnetate and both epimastigotes and trypomastigotes with [^{3}H]uridine, and measuring release of isotope from the protozoa (Thorne *et al.*, 1979, 1981). Human peripheral blood neutrophils and monocytes, in the absence of antiserum, are active against epimastigotes, but only neutrophils are active against trypomastigotes. Trypomastigotes are taken up by monocytes but escape into the cell cytoplasm. The activity of both types of effector cell is increased against both epimastigotes and trypomastigotes by the presence of antiserum to *T. cruzi*, to which *T. dionisii* is closely related (Baker, 1976). Neutrophils act very rapidly: phagocytosis is complete in 15 minutes and digestion within 1 hour in the presence of antiserum. Monocytes act more slowly, over several hours. Eosinophils in isolation are also able to phagocytose *T. dionisii*, but they are much less active than neutrophils and, in the presence of an equal number of neutrophils, very few protozoa are found in the eosinophils. Pregnancy alpha-2-glycoprotein inhibits phagocytosis of *T. dionisii* by normal human blood neutrophils (Persellin and Thorne, 1981), which could explain the observed increase in frequency and severity of protozoal infections during gestation.

Similar methods were used by Townsend and Duffus (1982) and Townsend *et al.* (1982) to show that neutrophils, eosinophils and macrophages obtained from bovine teat exudates killed *T. theileri* epimastigotes, but only in the presence of antibody.

The most extensively studied of the stercorarian trypanosomes is *T. cruzi*, because it is the aetiological agent of Chagas's disease. Two major groups of *T. cruzi* strains have been described; the myotropic strains, such as Ernestina and CL, are efficiently destroyed by mouse peritoneal macrophages (Dvorak and Schmuñis, 1972; Alcantara and Brener, 1978), but the reticulotropic strains, for example Tulahuén and Y, are able at least partially to evade the

microbicidal activity of the macrophage and survive and multiply in it (Nogueira and Cohn, 1976; Milder *et al.*, 1977). Most work has been done with reticulotropic strains in an attempt to elucidate the nature and extent of cell-mediated immunity and the resistance of the trypanosome to it.

The methods used for assessing cell-mediated killing of *T. cruzi in vitro* include phase contrast and electron microscopy, with measurement of loss of structure and motility (e.g. Dvorak and Schmuñis, 1972; Abrahamson and Da Silva, 1977; Nogueira and Cohn, 1976), and the loss of radioactive isotope from *T. cruzi* pre-labelled with [^{3}H]uridine (Sanderson *et al.*, 1978).

Epimastigotes, the dividing form from the gut of the insect vector, are effectively destroyed by human blood neutrophils, particularly in the presence of immune serum (Olabuenaga *et al.*, 1979; Rimoldi *et al.*, 1981; Madeira *et al.*, 1979), by rat peritoneal neutrophils and eosinophils in the presence of antiserum (Lopez *et al.*, 1978) and by mouse peritoneal macrophages (Behbehani, 1971a; Hoff, 1975; Nogueira and Cohn, 1976), provided that the ratio of effector cell to target is great enough (Tanowitz *et al.*, 1975; Kress *et al.*, 1977). Amastigotes, the dividing intracellular form, are also killed by mouse peritoneal macrophages (Carvalho *et al.*, 1981b).

Trypomastigotes are killed by human blood neutrophils, eosinophils and monocytes in the presence of immune serum (Kierszenbaum, 1979) and also by mouse and rat peritoneal neutrophils and eosinophils in the presence of specific antiserum (Kipnis *et al.*, 1981; Sanderson and DeSouza, 1979). Trypomastigotes are killed by macrophages only under certain conditions. Normal, proteose-peptone or thioglycollate induced peritoneal macrophages are able to phagocytose trypomastigotes but they do not kill them effectively and the protozoa escape into the cytoplasm of the host cell and multiply (Behbehani, 1971a, 1973; Nogueira and Cohn, 1976; Milder *et al.*, 1977). Macrophages from immune animals are somewhat more active against *T. cruzi* (Taliaferro and Pizzi, 1955; Hoff, 1975; Nogueira *et al.*, 1977a) and this enhanced activity is not specific since active macrophages can be obtained from animals immunized against *T. cruzi* or *Mycobacterium tuberculosis*. However, Nogueira and coworkers (1977a) report that these macrophages are trypanosomistatic and not trypanosomicidal.

Trypanosomicidal macrophages were obtained by incubating normal macrophages *in vitro* with lymphokines (Nogueira and Cohn, 1978). Both specific and nonspecific lymphokines are able to activate mouse and human macrophages to control *T. cruzi* trypomastigotes (Williams and Remington, 1977; Nogueira and Cohn, 1978).

Most of the work described above was done with trypomastigotes of *T. cruzi* obtained from culture. Trypomastigotes obtained directly from the blood of infected animals have increased resistance to the cytotoxic activity of mononuclear cells. They are not phagocytosed or killed by macrophages,

even after activation with lymphokines (Nogueira *et al.*, 1980). This resistance can, however, be overcome if the trypomastigotes are either pre-treated with trypsin or opsonized with specific antiserum (Nogueira *et al.*, 1980; Kierszenbaum, 1979; Kierszenbaum and Hayes, 1980).

Only epimastigotes, and not trypomastigotes, are susceptible to complement-mediated lysis (Nogueira *et al.*, 1975).

Considerable evidence from *in vivo* studies exists to show that lymphocytes play an important role in resistance to *T. cruzi* infection. Thymectomy or X-irradiation lowers resistance to infection with trypomastigotes of the Peru strain (Behbehani, 1971b) or the Tulahuén strain (Schmuñis *et al.*, 1971). Congenitally athymic homozygous (nu/nu) mice are more susceptible than heterozygous (nu/+) to infection, and transplantation of normal neonatal thymus into athymic mice restores normal levels of resistance (Kierszenbaum and Pienkowski, 1979). Transfer of splenic and lymph node lymphocytes from mice and rats infected with *T. cruzi* confers protection on uninfected animals (Roberson and Hanson, 1974; Burgess and Hanson, 1979; Trischmann and Bloom, 1980).

In vivo studies have also shown the importance of antibody in resistance to *T. cruzi*. A correlation was found between low antibody-forming capacity in Biozzi mice and susceptibility to infection with Tulahuén and Y strains (Kierszenbaum and Howard, 1976), and B-cell deficient rats were more susceptible to the acute phase of infection (Rodriguez *et al.*, 1981). Passive transfer of immune sera protected mice against infection with blood trypomastigotes of strains BG, M1 and Y (McHardy, 1980) and athymic mice against Tulahuén strain (Kierszenbaum, 1980).

2. *Leishmania*

In the vertebrate host, protozoa of the genus *Leishmania* assume the non-motile amastigote form and are obligate intracellular parasites of the mononuclear phagocyte system. Infection occurs via a bite from the sandfly vector in which the parasites exist extracellularly as flagellated motile promastigotes. Promastigotes grown *in vitro* in cell-free culture media closely resemble the insect form. Various Old and New World species of *Leishmania* elicit a broad spectrum of immunological responses in man similar to those observed in leprosy (Turk and Bryceson, 1971).

Towards the allergic end of the spectrum are the various forms of self-limiting cutaneous leishmaniases of different geographical origins (the *L. tropica* and *L. mexicana* complexes). Although these may exhibit many distinguishing epidemiological and clinical features, the histopathology and immunological characteristics of the disease are relatively uniform. Early in infection parasites multiply in histiocytes which accumulate at the site of the sandfly's bite. These eventually rupture and parasites are taken up by neigh-

bouring phagocytes. The core of the lesion ulcerates with extensive necrosis of parasite-laden macrophages (Ridley, 1979). A marked cell-mediated reactivity to parasite antigen develops, resulting in a strong delayed type hypersensitivity (DTH) reaction *in vivo* in response to parasite antigen (reviewed by Zuckerman, 1975; Preston and Dumonde, 1976) and blast transformation of peripheral lymphocytes cultured in the presence of parasite antigen *in vitro* (Witztum *et al.*, 1978; Wyler *et al.*, 1979). Interestingly, the conversion to a strong positive DTH occurs well before protective immunity is established (Zuckerman, 1975) at a time when parasites are still abundant in the lesions. Healing correlates with lymphocyte infiltration into the lesion and the draining lymph nodes and, in the last phase of infection, parasites disappear within the mononuclear phagocytes. Patients achieve radical cure and remain immune to reinfection (Guirges, 1971; Heyneman, 1971; Preston and Dumonde, 1976).

Two animal models which closely resemble the uncomplicated self-limiting cutaneous leishmaniases in man occur following low-dose subcutaneous (s.c.) inoculations of *L. enriettii* promastigotes into the guinea pig (Bryceson *et al.*, 1970) and *L. t. major* or *L. m. mexicana* promastigotes into resistant inbred mouse strains (Preston *et al.*, 1972; Perez *et al.*, 1978). Histologically and immunologically the course of infection *in vivo* is similar, with a strong positive DTH developing prior to healing (Bryceson *et al.*, 1970, 1974; Preston *et al.*, 1972; Perez *et al.*, 1978; Poulter, 1979). Although necrosis of antibody-coated infected macrophages may play a role in eliminating many parasites early in the healing process (Bryceson *et al.*, 1970; Monroy *et al.*, 1980), the essential role of T lymphocytes demonstrated by various T cell depletion experiments *in vivo* (Bryceson and Turk, 1971; Preston *et al.*, 1972; Howard *et al.*, 1980a), and the histological observation of parasite destruction within macrophages *in vivo* (Rezai *et al.*, 1972; Sordat and Behin, 1978), has led to the belief that the final effector mechanism for healing involves the non-specific microbicidal capacity of lymphokine-activated macrophages.

Initial *in vitro* studies on the guinea pig model (Behin *et al.*, 1975; Mauel *et al.*, 1975; see Table 3 for a summary of all the *in vitro* studies referred to below) demonstrated that, although 'immune' macrophages taken from animals recovering from *L. enriettii* infection or from those infected with BCG (Bacillus Calmette-Guérin) or *Toxoplasma* were activated to kill *Listeria monocytogenes*, they failed to kill *L. enriettii* amastigotes. Subsequent studies have shown, however, that it is possible to activate mouse macrophages to kill *L. enriettii* following incubation *in vitro* with lymphokine (Mauel *et al.*, 1978; Buchmüller and Mauel, 1979). Similarly, mouse macrophages may also be activated *in vitro* to kill *L. m. mexicana* (Lewis and Peters, 1977; Alexander, 1981a) and *L. t. major* (Behin *et al.*, 1979; Nacy *et al.*, 1981). The earlier results (Behin *et al.*, 1975; Mauel *et al.*, 1975)

TABLE 3

Fate of Leishmania *species inside host cells* in vitro[a]

Species	Parasite Strain	Parasite Stage	Host cell Species	Host cell Cell-type	Host cell Source	Culture conditions *in vitro* Treatment	Culture conditions *in vitro* H:P	Culture conditions *in vitro* Time	Intracellular fate Survive	Intracellular fate Multiply	Intracellular fate Killing	Assessment	Reference
L. donovani	I; S	Promastigote	Hamster	Macrophage	Spleen	—	—	1–20 d	+	+	–	LM	Hawking (1948)
L. donovani	S	Promastigote	Hamster	Macrophage	Hanks PE	—	1:4	3 d	+	+	–	LM	Miller and Twohy (1967)
L. donovani	I	Promastigote (virulent)	Hamster	Macrophage	Resident P	—	1:10 1:1	4 h–15 d	+	+	–	LM	Ebert *et al.* (1979)
L. donovani	I	Promastigote (avirulent)	Hamster	Macrophage	Resident P	—	1:10 1:1	4 h–15 d	–	–	+	LM	
L. donovani	1S	Promastigote	Human	Macrophage	Blood	—	1:20	6 d	+	+	–		Pearson *et al.* (1981)
L. donovani	1S	Promastigote	Human	Neutrophil	Blood	—	1:1 to 1:10	3 h	–	–	+		Pearson and Steigbigel (1981)
L. donovani	1S	Promastigote	CGD Human	Neutrophil	Blood	—	1:1 to 1:10	3 h	+	+	–		
L. donovani	1S	Promastigote	J774	Macrophage	Cell-line	—	—	2 d	+	+	–	LM	Murray (1981b)
						Con A LK	—	2 d	–	–	+	LM	
L. donovani	S	Amastigote	Hamster	Macrophage	Resident P	—	—	—	+	+	–	LM	Herman (1966)
L. donovani	1S	Amastigote	Hamster	Macrophage	Resident P	—	1:2 1:10 1:20 1:40	1–7 d	+	+	–	LM & EM	Chang and Dwyer (1976, 1978) Chang (1978a)
L. donovani	3S	Amastigote	Mouse	Macrophage	Hanks PE	—	1:4	3 d	+	+	–	LM	Miller and Twohy (1969)
L. donovani	3S	Amastigote	Super-inf Mouse	Macrophage	Hanks PE	—	1:4	3 d	–	–	+	LM	
L. donovani	1S	Amastigote	Mouse	Macrophage	Resident P	—	—	1–7 d	+	–	–	LM	Chang and Chiao (1981)
						Leish LK	—	1–7 d	–	–	+	LM	
L. donovani	1S	Amastigote	Inf mouse	Macrophage	Resident P	—	—	1–7 d	+	–	–	LM	
						Leish LK	—	1–7 d	–	–	+	LM	
L. donovani	1S	Amastigote	Mouse	Macrophage	Thiog PE	—	1:10 to 1:100	4 d	+	–	–	LM	Haidaris and Bonventre (1981)
						Con A LK	1:10 to 1:100	4 d	–	–	+	LM	
L. donovani	1S	Amastigote	Mouse	Macrophage	*C. parvum* PE	—	1:10 to 1:100	4 d	+	–	–	LM	
						Con A LK		4 d	–	–	+	LM	
L. donovani	1S	Amastigote	Mouse inf *M. tuberculosis*	Macrophage	PPD PE	—	1:10 to 1:100	4 d	+	–	–	LM	
						Con A LK		4 d	–	–	+	LM	
L. donovani	1S	Amastigote	Mouse	Macrophage	Resident P	—	—	3 d	+	–	–	LM	Murray *et al.* (1982)
						Leish or Con A LK	—	3 d	–	–	+	LM	
L. donovani	1S	Amastigote	Mouse inf *T. gondii*	Macrophage	Resident P	—	—	3 d	+	–	–	LM	
						Leish or Con A LK	—	3 d	–	–	+	LM	
L. donovani	1S	Amastigote	Mouse inf BCG	Macrophage	Resident P	—	—	3 d	+	–	–	LM	
						Leish or Con A LK	—	3 d	–	–	+	LM	
L. donovani	1S	Amastigote	Mouse	Macrophage	*C. parvum* PE	—	—	3 d	+	–	–	LM	
						Leish or Con A LK	—	3 d	–	–	+	LM	
L. donovani	1S	Amastigote	Human	Macrophage	Blood	37°C	1:5	6 d	+	+	–	LM	Berman *et al.* (1979)
L. donovani	1S	Amastigote	Human	Macrophage	Blood	35°C	1:7	6 d	+	+	–	LM	Berman and Neva (1981)
L. donovani	1S	Amastigote	Human	Macrophage	Blood	37°C	1:7	6 d	+	+	–	LM	
L. donovani	1S	Amastigote	Human	Macrophage	Blood	39°C	1:7	6 d	+	+	40%	LM	
L. donovani	2S	Amastigote	Human	Neutrophil	Blood	—	1:10	16 h	–	–	+	LM & EM	Chang (1978a, 1981a)
			CGD human	Neutrophil	Blood	—	1:10	16 h	+	+	–	LM & EM	

Species	Parasite Strain	Stage	Host cell Species	Cell-type	Source	Culture conditions *in vitro* Treatment	H : P	Time	Intracellular fate Survive	Multiply	Killing	Assessment	Reference
L. donovani	1S	Amastigote	P388D	Macrophage	Cell-line	—	—	—	+	+	−	LM	Berens and Marr (1979)
L. donovani	1S	Amastigote	J774	Macrophage	Cell-line	—	—	2 d	+	+	−	LM	Murray (1982)
						Con A LK	—	2 d	−	−	+	LM	
L. t. major	LRC-L137	Promastigote	Mouse	Macrophage	Resident P	—	1 : 2	4 d	+	+	−	LM	Handman and Burgess (1979)
						GM-CSF	1 : 2	4 d	−	−	+	LM	
L. t. major		Promastigote	Mouse	Macrophage	Starch PE	Ag + *T. gondii* Imm lymphocytes	1 : 4	3 d	+	+	−	LM; SDS	Behin *et al.* (1975)
L. t. major	Bokhara	Promastigote	Mouse	Macrophage	Resident P	—	1 : 3	3 d	+	+	−	LM	Behin *et al.* (1979)
						Con A LK	1 : 3	3 d	−	−	+	LM	
L. t. major	252 Iran	Promastigote	J774	Macrophage	Cell-line	—	—	2 d	+	+	−	LM	Murray (1981b)
						Con A LK	—	2 d	−	−	+	LM	
L. t. major	NIH 173	Amastigote	Mouse	Macrophage	Resident P adherent	—	1 : 5	4 d	+	−	−	LM	Nacy and Diggs (1981)
L. t. major	NIH 173	Amastigote	Mouse	Macrophage	Resident P suspension	—	1 : 5	4 d	+	+	−	LM	
L. t. major	NIH 173	Amastigote	Mouse	Macrophage	Resident P suspension	—	1 : 1–5	3 d	+	+	−	LM	Nacy *et al.* (1981)
L. t. major	NIH 173	Amastigote	Mouse	Macrophage	Resident P suspension	Con A LK	1 : 1–5	3 d	−	−	+	LM	
L. t. major	NIH 173	Amastigote	Mouse	Macrophage	Resident P suspension	CSF	1 : 1–5	3 d	+	+	−	LM	Ralph *et al.* (in press) (1982)
L. enriettii		Amastigote	Guineapig	Macrophage	Starch PE	—	1 : 10	3 d	+	+	−	LM	Bryceson *et al.* (1970)
L. enriettii		Amastigote	Inf guineapig	Macrophage	Starch PE	—	1 : 10	3 d	+	+	−	LM	
L. enriettii		Amastigote	Inf guineapig	Macrophage	Starch PE	Imm lymphocytes	1 : 10	3 d	−	−	+ (necrosis)	LM	
L. enriettii		Amastigote	Guineapig	Macrophage	Starch PE	—	1 : 4	3 d	+	−	−	LM; SDS	Mauel *et al.* (1975)
L. enriettii		Amastigote	Inf guineapig	Macrophage	Starch PE	—	1 : 4	3 d	+	−	−	LM; SDS	
L. enriettii		Amastigote	Inf guineapig	Macrophage	Starch PE	Imm lymphocytes	1 : 4	3 d	+	−	−	LM; SDS	
L. enriettii		Amastigote	HGG imm guineapig	Macrophage	Starch PE	—	1 : 4	3 d	+	−	−	LM; SDS	
L. enriettii		Amastigote	HGG imm guineapig	Macrophage	Starch PE	HGG	1 : 4	3 d	+	−	−	LM; SDS	
L. enriettii		Amastigote	BCG imm guineapig	Macrophage	Starch PE	PPD	1 : 4	3 d	+	−	−	LM; SDS	
L. enriettii		Amastigote	Mouse	Macrophage	Starch PE	Ag + *T. gondii* Imm lymphocytes	1 : 4	3 d	−	−	+	LM; SDS	Behin *et al.* (1975)
L. enriettii		Amastigote	Mouse	Macrophage	Starch PE	MLC	1 : 3·5 1 : 7	4 d	−	−	+	LM; SDS	Mauel *et al.* (1978)
L. enriettii		Amastigote	Mouse	Macrophage	Starch PE	Con A + lymphocytes	1 : 3·5 1 : 7	4 d	−	−	+	LM; SDS	
L. enriettii		Amastigote	Mouse	Macrophage	Starch PE	Con A LK	1 : 3·5 1 : 7	4 d	−	−	+	LM; SDS	Buchmüller and Mauel (1979)

[a] Abbreviations: Ag, antigen; CGD, chronic granulomatous disease; Con A, concanavalin A; CSF, colony stimulating factor; EM, electron microscopy; GM CSF, granulocyte macrophage colony stimulating factor; H: P, host cell: parasite ratio; I, Indian strain; Imm, immune; Inf, infected; Leish, *Leishmania*; LK, lymphokine; LM, light microscopy; MLC, mixed lymphocyte culture; PE, peritoneal exudate; PPD, purified protein derivative; S, Sudanese strain; SDS, host cells ruptured with sodium dodecyl sulphate, parasites transformed to amastigotes and enumerated; Thiog, thioglycollate.

may, therefore, have been due to failure to achieve a sufficient degree of activation rather than that guinea pig macrophages lacked the ability to become cidal for *L. enriettii per se*. In fact, comparison of *in vitro* lymphokine activation of naive versus 'immune' elicited macrophages suggests that macrophages from infected animals do appear to have been partially activated *in vivo* (Poulter, 1976). The necessity for a high degree of activation to achieve cidal activity correlates with the *in vivo* requirement for prolonged inflammation at the site of the lesion before parasites are finally eliminated (Behin *et al.*, 1977). This might explain the development of a strong DTH reaction long before healing is achieved.

At the anergic end of the spectrum of host responses to leishmanial infection, in diffuse cutaneous leishmaniasis (DCL) and clinical visceral leishmaniasis (the *L. donovani* complex), development of metastatic lesions in the skin or viscera is associated with impaired cell-mediated immunity. Patients do not respond to parasite antigen in DTH skin tests *in vivo* (Southgate and Manson-Bahr, 1967; Bryceson, 1970; Rezai *et al.*, 1978) or in blastogenesis assays *in vitro* (Wyler *et al.*, 1979; Carvalho *et al.*, 1981b). In both cases the impaired response is specific for leishmanial antigen and is reversible with effective chemotherapy.

Conditions resembling DCL immunologically can be induced in the *L. enriettii*/guinea pig and *L. t. major*/mouse models by s.c. inoculation of high doses of promastigotes (Bryceson *et al.*, 1974; Preston *et al.*, 1978). In addition, inoculation of low doses of *L. enriettii* already within macrophages (Poulter, 1980) and intravenous inoculation of *L. t. major* promastigotes at a range of doses into resistant C57BL/6 mice (Scott and Farrell, 1982), also induce DCL-type responses. In all cases, the induced non-healing condition is associated with diminished DTH reactivity. Genetically controlled susceptibility of certain mouse strains to s.c. *L. t. major* promastigote injection (Howard *et al.*, 1980b; De Tolla *et al.*, 1981; cf. Section VI A 2, p. 117) also results in an extreme non-healing condition correlated with an analagous diminution of DTH (Nasseri and Modabber, 1979; Howard *et al.*, 1980a).

Various animal models of visceral leishmaniasis (*L. donovani* complex) have also been examined. In hamsters (reviewed by Stauber, 1963), fatal visceral infection, similar to that observed in man, occurs, but little is known about the associated immunological status of the host. It does, however, correlate with the ability of hamster macrophages to support parasite multiplication *in vitro* (Hawking, 1948; Herman, 1966; Chang, 1978a; Chang and Dwyer, 1976, 1978; Ebert *et al.*, 1979). In inbred strains of mice, a broad spectrum of genetically controlled host responses has been observed (see Section VI A 2, p. 117). As with cutaneous leishmaniasis, the ability of some strains to self-cure is T cell-mediated (Skov and Twohy, 1974a, b) and correlates with a positive DTH response (De Tolla *et al.*, 1980; Rezai *et al.*,

1980). This is paralleled in man where the mild East African form (Manson-Bahr *et al.*, 1959) and subclinical cases (Manson-Bahr, 1961; A. D. Niazi and D. J. Bradley, personal communication) of visceral leishmaniasis may also be associated with positive DTH reactivity. Early studies *in vitro* indicated that macrophages from super-infected self-curing mice destroyed *L. donovani* amastigotes *in vitro* (Miller and Twohy, 1969). Recent *in vivo* and *in vitro* studies using mice (Chang and Chiao, 1981; Haidairas and Bonventre, 1981; Murray, H. W. *et al.*, 1982) show that cell-mediated responses correlate with lymphokine activation of macrophages.

Further *in vitro* studies of *Leishmania* inside macrophages may also bear on the survival of the parasite *in vivo*. Handman and Burgess (1979) have shown that addition of colony stimulating factor (CSF) to adherent resident peritoneal macrophages infected 24 hours previously with *L. t. major* promastigotes, or pretreatment of macrophages with CSF for 24 hours before and during infection, induces rapid microbicidal activity. In experiments using suspension cultures of resident peritoneal macrophages treated with CSF for 4 hours before or during infection with *L. t. major* amastigotes, Ralph *et al.* (in press) were unable to demonstrate CSF-induced amastigoticidal activity. These differences may have been due to the stage of the parasite used although, in the case of the post-infection CSF treatment, it would have to be postulated that the 24 hour period between promastigote infection and addition of CSF was insufficient for complete intracellular transformation of promastigotes to amastigotes. Alternatively, the differing culture conditions (suspension versus adherent) might have been responsible. Nacy and Diggs (1981) have also shown that, whereas *L. t. major* amastigotes show little or no multiplication in adherent macrophage populations, suspension cultures support a 5–10-fold increase in the parasite population over 72 hours. This might relate to the previously observed differences in steady-state and inducible oxidative activity between suspension and adherent cultures (Cohen *et al.*, 1981). Whether increased oxidative activity is associated with CSF-induced leishmanicidal activity is unknown, although Moore *et al.* (1981) have shown that CSF-stimulated macrophages generate higher levels of oxygen metabolites in response to phorbol myristyl acetate (PMA). Interestingly, the work of Ralph *et al.* (in press) demonstrated that the effects of three separate lymphokine fractions (Nacy *et al.*, 1981) were clearly separable from the CSF effect.

The CSF induced leishmanicidal activity observed by Handman and Burgess (1979) may relate to a change in the cell cycle. In studies with bone marrow-derived macrophages Alexander (1981a) demonstrated that 'immature' rapidly dividing cells destroy *L. m. mexicana* amastigotes whereas 'mature' slowly dividing cells support parasite multiplication. After 7–10 days, renewed macrophage multiplication in the mature macrophage populations is

accompanied by further leishmanicidal activity. Reinfection of these cultures results in a similar cycle of parasite multiplication followed by killing which again correlates with cell cycle turnover. Inhibition of mitosis with vinblastin or colchicine leads to increased parasite growth. When cell division is arrested in G_1S with mitomycin C, parasites are rapidly killed. Leishmanicidal activity may therefore be enhanced at certain stages of the cell cycle. Since CSF stimulates macrophage multiplication, increased microbicidal activity may correlate with re-entry of the cells from G_0 into active cell cycling (Bray and Alexander, in press).

Survival of *Leishmania* promastigotes and amastigotes has also been examined in macrophages derived from human monocytes and in various cell lines. Pearson *et al.* (1981) found that promastigotes of *L. donovani* were phagocytosed, transformed to amastigotes and multiplied within human monocyte-derived macrophages. Berman *et al.* (1979) found that *L. donovani* and *L. t. major* amastigotes multiply within human monocyte-derived macrophages infected 5–6 days after *in vitro* culture. Using this *in vitro* system Berman and Neva (1981) were able to demonstrate that *L. t. major* amastigotes multiplied more rapidly at 35°C than at 37°C and were almost completely eliminated at 39°C. In contrast, *L. donovani* amastigotes multiplied equally well at 35°C and 37°C and only 40% were eliminated at 39°C. Berman and Neva suggested that the localization of the two strains cutaneously and in the viscera may therefore be explained at least partially by this inherent temperature sensitivity of the parasite–macrophage unit. *Leishmania donovani* amastigotes (Berens and Marr, 1979) and *L. m. amazonensis* amastigotes (Chang, 1980) have been maintained in longterm *in vitro* culture in P388D and J774 macrophage-like cell lines, respectively. Murray (1981b, 1982) has also demonstrated multiplication of promastigotes of *L. t. major* and *L. donovani* and amastigotes of *L. donovani* in the J774 cell line. The potential use of these and other macrophage cell lines in leishmaniasis research is currently being explored (Bray and Alexander, in press).

3. *Giardia*

Giardia lamblia is an intestinal parasite infecting 2 to 25% of the world's population (Rendtorff, 1954; Faust *et al.*, 1970). Infection elicits specific serum IgG to surface antigens of *G. lamblia* trophozoites, which may play a role in resistance to infection by mediating complement fixation, opsonization, or cytotoxicity of the organism (Visvesvara *et al.*, 1980; Smith *et al.*, 1981). Smith and coworkers (1982) have also investigated the capacity of human peripheral blood effector cells to kill *G. lamblia* trophozoites *in vitro*. Isolated mononuclear leukocytes—granulocytes, lymphocytes and monocytes—were cultured for 16 hours with trophozoites which had been pulsed with [^{3}H]-thymidine. Spontaneous cytotoxicity for *G. lamblia* was observed at effector:

target cell ratios of 3:1 (13%) to 100:1 (56%) and was ablated by removal of the adherent cell (monocyte) population.

Humoral and cell-mediated responses to the self-limiting murine infection, *G. muris*, have also been studied in resistant and susceptible strains of mice (Section VI A 3, p. 119).

4. *Trichomonas*

The large flagellated extracellular protozoon *T. vaginalis* is a common parasite of the human urinogenital tract. Infection may elicit a profuse, acute, inflammatory discharge containing polymorphonuclear neutrophils. Rein and coworkers (1980) have shown that neutrophils isolated from the peripheral blood of uninfected antibody-negative humans are naturally cytotoxic to *T. vaginalis* at host cell:parasite ratios ranging from 1000:1 to 10 000:1. Killing was extracellular with fragments of the protozoon subsequently phagocytosed and digested by the neutrophils. Neutrophils killed trichomonads in fresh or absorbed serum but not in bovine serum albumin, in heat-inactivated serum, or in the presence of 1 mM trypan blue. This suggests a role for complement activated via the alternative pathway. The ability of *T. vaginalis* to activate complement by this pathway has been confirmed by Gillen and Sher (1981).

Landolfo and coworkers (1980) have also demonstrated natural cell-mediated cytotoxicity against *T. vaginalis* in the mouse. Resident lymphoid cells from the peritoneal cavity showed higher levels of cell-mediated cytotoxicity than cells from the bone marrow, spleen, lymph nodes, peripheral blood or thymus. Alveolar cells displayed high but variable levels of reactivity. Passage through nylon wool columns or adherence to a plastic surface removed the cytotoxic activity in the non-adherent population. Double treatment with carbonyl iron powder and a magnet also ablated the response. The cytotoxic response was unaffected by treatment of the cells with antisera to the lymphocyte surface antigens thy1,2 or Ia in the presence of complement, and was resistant to various doses of X-irradiation up to 2000 rads. The results suggest, therefore, that the natural cytotoxicity observed is mediated by a macrophage or macrophage-like cell.

B. SPOROZOEA

1. *Toxoplasma*

Toxoplasma is an obligate intracellular protozoon. Accidental infection with *T. gondii* is associated with marked tissue necrosis and an inflammatory infiltrate which contains mononuclear cells and, less commonly, neutrophils (Jones, 1980). In humans the infection is usually acquired by ingesting oocysts on contaminated food or otherwise and, in immunologically normal

individuals, is asymptomatic or clinically insignificant. All the work reviewed in this and subsequent sections refers to the endozoite (=tachyzoite) stage of *T. gondii*.

The ability of peritoneal macrophages from mice, rabbits, rats and guinea pigs to kill *T. gondii in vitro* was observed by Vischer and Suter (1954) by light microscopy of haematoxylin-stained preparations. Stainable *T. gondii* disappeared from the macrophage during the first 8 hours after infection. Those *T. gondii* that escaped the cytotoxic activity of the macrophages were, however, able to recover, divide and multiply until up to 8, or even 16, protozoa were present in a vacuole in the cell. The host cell then ruptured and the released *Toxoplasma* were free to infect further cells. Cell layers were covered with formvar to prevent loss of released *Toxoplasma* from the immediate environment. If the ratio of mouse macrophages to *T. gondii* was 5:3, extracellular protozoa in detectable numbers were seen after 19 hours. If the ratio was increased to 10:1, detectable release took about 43 hours. At a ratio of 20:1, all the protozoa were killed. Macrophages from rats, guinea pigs and rabbits were more active than those from mice. They phagocytosed *T. gondii* more rapidly, and it took longer for *T. gondii* to recover and multiply in macrophages from these animals. This correlated with greater sensitivity of mice than other species to infection with *T. gondii*. *Toxoplasma* survived less readily in macrophages from immune rats and guinea-pigs than in those from non-immune animals, and survival was reduced still further by the presence of specific antiserum (Table 4).

Investigation by electron microscopy showed that normal mouse macrophages kill about 50% of internalized *T. gondii* in the absence of antiserum (Jones *et al.*, 1972) and about 90% after coating with antibody (Jones *et al.*, 1975) (Table 5). It is of interest that after antibody-coating, *T gondii* loses its ability to enter HeLa cells or fibroblasts and is taken up only by macrophages. A number of different monoclonal antibodies against *T. gondii* render the trophozoites sensitive to killing by normal mouse macrophages (Sethi *et al.*, 1981; Hauser and Remington, 1981). In the absence of antibody, macrophages from chronically infected mice are microbistatic, without killing the ingested parasites; but after an intraperitoneal boost with *T. gondii* antigen, the macrophages display microbicidal activity, which lasts for 48 hours *in vitro* (Jones *et al.*, 1975).

An alternative method for studying the survival and replication of *T. gondii* in macrophages has been measurement of the incorporation of [^{3}H]deoxyuridine or [^{3}H]uracil into protozoal nucleic acids, either by scintillation counting or by autoradiography. A number of laboratories has used this technique to demonstrate and measure the survival and multiplication of *T. gondii* in macrophages (Table 4). Remington and coworkers (1972) showed that macrophages from mice infected with either *T. gondii* or *Bes-*

TABLE 4

Cell-mediated killing of Toxoplasma gondii[a,b].

Species	Effector cell Cell-type	Source	Treatment	Incubation E : T	Incubation Time	Killing Assessment	Reference
Rabbit	Macrophage	Casein PE	Inc 1 h	7 : 1	8 h	LM +	Vischer and Suter (1954)
Rat	Macrophage	Casein PE	Inc 1 h	7 : 1	8 h	LM +	
Guinea pig	Macrophage	Casein PE	Inc 1 h	7 : 1	8 h	LM +	
Mouse SW	Macrophage	Casein PE	Inc 1 h	7 : 1	8 h	LM +	
Mouse CFW	Macrophage	Resident P	Inc 24 h	1 : 2	3 h	EM 50%	Jones *et al.* (1975)
Mouse CFW	Macrophage	Resident P	Inc 24 h	1 : 2 +Ab	3 h	EM 90%	
Mouse SW	Macrophage	Resident P	Inc 1–3 h	1 : 1	2–4 h	LM 35%	McLeod *et al.* (1980·
Inf mouse	Macrophage	PE post Ag		1 : 1	24 h	LM ++	Murray *et al.* (1979)
Inf mouse	Macrophage	Resident P		1 : 2	18 h	LM +	Reikvam *et al.* (1975)
Inf hamster	Macrophage	PE post Ag		10 : 1	24 h	LM ++	Lindberg and Frenkel (1977)
Hamster	Macrophage	Casein PE	+sp LK	10 : 1	24 h	LM ++	
Man	Monocyte	Blood	Inc 1–4 h	1 : 1	2–4 h	LM 80%	McLeod *et al.* (1980)
Man	Macrophage	Blood	Inc 4 d	1 : 1	2–4 h	LM 80%	
Man	Macrophage	Spleen		1 : 1	2–4 h	LM 80%	
Man	Neutrophil	Blood		1 : 1	24 h	LM 50%	Wilson and Remington (1979)

[a] Abbreviations: EM, electron microscopy; E : T, effector: target cell ratio; LK, non-specific lymphokine; LM, light microscopy; PE, peritoneal exudate; Sp LK, parasite-specific lymphokine.

[b] All organisms were endozoites (=tachyzoites) of strain RH.

TABLE 5

Survival of Toxoplasma gondii *in macrophages* [a,b]

Species	Immune status	Source	Treatment	Assessment	Survive	Multiply	Reference
Mouse	Non-immune	Casein PE		LM (H)	+	+	Vischer and Suter (1954)
Mouse	Non-immune	Resident P		EM	50%	+	Jones *et al.* (1972)
Mouse	Non-immune	Resident P	Ab-coated target	EM	90%		Jones *et al.* (1972)
Mouse	Non-immune	Resident P	Sp LK	PCM	+	+	Jones *et al.* (1975)
Mouse	Infected *T. gondii*	Resident P		LM (G; AO)	+	−	Murray *et al.* (1979);
Mouse	Infected *T. gondii*	Resident P	Sp LK 24 h	LM (G; AO)	+	−	Jones *et al.* (1975)
Mouse	Infected *T. gondii*	Heat-killed *T. gondii* PE		LM (G; AO)	−	−	Murray *et al.* (1979)
Mouse	Non-immune	Resident P		[^{3}H]deoxy Ur		+	Remington *et al.* (1972);
Mouse	Infected *T. gondii*	Resident P		[^{3}H]deoxy Ur		−	McLeod and Remington (1977)
Mouse	Infected *B. jellisoni*	Resident P		[^{3}H]deoxy Ur		−	McLeod and Remington (1977)
Mouse	Infected *C. parvum*	Resident P		[^{3}H]deoxy Ur		−	McLeod and Remington (1977)
Mouse	Treated complete Freund's adjuvant	Resident P		[^{3}H]deoxy Ur		+/−	Remington *et al.* (1972)
Hamster	Non-immune	Casein PE		LM (G)	+	+	
Hamster	Non-immune	Casein PE	Ab-coated target	LM (G)		−	Lindberg and Frenkel (1977)
Hamster	Infected *T. gondii*	*T. gondii* PE		LM (G)		−	Lindberg and Frenkel (1977)
Hamster	Non-immune	Resident P	Sp LK	LM (G)		−	Lindberg and Frenkel (1977)
Hamster	Non-immune	Resident P	*B. jellisoni* LK	LM (G)		+	Lindberg and Frenkel (1977)
Rabbit	Non-immune	Resident P		LM (H)	+	+	Vischer and Suter (1954)
Guinea pig	Non-immune	Resident P		LM (H)	+	+	Vischer and Suter (1954)
Guinea pig	Infected *T. gondii*	Resident P		LM (H)		+/−	Vischer and Suter (1954)
Guinea pig	Infected *T. gondii*	Resident P	Ab-coated target	LM (H)		−	Vischer and Suter (1954)
Rat	Non-immune	Resident P		LM (H)	+	+	Vischer and Suter (1954)
Rat	Infected *T. gondii*	Resident P		LM (H)		+/−	Vischer and Suter (1954)
Rat	Infected *T. gondii*	Resident P	Ab-coated target	LM (H)		−	Vischer and Suter (1954)

Species	Immune status	Source	Treatment	Assessment	Survive	Multiply	Reference
Man	Non-immune	Blood		PCM	+	+	
Man	Non-immune	Blood	Ab-coated target	PCM	+	+	Borges and Johnson (1975)
Man	Non-immune	Blood	Sp LK	PCM		−	
Man	Non-immune	Blood	TB LK	PCM	+	+	
Man	Non-immune	Blood	Con A	PCM	+	+	
Man	Non-immune	Blood	Ab-coated target	[^{3}H]deoxy Ur		−	Anderson and Remington (1974)
Man	Non-immune	Blood	Sp LK	[^{3}H]deoxy Ur		−	
Man	Non-immune	Blood	Streptococcal LK	[^{3}H]deoxy Ur		+/−	
Man	Non-immune	Blood		[^{3}H]deoxy Ur	+	+	McLeod *et al.* (1980)
Man	Non-immune	Blood	Poly IC	[^{3}H]deoxy Ur		−	
Man	Infected *T. gondii*	Blood		[^{3}H]deoxy Ur	20%	+	
Man	Non-immune	Spleen		[^{3}H]deoxy Ur	20%	+	

[a] Abbreviations: Ab, antibody; AO, acridine orange; Con A, concanavalin A; EM, electron microscopy; G, Giemsa's stain; H, haematoxylin; LK, lymphokine; LM, light microscopy; PCM, phase contrast microscopy; PE, peritoneal exudate; Poly IC, polyinosinic-polycytidylic acid; Sp LK, parasite-specific lymphokine; TB LK, tuberculosis lymphokine; Ur, uridine.

[b] All organisms were endozoites (=tachyzoites) of strain RH.

noitia jellisoni had an enhanced capacity to kill *T. gondii*, and even Freund's complete adjuvant gave partial activation. These authors also used a plaque method for detecting survival, whereby the infected macrophage layer was overlaid with mouse embryo fibroblasts. Surviving *T. gondii* which were released from the macrophages entered and destroyed the overlying fibroblasts and produced a detectable plaque. McLeod and Remington (1977) also showed that infection with *Corynebacterium parvum* endowed macrophages with an enhanced ability to prevent the growth of *T. gondii*, and that irradiated *T. gondii* had some inducing capacity as long as they persisted in the infected animal. While these macrophages appeared to be activated non-specifically to control protozoa *in vitro*, only specific immunization protected the animal *in vivo*.

Peritoneal macrophages from hamsters have only a limited ability to control *T. gondii* as assessed by light microscopy of Giemsa stained preparations, but this is greatly enhanced if macrophages from immune animals are used (Hoff and Frenkel, 1974; Lindberg and Frenkel, 1977). Unlike the situation in mice, macrophages active against *T. gondii* can be induced specifically only by infection with *T. gondii*. This specificity of immunity was demonstrated both *in vivo* and *in vitro*. Lindberg and Frenkel (1977) also investigated different methods of eliciting macrophages and concluded that, if specific antigen is injected into the peritoneum of *Toxoplasma*-immune hamsters together with sodium caseinate, the macrophages become active more rapidly than if caseinate alone is used.

Human peripheral blood monocytes and, to a lesser extent, derived macrophages were shown to have a greater capacity to kill internalized *T. gondii* than mouse macrophages (McLeod *et al.*, 1980) (Table 5). However, those organisms that do survive in human cells proceed to multiply, divide and incorporate [^{3}H]deoxyuridine (Table 4). No difference in cytotoxic activity was detected between monocytes from infected and uninfected individuals. Spleen monocytes are as active as blood monocytes. Blood neutrophils have some activity, but this is much lower than the activity of mononuclear cells (Wilson and Remington, 1979; McLeod *et al.*, 1980) (Table 5). The increased susceptibility of human neonates to *T. gondii* cannot be explained by deficiencies in monocyte function since no difference was found between monocytes from adults and neonates (Berman and Johnson, 1978).

The mechanism of macrophage activation can be explained, at least partially, by the action of lymphocyte products. This will be discussed in more detail later (Section IV E, p. 103). A role for lymphocytes was first suggested by the observation of Frenkel (1967) that intact cells from spleen and lymph nodes transfer specific immunity to *T. gondii* and *B. jellisoni* in hamsters. When isolated macrophages are incubated *in vitro* for 3 hours with lymphocytes from animals infected with *T. gondii* together with *T. gondii* antigen they have an enhanced ability to prevent growth of *T. gondii*, but not of *B. jellisoni*

(Lindberg and Frenkel, 1977). Treatment with cortisol *in vivo* (20 mg per animal) and *in vitro* (5 μg ml^{-1}) inhibited this ability of lymphocytes specifically to 'arm' macrophages, but higher levels (20 μg ml^{-1}) were required to inhibit macrophage function.

In mice and in man the activation of macrophages by lymphocytes may be less specific than in hamsters (McLeod and Remington, 1977; Anderson and Remington, 1974). When human macrophages are incubated with sensitized lymphocytes and either the corresponding antigen from *T. gondii* or streptokinase-streptodornase they become more active against *T. gondii*. Supernatants from lymphocytes incubated with the corresponding antigen contain non-specific macrophage activating activity (Anderson *et al.*, 1976). However, Borges and Johnson (1975) found that human monocytes could be activated to kill *T. gondii* only by the specific supernatant from immune T-lymphocytes incubated with *T. gondii* antigen, and not by the supernatant from T-lymphocytes from tuberculin-positive subjects incubated with PPD (purified protein derivative), nor from lymphocytes stimulated with concanavalin A. Specific lymphokine was formed *in vitro* in 15 minutes and monocyte activation was complete in 2 hours. Lymphocytes from an infected individual produced lymphokine in response to *T. gondii* antigen only 7 months after the initial infection, by which time he had recovered from this illness, presumably by some other means (Johnson, 1981).

2. *Plasmodium*

Plasmodium is vulnerable to cell-mediated attack both during the intra-erythrocytic stage and in the brief time when it exists as a free merozoite, liberated from one red cell and before invasion of another. Macrophages have been implicated as cytotoxic cells which attack both free merozoites and parasitized erythrocytes. Enlarged populations of macrophages are seen after plasmodial infection (Singer, 1954), particularly in the liver and spleen. These macrophages were observed *in vivo* to have avidly phagocytosed both free parasites and parasitized erythrocytes 1 week after infection (Taliaferro and Canon, 1936; Singer, 1954). On reinfection, or with a massive infecting dose, phagocytosis is seen within 1 hour.

The phagocytosis of free merozoites of *P. berghei* by rat peritoneal macrophages *in vitro* was investigated by electron microscopy by Brooks and Kreier (1978). They reported that the presence of a thick capsule on the merozoite inhibits phagocytosis, but that this antiphagocytic surface coat can be neutralized with opsonizing antibody. Free merozoites of *P. falciparum* are phagocytosed by blood neutrophils in humans with established infection (Trubowitz and Masek, 1968).

Plasmodium-infected erythrocytes are phagocytosed by monolayers of mouse peritoneal macrophages *in vitro* (Table 6), but again only in the presence of specific antiserum (Brown, 1971; Tosta and Wedderburn, 1980)

TABLE 6

Cell-mediated killing of Plasmodium *and* Babesia *species*[a]

Organism		Effector cell			Incubation		Killing Assessment		
Species	Stage	Species	Cell-type	Source	E : T	Time			Reference
P. brasilianum	Ic and free merozoite	Monkey	Macrophage	Liver; spleen	*In vivo*	7 d	LM	Ph	Taliaferro and Cannon (1936)
P. berghei	Ic and free merozoite	Mouse	Macrophage	Liver; spleen	*In vivo*	6 d	LM	Ph	Singer (1954)
P. berghei	Free merozoite	Rat	Macrophage	Resident P	+Ab	15 m	EM	Ph	Brooks and Kreier (1978)
P. berghei	Ic	Imm mouse	Macrophage	Resident P		15 m	LM + EM	Ph	Seitz *et al.* (1977)
P. berghei	Ic	Imm mouse	Macrophage	Spleen	+Ab	45 m	LM	Ph	Seitz *et al.* (1977)
P. falciparum	Merozoite	Imm man	Neutrophil	Blood	*In vivo*	—	LM	Ph	Trubowitz and Masek (1968)
P. falciparum	Ic	Imm man	Neutrophil	Blood	10:1	24 h	LM	50%	Brown and Smalley (1981)
P. knowlesi	Ic	BCG mouse	Macrophage	Resident P	+Ab	2 h	LM	Ph	Brown (1971)
P. yoelii	Ic	Mouse	Macrophage	Resident P	1 : 5 + Ab	45 m	LM	Ph	Tosta and Wedderburn (1980)
P. yoelii	Ic	Mouse	Cells	Spleen	1000:1	16 h	Infect	80%	Taverne *et al.* (1982)
P. yoelii	Ic	Mouse	Macrophage	Resident P	80:1	16 h	Infect	97%	Taverne *et al.* (1982)
P. yoelii	Ic	Mouse	Macrophage	Resident P	90:1 + Ab	16 h	Infect	99·9%	Taverne *et al.* (1982)
P. yoelii	Ic	Mouse	Monocytes & neutrophils	Blood	50:1	16 h	Infect	99%	Taverne *et al.* (1982)
P. yoelii	Ic	Mouse	Monocytes & neutrophils	Blood	50:1 + ILC	16 h	Infect	99.7%	Taverne *et al.* (1982)
B. microti	Ic and free merozoite	Hamster	Macrophage	Resident P	+Ab	48 h	LM	Ph	Bautista and Kreier (1980)
B. microti	Ic and free merozoite	Imm hamster	Macrophage	Resident P		48 h	LM	Ph	Bautista and Kreier (1980)
B. rodhaini	Ic	Rat	Macrophage	Resident P	50:1 + Ab	2 h	LM	Ph	Rogers (1974)

[a] Abbreviations: Ab, antibody; EM, electron microscopy; E : T, effector: target cell ratio; Ic, intraerythrocytic forms; ILC, immune lymphocytes; Imm, immune; Infect, infectivity; LM, light microscopy; P, peritoneal; Ph, phagocytosis.

or if the macrophages are from immune mice (Seitz *et al.*, 1977) It has been well established that parasitized erythrocytes have a modified antigenic surface. New surface antigens of protozoal origin have been identified (Schmidt-Ullrich *et al.*, 1979) and shown to be recognized by circulating immunoglobulins. Phagocytosis of parasitized erythrocytes is inhibited by protein A (Shear *et al.*, 1979). Brown (1971) showed that peritoneal macrophages from BCG-infected mice have a greatly enhanced phagocytic activity towards antibody-coated *P. knowlesi*-infected erythrocytes. This may be one reason why Brooks and Kreier (1978) had difficulty in demonstrating phagocytosis by normal rat macrophages of rat erythrocytes infected with *P. berghei in vitro*, with or without antiserum. Prior infection of mice with *P. chabaudi* or with *P. vinckei* enhances the phagocytic activity of macrophages towards carbon particles (Lucia and Nussenzweig, 1969).

The killing of intra-erythrocytic *P. yoelii in vitro* by both macrophages and neutrophils has been demonstrated by Taverne *et al.* (1982). Parasite-infected blood was incubated with partially purified leucocytes for 16 hours. Loss of parasite viability was assessed by reinjection into mice and measurement of parasitaemia. Neutrophils and macrophages from blood and from the peritoneal cavity had similar cytotoxic activities, but eosinophils appeared to be relatively unimportant. Eosinophils have only a limited ability to phagocytose antibody-coated *P. yoelii* (Tosta and Wedderburn, 1980). It is possible that neutrophils react more rapidly than macrophages, but this would not have been detectable after 16 hours incubation. The activity of the peritoneal macrophages was enhanced by addition of immune serum. An absolute requirement for antibody was not demonstrated, but the possibility that immunoglobulin was introduced into the incubation mixture together with the infected blood which was the source of parasites cannot be excluded. Addition of lymph node cells from immune animals enhanced the activity of blood leucocytes, presumably by a lymphokine-mediated activation of the monocytes. Peripheral blood cells were more active than peritoneal cells, which were more active than spleen cells. *In vivo*, spleen and liver macrophages were reported to be the most active against *P. berghei* (Singer, 1954) and *P. brasilianum* (Taliaferro and Cannon, 1936).

These experiments *in vitro* help to explain the observations *in vivo* that protective immunity can be transferred with both B and T lymphocytes, but is maximal with both (Graveley and Kreier, 1976). The role of B lymphocytes would be to produce specific antibody to opsonize both free merozoites and parasitized erythrocytes, and thereby allow uptake into macrophages. T lymphocytes may act both as helper cells and as a source of lymphokine for macrophage activation. Monkeys show faster antibody-producing responses to later infections with antigenic variants of *P. knowlesi* than they do to the first infection (Brown, 1971). Brown ascribes this more rapid response to new

antigens to the presence on the parasitized erythrocyte of common antigenic determinants which pre-sensitize responder lymphocytes to react rapidly when a new variant antigen is presented simultaneously with the familiar common antigen. Helper T cells may be involved. Some common antigens also occur on the merozoite surface (Langreth and Reese, 1979). Macrophages activated by plasmodial infection or with BCG have an enhanced capacity to kill *Plasmodium* spp. T lymphocytes from animals infected with *Plasmodium* will respond to a new infection by secreting lymphokine, which will activate macrophages and thereby enhance killing.

Optimal cell-mediated immunity to *Plasmodium* requires macrophages, specific antibody-secreting B lymphocytes and pre-sensitized T lymphocytes. T cells are required to facilitate the response of B cells to new antigenic variants and to secrete lymphokine, in response to plasmodial antigen, for macrophage activation.

3. *Babesia*

Rogers (1974) observed antibody-dependent phagocytosis of *B. rodhaini*, both as free merozoites and within erythrocytes, by rat macrophages. Bautista and Kreier (1980) showed that free merozoites of *B. microti* and parasitized hamster erythrocytes disappear during culture for a few days with hamster macrophages, either in the presence of immune serum, or if macrophages from immune animals are used.

Cell-free supernatants from cultures of *B. microti* incubated either with immune macrophages, or with normal macrophages and immune serum, also inhibit the growth of *B. microti* (Bautista and Kreier, 1980). Clark *et al.* (1977) suggest that activated macrophages produce soluble factors which can kill both *Babesia* and *Plasmodium*.

The inability of *nu/nu* mice to control *B. microti* infections (Clark and Allison, 1974), and the transfer of immunity to naive mice with lymph nodes and with spleen cells from infected mice (Ruebush and Hanson, 1980), implicate T lymphocytes in immunity to *Babesia*.

4. *Theileria*

Protozoa of the genus *Theileria* produce diseases, for example East Coast fever (*T. parva*) and corridor disease (*T. lawrencei*), which cause the death of several million cattle in East and Central Africa each year. Sporozoites are introduced into the skin with saliva from the tick vector and invade the lymphoid cells, where they appear as macroschizonts in the cytoplasm. The subject of cell-mediated and humoral immunity in theileriosis was reviewed recently by Allison and Eugui (1980).

Theileriosis is interesting because, like Epstein Barr virus, the parasites transform lymphoid cells into cells with uncontrolled proliferation. There is

widespread infiltration of transformed cells into the tissues and a strong cell-mediated immune response develops. In primary infections, cells capable of lysing allogeneic infected cells appear, which may be analogous to natural killer cells observed in other host species. In immune animals, rapid proliferation of clones of specific cytotoxic T cells, capable of lysing syngeneic infected cells, is observed.

C. LOBOSEA

1. *Naegleria*

Naegleria fowleri, a free-living amoeboflagellate found in most soil and fresh water habitats, produces fatal meningoencephalitis in humans and laboratory animals. Although humans are frequently exposed to the organism, very few clinical cases have been reported. Mice immunized with live or formalinized amoebae are protected against subsequent challenge (John *et al.*, 1977) and, since transfer of immune serum confers protective immunity (Thong *et al.*, 1978), humoral immunity is implicated. Interestingly, however, experiments *in vitro* have shown that *N. fowleri* is not killed by antibody plus complement and that it can free itself from surface-bound antibody (Ferrante and Thong, 1979; Section V C 2, p. 114). Hence other immunological mechanisms in addition to antibody and complement may be involved *in vivo*. Guinea pigs inoculated subcutaneously have been shown to develop delayed hypersensitivity reactions in response to parasite antigen (Diffley *et al.*, 1976). Little is known, however, about the possible role of T cell-mediated immune mechanisms. In a recent paper Ferrante and Thong (1980) examined neutrophil-mediated killing of *N. fowleri in vitro*. In addition to the usual contact-mediated lysis of these large amoebae, a unique phagocytic process was observed where a portion of amoeba was pinched off after an apparently unsuccessful attempt to phagocytose the whole amoeba. The role of neutrophil-mediated killing *in vivo* has not been evaluated.

IV. MECHANISMS OF KILLING

A. ASSESSMENT OF INTRACELLULAR DEATH

Numerous methods have been used to measure the killing activity of cytotoxic cells and of their isolated enzymes. The most direct demonstration of the death of protozoa is the loss of their ability to infect, multiply in and ultimately kill a susceptible animal. The disadvantage of this method is that, like many biological assays, it is difficult to measure, slow and expensive in animals. In addition, it is possible that certain treatments of protozoal cells

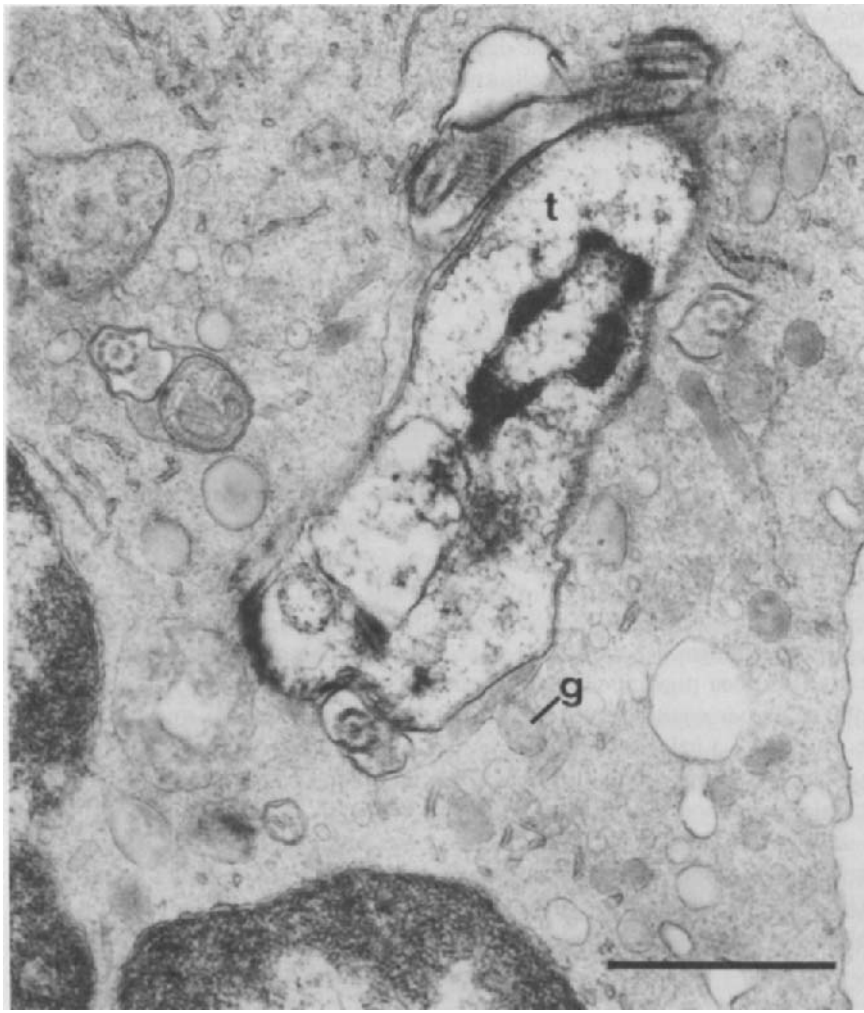

FIG. 1. Killing of antibody-coated trypomastigote (t) of *T. dionisii* by human blood neutrophil. A neutrophil lysosomal granule (g) has fused with the phagocytic vacuole. Incubation time 2 hours. Bar line 1 μm.

may eliminate their infectivity without directly impairing viability, for example by increasing their susceptibility to the host immune responses.

An '*in vitro*' biological assay for *T. gondii* was developed by Remington *et al.* (1972). Infected mouse macrophages are overlaid with mouse embryo

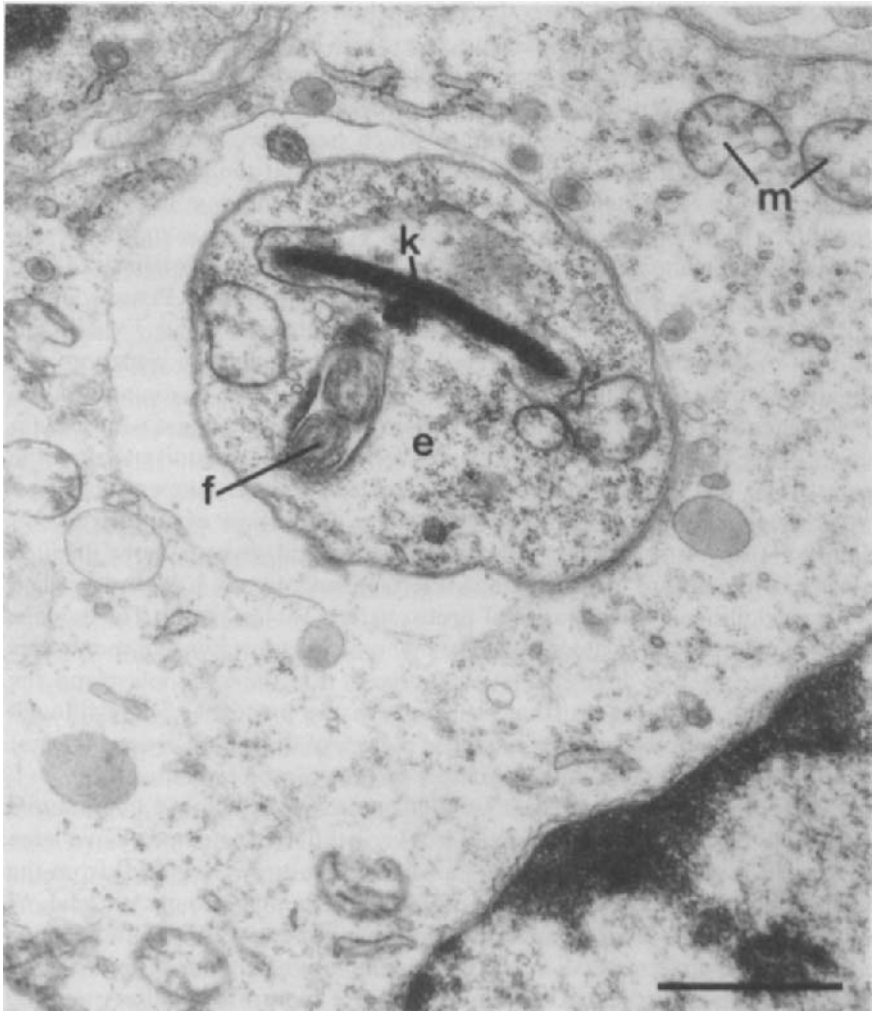

FIG. 2. Killing of antibody-coated epimastigote (e) of *T. dionisii* by human blood monocyte. The most resistant organelles in the epimastigote are the kinetoplast (k) and the flagellum (f). The monocyte is itself damaged during the interaction and has swollen mitochondria (m). Incubation time 5 minutes. Bar line 1 μm.

fibroblasts. Surviving *T. gondii*, which are released from the macrophages, enter and destroy the overlying fibroblasts and produce a detectable plaque.

An alternative method of measuring viability is direct observation of growth in cells infected *in vitro*, or occasionally *in vivo*, by light microscopy of

stained samples, taken at intervals. This method gives a quantitative measure of the ability of intracellular parasites to grow in cytotoxic cells, but does not necessarily distinguish between microbistatic and microbicidal activity. Sometimes, however, dead parasites disappear and are replaced by debris.

Loss of motility is another feature which has been used to assess cell death. It is important with this method to check that the loss of motility is irreversible since flagellates will sometimes stop moving under adverse conditions. For example, trypomastigotes of *T. dionisii* remain stationary in a protein-free medium, but recover their motility when protein is added (K. J. I. Thorne and D. Franks, unpublished observation). Phase-contrast microscopy is used to observe motility and it is interesting that *T. cruzi* and *T. dionisii* can be seen moving even within macrophages, and continue moving until they are killed (Dvorak and Schmuñis, 1972; Liston and Baker, 1978). Mauel *et al.* (1978) released *L. enriettii* from macrophages with sodium dodecyl sulphate and counted the number of motile organisms after the amastigotes had transformed to promastigotes at a lower temperature.

Although light microscopy can give some indication of ultrastructural damage, electron microscopy is more sensitive and gives a more detailed picture of the nature of the damage. Electron microscopy has been used to study the killing of a wide range of protozoa (see Tables 2 to 6). The damage inflicted on antibody-coated *T. dionisii* by human neutrophils and monocytes is illustrated in Figs 1 and 2. It is of interest that the kinetoplast and the flagellum are the most resistant structures in the protozoal cell. Although electron microscopy gives a very detailed picture of the killing process it is not quantitative since only a limited number of cells can be examined.

Radioactive isotopes have been used in an attempt to obtain a quantitative measure of cell-mediated killing. Two very different methods have been developed. Either the ability of the parasites to multiply is assessed from the level of incorporation of [^{3}H]deoxyuridine or [^{3}H]uracil into nucleic acid, or their viability is assessed from their ability to retain incorporated radioactive isotopes. The first method is used in combination with light microscopy and is a more sophisticated version of counting the protozoa after staining, but with the advantage that dead cells do not incorporate isotope. Incorporated radioactivity is determined either by autoradiography and grain counting, or by scintillation counting of dissolved samples. It gives a measure of the number of living protozoa and, if continued over a period of time, their ability to proliferate. This method has proved particularly useful in studying the survival of *T. gondii* in macrophages (e.g., by Remington *et al.*, 1972).

Retention of isotope has been assayed in protozoa prelabelled with [^{3}H]-uridine, [^{99m}Tc]pertechnetate, or [^{32}P]phosphate. The use of [^{3}H]uridine to prelabel ribonucleic acid (RNA) was developed by Sanderson and coworkers

(1978) into a sensitive assay for cell-mediated cytotoxicity of *T. cruzi*. Release of ^{3}H-labelled RNA was detectable within an hour after addition of rat spleen cells to antibody-coated *T. cruzi*. As little as 10 μCi of [5-^{3}H]uridine (40–50 Ci mmol^{-1}) was added to 10^{6} parasites and after 5 days they had incorporated at least 10 c.p.s. per 10^{5} parasites. About 95% of this radioactivity was released when they were lysed by freezing and thawing, while spontaneous release was less than 20% in a 4 hour incubation. This method has also been used for studying cell-mediated cytotoxicity to epimastigotes of *T. theileri* (Townsend and Duffus, 1982) and trypomastigotes of *T. dionisii* (Thorne *et al.*, 1981) and cultured *T. brucei* (D. Paton and D. Franks, personal communication).

[^{99m}Tc]Pertechnetate is a metastable gamma emitter of high specific activity, with a half life of 6 hours. In the presence of unlabelled sodium chromate it is taken up and retained by certain cells where it forms relatively stable chelates with protein (Barth *et al.*, 1977). The loss of ^{99m}Tc from damaged protozoa has been used by Mkwananzi *et al.* (1976) and by Thorne and coworkers (1979) to study cell-mediated cytotoxicity to epimastigotes of *T. dionisii*. Uptake is rapid and labelling is high, but not all protozoa retain the isotope and, notably, trypomastigotes of *T. dionisii* could not be labelled by this method (Thorne *et al.*, 1981).

[^{32}P]Orthophosphate was used by Ferrante and Jenkin (1979) to prelabel *T. lewisi*. Loss of label could be detected after incubation with rat or mouse macrophages.

Since cell-mediated killing occurs within a phagocytic vacuole the radioactive isotope released from damaged protozoa must escape from the effector cells before it can be detected in the medium. Electron microscopy reveals that monocytes and neutrophils become damaged themselves after they have killed internalized protozoa (see Figs 2, 3, 5). Sufficient loss of structural integrity of the effector cell may occur to allow leakage of radioactive protozoal macromolecules out into the medium. The apparent time lag in release of radioactive protozoal macromolecules of about 1 hour after uptake by neutrophils, and several hours after uptake by mononuclear cells, may reflect the time necessary for the isotope to escape from the effector cell and may not, therefore, be a reflection of the relative trypanosomicidal activity of the two cells. Direct incubation of *T. dionisii* with lysosomal enzymes from neutrophils results in rapid release of isotope (Thorne *et al.*, 1979, 1981). It is interesting that Townsend and Duffus (1982) detected release of [^{3}H]RNA from *T. theileri* within 15 minutes after adding bovine neutrophils. This protozoon is very large (130 μm) and therefore difficult to phagocytose, and it appears that it can be killed before complete closure of the phagocytic vacuole, which allows early detection of isotope release (Fig. 4).

B. OXIDATIVE REACTIONS

The phagocytosis of bacteria by neutrophils is accompanied by an increased production of hydrogen peroxide (Paul and Sbarra, 1968). This hydrogen peroxide is believed to be generated by NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) oxidase located in the neutrophil plasma membrane (DeChatelet, 1978), and it can be subsequently detected in the phagocytic vacuole (Briggs *et al.*, 1977; Chang, 1981a). Hydrogen peroxide together with myeloperoxidase from neutrophil granules and a halide forms a potent anti-bacterial system (Klebanoff, 1968). The role of this system and of other reactive oxygen intermediates in the killing of protozoa by neutrophils and macrophages has been explored in some detail.

1. *Generation of reactive oxygen intermediates*

Molecular oxygen is reduced by NADPH oxidases to the superoxide radical O_2^- (Fig. 6). NADPH oxidase is a plasma membrane enzyme with a flavin cofactor, which generates superoxide in a one-electron transfer (Light *et al.*, 1981). A cytochrome *b* of redox potential —235 mV associated with the partially purified enzyme is not reduced by NADPH but may participate in the univalent reduction of oxygen (Segal and Jones, 1978; Light *et al.*, 1981). Superoxide is converted either spontaneously into hydrogen peroxide and oxygen, some of which may be in the singlet state (Khan, 1970) or, in the presence of superoxide dismutase (SOD), into hydrogen peroxide and stable triplet oxygen (McCord and Fridovich, 1968). Below pH 6 further NADPH is apparently oxidized non-enzymically with the formation of hydrogen peroxide in a self-sustaining chain reaction, possibly initiated by superoxide (Bellavite *et al.*, 1980; Suzuki and Lehrer, 1980; DeChatelet and Shirley, 1981), and enhanced by divalent manganese ions, Mn^{2+} (Curnutte *et al.*, 1976).

Hydrogen peroxide reacts with further superoxide in the presence of trivalent iron (Fe^{3+}) ions to give singlet oxygen and hydroxyl radicals (Kellogg and Fridovich, 1977). Lactoferrin, an iron-binding protein from neutrophil-specific granules may, after discharge into the phagocytic vacuole, act as a protein-bound source of iron to catalyse this reaction (Ambruso and Johnston, 1981). Any of the intermediates superoxide, hydrogen peroxide, singlet oxygen or hydroxyl radicals (Fig. 6) may be directly toxic to cells. In addition, Kong and Davison (1980) have proposed that hydrogen peroxide interacts with hydroxyl radicals to produce an even more potent species. Hydrogen peroxide will, in the presence of the relevant peroxidase, decarboxylate amino acids (Zgliczynski *et al.*, 1968; Adeniyi-Jones and Karnovsky, 1981), iodinate proteins (Klebanoff, 1967) and form hypochlorous acid (Klebanoff, 1975), which is both a strong oxidizing agent and will react with hydrogen peroxide to give singlet oxygen (Seliger, 1960; Rosen and Klebanoff, 1977), or with superoxide to give hydroxyl radicals (Fig. 6). Major effects of hypochlorous acid are the oxidation of cytochromes, which inhibits electron

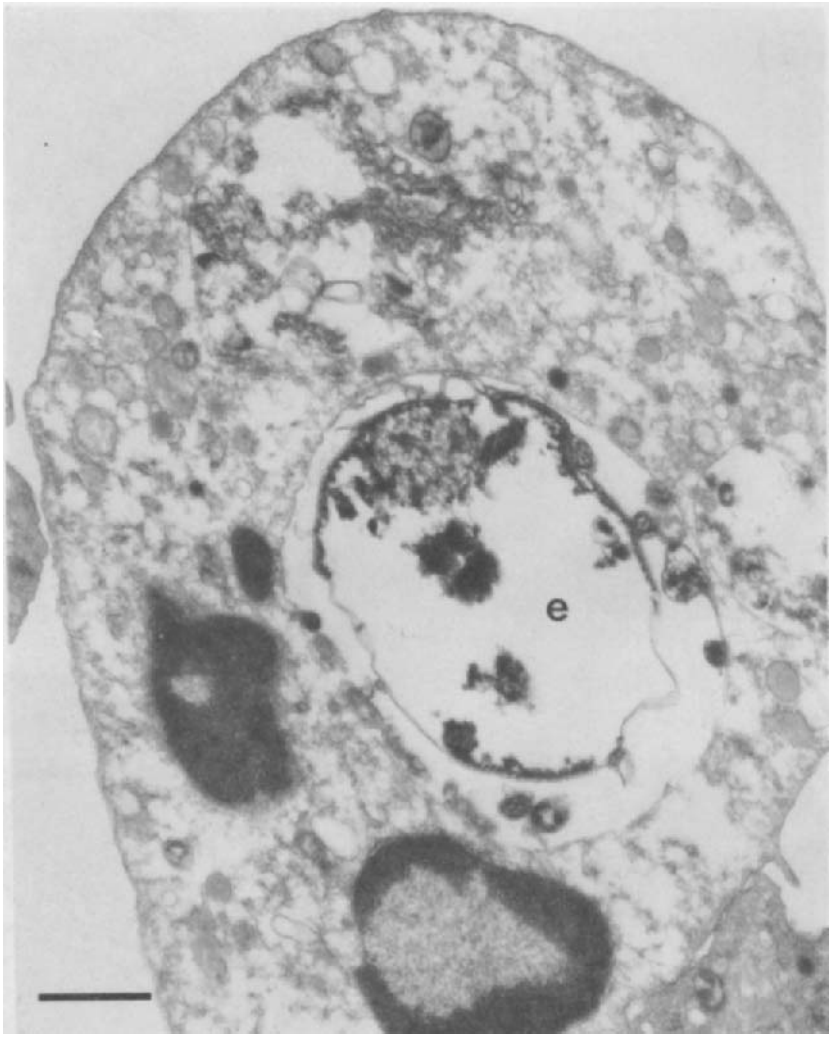

FIG. 3. Damage to neutrophil during the killing of epimastigote (e) of *T. dionisii*. The neutrophil cytoplasm is vacuolated (cf. Fig. 1). Incubation time 3 hours, without antibody. Bar line 1 μm.

transport, and the oxidation of thiols, which inhibits numerous enzymes (Albrich *et al.*, 1981). Free radicals initiate the oxygenation of polyunsaturated fatty acids to peroxides which decompose to aldehydes (Halliwell, 1978).

Protection from these intermediates is afforded by a number of enzymes. Catalase will break down hydrogen peroxide but it has an apparently high K_m value (Halliwell, 1974) and is located principally in peroxisomes.

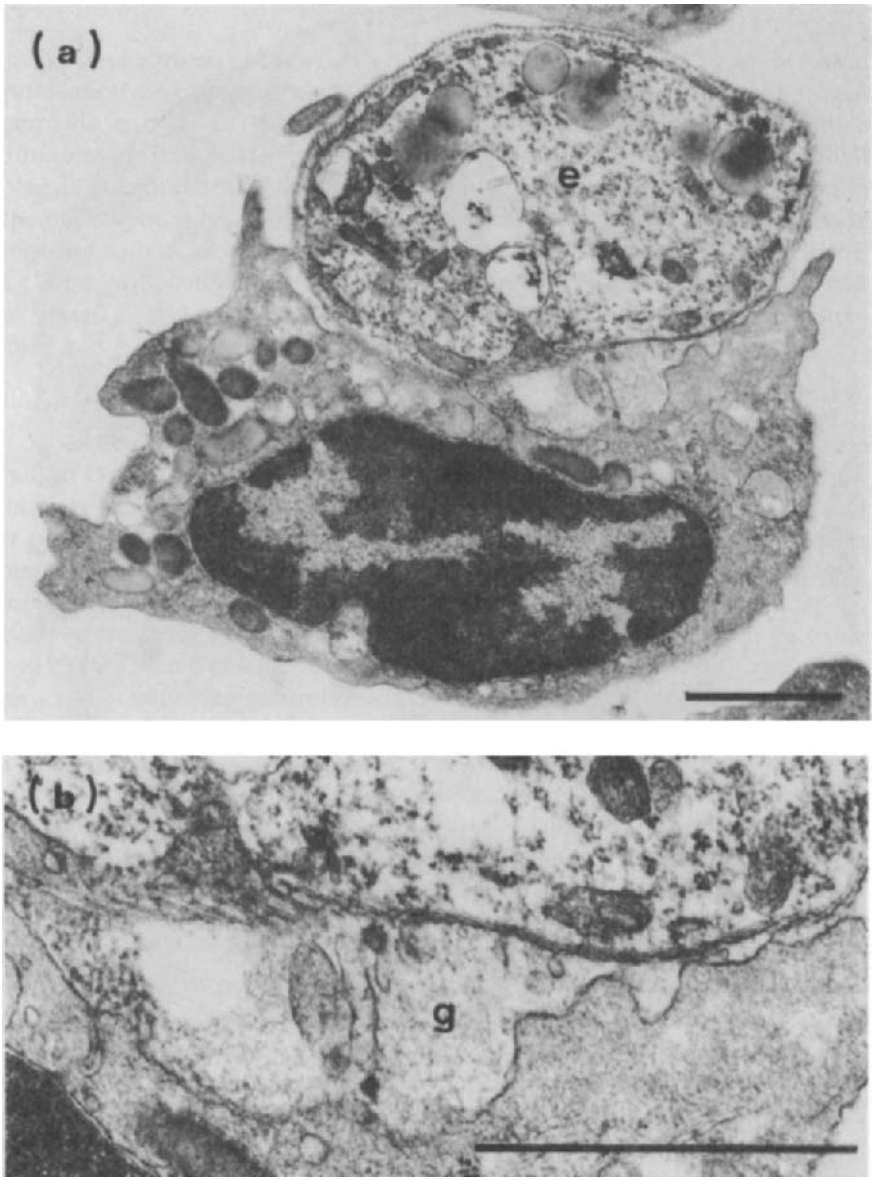

FIG. 4. (a) Damage to antibody-coated epimastigote (e) of *T. theileri* by bovine exudate neutrophil, before complete closure of phagocytic vacuole. (b) Lysosomal granule contents (g) can be seen in the enclosed phagocytic vacuole. Incubation time 1 minute. Bar lines 1 μm. (Reproduced with permission from Townsend *et al.* (1982). *Journal of Cell Science*, **56**, 389–407.

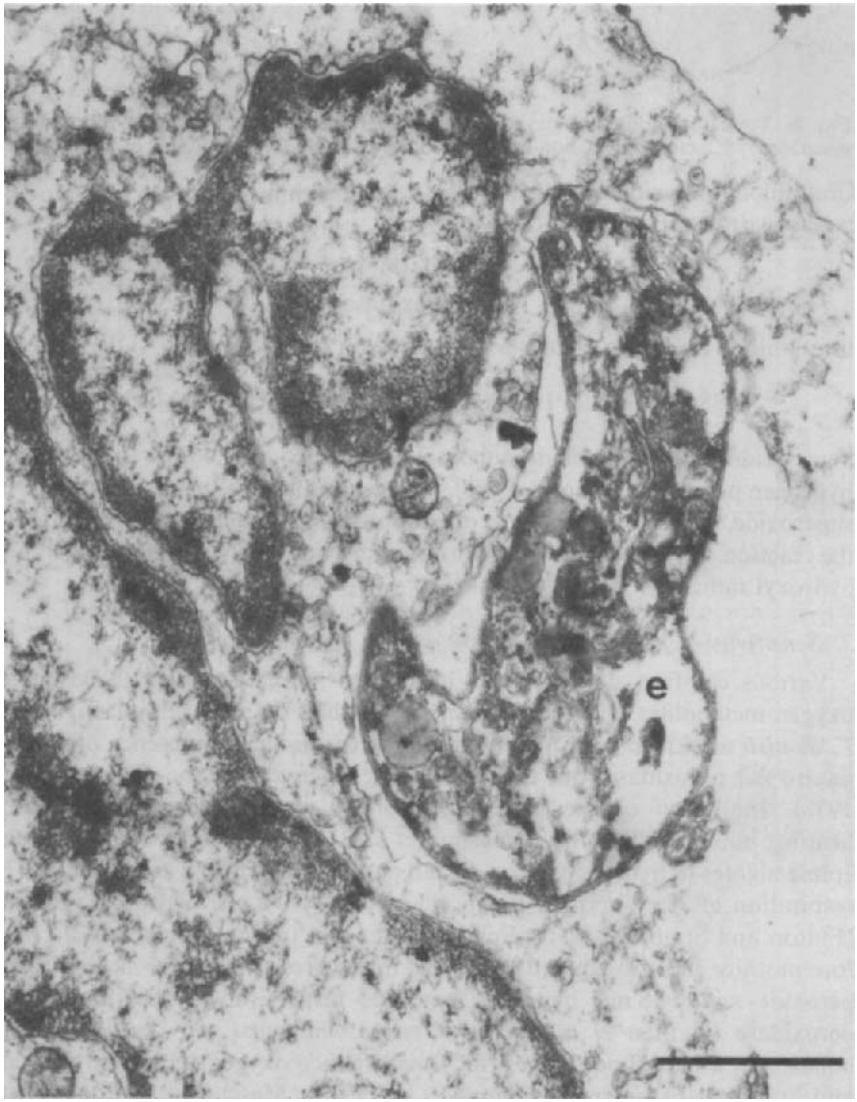

FIG. 5. Release of partially digested epimastigote (e) of *T. dionisii* from human peripheral blood monocyte. The monocyte has disintegrated during the killing of the epimastigote. Incubation time 1 hour without antibody. Bar line 1 μm.

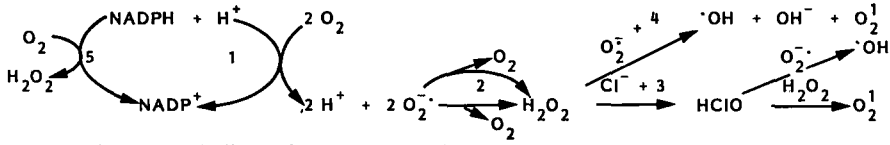

FIG. 6. Toxic metabolites of oxygen. **1**, NADPH oxidase; **2**, superoxide dismutase; **3**, peroxidase; **4**, lactoferrin; **5**, non-enzymic oxidation of NADPH (initiated by superoxide?).

Glutathione peroxidase appears to be a more significant means of removing excess hydrogen peroxide (Reed, 1969):

$$2GSH + H_2O_2 \xrightarrow[\text{peroxidase}]{\text{glutathione}} GSSG + 2H_2O$$

Glutathione reductase catalyses the reduction of GSSG by NADPH thus:

$$GSSG + NADPH + H^+ \xrightarrow[\text{reductase}]{\text{glutathione}} 2GSH + NADP^+.$$

Superoxide dismutase binds superoxide radicals and converts them into hydrogen peroxide and normal triplet oxygen. This prevents direct damage by superoxide, avoids the formation of possibly toxic singlet oxygen and inhibits the reaction of superoxide with hydrogen peroxide to give singlet oxygen and hydroxyl radicals.

2. *Sensitivity of protozoa to reactive oxygen intermediates*

Various cell-free assay systems have been adopted to test the effect of oxygen metabolites on protozoan species (Tables 7 and 8). Epimastigotes of *T. dionisii* are killed by 1 mM hydrogen peroxide, in the presence of human neutrophil peroxidase, but not by hydrogen peroxide alone (Thorne *et al.*, 1978). Inhibition of the peroxidase with diethyldithiocarbamate, or by heating, inhibits killing. Trypomastigotes of *T. dionisii* are more resistant than epimastigotes to hydrogen peroxide and peroxidase (Thorne *et al.*, 1981). The respiration of *T. rhodesiense* is inhibited 50% by 0·3 mM hydrogen peroxide (Fulton and Spooner, 1956). Epimastigotes and trypomastigotes of *T. cruzi* lose motility and subsequently are lysed in the presence of 0·39 mM hydrogen peroxide and 0·56 mM hydrogen peroxide respectively in the absence of peroxidase (Nathan *et al.*, 1979). *Trypanosoma cruzi* can be killed by β-lapachone, a naphthoquinone which may reduce oxygen (O_2) to superoxide and hydrogen peroxide (DoCampo *et al.*, 1977). Meschnick and coworkers (1977) suggest that the trypanosomicidal activity of haem towards *T. brucei* is due to its ability to convert endogenously-generated hydrogen peroxide into hydroxyl and superoxide radicals.

Toxoplasma gondii is killed by hydrogen peroxide provided that peroxidase and iodide are also present (Murray and Cohn, 1979). *Toxoplasma gondii* can also be killed by xanthine and xanthine oxidase. Both hydrogen peroxide and superoxide are required in this system since destroying the former with catalase or the latter with superoxide dismutase abolishes the activity. It

TABLE 7

Sensitivity of protozoa to oxidative intermediates in vitro[a]

Species and stage	LD_{50} (LD_{100})	Postulated toxic agent	Inhibitors	Reference
T. dionisii				
epimastigote	0·5 mM H_2O_2 + MPO	HClO	DETC	Thorne *et al.* (1978)
T. rhodesiense				
trypomastigote	0·3 mM H_2O_2	H_2O_2	Catalase	Fulton and Spooner (1956)
T. equiperdum				
trypomastigote	(1 μM H_2O_2)	H_2O_2	Catalase	Strangeways (1939)
T. cruzi				
epimastigote	7 μM H_2O_2 min^{-1}	H_2O_2	Catalase	Nathan *et al.* (1979)
trypomastigote	9 μM H_2O_2 min^{-1}	H_2O_2	Catalase	Nathan *et al.* (1979)
T. brucei				
trypomastigote	(70 μM H_2O_2)	Hydroxyl radical	Riboflavin	Meschnick *et al.* (1977)
T. gondii				
	10 mM H_2O_2	H_2O_2	Catalase	Murray and Cohn (1979)
	5 μM H_2O_2 + LPO + KI	H_2O_2	Catalase	Murray and Cohn (1979)
	0·15 mM xanthine + 5 μg ml^{-1} XO	Hydroxyl radical singlet oxygen	Catalase; SOD; mannitol; benzoate; DABCO; histidine	Murray and Cohn (1979)
L. donovani[b]				
promastigote (sub 19)	0·1 mM H_2O_2	H_2O_2	Catalase	J. Y. Channon and J. M. Blackwell (unpublished observations)
promastigote (sub 1)	0·5 mM H_2O_2	H_2O_2	Catalase	J. Y. Channon and J. M. Blackwell (unpublished observations)
amastigote	0·75 mM H_2O_2	H_2O_2	Catalase	J. Y. Channon and J. M. Blackwell (unpublished observations)
L. m. mexicana[b]				
promastigote	0·1–0·2 mM H_2O_2	H_2O_2	Catalase	J. Alexander (personal communication)
promastigote	10–20 μM H_2O_2 + LPO + KI	H_2O_2	Catalase	J. Alexander (personal communication)
amastigote	1–2 mM H_2O_2	H_2O_2	Catalase	J. Alexander (personal communication)
amastigote	0·1–0·2 mM H_2O_2 + LPO + KI	H_2O_2	Catalase	J. Alexander (personal communication)

[a] Abbreviations: DABCO, diazabicyclooctane; DETC, diethylthiocarbamate; HClO, hypochlorous acid; LPO, lactoperoxidase; MPO, myeloperoxidase; SOD, superoxide dismutase; Sub 1, sub 19; 1 and 19 weeks after isolation from hamster; XO, xanthine oxidase.

[b] See also Table 8 for sensitivity to glucose oxidase-generated hydrogen peroxide.

TABLE 8

Sensitivity of Leishmania *species to catalase-inhibitable glucose oxidase-generated hydrogen peroxide*[a].

Species and stage	Parasite density (ml^{-1})	Glucose concentration (mM)	Incubation time (h)	Viability assay	GO (mU ml^{-1})	LD_{50} (nmol H_2O_2 min^{-1} ml^{-1})[b]	Reference
L. donovani							
promastigote	5×10^6	5·5	1	M; C	—	1·5 0·4 + LPO + KI	Murray (1981a)
promastigote	2×10^6	13·0	24	M	32	8·8[c]	Pearson and Steigbigel (1981)
promastigote (sub 19)	5×10^6	5·5	1	M; C; FDA/EB Eryth B	15 0·5	3·1 0·09 + LPO + KI	J. Y. Channon and J. M. Blackwell unpublished observations
promastigote (sub 1)	5×10^6	5·5	1	M; C; FDA/EB Eryth B	25	4·7	
amastigote	$5–10 \times 10^6$	5·5	1	Trans	—	8·1 0·9 + LPO + KI	Murray (1982)
amastigote	5×10^6	5·5	1	Trans; FDA/EB	50 5	8·7 0·9 + LPO + KI	J. Y. Channon and J. M. Blackwell unpublished observations
L. t. major							
promastigote	5×10^6	5.5	1	M; C	—	0·5	Murray (1981a)
L. m. mexicana							
promastigote	5×10^6	5·5	1	TB	10–15	1·7–3·1[c]	J. Alexander (personal communication)
amastigote	5×10^6	5·5	1	TB	50–100	8·7–16·2[c]	

[a] Abbreviations: C, subsequent culture for 1–3 days; Eryth B, erythrosin B uptake by non-viable parasites; FDA/EB, combined fluorescein diacetate esterase staining for viable parasites and ethidium bromide for non-viable parasites; GO, glucose oxidase; LPO, lactoperoxidase; M, motility; Sub 1, sub 19; 1 and 19 weeks after isolation from hamster; TB, trypan blue uptake by non-viable parasites; Trans, transformation of amastigotes to promastigotes in subsequent culture over 42 hours.

[b] Initial rate.

[c] Calculated from the original authors' data.

seems likely that both hydroxyl radicals and singlet oxygen are involved since scavenging either the former with mannitol or benzoate, or the latter with diazabicyclooctane (DABCO) or histidine, inhibits killing.

The susceptibility of *Leishmania* promastigotes and amastigotes to reagent hydrogen peroxide and to hydrogen peroxide generated from glucose oxidase has been studied in several different laboratories (Tables 7 and 8). Variation in LD_{50} (lethal dose 50%) values obtained in different laboratories for the same species and stage of *Leishmania* may not necessarily be due to strain differences in susceptibility to hydrogen peroxide. More likely, they reflect variations in experimental conditions, for example parasite density, treatment time, activity of glucose oxidase, glucose concentration, or degree of agitation of the reaction mixture (unpublished observations of J. Y. Channon and J. M. Blackwell and of J. Alexander). Nevertheless, from examination of results obtained within the same laboratory, it appears that (a) *L. t. major* promastigotes are more sensitive to hydrogen peroxide than *L. donovani* promastigotes; (b) within each species, promastigotes are more sensitive to hydrogen peroxide than amastigotes; and (c) the sensitivity of *Leishmania* promastigotes and amastigotes to hydrogen peroxide is always markedly enhanced by the addition of peroxidase and a halide. Promastigotes of *L. donovani* and *L. t. major* are also killed by hydrogen peroxide generated from xanthine and xanthine oxidase (Murray, 1981a). Since scavengers of superoxide, singlet oxygen and hydroxyl radical do not prevent killing it seems unlikely that these intermediates are involved.

There thus appears to be considerable variation in the susceptibility of different protozoa to different oxidative intermediates. *Trypanosoma dionisii* and *T. gondii* are resistant to hydrogen peroxide while *T. cruzi* and *Leishmania* promastigotes and amastigotes are, to varying degrees, sensitive. This may, in part, reflect differences in levels of endogenous scavengers of hydrogen peroxide. For example, Murray and Cohn (1979) have shown that *T. gondii* has high levels of both catalase and glutathione peroxidase. *Leishmania donovani* amastigotes have much lower levels of these enzymes (Murray, 1982) and promastigotes even less (Murray, 1981a). The role of other antioxidants, for example vitamins C and E, cysteine, unsaturated fatty acids, has not, however, been examined in protozoa. Peroxidase-mediated killing by hydrogen peroxide has been demonstrated with epimastigotes and, less effectively, with trypomastigotes of *T. dionisii*, and with *T. gondii* and *Leishmania* species. Hydroxyl radicals damage both *T. brucei* trypomastigotes and *T. gondii* but do not appear to be involved in killing *Leishmania*.

3. *Oxygen metabolite mediated intracellular killing*

Two experimental approaches have been used to demonstrate that oxidative metabolites are involved in the killing of a particular protozoal species by a

particular effector cell. Firstly the specific target needs to induce the formation of the relevant oxidative intermediate. Secondly inactivating the oxidative intermediate with a selective scavenger should inhibit killing by competing for the toxic agent.

(*a*) *Neutrophils.* Thorne and others detected an increase in the rate of formation of hydrogen peroxide from human neutrophils when they reacted with epimastigotes or trypomastigotes of *T. dionisii* in the presence or absence of antibody (Thorne *et al.*, 1978, 1981). Hydrogen peroxide was assayed by measuring the peroxide-dependent formation of fluorescent dimers from *p*-hydroxyphenylacetate in the presence of lactoperoxidase (Guibault *et al.*, 1968). Fluorescence assays suffer from interference from other fluorescent products of the cells present. For this reason assays should be repeated without peroxidase to eliminate peroxidase-independent fluorescence. Serum proteins also interfere with this assay. More hydrogen peroxide was detected when superoxide dismutase was added to the neutrophil–T. *dionisii* system, which suggests that superoxide is an intermediate in the formation of hydrogen peroxide.

Trapping of superoxide with superoxide dismutase and converting it to hydrogen peroxide did not inhibit neutrophil cytotoxicity to *T. dionisii.* It therefore seems unlikely that superoxide itself or either of its products, singlet oxygen or hydroxyl groups, is responsible for the cytotoxicity. Inhibition of hydrogen peroxide production with diazobenzenesulphonic acid and inhibition of peroxidase with diethyldithiocarbamate did, however, inhibit cytotoxicity. This confirms that it is the hydrogen peroxide–peroxidase system which is operative when neutrophils kill *T. dionisii.*

The hydrogen peroxide–peroxidase–halide microbicidal system is also believed to be responsible for killing of *L. donovani* promastigotes (Pearson and Steigbigel, 1981) and amastigotes (Chang, 1978b, 1981a) by human neutrophils. For the promastigote study, production of superoxide anion during phagocytosis was assessed by reduction of ferricytochrome *c.* In the amastigote study, reaction products of hydrogen peroxide were detected in the parasitophorous vacuoles in electron micrographs. In both cases, leishmanicidal activity was assessed by light and electron microscopy and was found to be absent from neutrophils from patients with chronic granulomatous disease.

Oxidative microbicidal systems have also been implicated in the *in vitro* extracellular killing of *Trichomonas vaginalis* by human neutrophils (Rein *et al.*, 1980). After 60 minutes of aerobic incubation, light and electron microscopic observation revealed that 100% of trichomonads were killed and that nitroblue tetrazolium was reduced at the interface between the neutrophil and the trichomonad. Killing was eliminated by addition of catalase or superoxide dismutase and under anaerobic conditions only about

12% of trichomonads were killed. Interestingly, neutrophils from chronic granulomatous disease patients killed trichomonads as effectively as normal neutrophils. Rein and coworkers (1980) propose that, as with the microbicidal activity of neutrophils against some bacteria, the oxygen metabolites are produced by the microbe (Kaplan *et al.*, 1968; Mandell and Hook, 1969a, b). Presumably the peroxidase of the neutrophil increases the potency of the microbial source of hydrogen peroxide although the inhibition by both superoxide dismutase and catalase suggests that both superoxide and hydrogen peroxide are required and that singlet oxygen and hydroxyl radicals may be involved in the killing.

Toxoplasma gondii induced only low levels of oxidative activity in human neutrophils (Wilson *et al.*, 1980), as assessed by oxidative decarboxylation of [1-^{14}C]glucose to $^{14}CO_2$, by chemiluminescence which is believed to result from the action of more than one oxygen species (McPhail *et al.*, 1979), and by the reduction of nitroblue tetrazolium to insoluble formazan. Reduction of nitroblue tetrazolium by neutrophils, induced by phagocytosis, has been ascribed to superoxide radicals from its dependence on molecular oxygen and its sensitivity to SOD (Baehner *et al.*, 1976). The lack of response to *T. gondii* may explain why the neutrophil is not important in cell-mediated killing of *T. gondii*.

The mechanism whereby contact with protozoa activates plasma membrane oxidase to initiate the production of oxidative intermediates is not known. Investigations have been made, however, of the mechanism of activation of neutrophil NADPH oxidase by the membrane perturbant phorbol myristyl acetate (PMA). The state of the membrane environment appears to be particularly important in determining the activity of the enzyme. A sharp discontinuity at 30°C in measurement of enzyme activity at different temperatures indicates that it may be influenced by membrane fluidity (Cohen *et al.*, 1980). The membrane active anaesthetics chlorpromazine, trifluoroperazine, prochloroperazine, and, to a lesser extent, tetracaine and lidocaine inhibit superoxide production when neutrophils are triggered by PMA or by phagocytosis of zymosan (Cohen *et al.*, 1980). Chlorpromazine also inhibits the activity of NADPH oxidase of an isolated particulate fraction, as well as its activation in whole cells. The activation of the enzyme by PMA is accompanied by depolarization of the membrane (Whitlin *et al.*, 1980). Chlorpromazine has no effect on the resting potential but affects the rate and extent of depolarization induced by PMA. A role for calcium is suggested by the observations of Smith and Iden (1981) that TMB-8, an intracellular calcium antagonist, causes a dose-dependent inhibition of PMA-induced superoxide production in calcium-free medium, and that this could be reversed by the addition of calcium. Takeshige and coworkers used the fluorescent probe chlortetracycline to demonstrate that, when *E. coli* is used as a stimulus,

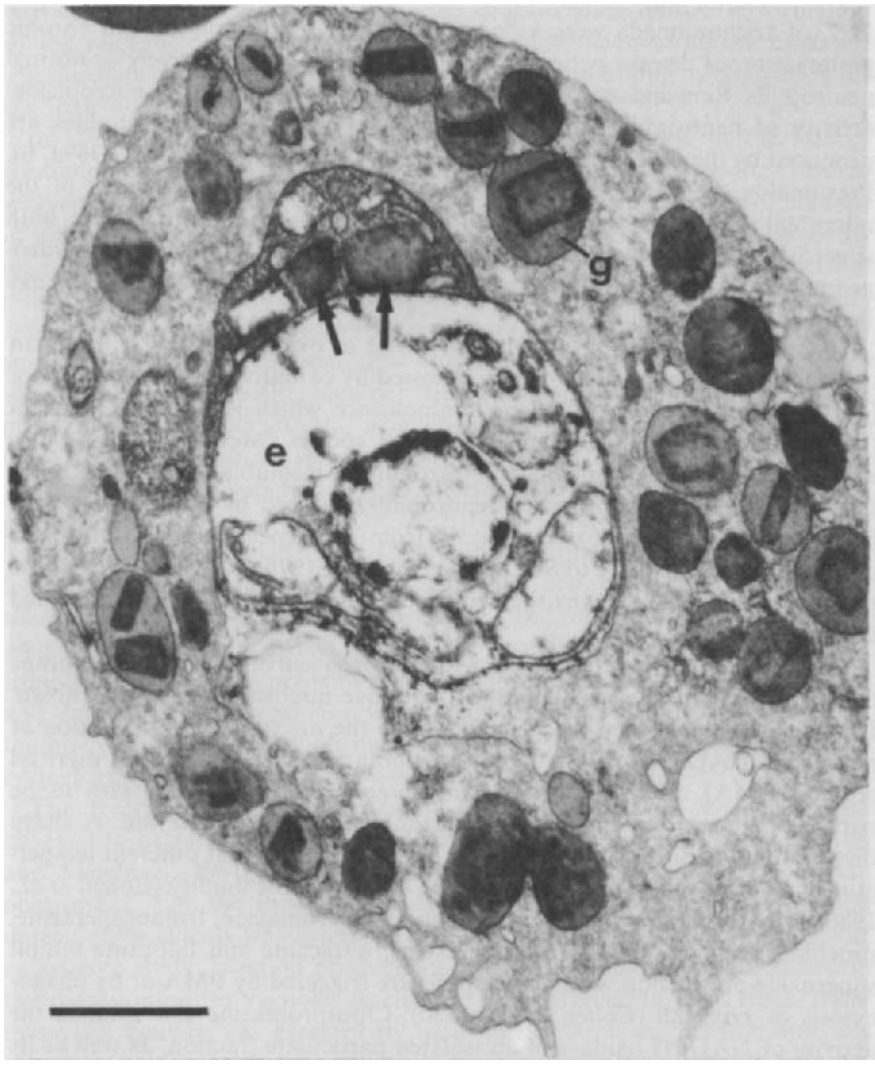

FIG. 7. Killing of antibody-coated epimastigote (e) of *T. dionisii* by human blood eosinophil. Crystalline cores from the eosinophil granules (g) can be seen in the phagocytic vacuole (arrows). Incubation time 30 minutes. Bar line 1 μm.

calcium is released from membranes in parallel with the production of superoxide (Takeshige *et al.*, 1980). Anion uptake may also be important since inhibition of anion transport with 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid (SITS) or with 1-anilino-8-naphthalenesulphonate inhibits the generation of superoxide by phagocytosing granulocytes (Gennaro and Romeo, 1979).

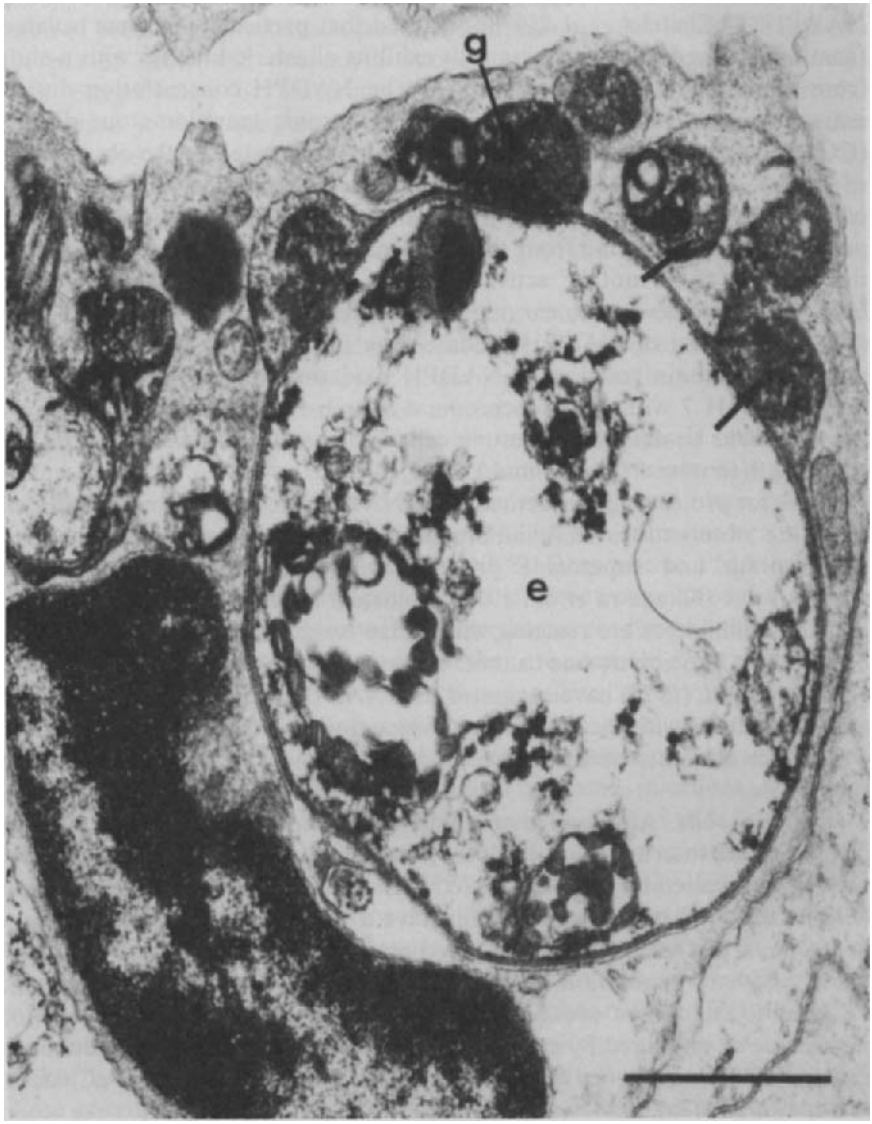

FIG. 8. Killing of antibody-coated epimastigote (e) of *T. theileri* by bovine exudate eosinophil. Eosinophil granules (g) have fused with the phagosome and granule contents (arrows) can be seen in the phagocytic vacuole. Incubation time 30 minutes. Bar line 1 μm. (Reproduced with permission from Townsend *et al.* (1982) *Journal of Cell Science* **56**, 389–407).

Conflicting observations have been made on the changes in the binding constant of NADPH oxidase for NADPH in response to an activating stimulus. Patriarca *et al.* (1971) found that initiation of phagocytosis is

associated with a tenfold decrease in the K_m value of NADPH oxidase for NADPH. DeChatelet *et al.* (1978b) showed that particulate enzyme isolated from normal and phagocytosing cells exhibits allosteric kinetics with a shift from sigmoid to hyperbolic dependence on NADPH concentration during activation; this was not seen in a case of chronic granulomatous disease (CGD). Evidence for an allosteric inhibitor was suggested by the observation of DeChatelet *et al.* (1980) that a similar kinetic modification could be obtained by dialysis of the partially purified enzyme. It is interesting that the partially purified enzyme from the CGD patient could also be activated by dialysis, but could not be activated in the cell by phagocytosis. In these experiments, however, the enzyme was assayed at pH 6, under which conditions triggering of NADPH oxidase may initiate a superoxide-mediated non-enzymic chain reaction of NADPH oxidation. When the enzyme was assayed at pH 7 with ferricytochrome *c* to trap the superoxide formed, no activity could be detected in resting cells and therefore no value for its K_m could be determined (Suzuki and Lehrer, 1980).

A role for proteases in the activation of NADPH oxidase has been suggested from the observations that inhibitors of serine proteases inhibit oxygen consumption and superoxide production by neutrophils stimulated in various ways (Kitagawa *et al.*, 1979; Goldstein *et al.*, 1979). The possibility that these inhibitors are reacting with serine residues to block some activity other than a proteolytic one cannot, however, be excluded.

Millard *et al.* (1979) have suggested that NADPH oxidase of a cytoplasmic membrane fuses with the plasma membrane during phagocytosis to combine with other elements of the electron transport chain and thereby generate superoxide.

(*b*) *Eosinophils.* Although eosinophils appear to be less effective against trypanosomes than are neutrophils they do, nevertheless, have some cytotoxic activity as assessed by loss of radioactive isotope and by electron microscopy (Figs 7 and 8). Although eosinophils have a lower phagocytic activity than neutrophils, phagocytic stimuli induced greater production of hydrogen peroxide, assayed by oxidation of [^{14}C]formate to $^{14}CO_2$ (Baehner and Johnston, 1971) or iodination of protein (DeChatelet *et al.*, 1977), a similar level of superoxide as measured by cytochrome *c* or nitroblue tetrazolium reduction, higher chemiluminescence and higher levels of NADPH oxidase (DeChatelet *et al.*, 1977, 1978a).

Eosinophil granules contain high levels of a peroxidase which differs from the myeloperoxidase of neutrophils. Unlike myeloperoxidase, eosinophil peroxidase is unable to decarboxylate amino acids, but it does catalyse the incorporation of iodine into protein (Migler *et al.*, 1978). In the absence of iodide, eosinophil cell-free preparations were unable to kill bacteria unless the enzyme was present at high concentrations and the pH was low (Jong *et al.*,

1980). The optimum pH of neutrophil peroxidase, when assayed by iodination of protein, is 5, whereas that for eosinophils is 6 (Bos *et al.*, 1981). Since the pH of neutrophil phagocytic vacuoles drops to 4 within 10 minutes after phagocytosis of bacteria (Jensen and Bainton, 1973), neutrophil peroxidase is well adapted to operate within this environment. Similar studies have not been done with the eosinophil, but it seems likely that the peroxidase, like the other eosinophil granule proteins, acts mainly in the extracellular environment. Kierszenbaum and coworkers (1981, 1982) report that the cationic protein from eosinophil granules can kill bloodstream trypomastigotes of *T. cruzi*.

(*c*) *Macrophages.* It has been clearly demonstrated that oxidative reactions, particularly those involving hydrogen peroxide and peroxidase, play a major role in the microbicidal activity of neutrophils, but the importance of these reactions in macrophages is less well understood.

The level of oxidative activity inducible in macrophages is related to the state of the macrophage. Human blood monocytes generate between 20% and 50% as much hydrogen peroxide, superoxide and chemiluminescence as do neutrophils in response either to a phagocytic stimulus or to PMA (Johnston *et al.*, 1976; Reiss and Roos, 1978). Response to PMA has been used to compare the oxidative potential of different populations of macrophages (Table 9). Mouse peritoneal macrophages produce significant levels of superoxide in response to PMA only if they are prepared from mice treated with BCG or, less effectively, with thioglycollate or lipopolysaccharide (Johnston *et al.*, 1978). Production of hydrogen peroxide is also substantial in macrophages from mice elicited with BCG, or with casein or *Corynebacterium parvum*, but not with thioglycollate unless repeated injections are given (Nathan and Root, 1977). Macrophages from mice infected with *T. gondii* are inactive, but macrophages from similarly infected mice given a boost with *T. gondii* antigen become responsive to PMA (Murray *et al.*, 1979). Similarly, mice infected with *T. cruzi* produce responsive macrophages if the animals are boosted with *T. cruzi* antigen. Resident peritoneal macrophages can also be activated *in vitro* with supernatants from lymphocytes harvested from mice infected with *T. cruzi*, and then cultured for 48 hours with *T. cruzi* antigen (Nathan *et al.*, 1979). This ability to produce hydrogen peroxide in response to PMA correlates well with the observations that *T. cruzi* is killed by only those macrophages that have been activated *in vivo* or *in vitro* with lymphokines (Nogueira *et al.*, 1977b; Nogueira and Cohn, 1978).

The susceptibility of different macrophages to protozoal stimuli has also been studied (Table 10). Wilson *et al.* (1980) have correlated the susceptibility or resistance of *T. gondii* to different macrophages with the ability of the macrophages to generate an oxidative response to *T. gondii*. For example, while human blood monocytes were active against *T. gondii* and corres-

TABLE 9

Oxidative responses of macrophages to phorbol myristyl acetate

Cell type[a]	Reactive oxygen intermediate[b]	Assay method	Reference
Human blood monocytes	$2{\cdot}3/10^6$ cells	Cytochrome *c* reduction	Johnston *et al.* (1976)
Human blood monocytes (cultured 1 day)	0·05/100 μg protein	Cytochrome *c* reduction	Johnston *et al.* (1978)
Human blood monocytes (cultured 3 days)	1·0/100 μg protein	Cytochrome *c* reduction	Johnston *et al.* (1978)
Mouse peritoneal cells (resident)	0·1/100 μg protein	Cytochrome *c* reduction	Johnston *et al.* (1978)
Mouse peritoneal cells (thioglycollate elicited)	1·0/100 μg protein	Cytochrome *c* reduction	Johnston *et al.* (1978)
Mouse peritoneal cells (lipopolysaccharide elicited)	1·4/100 μg protein	Cytochrome *c* reduction	Johnston *et al.* (1978)
Mouse peritoneal cells (BCG elicited)	2·0/100 μg protein	Cytochrome *c* reduction	Johnston *et al.* (1978)
Mouse peritoneal cells (BCG mice + BCG Ag boost)	2·4/100 μg protein	Cytochrome *c* reduction	Johnston *et al.* (1978)

Cell type[a]	Reactive oxygen intermediate[b]	Assay method	Reference
Mouse peritoneal cells (resident)	0·06/10^6 cells	Scopoletin fluorimetry	Nathan and Root (1977)
Mouse peritoneal cells (thioglycollate elicited)	0.06/10^6 cells		
Mouse peritoneal cells (*C. parvum* elicited)	0·6/10^6 cells		
Mouse peritoneal cells (casein elicited)	1·0/10^6 cells		
Mouse peritoneal cells (BCG elicited)	1·6/10^6 cells		
Mouse peritoneal cells (resident)	0/100 μg protein		Murray *et al.* (1979)
Mouse peritoneal cells (*T. gondii* infected mice)	0/100 μg protein		
Mouse peritoneal cells (*T. gondii* infected + Ag boost)	0·6/100 μg protein		
Mouse peritoneal cells (resident)	0·04/100 μg protein		Nathan *et al.* (1979)
Mouse peritoneal cells (*T. cruzi* Ag boost)	0·04/100 μg protein		
Mouse peritoneal cells (*T. cruzi* infected + Ag boost)	0·4/100 μg protein		
Mouse peritoneal cells (resident + *T. cruzi* LK)	0·2/100 μg protein		

[a] Abbreviations: Ag, parasite specific antigen; LK, lymphokine.
[b] O_2^- for cytochrome *c* reduction; H_2O_2 for scopoletin fluorimetry; calculated from data in cited papers; values in nmol $min.^{-1}$

TABLE 10

Stimulation by protozoa of the formation of oxidative intermediates by cytotoxic cells

Cell type	Stimulus[a]	Reactive oxygen intermediate[b]	Assay method	Reference
Human blood neutrophils	*T. dionisii*			
	epimastigote	0·1		
	Ab-epimastigote	0·09		
	trypomastigote	0·08		
	Ab-trypomastigote	0·07		
Human blood monocytes	*T. dionisii*		*p*-hydroxyphenyl acetate fluorimetry	Thorne *et al.* (1981)
	epimastigote	0·03		
	Ab-epimastigote	0·03		
	trypomastigote	0·04		
	Ab-trypomastigote	0·03		
Human blood monocytes	*T. gondii*	0·4		
	Ab-*T. gondii*	0·8		
	Candida	1·5		
Human blood macrophages	*T. gondii*	0·06		
Mouse macrophages (resident peritoneal)	*T. gondii*	0·06	nitroblue tetrazolium reduction	Wilson *et al.* (1980)
Mouse macrophages (*C. parvum* elicited)	*T. gondii*	0·22		
Mouse macrophages (infected *T. gondii*)	*T. gondii*	0·24		

[a] Ab, antibody-coated.

[b] Calculated from data in cited papers as nmole per 10^6 cells per min, H_2O_2 for fluorimetry and O_2^- for reduction assays.

pondingly responded to *T. gondii* by producing superoxide radicals, after culturing for several days they became macrophage-like and lost their ability to respond to *T. gondii*. Interestingly, they did not lose their ability to respond to *Candida* or to latex particles. Resident mouse peritoneal macrophages did not respond to *T. gondii* while those from mice treated with *C. parvum*, or infected with *T. gondii*, did. In all situations *T. gondii* was a less effective stimulus of oxidative metabolism than was *Candida*. Antibody-coating *T. gondii* made it twice as effective as an activator of oxidative metabolism while boiled *T. gondii* was a poor stimulus.

No significant difference was found between the ability of epimastigotes and trypomastigotes of *T. dionisii* to induce hydrogen peroxide formation from human blood monocytes, or between antibody-coated and normal cells (Thorne *et al.*, 1981); however, the level of hydrogen peroxide detected was less than that from neutrophils. While neutrophils kill both epimastigotes and trypomastigotes effectively and rapidly, monocytes are active against epimastigotes only, and appear not to produce enough hydrogen peroxide to kill trypomastigotes.

Work from several laboratories indicates that promastigote and amastigote forms of various *Leishmania* species do differ in their ability to trigger the respiratory burst in resident macrophage populations infected *in vitro*. Using the qualitative nitroblue tetrazolium reduction technique with resident peritoneal macrophages, Murray (1981a, 1982) obtained 78–85% formazan-positive infected macrophages in response to *L. donovani* and *L. t. major* promastigotes, and 58–63% in response to a crude preparation of *L. donovani* amastigotes isolated from hamster spleen. With promastigotes (Murray, 1981a), the extensive triggering correlated with hydrogen peroxide production and the prompt killing and digestion of ingested parasites. With amastigotes (Murray, 1982), the reduced triggering response did not generate sufficient hydrogen peroxide for measurable amastigoticidal activity as assessed by light microscope counts. Using similar experimental conditions J. Y. Channon and J. M. Blackwell (unpublished observations) found that 85–100% of infected resident peritoneal macrophages were triggered by *L. donovani* promastigotes while only 17–72% responded to amastigotes 'purified' by Percoll treatment. In these assays it was possible to distinguish individual formazan-positive parasites. With promastigotes there was seldom more than one ingested parasite per infected macrophage, so the proportion of individual formazan-positive promastigotes (85–100%) invariably matched the proportion of infected macrophages triggered. With equivalent amastigote inocula, however, uptake was greater and many macrophages contained several formazan-negative parasites, together with only one or two which were formazan-positive. Hence, the proportion of individual formazan-positive amastigotes (12–40%) was always lower than that of infected macrophages

triggered. This indicates an even greater differential triggering response between promastigotes and amastigotes than Murray's (1981a, 1982) data suggest. It was not possible to determine whether the hydrogen peroxide generated locally was sufficient to kill the individual amastigotes which triggered the response. It is possible, however, that for both forms of the parasite it is the individual parasites which do not trigger that are important in initiating and maintaining the infection. Opsonization of amastigotes with anti-leishmanial antisera resulted in an increased triggering response (J. Y. Channon and J. M. Blackwell, unpublished observations).

Other workers have confirmed the differential response of promastigotes and amastigotes in resident peritoneal macrophages infected *in vitro* with *L. t. major* (Stokes, 1981) and *L. m. mexicana* (J. Alexander, personal communication). Interestingly, J. Y. Channon and J. M. Blackwell (unpublished observations) found that resident tissue macrophages (spleen and Kupffer cells) showed a reduced triggering response to both promastigotes and amastigotes compared to resident peritoneal macrophages although, again, promastigotes tended to trigger more cells than amastigotes. Considered together with the increased resistance of amastigotes, compared to promastigotes, to hydrogen peroxide generated in a cell-free system (Section IV B 2, p. 85), the combined findings from all laboratories offer a possible explanation for (i) the reduced infectivity of promastigote compared with numerically equivalent amastigote inocula both *in vivo* (especially with intravenous injections) and *in vitro*, and (ii) the different infection and survival rates of the various *Leishmania* species in different subpopulations of tissue macrophages.

Recently-derived bone marrow macrophages, blood monocytes and macrophages freshly derived from them, and various elicited peritoneal macrophages, all contain granular peroxidase (Van Furth *et al.*, 1970; Daems *et al.*, 1973, 1976; Alexander and Smith, 1978). Studies on immunological cross-reactivity (Salmon *et al.*, 1970), the similarity of patterns of genetic deficiency (Lehrer and Cline, 1969), electron paramagnetic resonance, electron proton resonance, absorbance spectra, utilization of *o*-dianisidine and iodide, and the pH optimum of 5·5 (Bos *et al.*, 1978), all indicate that the enzyme resembles neutrophil myeloperoxidase. It is situated in the lysosomes and, following phagocytosis, is delivered into the phagocytic vacuole (Daems *et al.*, 1973, 1975; Daems and van der Rhee, 1980) where, in the presence of hydrogen peroxide, it can participate in the microbicidal activity of the cells. Older bone marrow- and monocyte-derived macrophage cultures and resident peritoneal and tissue macrophages lack detectable granular peroxidase. Peroxidase activity in these cells is restricted to the rough endoplasmic reticulum and perinuclear cisterna (Daems *et al.*, 1973; Bodel *et al.*, 1977). Characterization of the enzyme in Kupffer cells (Van Berkel, 1974) showed that it reacts with diaminobenzidine but not with guiacol or iodide, and only

poorly with *p*-phenylene diamine, and that it has a pH optimum greater than 8. Since it is not delivered into phagocytic vacuoles (Fahimi, 1970; Daems *et al.*, 1973), it is unlikely to participate in microbicidal activity.

In macrophages that do possess granular peroxidase, enhanced microbicidal activity would be expected in response to infection with a protozoon which can induce hydrogen peroxide formation and which is sensitive to the peroxidase–hydrogen peroxide system. Although nothing is known of the associated triggering response and hydrogen peroxide production, this may account for the more rapid destruction of amastigotes of *L. m. mexicana* (Alexander, 1981a) and *L. donovani* (Slackmuyldert-Otte and Trouet, 1980) in bone marrow-derived macrophages than in resident peritoneal cells (Bray and Alexander, in press). It would also explain why monocyte-derived macrophages do not support *L. donovani* amastigotes until 6 days after *in vitro* culture (Berman *et al.*, 1979). Buchmüller and Mauel (1981) found that amastigotes of *L. enriettii* failed to trigger the respiratory burst in starch-elicited mouse macrophages. Following *in vitro* activation with concanavalin A-induced supernatants from spleen cells, the macrophages produced an oxidative response and became leishmanicidal. The leishmanicidal activity was lost when the activated macrophages were treated with two peroxidase inhibitors, amino triazole and trypan blue. Resident peritoneal macrophages, which do not contain granular peroxidase, can also be activated to kill *L. donovani* amastigotes (Chang and Chiao, 1981; Murray, 1982) by *in vitro* exposure to concanavalin A lymphokine before infection. Glucose deprivation and pretreatment with PMA markedly impaired macrophage oxidative activity and abolished the killing response (Murray, 1982). Catalase (but not scavengers of superoxide anion, singlet oxygen or hydroxyl radical) achieved similar results, confirming a primary leishmanicidal role for hydrogen peroxide. Differential sensitivity to lymphokine activation by peroxidase-positive and peroxidase-negative cells is indicated by the fact that bone marrow-derived macrophages activated before or after infection rapidly kill *L. m. mexicana* while resident peritoneal macrophages can be activated to kill *L. m. mexicana* only if treated with lymphokine before infection (Bray and Alexander, in press).

Catalase is seen in peroxisomes of Kupffer cells, alveolar macrophages and peritoneal macrophages. Evidence has been obtained that this enzyme is discharged into the phagocytic vacuole of alveolar macrophages (Stossel *et al.*, 1972) and peritoneal macrophages (Eguchi *et al.*, 1979) but not Kupffer cells (Fahimi *et al.*, 1976). There is an increase in the oxidation of formate by catalase of alveolar macrophages during phagocytosis (Gee *et al.*, 1970) which is inhibited by amino triazole. Catalase could, in the presence of low concentrations of hydrogen peroxide, act as a peroxidase (Nicholls and Schonbaum, 1963) and use hydrogen peroxide to kill microbes (Klebanoff, 1969). This mechanism has not yet been reported for protozoa.

Inhibition or enhancement of monocyte- or macrophage-mediated killing by scavengers of oxidative intermediates and by enzyme inhibitors has proved valuable in the elucidation of the mechanisms involved. Inhibition of the killing of *T. dionisii* epimastigotes by mononuclear cells resulting from inhibition of hydrogen peroxide formation with diazobenzene-sulphonic acid, and of peroxidase with diethyldithiocarbamate, confirmed that the mechanism of human blood monocyte cytotoxicity to this organism resembles that of neutrophils in involving hydrogen peroxide and peroxidase (Thorne *et al.*, 1978). As discussed above, scavengers of singlet oxygen, superoxide and hydroxyl radicals had little effect on the killing of *L. donovani* or *L. t. major*, whereas dissipation of hydrogen peroxide by incubation of macrophages with catalase both for 3 hours and during exposure to the parasites reduced the leishmanicidal activity (Murray, 1981a, b, 1982). Aminotriazole and trypan blue inhibited the killing of *L. enrietti* by starch-elicited macrophages activated with macrophage activating factor (MAF), presumably by inhibiting macrophage peroxidase (Buchmüller and Mauel, 1981). The anti-*T. gondii* activity of microbistatic and microbicidal macrophages appears to be different since it was reduced by scavenging with mannitol, histidine, DABCO, SOD and catalase provided that they were added before the *T. gondii* (Murray *et al.*, 1979). This suggests a role for superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen.

In summary, *T. dionisii* appears to be killed by blood monocytes by hydrogen peroxide and peroxidase. *Trypanosoma cruzi* is killed by activated mouse macrophages by hydrogen peroxide in a reaction which may or may not be mediated by peroxidase. *Leishmania* are susceptible to high levels of hydrogen peroxide alone, but they may be killed by MAF-activated starch-elicited macrophages with the intervention of a peroxidase. *Toxoplasma gondii* is susceptible to other oxidative intermediates and these may be generated by lymphokine-activated macrophages.

C. LYSOSOMES

Indirect evidence for the role of lysosomal contents in microbistatic and microbicidal activity against some protozoan species is given by the results of (i) Alexander (1981b) who demonstrated that peritoneal and bone marrow-derived macrophages treated with poly-D-glutamic acid, which inhibits phagolysosomal fusion, support greater multiplication of *L. m. mexicana*, and (ii) Mauel (1980) who found that treatment with hydrocortisone, which also inhibits phagolysosome fusion, protects parasites from destruction in activated elicited macrophages. While the latter result may be explained as decreased exposure to lysosomal peroxidase, the results obtained by Alexander (1981b) with resident peritoneal macrophages suggest the involvement of enzymes other than peroxidase.

1. *Evidence for phagolysosome fusion*

After uptake of the protozoa by phagocytosis the lysosomes of the phagocytic cells fuse with the phagosome and discharge their contents into it. This has been demonstrated in macrophages by pre-loading secondary lysosomes with thorotrast and observing the appearance of thorotrast in the phagosome by electron microscopy. Discharge of thorotrast from the lysosomes into the phagosome has been seen after phagocytosis of antibody-coated *T. gondii* (Jones *et al.*, 1975), of *T. cruzi* (Kress *et al.*, 1975, 1977; Nogueira and Cohn, 1976; Milder and Kloetzel, 1980), and of *L. mexicana* (Lewis and Peters, 1977; Alexander, 1981b) by mouse macrophages; of *L. donovani* by hamster macrophages (Chang and Dwyer, 1976, 1978) and of *L. donovani* and *L. t. major* (Berman *et al.*, 1979) and *T. dionisii* (A. M. Glauert, R. C. Oliver and K. J. Thorne, unpublished observations: see Fig. 9) by human adherent mononuclear cells.

An alternative electron-dense stain which is taken up into secondary lysosomes by macrophages is saccharated iron oxide. This has been used to demonstrate the fusion of lysosomes with phagocytic vacuoles containing *L. m. mexicana* (Alexander and Vickerman, 1975; Lewis and Peters, 1977) or *T. dionisii* (Liston and Baker, 1978). Carvalho and coworkers (1981a) used horseradish peroxidase to prelabel lysosomes of mouse macrophages and watched it discharge into phagosomes containing amastigotes of *T. cruzi.*

Acridine orange can be used for fluorescence microscopy of lysosomal contents since it concentrates in lysosomes (D'Arcy Hart and Young, 1975). It has been used as an alternative method for the demonstration of fusion of lysosomes with phagocytic vacuoles of macrophages containing *T. dionisii* (Liston and Baker, 1978) and *L. m. mexicana* (Alexander, 1981b). Another fluorescent probe, fluorescein isothiocyanate (FITC) coupled to dextran, was used by Chang (1980) to demonstrate fusion of lysosomes with phagosomes containing amastigotes of *L. donovani* or *L. m. mexicana* in mouse macrophages.

Several authors have reported that, after fusion of lysosomes with phagocytic vacuoles containing protozoa, electron-dense material can be seen in the phagocytic vacuole directly after staining with uranyl acetate, osmium tetroxide or lead citrate. It has been seen with *Leishmania* in macrophages (Gardener, 1974; Trager, 1974; Alexander and Vickerman, 1975), with *T. cruzi* in macrophages (Gardener, 1974), with *T. cruzi* in eosinophils and neutrophils (Sanderson and DeSouza, 1979), with *T. dionisii* in neutrophils (see Fig. 1) and eosinophils (see Fig. 7) (A. M. Glauert, R. C. Oliver and K. J. Thorne, unpublished observations), and with *T. theileri* in bovine eosinophils (see Fig. 8; Townsend *et al.*, 1982). Gardener (1974) believed this material in macrophages to be secreted digestive enzyme from the parasite which was subsequently sequestered into macrophage vesicles. Trager (1974), however,

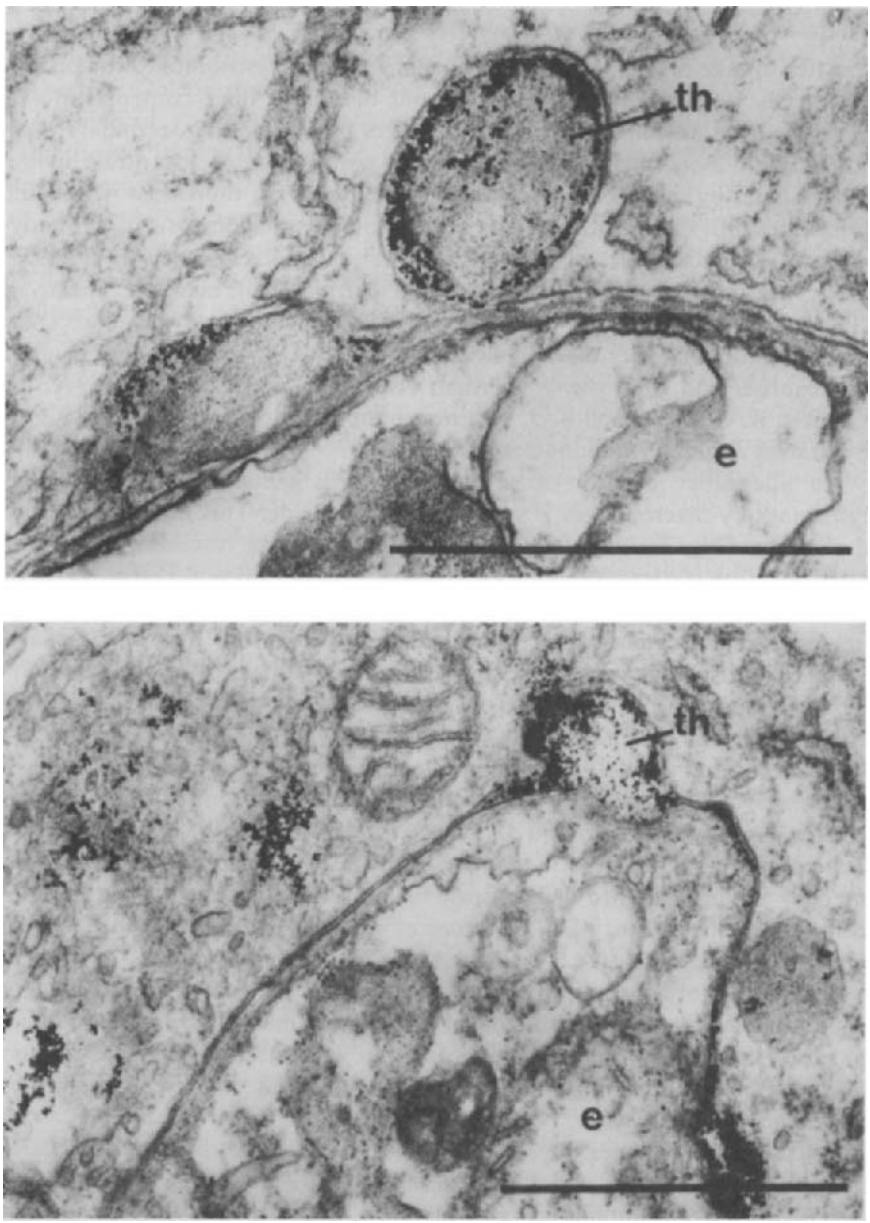

FIG. 9. Fusion of thorotrast-labelled lysosomes (th) with the phagocytic vacuole during the killing of epimastigotes (e) of *T. dionisii* by human blood monocytes. Incubation time 60 minutes. Bar lines 1 μm.

believed that it originated from the lysosomes and could be used to follow discharge of lysosomal contents into the vacuole.

Primary lysosomes can be stained for acid phosphatase with glycerol phosphate and lead nitrate to give an electron-dense precipitate (Bainton and Farquhar, 1968). This not only detects lysosomal contents, but also shows that the lysosomal enzyme is active. Only a low level of acid phosphatase reaction product was detected in phagosomes of macrophages containing *L. m. mexicana* by electron microscopy (Alexander and Vickerman, 1975; Lewis and Peters, 1977). The former authors suggest that these protozoa produce enzyme inhibitors to protect them from the lysosomes, which interfere with the histochemistry. Peroxidase from neutrophil and eosinophil granules was detected in phagosomes containing amastigotes of *L. donovani* by staining with diaminobenzidine and hydrogen peroxide (Chang, 1981a).

2. *Role of hydrolytic enzymes*

The hydrolytic enzymes of lysosomes play a variety of roles in the killing of different species of bacteria. Cathepsin D and elastase, for example, are directly bactericidal to the Gram-negative *Acinetobacter* sp. 199A. These proteases and cathepsin B also render *Acinetobacter* sensitive to lysozyme (Thorne *et al.*, 1976). Similar experiments on the killing of protozoa by proteases have not been done, but it is noteworthy that azurophil granules, which contain hydrolases and lysozyme as well as peroxidase (Bretz and Baggiolini, 1974) kill *T. dionisii* in a hydrogen peroxide-dependent reaction only (Thorne *et al.*, 1978). Possibly some form of synergistic activity between proteases and hydrogen peroxide exists, which would explain even the peroxidase-independent observation of Alexander (1981b) referred to above. With macrophage tumoricidal activity, Adams *et al.* (1981) have shown that hydrogen peroxide at non-lytic concentrations synergistically potentiates the lytic effects of a cytolytic protease secreted by activated macrophages.

Further indirect evidence for the involvement of proteases is suggested by the observed inhibition of the killing of *T. gondii* by activated macrophages with tosyl lysyl chloromethyl ketone (TLCK) (McLeod and Remington, 1980). As discussed previously (Section IV B 3a, p. 90), however, this inhibitor also inhibits activation of the oxidative burst and it may react with serine residues of proteins other than proteases. Indeed, McLeod and Remington (1980) showed that the other protease inhibitors tosyl phenylalanyl chloromethyl ketone (TPCK), tosyl arginine methyl ester (TAME) and soyabean trypsin inhibitor (SBTI) did not interfere with the microbicidal activity.

Although direct evidence of a role for hydrolytic enzymes in killing protozoa has not, so far, been demonstrated, they are important in digesting killed organisms. After *T. gondii* have been killed with xanthine and xanthine oxidase they are rapidly digested by macrophages (Murray and Cohn, 1979).

Electron micrographs reveal that the structures in *T. dionisii* most resistant to lysosomal digestion are the kinetoplast and the flagellum (Thorne *et al.*, 1981; see Fig. 2).

3. *Role of cationic proteins*

The antibacterial and antifungal activity of cationic proteins from neutrophil lysosomes has been known for some time (Hirsch, 1956; Zeya and Spitznagel, 1966; Drazin and Lehrer, 1977). Several cationic proteins have been purified and it has been shown that they kill bacteria through their cationic properties and not through proteolytic activity (Thorne *et al.*, 1976). They are particularly potent against Gram-negative bacteria and appear to react with the lipopolysaccharide of the outer membrane (Modrzakowski and Spitznagel, 1979) and to increase cell permeability (Weiss *et al.*, 1978). Microbicidal cationic proteins have also been found in rabbit alveolar macrophages (Patterson-Delafield *et al.*, 1980). They are of low molecular weight and high arginine and cysteine content (Patterson-Delafield *et al.*, 1981) and are enhanced in level after eliciting with mycobacterial antigen (Lehrer *et al.*, 1981).

While there is no evidence that cationic proteins from neutrophils or macrophages kill protozoa, there is a report that bloodstream forms of *T. cruzi* Tulahuén are killed by the major basic protein (MBP) of eosinophil granules (Kierszenbaum *et al.*, 1981), as assessed by loss of motility and morphological integrity. The cytotoxic activity of eosinophils is inhibited by heparin (300 units ml^{-1}) (Kierszenbaum *et al.*, 1982), which neutralizes the effect of MBP. MBP is known to be highly toxic to larvae of helminths such as *Schistosoma mansoni* (Butterworth *et al.*, 1979) and *Fasciola hepatica* (Duffus *et al.*, 1980), but it has not been found to have activity against other protozoa (K. J. Thorne and D. Franks, unpublished observation).

4. *Role of phagolysosomal pH*

The acidity of the neutrophil phagolysosomes (Jensen and Bainton, 1973) provides an adverse environment for many microbes. Exposure of *T. gondii* to pH 5 for 30 minutes kills 90% of the protozoa (Murray and Cohn, 1979). Low pH also favours prolonging the formation of hydrogen peroxide in the superoxide-initiated non-enzymic oxidation of NADPH discussed previously (Section IV B 1, p. 78). Myeloperoxidase has a pH optimum of 5·5 and catalase can act as a peroxidase at low pH in cells which lack the appropriate peroxidase (Klebanoff, 1969). Cathepsins B and D and exopeptidases, a wide range of lysosomal glycosidases, phospholipases A_1, A_2 and C, DNAase, RNAase, phosphodiesterase and a number of mono-esterases all have acidic pH optima. The main effect of low pH appears, therefore, to be in enhancing other microbicidal mechanisms rather than as a direct toxic agent.

D. CYCLIC NUCLEOTIDES

It has been reported that elevation of the cAMP (adenosine cyclic monophosphate) content of neutrophils inhibits antibody-mediated cellular cytotoxicity while elevation of cGMP (guanosine cyclic monophosphate) facilitates it (Gale and Zighelboim, 1974). cAMP inhibits lysosomal enzyme release while cGMP enhances it (Zurier *et al.*, 1974). cAMP levels can be raised by inhibition of phosphodiesterase with aminophylline and this treatment lowers the ability of activated mouse macrophages to inhibit or kill *T. gondii* (McLeod and Remington, 1980). Since guanylate cyclase is activated by hydroxyl radicals (Mittal and Murad, 1977), initiation of the oxidative burst by protozoa could lead to elevated levels of cGMP, which would stimulate lysosomal enzyme release. Conversely, the adenylate cyclase activity of macrophages from mice infected with *T. gondii* is markedly less than that from uninfected mice (Droller and Remington, 1975).

E. MACROPHAGE ACTIVATION

The activation of macrophages is a very important part of the anti-protozoal immune defence system. We have already discussed how activated macrophages can kill *Leishmania*, *Trypanosoma* and *Toxoplasma*. This can be attributed largely to their enhanced ability to generate oxidative intermediates, but perhaps partially to their increased levels of lysosomal enzymes. Mouse macrophages activated *in vivo* have an enhanced ability to control *T. gondii* and also enhanced levels of acid phosphatase and β-glucuronidase (Reikvam *et al.*, 1975). Cohn (1978) has summarized the known metabolic differences between normal and inflammatory peritoneal macrophages and has concluded that activated macrophages have increased levels of hydrolases, ATP (adenosine triphosphate) and superoxide-generating activity, increased numbers of C3b and, to a lesser extent, Fc receptors, and decreased ecto-enzymes such as 5′-nucleotidase. In addition, the extent of fusion of lysosomes with phagosomes is increased (Kielian and Cohn, 1981). Activated macrophages may thus represent the ultimate in synergistic activity of all the killing mechanisms we have discussed, the infected host thereby mustering its 'last defence' to control the infection.

Lymphocytes have been activated to produce macrophage activating factor (MAF) in a number of ways. They can be activated with concanavalin A or lipopolysaccharide to produce a supernatant which activates macrophages to kill *L. enriettii* (Buchmüller and Mauel, 1979) or *T. cruzi* (Nogueira and Cohn, 1978). Mixed lymphocyte cultures (MLC) produce MAF (Newman *et al.*, 1978), as assessed by their ability to activate macrophages to produce plasminogen activator which can be assayed quantitatively from the lysis of ^{125}I-labelled fibrin. Protozoal antigens react with lymphocytes from specifically

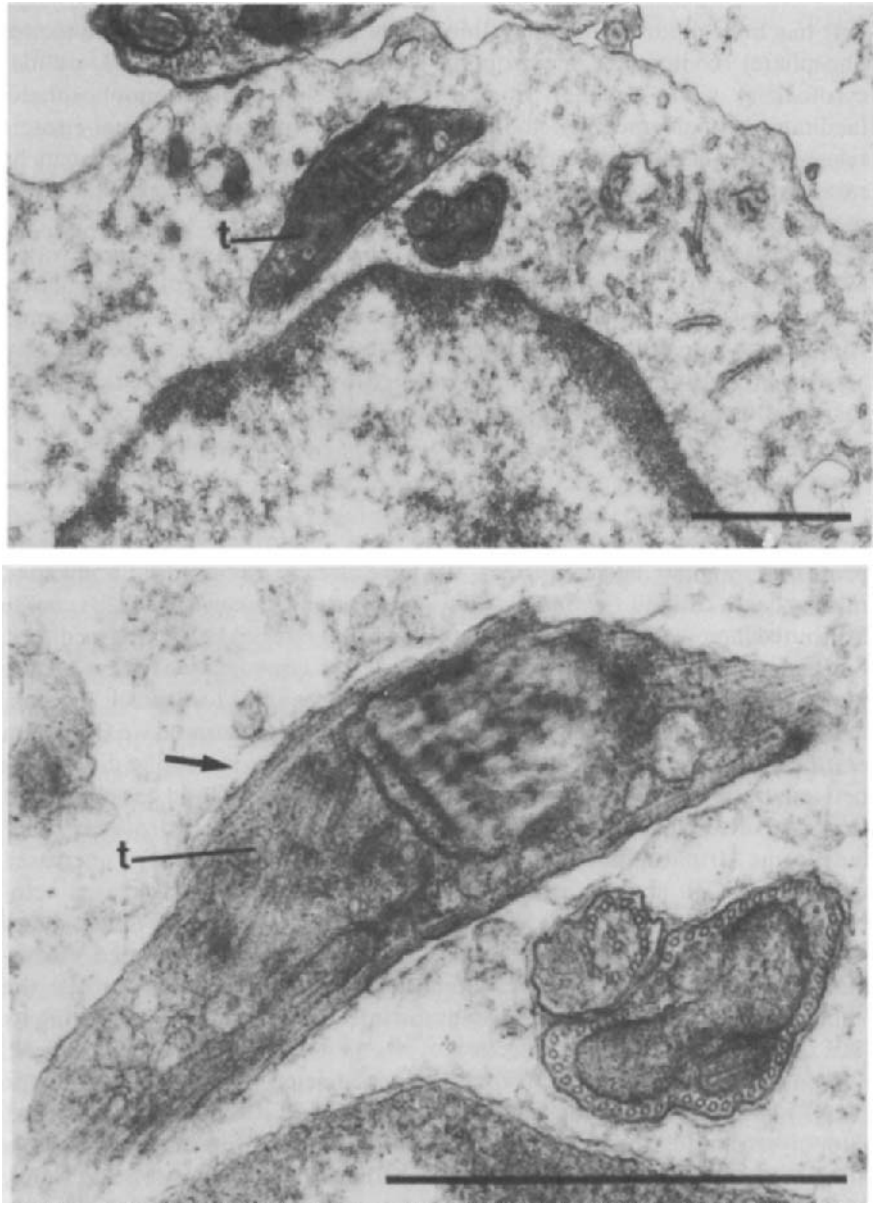

FIG. 10. Damage to phagosome membrane (arrow) of human blood monocyte by phagocytosed trypomastigote (t) of *T. dionisii*. Incubation time 4 hours, without antibody. Bar lines 1 μm.

sensitized animals to produce MAF. The lymphocytes have optimal sensitivity to antigen 3 weeks after infection in mice (Nogueira *et al.*, 1977b) but not until 6 months after infection in a human patient (Johnson, 1981). Lymphocytes from mice infected with the Y strain of *T. cruzi* responded to antigen from the Tulahuén strain (Nogueira *et al.*, 1977b).

T lymphocytes purified on nylon wool columns retain the lymphokine-forming activity (Nogueira *et al.*, 1977b) and this activity is lost when T cells are killed by anti-thy1,2 serum and complement. There is evidence that antigen-induced lymphokine production may be achieved only by Lyt-1$^+$ cells (Adelman *et al.*, 1979) while concanavalin A or MLC induce lymphokine from Lyt-1$^+$ and Lyt-2$^+$3$^+$ cells (Newman *et al.*, 1978). It has been suggested that macrophage handling of antigen is necessary for induction of lymphokine synthesis by T cells (Wahl *et al.*, 1975). No correlation has been demonstrated between histocompatibility type and ability to produce MAF in response to antigen (Nogueira *et al.*, 1977b).

Nacy and coworkers (1981) separated lymphokine supernatants, obtained either from BCG-immunized mouse lymphocytes triggered with PPD (purified protein derivative) or from concanavalin A-stimulated spleen cells, into three active fractions by gel filtration. All three fractions, molecular weights (M_r) 130 000, 45 000 and $< 10\,000$, activated resident peritoneal macrophages to kill intracellular amastigotes of *L. t. major*. Only one of these fractions, M_r 50 000, also increased the resistance of macrophages to infection with *L. t. major*. This fraction co-chromatographs with the lymphokine activity for induction of macrophage mediated tumour cytotoxicity, and also has the same molecular weight as mouse interferon (Schultz, 1980).

The receptor for MAF on macrophages is sensitive to both trypsin and neuraminidase (Buchmüller and Mauel, 1980; Jones, 1980) and the effect of MAF is enhanced by α_1-antitrypsin. To activate macrophages to kill *L. enriettii* the lymphokine need only be present for 20 h, and indeed the receptor can be destroyed with trypsin after 9 h without the macrophage losing anti-leishmanial activity (Buchmüller and Mauel, 1980). Killing of *T. cruzi* is achieved only if the lymphokine is present continuously and is constantly replenished (Nogueira and Cohn, 1978). However the lymphokine can activate macrophages to kill *T. cruzi* which have escaped from the phagosome into the cytoplasm.

While activation of macrophages with lymphocyte supernatant lymphokines is non-specific, a specific macrophage 'arming' factor (SMAF) has been described (Evans and Alexander, 1970). Macrophages armed by SMAF are activated only in the presence of the specific antigen which acted on the lymphocytes to produce the specific SMAF. Some of the observed macrophage activation by supernatants from antigen-activated immune lymphocytes which occurs in the presence of protozoa may be of this type. Hence, as we

TABLE 11
Summary of evasion mechanisms

Organism	Stage	Evasion mechanism	Reference
Toxoplasma gondii	Trophozoite	Inhibition of lysosomal fusion	Jones and Hirsch (1972)
		Capping and shedding of surface antigen–antibody complexes	Dzbenski *et al.* (1976)
		Immunosuppression	Huldt *et al.* (1973)
Trypanosoma brucei	Trypomastigote	Antigenic variation	Cross (1978)
		Immunosuppression	Goodwin *et al.* (1972)
T. cruzi	Trypomastigote	Escape into cytoplasm	Nogueira and Cohn (1976)
		Immunosuppression	Cunningham and Kuhn (1980a)
		Capping and shedding of surface antigen–antibody complexes	Schmuñis *et al.* (1980)
		Bound host serum protein	Snary (1980)
		? Digestion of phagosome membrane	
T. dionisii	Trypomastigote	Escape into cytoplasm	Thorne *et al.* (1981)
		Peroxidase resistant surface	Thorne *et al.* (1981)
Leishmania donovani	Promastigote	Concealment within fibroblasts	Chang (1978a)
L. donovani	Amastigote	Resistant surface	Chang and Dwyer (1978)
		Immunosuppression	Kutisch and Janovy (1981)
L. m. mexicana	Amastigote	Inhibition of lysosomal enzymes	Alexander and Vickerman (1975)
L. t. major		Immunosuppression	Howard *et al.* (1980a)

Organism	Stage	Evasion mechanism	Reference
L. enriettii	Amastigote	Immunosuppression Capping and shedding of surface antigen–antibody complexes	Bryceson *et al.* (1974) Doyle *et al.* (1974)
Plasmodium spp.	Schizont	Concealment in hepatocytes	Cohen (1980)
P. berghei		Immunosuppression	Lelchuk and Playfair (1980)
		Antigenic variation	Cox (1959)
	Merozoite	Antiphagocytic capsule	Brooks and Kreier (1978)
P. yoelii		Immunosuppression	Lelchuk and Playfair (1980)
P. cynomolgi		Antigenic variation	Voller and Rossan (1969)
P. knowlesi		Antigenic variation	Brown and Brown (1965)
P. falciparum		Antigenic variation	Wilson and Phillips (1976)
Babesia spp.		Antigenic variation	Phillips (1969)
		Immunosuppression	Phillips and Wakelin (1976)
Naegleria fowleri		Capping and endocytosis of surface antigen–antibody complexes	Ferrante and Thong (1979)
Entamoeba histolytica		Capping and endocytosis of surface antigen–antibody complexes	Aust-Kettis and Sundqvist (1978)

observed previously (Section III B 1, p. 69), Borges and Johnson (1975) found that human monocytes could be activated to kill *T. gondii* only by the specific supernatant from immune T lymphocytes incubated with *T. gondii* antigen, and not by the supernatant from T lymphocytes from tuberculin-positive subjects incubated with PPD, nor from concanavalin A-stimulated lymphocytes.

The mechanisms whereby lymphokines activate macrophages to produce enhanced levels of lysosomal enzymes, oxidative capability and perhaps lysosomal fusion are not known. Cyclic nucleotides may play a role. The level of adenylate cyclase is lower in macrophages from mice infected with *T. gondii* than in control animals and it increases poorly in response to prostaglandin E_1 (Droller and Remington, 1975). Possibly reduced levels of cAMP and enhanced levels of cGMP mediate an activation of the macrophage cytotoxicity. However, Masur *et al.* (1981) found elevated levels of cAMP in *Toxoplasma*-immune macrophages and in normal macrophages incubated with *Toxoplasma*-induced lymphokine and heart infusion broth. Hydrocortisone treatment (100 $\mu g\ ml^{-1}$) renders macrophages unresponsive to lymphokine from *T. gondii*-triggered immune lymphocytes (Masur *et al.*, 1982), but this cannot be explained by an inhibition of prostaglandin synthesis since neither indomethacin nor aspirin interferes with the macrophage activation. Indeed activated macrophages from mice treated with BCG or *C. parvum* show *reduced* formation of arachidonic acid metabolites, in response to a zymosan trigger, and the reduced capacity to form prostaglandins correlates with increased anti-*Toxoplasma* activity (Scott *et al.*, 1982). A similar modification of arachidonic acid metabolism when resident macrophages are exposed to lymphokine *in vitro* was not observed.

F. INTERFERON

Interferon inhibits the development of *P. berghei* in mice (Jahiel *et al.*, 1969) and *T. gondii* in chick embryo fibroblasts and mouse L cells (Remington and Merigan, 1968). Enhanced levels of interferon are found after infection with *P. berghei* (Huang *et al.*, 1968) and *T. gondii* (Rytel and Jones, 1966). Elevation of interferon levels in C57BL/6J mice by treatment with tilerone enhances resistance to bloodstream trypomastigotes of *T. cruzi* (James *et al.*, 1982). Resident peritoneal macrophages from these animals have an enhanced ability to take up *T. cruzi* and to control its multiplication. Interferon may be acting as a macrophage activating factor (Schultz, 1980).

V. EVASION OF HOST MICROBICIDAL ACTIVITY

Different protozoa have evolved a remarkable variety of different mechanisms to evade the immunological defences of the host. These are summarized in Table 11.

A. ZOOMASTIGOPHOREA

1. *Trypanosoma*

(*a*) *Salivarian trypanosomes.* Since cell-mediated immunity to salivarian trypanosomes is antibody-dependent, evasion involves interference with the formation of specific antibodies. Antigenic variation with the appearance of different glycoproteins on the cell surface (Cross, 1978) allows *T. brucei* to evade recognition by circulating antibody and thereby evade antibody-dependent killing. Surface glycoprotein antigen can be lost from *T. brucei* trypomastigotes spontaneously from the entire surface when transferred into culture, but this is not in response to antibody (Barry and Vickerman, 1979). Since, in addition, new variants can appear in the absence of antibody (Doyle *et al.*, 1980), it seems unlikely that antibody induces variant formation. Instead, host antibody would 'weed out' the susceptible homologous variants and allow overgrowth of new variants to which the host has not yet developed immunity. The mechanism of formation of new antigens appears to involve differential expression of a large series of different genes (Hoeijmakers *et al.*, 1980). The existence of a basic gene copy in each variant, together with an expression-linked copy in a variant expressing that gene, has been described.

Both acute and chronic infections with salivarian trypanosomes are enhanced as a consequence of trypanosome-induced immunosuppression. *Trypanosoma brucei* infection in mice and rabbits ultimately depresses antibody responses even to unrelated antigens (Goodwin *et al.*, 1972). Spleens from mice infected with *T. brucei* contain suppressor cells, as assessed by lack of responsiveness to T and B cell mitogens and inhibition of these responses in normal cells (Jayawardena and Waksman, 1977). Normal spleen cells lose their ability to mount a plaque-forming cell response to sheep red blood cells when incubated with suppressor spleen cells from mice infected with *T. rhodesiense* (Wellhausen and Mansfield, 1980). Evidence for exhaustion of B cell potential and possible involvement of suppressor T cells and macrophages has been presented (Corsini *et al.*, 1977). A particulate fraction from *T. brucei* will mimic the immunosuppressive effect of the whole parasite (Clayton *et al.*, 1979). A high molecular-weight soluble fraction from the parasite has been further purified and the lipid component has been found to suppress significantly a primary immunoglobulin G (IgG) anti-sheep erythrocyte response in mice (Sacks *et al.*, 1982).

(*b*) *Stercorarian trypanosomes.* Intracellular trypanosomes are phagocytosed by macrophages, but subsequently escape from the phagocytic vacuole into the cytoplasm where they survive and multiply (Kress *et al.*, 1975, 1977). Trypomastigotes of *T. cruzi* (Nogueira and Cohn, 1976) and *T. dionisii* (Thorne *et al.*, 1981) escape destruction in the phagocytic vacuole of macrophages more readily than do epimastigotes. Interestingly, Milder and

coworkers (1977) showed that only trypomastigotes of the Y strain (slender) of *T. cruzi* escape into the cytoplasm of hamster macrophages, whereas trypomastigotes of the F strain (broad) are killed. They postulate that antibody may be formed to the broad F strain but not to the slender Y strain. Certainly trypomastigotes of *T. cruzi* (Kierszenbaum, 1979) and of *T. dionisii* (Thorne *et al.*, 1981) are killed more readily by human monocytes in the presence of antibody than in its absence. However, trypomastigotes isolated from the blood stream of mice carry bound mouse serum protein which interferes with antibody bonding (Snary, 1980), and attached antibody can be shed by capping of the surface antigen and subsequent loss into the medium (Schmuñis *et al.*, 1980). In the latter experiments, peroxidase-labelled anti-IgG was added only after capping to detect the localization of anti-trypanosome IgG, and did not therefore itself induce capping artificially. Surface antigen is regenerated during the following few hours. The cell surface glycoprotein of *T. cruzi* does not exhibit the antigenic variation observed in *T. brucei* (Snary, 1980).

Trypomastigotes of *T. cruzi* (Milder and Kloetzel, 1980) and *T. dionisii* (Liston and Baker, 1978) do not inhibit lysosomal fusion and they are therefore exposed to the degradative activity of the lysosomal enzymes. Trypomastigotes of *T. dionisii* are, however, more resistant to hydrogen peroxide and peroxidase than are epimastigotes (Thorne *et al.*, 1981). Possibly the higher peroxidative activity of neutrophils and activated macrophages (Nathan *et al.*, 1979) is sufficient to kill trypomastigotes while the lower activity of blood monocytes and resting macrophages is insufficient, thus allowing them to escape and make their way into the cytoplasm. Disintegration of the phagosome membrane has been observed in macrophages infected with *T. cruzi* (Nogueira and Cohn, 1976) and *T. dionisii* (Liston and Baker, 1978; Thorne *et al.*, 1981). It is not clear if this breakdown of the membrane is caused by autodigestion by the macrophage lysosomal enzymes or if the trypomastigote releases extracellular degradative enzymes. Cell-free extracts from culture forms of *T. cruzi* have been prepared which lyse mammalian cells (O'Daly and Aso, 1979). The active factor is heat stable and lipid soluble. Trypomastigotes of *T. cruzi* contain high levels of acid protease and α-mannosidase (Steiger *et al.*, 1979), but it is not known if any of these substances are secreted extracellularly.

Macrophages require prior activation with lymphokine to kill trypomastigotes of *T. cruzi*. *Trypanosoma cruzi* infection can impair T cell function (Teixeira *et al.*, 1978). Cunningham and Kuhn (1980a) have isolated a protein of M_r 200 000 from the serum of mice infected with *T. cruzi* which activates spleen suppressor cells. The suppressor cells are adherent, non-thy1,2 and therefore appear to be of macrophage lineage (Rowland and Kuhn, 1978; Cunningham and Kuhn, 1980b). Immunosuppression by *T. musculi* appears

to involve inhibition of antibody producing cells (Albright and Albright, 1981) and is independent of suppressor T cells. When an extract of *T. musculi* was incubated for 2 hours with normal mouse spleen cells they lost their ability to respond to antigen. The trypanosome-derived substance was detected, using specific antibody, on the surface of a substantial proportion of infected mouse spleen cells and treatment with the antibody eliminated the inhibition of the spleen cell response to lipopolysaccharide. *Trypanosoma lewisi* did not inhibit the mouse immune response (Albright and Albright, 1981).

Bloodstream trypomastigotes resist phagocytosis by mouse macrophages, possibly by the presence of a surface glycoprotein of M_r 90 000 and isoelectric point 5·0 (Nogueira *et al.*, 1981a). The anti-phagocytic material is neutralized by specific antiserum. Since *T. cruzi* needs to enter macrophages to multiply in the host cell cytoplasm it is not clear how the anti-phagocytic substance helps it to survive. It would be of interest to know if it prevents phagocytosis of *T. cruzi* by the more dangerous neutrophils.

2. *Leishmania*

Amastigotes of *Leishmania* sp. live and multiply in macrophage phagocytic vacuoles with a high natural rate of phagolysosomal fusion (Alexander and Vickerman, 1975; Chang and Dwyer, 1976, 1978; Berman *et al.*, 1979; Alexander, 1981b). Promastigotes are more susceptible than amastigotes to killing by macrophages and need to revert to amastigotes to survive (Lewis and Peters, 1977). Chang (1978c) has shown that promastigotes of *L. donovani* can avoid macrophages by entering non-phagocytic cells such as fibroblasts and there transform into the more resistant amastigotes which may subsequently escape and colonize macrophages. It has been suggested (Alexander and Vickerman, 1975; Lewis and Peters, 1977) that amastigotes of *L. mexicana* survive by inhibiting lysosomal enzymes, since the detectable acid phosphatase activity is very low in vacuoles that contain living protozoa and since the parasitophorous vacuoles become distended, presumably from fluid influx in response to the presence of much indigestible material. Amastigotes of *L. mexicana* have high levels of an endogenous soluble cysteine proteinase which is not found in promastigotes and which could inactivate macrophage enzymes (Coombs, 1982).

Chang and Dwyer (1978) believe that *L. donovani* amastigotes do not inhibit lysosomal enzymes since other material in the same parasitophorous vacuoles is digested. They therefore attribute the resistance of *L. donovani* amastigotes to a particularly refractory surface. El On *et al.* (1980) have shown that anionic carbohydrates of M_r 18 000 and 27 000 secreted by *L. donovani* and *L. t. major* promastigotes (Slutzky *et al.*, 1979) inhibit β-galactosidase but not acid phosphatase. It would be of interest to know if the more resistant amastigotes also produce soluble lysosomal enzyme inhibitors.

Leishmanial infection can lead to a generalized suppression of macrophage effector function. Bryceson *et al.* (1974) reported that large inocula of *L. enriettii* in guinea pigs produced desensitization of cell-mediated immunity and development of large ulcerated primary lesions with widespread metastases. *In vitro* infection of resident hamster macrophages with the virulent strain 2S of *L. donovani* inhibits their ability to kill the kinetoplastid *Leptomonas costons*, but infection with an avirulent Khartoum strain has no effect (Kutisch and Janovy, 1981). *In vivo* infection with *L. enriettii* produces an initial resistance to re-infection which begins to decay after 5 weeks and cannot be re-established by infusion of immune lymphocytes (Poulter and Pearce, 1980). Antibody levels remain high but macrophage-mediated cytotoxic activity against *L. enriettii* and against *Listeria monocytogenes* is suppressed.

Howard and coworkers (1980a) attribute the suppression of cell-mediated immunity by *L. t. major* in genetically susceptible mice to the formation of a potent specific suppressor T cell population. In this model, development of suppression was independent of the inoculating dose, could be abrogated by adult thymectomy, X-irradiation and bone marrow reconstitution (ATxXBM) or by sublethal irradiation and was transferable with T enriched lymphocyte populations (Howard *et al.*, 1980a, 1981). Howard and coworkers (1980c) believed, however, that the development of suppression was a secondary effect of the high antigenic load created by innately susceptible macrophages. *Leishmania*-induced immunosuppression thus appears to require high concentrations of leishmanial antigen, as a consequence of either inefficient destruction by macrophages or the use of large inocula, as in the earlier work (Bryceson *et al.*, 1974). The role of antibodies in resistance to leishmanial infection is unclear, but since the binding of immunoglobulin to leishmanial surfaces induces capping and shedding of surface antigen (Doyle *et al.*, 1974; Dwyer, 1976), antibodies may have the adverse effect of enhancing immunosuppression by causing accumulation of high concentrations of leishmanial antigen. Antibody appears to inhibit phagocytosis of *L. donovani* amastigotes by neutrophils and monocytes (Chang, 1981b), possibly because the shed antigen–antibody complexes block the Fc receptors.

B. SPOROZOEA

1. *Toxoplasma*

Toxoplasma gondii survive and multiply in the phagocytic vacuoles of mouse macrophages. They avoid exposure to the degradative enzymes of the macrophage lysosomes by inhibiting fusion of lysosomes with the phagosome (Jones and Hirsch, 1972). The mechanism whereby *Toxoplasma* exerts this

effect is not known but it occurs only with living *T. gondii*. In experiments with amastigotes and promastigotes of *L. mexicana*, which inhibit lysosomal fusion by only about 10%, Alexander (1981b) showed that the polyanion polyglutamic acid greatly enhanced this small inhibition of fusion and thereby allowed survival even of promastigotes. Fusion can also be inhibited by anionic glycolipids from *M. tuberculosis* (Goren *et al.*, 1976). Perhaps the inhibition of fusion by *T. gondii* also involves polyanions. Electron micrographs reveal that when fusion is inhibited by *T. gondii* the phagosome becomes surrounded by endoplasmic reticulum and mitochondria and lysosomes are excluded from this region of the cell. The requirement for lysosomal enzymes, in addition to the initial product of a membrane NADPH oxidase, superoxide and hydrogen peroxide, to kill the protozoa confirms the observations that *T. gondii* is susceptible to hydrogen peroxide only in the presence of peroxidase or after its conversion into hydroxyl radicals.

If the infected host produces antibodies to *T. gondii*, the antibody-coated protozoa lose their ability to inhibit lysosomal fusion and hence their ability to avoid the toxic agents of the infected cell (Jones *et al.*, 1975). Subsequently, however, *T. gondii* may shed the attached immunoglobulin by capping to the anterior pole followed by discharge of antigen–antibody complexes into the external medium (Dzbenski *et al.*, 1976). Since in this study capped and shed immunoglobulins were detected with a second antibody, labelled with fluorescein, peroxidase or ^{125}I, it is possible that the second antiserum artificially induced cross-linking and capping and that the immunoglobulin may not be shed as readily *in vivo*.

Toxoplasma gondii can also inhibit antibody formation by a generalized immunosuppression (Huldt *et al.*, 1973). Interestingly, *T. gondii* induces immunosuppression in genetically low-responder mice (C57BL/6J) only, not in a high-responder strain (Nya:Nylar) (Macario *et al.*, 1980).

2. *Plasmodium*

Since the clearance of both free merozoites and infected erythrocytes by macrophages is antibody-mediated, evasion mechanisms are directed primarily towards avoidance of antigenic recognition.

Physical protection by concealment in host cells may occur with schizonts living in hepatic parenchymal cells, since plasmodial antigens are probably not expressed on liver cell surfaces (Cohen, 1980). Mature parasitized erythrocytes carry surface plasmodial antigen (Schmidt-Ullrich and Wallach, 1978) which could bind antibody and mediate macrophage endocytosis, but the parasite is protected from T cells owing to the absence of major histocompatibility complex (MHC) determinants on the erythrocyte cell surface.

The principal means of evasion involves the development of antigenic variants. This has been observed in *P. berghei* (Cox, 1959), *P. cynomolgi*

(Voller and Rossan, 1969), *P. knowlesi* (Brown and Brown, 1965) and *P. falciparum* (Wilson and Phillips, 1976).

Immunosuppression is observed both in human and murine malaria and has been investigated in some detail. Lelchuk and Playfair (1980) described two distinct types of non-specific immunosuppression in murine malaria. *Plasmodium berghei* infection induces cells that suppress cell-mediated DTH responses and also proliferation in response to mitogen. *Plasmodium yoelii* infection suppresses only the proliferative response. The anti-proliferative effect may be due to impairment of macrophage function. Warren and Weidanz (1976) found that a splenic adherent cell, probably the macrophage, from mice infected with *P. yoelii*, is functionally defective as an accessory cell in plaque-forming response to horse red blood cells. Both *P. yoelii* and *P. berghei*, but not *P. chabaudi*, inhibit the bactericidal activity of mouse liver and spleen macrophages *in vivo* (Murphy, 1981). A high molecular-weight factor released from erythrocytes infected with *P. berghei* suppresses development of cell-mediated response to sheep red blood cells, but enhances the humoral response (Liew *et al.*, 1979). This factor was not found in the serum of infected mice.

The extracellular merozoite of *P. berghei* protects itself from phagocytosis with an anti-phagocytic capsule (Brooks and Kreier, 1978), the activity of which is neutralized by specific antiserum.

3. *Babesia*

The evasive steps taken by *Babesia* to avoid immune reactions resemble those of *Plasmodium*. Antigenic variation has been detected in *B. rodhaini* (Phillips, 1969), *B. bovis* (Curnow, 1973) and *B. bigemina* (Callow, 1967). Immunosuppression is seen after infection with *B. microti* and *B. hylomysci* (Phillips and Wakelin, 1976). Antibody production is decreased but cell-mediated responses are unaffected. Phagocytic activity is, however, increased (Purvis, 1977).

C. LOBOSEA

1. *Entamoeba*

Capping followed by internalization of surface antibody has been detected in *E. histolytica* with fluorescent anti-IgG (Aust-Kettis and Sundqvist, 1978). These authors also detected loss of surface IgG even without a second cross-linking antibody.

2. *Naegleria*

Resistance to *Naegleria fowleri* is antibody-dependent. One mechanism of evasion involves antibody-induced capping of surface antigens (Ferrante and

Thong, 1979). Specific antibody initially immobilizes the amoebae, but after further incubation the amoebae return to their original state of amoeboid movement. Antibody-induced capping was, however, detected only when the antibody-coated amoebae were incubated with a second, fluorescent, anti-IgG. It is therefore probable that univalent antigens on the *Naegleria* surface are not capped directly by antibody but that a second agent such as rheumatoid factor is necessary to cross-link the antigen–antibody complexes *in vivo*. Capping was followed by endocytosis of antigen–antibody complexes and not by shedding.

VI. Genetic Control of Resistance Mechanisms

In recent reviews Wakelin (1978) examined the whole topic of genetics of resistance to parasite infections whilst Bradley (1980) dealt specifically with protozoal infections. Blackwell (1981) summarized data on patterns of mouse strain susceptibility to infections including the protozoan parasites. Although the genetics is, in many cases, very well defined little is known as yet of the mode of action of resistance genes at the molecular level. We include here a brief account of genetically-controlled host resistance to protozoal infection and the associated differences in the host's response to the infection.

A. ZOOMASTIGOPHOREA

1. *Trypanosoma*

Strain differences in resistance and susceptibility of mice have been recorded to both salivarian [*T. brucei* (Clarkson, 1976; Clayton, 1978), *T. congolense* (Morrison *et al.*, 1978; Morrison and Murray, 1979, 1982) and *T. rhodesiense* (Levine and Mansfield, 1981)] and stercorarian species [*T. cruzi* (Cunningham *et al.*, 1978; Trischmann *et al.*, 1978; Nogueira *et al.*, 1981b; Trischmann and Bloom, 1982) and *T. musculi* (Jarvinen and Dalmasso, 1977)]. Trypanotolerance to salivarian trypanosomes has recently been reviewed by Murray, M. *et al.* (1982). Mouse strain distribution patterns are similar for all four trypanosome infections, with a broad spectrum of resistance phenotypes from highly susceptible (C3H), through intermediate (BALB), to highly resistant (C57BL/6, C57BL/10). Resistance is dominant in F1 crosses between resistant and susceptible mice (Morrison and Murray, 1979; Trischmann and Bloom, 1982), with evidence for complementation between several resistance genes. Analysis of F1, F2 and backcross generations indicates polygenic control (Morrison and Murray, 1979; Trischmann and Bloom, 1982) with the influence of *H*-2 observed only for *T. cruzi* on the intermediate BALB

genetic background (Trischmann and Bloom, 1982). In all cases resistance and susceptibility were assessed as time to death. Since mortality represents the summation of many events, each of which may exert only a small but additive influence on survival, it is not surprising that polygenic control is indicated. A more careful analysis of the sequence of events which follow infection might eventually reveal single gene-controlled mechanisms which have a major regulatory influence on the subsequent course of infection. Similar strain distribution patterns for all the trypanosome infections examined is consistent with the notion of a single major event which occurs early in infection and regulates the subsequent progression of the disease whether it be due to a salivarian or stercorarian species. Events that correlate with susceptibility and resistance have been recorded but so far no direct causal relationship has been proven.

In *T. brucei* infection, Clarkson (1976) demonstrated a marked rise in serum IgM in strains that survived longest, although differential parasitaemias were not observed. With the NIM6 antigenic clone of *T. brucei*, Clayton (1978) found that there was usually an inverse relationship between survival time and parasite load during the first peak of parasitaemia. Cunningham and co-workers (1978) found that, although several major differences in phenomena related to immunosuppression between susceptible and resistant mice were observed, there was no apparent relationship between non-specific suppression of the humoral response and resistance to *T. cruzi* infection. Tanowitz and coworkers (1981) have shown, however, that inhibition of the primary antibody response to intravenously administered sheep erythrocytes correlates with susceptibility, whereas no inhibition or, indeed, augmentation of the response correlates with natural as well as acquired resistance. The higher antigenic load in susceptible mice possibly enhances suppressor cells, whilst in resistant mice helper cell function may be amplified. In *T. congolense* infection, Morrison *et al.* (1978) found that, although non-specific polyclonal activation of lymphocytes was observed in all strains, the increase in splenic B and null lymphocyte populations was less marked in the resistant (longest surviving) strains. The capacity of certain strains to survive longer than others appeared to be related to their ability to limit the number of trypanosomes in the circulation. Whether the smaller increase in splenic B and null cells is associated with a decreased sensitivity of these strains to polyclonal activation induced by trypanosomes, or whether this is merely the result of lower levels of parasitaemia, remains to be determined.

Kierszenbaum and Howard (1976) showed that the Biozzi high and low antibody-responding lines of mice (Ab/H and Ab/L) differ in their response to *T. cruzi* infection. Low-responder mice were significantly more susceptible to infection in terms of shorter survival times, increased mortality rate, higher parasitaemias and lower LD_{50}. Although this correlated with much lower

anti-*T. cruzi* antibody titres in the Ab/L mice, other studies have shown that Ab/H and Ab/L lines differ functionally in many responses including differential macrophage function (Mouton *et al.*, 1976).

Impairment of the immune system in resistant C57BL/10 mice by X-irradiation, splenectomy, or treatment with silica led to high and often fatal *T. cruzi* parasitaemias (Trischmann *et al.*, 1978). Athymic mice also attained exceptionally high parasitaemias before dying. Similarly, athymic and splenectomized mice were more susceptible to *T. congolense* infection (Morrison *et al.*, 1978), although the effect of splenectomy was less pronounced for the resistant C57BL/6 strain. Nogueira *et al.* (1981b) demonstrated that resistance and susceptibility to *T. cruzi* in C57BL/6 and C3H mice correlated with earlier *in vivo* lymphokine production in the resistant strain, although there was no differential sensitivity of macrophages to lymphokine activation *in vitro*.

Boyer *et al.* (in press) have recently shown that four inbred strains of mice bearing the single autosomal recessive gene, *lpr* (lymphoproliferation), are significantly more susceptible to acute infection with the Y strain of *T. cruzi* than their normal congenic partner strains. This was true for resistant (C57BL/6), intermediate (AKR) and susceptible (C3H) genetic backgrounds. The gene controls certain autoimmune manifestations and provides a model for studying the kind of autoimmune aberrations which may contribute to the immunopathological consequences of Chagas's disease.

2. *Leishmania*

Innate resistance or susceptibility to *L. donovani* infection in mice (measured as liver parasite burdens over the first 2 weeks of infection) is under the control of a single autosomal gene (*Lsh*) segregating for incompletely dominant resistant (r) and recessive susceptible (s) alleles (Bradley, 1974, 1977). *Lsh* maps to a position near *Idh-1* on mouse chromosome 1 (Bradley *et al.*, 1979) and appears to be identical with the genes *Ity* and *Bcg* which control the innate responses to *Salmonella typhimurium* and *Mycobacterium bovis* infections in mice (Plant *et al.*, 1982). Resistance is unaltered by thymectomy (Bradley and Kirkley, 1972), ATxXBM treatment (J. M. Blackwell, unpublished observation), pretreatments with cyclophosphamide or sublethal irradiation (Ulczak and Blackwell, in press), or lethal irradiation (P. Crocker and J. M. Blackwell, unpublished observations). Autoradiographic studies demonstrate much lower parasite proliferation rates in the livers of resistant mice than in susceptible animals as early as 3 days after infection (Bradley, 1979). Similarly, studies of early *in vivo* multiplication of *S. typhimurium* (Hormaeche, 1980) demonstrate lower bacterial multiplication rates in resistant mice than in susceptible animals. Nevertheless, current studies following the *in vitro* course of *L. donovani* infection in Kupffer cells isolated

from mice at various intervals following *in vivo* infection, indicate that the naive state of the macrophages from both susceptible and resistant mice is 'susceptible' and that 1–3 days are required before *Lsh* gene activity is expressed (P. Crocker, unpublished observations). In reciprocal radiation chimaeras between histocompatible strains, resistance and susceptibility can be transferred with the donor haematopoietic system (P. Crocker and D. J. Bradley, unpublished observations). Similarly, resistance to *S. typhimurium* can be transferred with the bone marrow (Hormaeche, 1979; J. E. Plant, personal communication). All the available evidence suggests, therefore, that *Lsh* (*Ity*/*Bcg*) gene activity is expressed at the level of the infected resident tissue macrophage.

Amongst homozygous recessive Lsh^s strains of mice different phenotypic patterns of recovery are observed when the course of infection is followed over a longer term (Bradley and Kirkley, 1977). In some strains a dramatic fall in parasite numbers with histological liver damage occurs, while other strains maintain immense parasite loads for up to 2 years involving mononuclear phagocytes throughout the body. On B10 and BALB genetic backgrounds this difference in longterm response (measured as liver parasite burden over 130 days of infection) is largely controlled by one or more gene(s) situated in the K end of the *H*-2 complex (Blackwell *et al.*, 1980; Blackwell, 1982). Three phenotypic patterns of recovery are observed, early cure ($H\text{-}2^{s,r}$), cure ($H\text{-}2^b$) and non-cure ($H\text{-}2^{d,f,g}$), with 'cure' behaving as a recessive trait in $H\text{-}2^{b/d}$ mice. 'Cure' is T cell-mediated (Skov and Twohy, 1974a, b) and correlates with a positive DTH response (DeTolla *et al.*, 1980; Rezai *et al.*, 1980). 'Non-cure' is dose-dependent and correlates with reduced DTH reactivity and the generation of suppressor cell populations during infection (Ulczak and Blackwell, in press). Whether *H*-2 directly controls differential suppressor and/or effector cell function in the three phenotypes has yet to be determined. Non-*H*-2 linked genes also play a role in acquired resistance to *L. donovani* infection (DeTolla *et al.*, 1980; Semprevivo *et al.*, 1981; Blackwell, 1982). On a B10($H\text{-}2^b$) genetic background, strains carrying the alternative $Ir\text{-}2^b$ allele mimic the *H*-2 linked 'early cure' haplotypes, while strains carrying the alternative $H\text{-}11^b$ allele mimic the 'non-cure' haplotypes. In CXB recombinant inbred strains between BALB/c and C57BL/6 mice, other non-*H*-2 linked genes influence the rate of recovery in some strains, although *H*-2 appears to control the eventual outcome.

The extreme non-healing response of certain mouse strains to subcutaneous *L. t. major* promastigote infection has also been shown to be under single gene control (Howard *et al.*, 1980b; DeTolla *et al.*, 1981) and is associated with a diminished DTH response (Nasseri and Modabber, 1979; Howard *et al.*, 1980a). In attempting to explain the diminution of cell-mediated immunity in anergic forms of leishmaniasis, Garnham and Humphrey (1969) suggested

that it might be due to the blocking effect of humoral antibody. The development of higher anti-leishmanial antibody titres does not, however, appear to determine either mouse strain susceptibility to *L. t. major* (Handman *et al.*, 1979; Olobo *et al.*, 1980) or inhibition of DTH during infection (Hale and Howard, 1981). Recent studies (Howard *et al.*, 1980a) have shown, in fact, that the extreme non-healing response of BALB/c mice to *L. t. major* infections corresponds with the generation of a potent specific T suppressor (T_S) cell population (cf. Section V A, p. 112). In reciprocal radiation chimaeras between histocompatible pairs of susceptible and resistant strains, susceptibility is determined by descendents of the donor haematopoietic cells (Howard *et al.*, 1980c). Generation of T_S cells in susceptible mice is seen, therefore, as a secondary effect of the high antigenic load created by an innately susceptible macrophage population. *In vitro* studies with peritoneal macrophage populations suggest, in fact, that there is a correlation between *in vivo* susceptibility and both quantitative (Behin *et al.*, 1979) and qualitative (Nacy *et al.*, in press) responses to lymphokine activation *in vitro*. In addition, *in vitro* studies by Gorczynski and MacRae (1981), using macrophages isolated from skin, have shown that, even in the absence of lymphokine activation, susceptible macrophages support greater multiplication of the parasites than macrophages from resistant mice. Expression of antigen Ia and antigen-presenting function also differed in skin macrophages isolated from resistant and susceptible mice.

3. *Giardia*

Roberts-Thomson and Mitchell (1978) observed marked variation in the time course of spontaneous elimination of *G. muris* amongst inbred mouse strains. Analysis of F1 hybrids indicates that resistance is dominant, but the results of further genetic analyses have yet to be reported. Humoral and cell-mediated immune responses have been evaluated in resistant (BALB/c) and susceptible strains (C3H/He) by Anders *et al.* (1982), who examined IgG and IgA antibody levels in parasite-specific antiserum, IgA levels in intestinal washes, and DTH reactivity in response to parasite antigen. Some difference in the degree of antibody responses was observed but there did not appear to be any defect in either humoral or cell-mediated immune responses in the susceptible strain. Both humoral and cell-mediated responses were assessed using whole sonicated trophozoites as antigen and the authors suggest that the strains may, in fact, differ in their responses to individual parasite antigens.

4. *Trichomonas*

Strain differences in resistance and susceptibility to the extracellular protozoon *T. vaginalis* measured *in vivo* appear to be dependent upon non-*H*-2

linked genes (Landolfo *et al.*, 1981). Resistance varies according to the inoculation route and the age and sex of the host. Formal genetic studies have not been reported. Natural cell-mediated cytotoxicity against *T. vaginalis* measured *in vitro* also varies amongst strains of mice (Landolfo *et al.*, 1980) and is mediated primarily by a macrophage or macrophage-like cell. Strain distribution patterns differ for the *in vivo* and *in vitro* responses, the *in vivo* response probably reflecting both natural and acquired immune mechanisms.

B. SPOROZOEA

1. *Toxoplasma*

Susceptibility of outbred and inbred strains of mice to infection with endozoites (=tachyzoites) of *T. gondii* at different doses has been examined by Araujo *et al.* (1976). Striking strain differences and changes in susceptibility with dosage were observed. Genetic analysis of F1 and F2 progeny from crosses between resistant and susceptible strains, and of congenic and recombinant inbred strains (Williams *et al.*, 1978), indicated polygenic control with *H-2* and *H-13* linked genes implicated. As with the trypanosome studies, resistance and susceptibility were based on cumulative mortality data which clearly represented the summation of more than one resistance mechanism.

2. *Eimeria*

Differences in susceptibility of strains of mice to *Eimeria ferrisi* have been observed by infecting eight strains of mice with six infectious dose levels and comparing mortality rates among the strains over 12 days (Klesius and Hinds, 1979). Strains were clearly separable into resistant and susceptible categories, with resistance being dominant in F1 hybrids. Further genetical analysis was not carried out.

3. *Plasmodium and Babesia*

On the basis of early parasitaemia, similar strain distribution patterns are observed in mice infected with *P. berghei* (Greenberg and Kendrick, 1957; Most *et al.*, 1966) and *B. microti* (Ruebush and Hanson, 1979), although with *P. berghei* all mice eventually die whilst with *B. microti* all strains ultimately eliminate the parasite. Greenberg and Kendrick (1957) suggest that the degree of parasitaemia and the extent of mortality 1 week after *P. berghei* infection are two partially independent variables under separate gene control. Analysis of F1, F2 and backcross progeny from crosses between short and long surviving strains (Greenberg *et al.*, 1953; Nadel *et al.*, 1954, 1955) and between high and low parasitaemia strains (Greenberg and Kendrick, 1958, 1959) indicates that both characters are under polygenic control.

Eugui and Allison (1980) examined susceptibility to *P. chabaudi* and *B. microti* in different strains of mice. They observed a positive correlation between resistance and natural killer cell activity and suggested that the two were causally related. By analysis of F1, F2 and backcross progeny E. Skamene and coworkers (personal communication) have been able to show (a) that resistance or susceptibility to *P. chabaudi* appears to be under single gene control, and (b) that this gene is inherited independently of natural killer cell activity.

C. LOBOSEA

1. *Entamoeba*

Neal and Harris (1977) reported C3H/mg and CBA/Ca mice to be susceptible to *E. histolytica* by intracardial inoculation. To investigate possible genetical and immunological differences among mice, Gold and Kagan (1978) inoculated *E. histolytica* into the right ventral lobe of the liver in one outbred and several inbred strains of mice. Only eight of 209 mice examined had lesions as well as live amoebae in the liver and no single strain showed greater susceptibility.

VII. Summary and Conclusions

Cell-mediated immunity represents an important host defence mechanism against protozoal infections. The effector cells directly involved are neutrophils, macrophages and, ultimately, activated macrophages. Within this simple scheme there are, however, considerable variations in activity. Effector cells from different animal species, and even from different strains of the same species, may be more or less effective in controlling a certain protozoal infection. Different protozoa differ in their susceptibility to cell-mediated killing according to genus, species, strain and morphological form.

The most susceptible morphological form is that which occurs in the insect vector, and which has not yet adapted to protect itself from the vertebrate host. Epimastigotes of *Trypanosoma* and promastigotes of *Leishmania* are readily killed by phagocytic cells, while the corresponding trypomastigote and amastigote forms are considerably more resistant. Protozoa which live in macrophages, such as amastigotes of *Leishmania*, endozoites (tachyzoites) of *Toxoplasma* and amastigotes of reticulotropic strains of *T. cruzi*, have developed a remarkable resistance to the microbicidal activity of the host cell. Conversely, amastigotes of myotropic strains of *T. cruzi*, which live in muscle cells, have not developed this resistance to cell-mediated killing by macrophages. Readily accessible protozoa, such as

T. brucei trypomastigotes and *Plasmodium* merozoites in the bloodstream, while they lack the marked resistance developed by reticulotropic protozoa, have a partial protection since they are attacked by phagocytic cells only when specific antibody is present.

Granulocyte-mediated killing can be largely attributed to neutrophils. Eosinophils appear to play only a minor role and compete ineffectually when neutrophils are also present. The only group of protozoal species which may be significantly controlled by eosinophils are the stercorarian species of *Trypanosoma*. *In vitro* experiments show that antibody-coated trypomastigotes of *T. cruzi* can be killed by eosinophils, although there is little evidence that this occurs *in vivo*. Interestingly, this is the only species that has been reported to be susceptible to the major basic protein of eosinophils, a toxic component of the lysosomal granules which is very active against helminths.

Neutrophils are not very active against endozoites of *Toxoplasma gondii*, but they are active against epimastigotes and trypomastigotes of stercorarian *Trypanosoma*, trypomastigotes of salivarian *Trypanosoma*, free merozoites of *Plasmodium*, and promastigotes and amastigotes of *Leishmania*. The anti-protozoal activity of neutrophils can be attributed largely to the toxic effect of the hydrogen peroxide–peroxidase–chloride system first characterized by Klebanoff (1968) as one means by which neutrophils kill bacteria. This has been demonstrated for epimastigotes and trypomastigotes of *T. dionisii* and the system is probably involved in killing promastigotes and amastigotes of *L. donovani*. Endozoites of *T. gondii*, however, induce low levels of oxidative activity in human blood neutrophils and this may explain why they are inefficiently killed by these cells.

Macrophages vary greatly in their ability to kill different protozoa. Blood monocytes can kill epimastigotes of *T. dionisii* but not trypomastigotes, and they can kill trophozoites of *T. gondii*. In the presence of antibody, blood monocytes kill a wider range of organisms, including trypomastigotes of *T. dionisii*, *T. cruzi* and *T. brucei*. Resident peritoneal macrophages are active against promastigotes of *Leishmania*, but have only limited activity against amastigotes or against *T. gondii*, *Plasmodium*, or trypomastigotes of *Trypanosoma*. Specific antibodies to *T. gondii*, *Plasmodium* and *Trypanosoma* enhance their susceptibility to resident peritoneal macrophages. Elicited peritoneal macrophages are no more active than resident macrophages, with the possible exception of those from animals treated with BCG or *C. parvum*, where the formation of lymphokines cannot be excluded.

For optimal activity against protozoa, macrophages require prior activation with lymphokines. This occurs where the lymphocytes of a previously infected or immunized animal are triggered with the same antigen, either *in vivo* or *in vitro*, and secrete a number of proteins which activate macrophages to an enhanced level of microbicidal capability.

The observed differences between different macrophages can be explained, at least partly, by differences in their oxidative activities. Differences are found both in their potential capability to mount an oxidative response and in their ability to recognize a protozoal cell as an alien stimulus. The oxidative response has two components: formation of superoxide and hydrogen peroxide during the oxidation of NADPH by NADPH oxidase, and delivery of peroxidase from the lysosomes to the phagocytic vacuole. While the levels of oxidative intermediates produced by macrophages triggered by the membrane perturbant PMA are considerably lower than those produced by neutrophils, more are produced from lymphokine-activated macrophages than from resident or thioglycollate-elicited cells. A microbicidal peroxidase is present, although in lower amounts than in neutrophils, in blood monocytes and in both elicited and activated macrophages, but not in resident peritoneal or tissue macrophages. Not only is the potential microbicidal capacity of resident macrophages lower than that of activated macrophages, but they also vary in their ability to be triggered by different protozoa. Activated macrophages produce more superoxide in response to *T. gondii* and to amastigotes of *Leishmania* than do elicited or resident macrophages. Tissue macrophages respond even less than peritoneal macrophages to *Leishmania*. Promastigotes of *Leishmania*, which are effectively killed even by resident macrophages, do trigger the oxidative response in these cells. It is possible that mannose receptors, which have been shown to be involved in the attachment of *Leishmania* to the resident macrophage, may be involved in triggering the respiratory burst. Antibody-coating of *L. donovani* and *T. gondii* enhances the oxidative response.

Activated macrophages have not only an enhanced capacity to produce superoxide and hydrogen peroxide and higher levels of peroxidase, they also have phagocytic activity and greater concentrations of other lysosomal enzymes, which may act either independently or synergistically with the oxidative system to kill the internalized protozoa.

The importance of antibody in cell-mediated immunity varies from its being an essential requirement, as in the killing of salivarian *Trypanosoma* and *Plasmodium*, or an enhancing agent, as in cell-mediated activity against stercorarian *Trypanosoma* and *Toxoplasma*, to its having no essential *in vivo* role, as in the killing of *Leishmania*. There are several ways in which antibody acts to promote cell-mediated cytotoxicity. It is required as an opsonin for *Trypanosoma* and *Plasmodium*. It enhances the oxidative response of macrophages to *Toxoplasma* and *Leishmania*. It prevents the inhibition of lysosomal granule fusion induced by *T. gondii*. It may also make the protozoa more susceptible to oxidative damage.

The host mounts both non-specific resistance through neutrophils, which are highly active but short-lived, and macrophages, of lower microbicidal

activity, and specific resistance through lymphocytes and their products, which enhance the activity of the macrophages and arm the host to withstand repeated infection with the same organism. Protozoa have evolved numerous, different evasive tactics enabling them to survive under these antagonistic conditions. The mechanism of generalized immunosuppression, characteristic of many protozoal infections, is still being elucidated; it may involve both B and T cells as well as macrophages. There is also considerable evidence that some protozoa induce antigen-specific suppressor cell populations. If active antibody is formed, the parasite may avoid it either by producing antigenic variants (salivarian *Trypanosoma*, *Plasmodium*) or by capping bound surface antigen–antibody complexes and either shedding them (stercorarian *Trypanosoma*, *Leishmania*) or internalizing them (*Naegleria*, *Entamoeba*). Some protozoa physically hide themselves from the cytotoxic cells in inactive cells such as hepatocytes (*Plasmodium* extra-erythrocytic schizonts) and muscle cells (myotropic *T. cruzi*). Those protozoa that live in macrophages require particular protection. Hence, the intracellular forms of *Leishmania* and some stercorarian *Trypanosoma* species are intrinsically more resistant to oxidative attack than are the insect vector forms, and can survive the low activities generated in resident macrophages. As previously noted, amastigotes of *Leishmania* are also less effective triggers of the production of oxidative activity than are promastigotes. *Toxoplasma gondii* protects itself by inhibiting fusion of lysosomes with the parasitophorous phagocytic vacuole, thereby preventing the arrival of the degradative lysosomal enzymes.

Genetic analyses with inbred strains of mice indicate that several cellular and molecular mechanisms are important in controlling an individual's resistance to protozoal infection. Of prime importance are genes controlling macrophage function, both in terms of the innate ability of the macrophage to handle the parasite and to present antigen, and in the ability of the macrophage to respond to lymphokine activation. Secondary effects include B cell function and antibody-production, T cell function and lymphokine production, and the induction of suppressor T cells.

The principal mechanism whereby phagocytic cells kill protozoa appears to be oxidative, involving hydrogen peroxide generated by a cell-surface oxidase in response to contact with the parasite, peroxidase from neutrophil or macrophage lysosomes and probably chloride ions. The susceptibility or resistance of different protozoa to neutrophils and to macrophages in different states of activation can be explained in terms of their susceptibility to peroxidative attack and the oxidative activity of the effector cell which they induce. While other lysosomal enzymes may play a contributory role and have considerable degradative capability, the initial lethal 'hit' appears to be administered by an oxidative pathway.

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Sexual Development of Malarial Parasites

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I. Introduction

In the genus *Plasmodium* sexual development begins when a merozoite invades the host erythrocyte, where it differentiates into a mature gametocyte. The sexual phase is completed only on the formation of a uninucleate zygote within the bloodmeal of the insect vector. This fascinating transformation invokes four critical developmental "decisions", these are: (a) the invading merozoite may develop into a sexual or asexual parasite; (b) the sexual parasite may be either male or female; (c) the mature gametocyte may remain within the vertebrate host where it will eventually die, or be taken into the mosquito bloodmeal where it must rapidly produce gametes; (d) the gametes may disperse, associate and fuse to produce the zygote, or they may be killed within the bloodmeal of the vector. When reaching each of these four "decisions" the parasite must respond to some specific stimulus or the transmission cycle will be broken. Needless to say none of these 'stimuli' is understood, but all are the subject of lively controversy.

Studies on the sexual development of malarial parasites began with distinction for it was the rapid development of the male gametes of *Plasmodium* which, when observed by Laveran (1881), confirmed his suspicions that the disease malaria was indeed caused by a living organism. Since that time the single major limitation to studies on the sexual cells has been the difficulty in purifying either gametocytes or gametes for subsequent study. Currently, however, a concerted effort is being made in a number of laboratories to study the biology of the gametocyte, particularly of the important human pathogen *P. falciparum*. These studies, which form the basis for much of this personal review, have overcome many of the technical difficulties which limited the otherwise penetrating early studies. Further, they are revealing the gametocyte to be an exceptional, if not unique, cell whose coordination and molecular strategy is remarkably adapted to the considerable and various demands of host–vector transmission.

Acknowledging the universal occurrence of sexual differentiation in malarial parasites, an intriguing question, seldom addressed, is 'What is the purpose of sex?'. As we will see it is, in energetic terms, a most costly form of development and must therefore confer some advantage to the parasite to be retained in the life cycle. Sex is not essential for the transmission of the parasite between the vertebrate to invertebrate host, for the "reverse" phase of infection, i.e. from the mosquito to man, is achieved by an asexual sporozoite. It has been argued (Williams, 1975) that both inter- and intrachromosomal reassortment of genes resulting from the events of meiosis allows a rapid selection of advantageous combinations of phenotypic characters and hence an evolutionary advantage to the species.

However, to date, there is no evidence for *intra*-chromosomal recombination in the Sporozoa. All evidence currently available suggests that there is an immediate reduction division following zygote formation (Canning and Morgan, 1975; Sinden, 1978; Cornelissen, 1982) which necessarily precludes recombination within sister chromatids. If this should prove to be the case it must be argued that the potential for *inter*-chromosomal reassortment alone confers adequate selective advantage to ensure the retention of the sexual process. However, it would be unwise on the current limited cytological evidence, and with malarial genetics still in their infancy, to dismiss totally the possibility that malarial parasites do indeed have the full capacity for genetic exchange.

Whatever the evolutionary justification for sexual development in malarial parasites, its situation in the life cycle conforms to the maxim 'sex in adversity' for clearly the complex defence mechanisms of the vertebrate host offer a considerable potential hazard to the lingering and trapped asexual parasite. However, the escape mechanism chosen, i.e. into the bloodmeal within the stomach of the mosquito, is not without its own problems. It is perhaps this combination of exacting demands exerted by both host and vector which have resulted in the evolution of cells with such exceptional capacities.

If this article can provoke just one reader to wonder at the almost fabulous cunning and guile of the gametocyte it will have served a useful purpose. If it stimulates others to study them, be forewarned! The unique constraints placed upon gametocytes have produced remarkably efficient, but most unusual, cell strategies to achieve their objectives.

II. Sexual Development *in vivo*

A. Temporal Distribution of Gametocytes within the Infected Host and Population

Gametocytes may arise in the blood of an infected host directly from the merozoites released by the exoerythrocytic schizonts, appearing at the same time as, or slightly later than, the initial asexual parasitaemia, e.g. *P. chabaudi*, *P. yoelii* (Landau and Killick-Kendrick, 1966) and *P. gallinaceum* (Adler and Tchernomoretz, 1943). Alternatively there may be a brief interval of purely asexual reproduction in the blood prior to the onset of sexual development, for example *P. cynomolgi* (R. Gwadz, personal communication). In the natural situation it must be assumed that at some point every infection will give rise to gametocytes. This assumption is not true for laboratory infections, some of which rapidly lose their sexual potential either temporarily

or irrevocably when maintained by direct blood passage. Observations on the highly variable spatial and temporal distribution of sexual parasites in the vertebrate host have inevitably concentrated on human parasites and have been exhaustively reviewed by Carter and Gwadz (1981). For *P. falciparum* it was originally contended that gametocytes appeared at the same time relative to the onset of asexual parasitaemia in both blood-induced and sporozoite-induced infections (Boyd and Kitchen, 1937). Later, however, Shute and Maryon (1951) suggested that the gametocytes appeared 7–14 days earlier relative to the symptoms of disease in blood-, compared to sporozoite-induced infection. The discrepancy in the time of onset of malarial symptoms may reflect the use of non-immune patients in Shute and Maryon's study. In marked contrast to the 48 hour cycle of asexual development, gametocyte maturation in species of the subgenus *Laverania*, for example *P. (L.) falciparum*, takes between 10 and 12 days *in vivo* (Field and Shute, 1954) and, having appeared in the peripheral circulation, the mature sexual cell has a half life of about 2·5 days and any one wave of gametocytes may persist for up to 3 weeks (Smalley and Sinden, 1977).

Other species of malarial parasite have gametocytes with a relatively short maturation period which is usually only a few hours longer than that of the asexual parasite, for example 24 hours and 22–24 hours respectively for *P. yoelii;* 40 hours and 46 hours for *P. gallinaceum;* and 36–96 hours and 48 hours for *P. cynomolgi* (see Garnham, 1966). Species with short maturation periods appear also to have a brief life span of less than 24 hours, although *P. vivax* has been reported to persist for 2 to 3 days (Jeffrey, 1958).

Upon loss of infectivity gametocytes appear to be rapidly cleared from the peripheral circulation, nonetheless, some authors have described persistent circulating parasites of various species which they consider to be either senile or degenerate (e.g. Cantrell and Jordan, 1946; Eyles, 1952; Dei Cas *et al.*, 1980a, b). Thus the peak gametocytaemia may be considered to follow the first asexual parasitaemia by a brief period equal to the difference in maturation times of the two classes of parasite. The courses of asexual and sexual parasite development in various *Plasmodium* species are illustrated in Figs 1 and 2.

In those hosts that recover from the initial asexual parasitaemia, and which may therefore be regarded as semi-immune, one might expect to find a persistent gametocyte production representing a constant proportion of the successive waves of asexual parasitaemia. This is particularly emphasized in the studies on the human parasites *P. falciparum* (Fig. 1a) and relapsing *P. vivax* (Fig. 2; see Field and Shute, 1954). However, contrasting observations on the distribution of gametocytes have been made in epidemic and endemic situations; in the latter, children show higher gametocyte rates than adults (Bray and Burgess, 1964) whereas in epidemic areas gametocyte rates are high

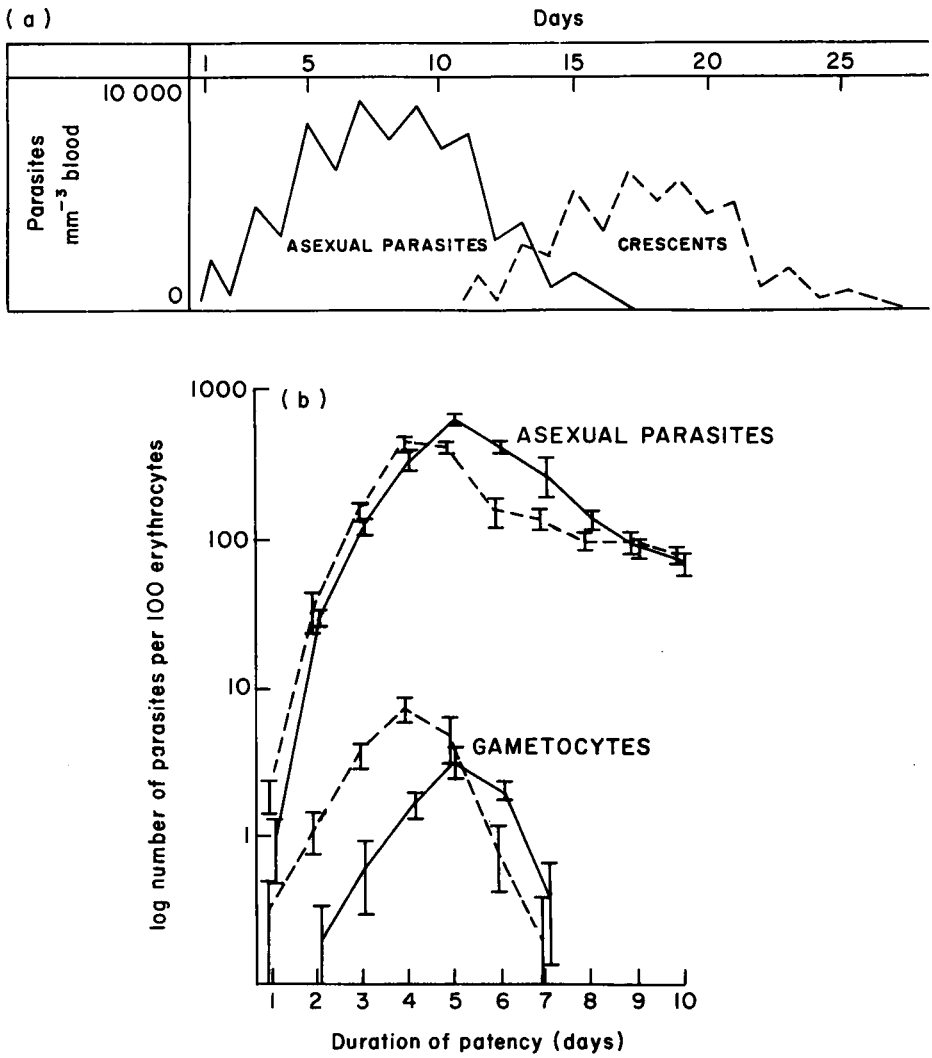

FIG. 1. The course of asexual and sexual parasitaemias of *Plasmodium* spp. (a) *Plasmodium falciparum* trophozoites in peripheral circulation; ——, Mature gametocytes (crescents); – – –, in peripheral circulation of an infected patient (taken from Field and Shute, 1954). (b) *Plasmodium gallinaceum*: ——, sporozoite-induced parasitaemia, – – – –, blood-induced parasitaemia. Taken from Turner, D. Ph.D. Thesis University of London.

in both groups. Carter and Gwadz (1981) have summarized much of the early data, for example Muirhead-Thomson (1957), on the distribution of *P. falciparum* gametocyte carriers in longitudinal population surveys in endemic areas and conclude that, although infants (0–5 years) are unquestion-

ably the most heavily infected individuals and are highly infective to mosquitoes, their number in the population could hardly have contributed more than the older age groups to the total reservoir of infection, and they further suggest that it is the older asymptomatic individuals producing gametocytes at the threshold of infectiousness, i.e. less than 100 gametocytes mm^{-3}, which constitute the main reservoir of infection. Little work has been done to determine the natural rate of conversion of the asexual parasites into gametocytes. Smalley (1976) showed that successive populations of asexual parasites from one individual do indeed produce gametocytes *in vitro* though with variable efficiency. This was followed by a study (Smalley *et al.*, 1981b) which showed that in children aged between 2 and 3 years, those with 'older' infections (recognized as those already bearing circulating mature gametocytes) there was a higher conversion rate to gametocytes *in vitro* than in the 'younger' infections (i.e. those not producing gametocytes). If substantiated with patients of a wider age range this would lend further weight to the notions that gametocytes are not produced as a constant proportion of the total parasitaemia and that it is the older asymptomatic patient who contributes the most to the potential reservoir of infection.

In *P. vivax* the situation appears somewhat different (Fig. 2). In untreated cases gametocytes arise only once in significant numbers early in the infection (i.e. 15–35 days); if, however, the primary parasitaemia and each successive

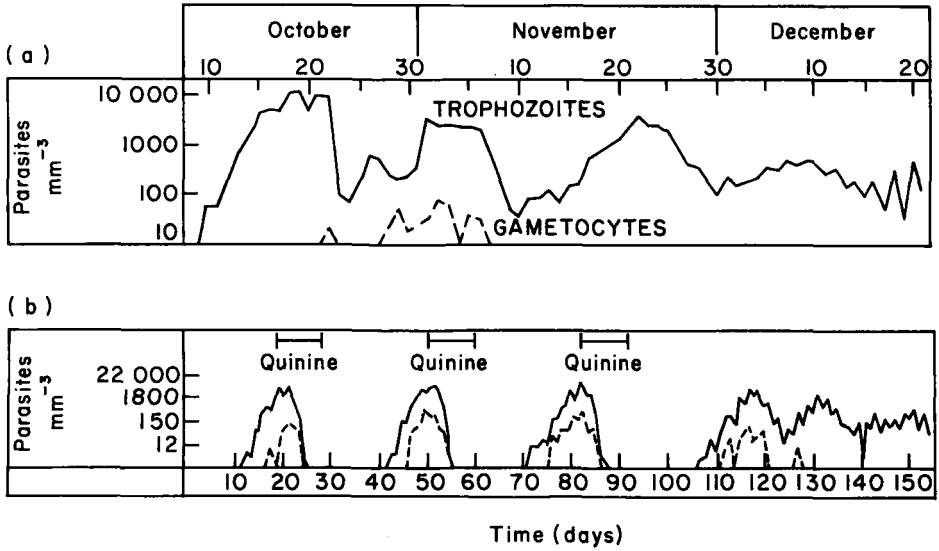

FIG. 2. The course of asexual (——) and sexual (– – –) parasitaemia of *P. vivax* in patients without (a) and with (b) chemotherapeutic suppression of patent parasitaemia (taken from Field and Shute, 1954).

relapse is treated, each resulting parasitaemia produces a constant proportion of sexual stages. If a relapse is then left untreated the gametocytaemia again is lost after a period of about 20 days (see Field and Shute, 1954). Whilst confirming the suspicion that gametocytes are a highly variable fraction of the population, in contrast to the evidence from *P. falciparum*, it seems that in older untreated infections of *P. vivax* there is a reduction in the conversion of parasites into gametocytes.

Clearly no generalization can be made as to the mechanism of induction of gametocytogenesis *in vivo*. Not surprisingly the stimuli proposed have been wide-ranging and sometimes bizarre. These include: the rise in asexual parasitaemia *per se*; a rising immunity to the asexual infection (Miller, 1958; Thomson, 1914); a fall in the immunity to the sexual stages (Sinton *et al.*, 1926); a rise in immunosuppressive agents (Ward *et al.*, 1972); C_{3b} hypocomplementaemia (Ade-Serrano *et al.*, 1981); the presence of parasite cell debris (Mons and Van der Kaay, 1980); sub-curative drug levels, for example quinine and sulphamethazine (Shute and Maryon, 1954); and a change in the pH of the spleen (Sinton *et al.*, 1926)! None of these possibilities alone has been shown conclusively to be the natural stimulus. It is most probable that a variety of conditions, all of which are adverse to the survival of the asexual parasite, are capable of triggering the parasite to a sexual path, and that this trigger must ultimately be operated by a common 'secondary-messenger' within the parasite. It should always be borne in mind, however, that some Haemosporidia are exclusively sexual in their erythrocytic development. Perhaps we are better to ask 'What is it that diverts the *Plasmodium* merozoite to an asexual growth strategy?'.

Within any one wave of gametocyte formation, brief and cyclic periods of infectivity have been described for *P. falciparum*, *P. cynomolgi*, *P. vinckei*, *P. chabaudi*, *P. gallinaceum*, *P. knowlesi*, *P. cathemerium*. Of these studies, one (*P. falciparum*) examined the ability of gametocytes to exflagellate *in vitro*, the others examined infectivity to mosquitoes (Hawking *et al.*, 1966, 1968, 1971, 1972).

Cyclic periods of infectivity have also been described in *P. cynomolgi* by Coatney *et al.* (1971), but in only one of five monkeys observed by Garnham and Powers (1974). This periodic infectivity, it was suggested, resulted from the finite maturation period of successive waves of asexual parasites which were themselves synchronized on a circadian pattern 48 hours previously. However, R. Gwadz (unpublished results) considers these observations of periodic infectivity in *P. cynomolgi* to be exceptional, and believes a continuous infectivity is the norm.

Independent observations on *P. falciparum* would not appear to concur with Hawking's otherwise most attractive hypothesis. In this species the gametocytes are of great persistence (Smalley and Sinden, 1977), and *in vitro* they

are certainly able to exflagellate and infect mosquitoes for many days, showing no sign of a circadian rhythm. Indeed Bray *et al.* (1976) could not demonstrate a circadian pattern of infectivity of *P. falciparum*-infected Gambian patients to *Anopheles gambiae*. Thus if Hawking's hypothesis is to be accepted it must be confined to those parasites in which the mature gametocytes have a longevity of much less than 24 hours and have at some time in their asexual cycle a circadian pattern of development. The evolutionary wisdom of the "Hawking strategy" of directed infectivity is questionable for it is far more susceptible to environmental disruption than the contrasting, almost casual, approach of persistent exposure of gametocytes to the vector which obviously prevails in the highly successful species *P. falciparum*.

B. SPATIAL DISTRIBUTION OF GAMETOCYTES WITHIN THE INFECTED HOST

With increasing gametocyte maturity the parasite can show remarkable changes in spatial distribution in the vertebrate host. In the human parasite *P. falciparum* the sexually determined ring stage classically disappears from the peripheral circulation and undergoes its maturation in the deep tissues of the spleen and bone marrow (Garnham, 1931; Thomson and Robertson, 1935; Smalley *et al.*, 1981a), and has been reported also in hepatoma ascites fluid (Swanson-Beck *et al.*, 1970). Subsequently they re-emerge in the peripheral ciculation as fully formed crescents some 10–12 days later, although possibly requiring a further 1 to 2 days to achieve their full maturity (Jeffrey and Eyles, 1955; Ward *et al.*, 1972). Despite being freely available within the peripheral circulation, the mature gametocytes of *P. falciparum* are surprisingly *not* found in the cavernous blood spaces of the placenta (Bray and Sinden, 1979). Similar migrations of the young sexual parasite to the deep tissues have been claimed for *P. vivax* (Field and Shute, 1954).

Recent work by Dei Cas *et al.* (1980a, b) has shown that the population of gametocytes of *P. inui* and *P. yoelii* found within a mosquito bloodmeal (predominantly of types O and I, but with some type II and III) differ from those in the veins of the infecting host (types II and III). They suggest that gametocytes of types O and I are distributed preferentially within the capillary beds of the host from which the vector feeds, and it is these younger stages alone that are infective. Despite the technical problems in this assay it offers the intriguing possibility that the localized distribution of the infective stages (which are slightly larger than the non-infective, and therefore remain in the capillary bed) is singularly adapted to offer the best possible site for potential transmission from host to vector.

Interestingly, Gore, Pittman and Noblet (1978) have shown that host body temperature which is controlled by neurohormones and influenced by the

day/night cycle, changes the peripheral distribution of gametocytes of *Haemoproteus* in the turkey. They suggest this may occur by the direct action of the host neurohormone on the parasite. In combination with the findings of Dei Cas *et al.* (1980a, b) these mechanisms offer an attractive alternative to the 'Hawking-phenomenon' as an explanation for the circadian patterns of infectivity observed in some species of malarial parasite. This model does not rely on an exceptionally brief period of innate gametocyte infectivity, but simply controls gametocyte distribution within the host and hence their availability to the vector.

C. GAMETOCYTE INFECTIVITY

Gametocyte infectivity to the vector is a complex phenomenon which has been extensively reviewed by Carter and Gwadz (1981) but is best summarized in the tantalizing recommendation of P. C. C. Garnham "Always feed the mosquitoes the day *before* you see the first gametocytes in the blood film"!

The concept of infectivity must encompass the following functions: (1) the maturity of the gametocyte, i.e. its ability to make gametes (see page 159); (2) the distribution of the gametocytes in the host (see page 160); (3) the availability of host–vector contact; (4) the immunological status of the host with respect to the sexual parasites; and (5) other aspects of host physiology which impinge on gametocyte development within the mosquito blood meal.

Following the suggestions of Eyles (1952), Huff *et al.* (1958), and others, there is now evidence that in some infections antibodies are produced which are capable of binding to the extracellular microgametes and agglutinate or otherwise inhibit their capacity to fuse with the macrogamete and thus infect the mosquito (Mendis and Targett, 1981). However, Carter *et al.* (1979b) consider gamete agglutinating (AG) antibody to be ineffective in blocking transmission and show that in their system (*P. gallinaceum*–chicken) this antibody is synthesized throughout the period of infectiousness. They have, however, shown that the suppression of mosquito transmission immediately following the peak of sexual parasitaemia in blood-induced infections of *P. gallinaceum* is related to the production of antibody they classify as surface fixing (SF). Antigamete antibodies which react with gametocyte and gamete preparations of *P. falciparum* in the IFA technique can be detected in a significant proportion of an endemic population (Voller and Bray, 1962; Smalley and Sinden, 1977; Carter *et al.*, 1979c). However, no correlation has been found between the presence of these antibodies and the lack of infectiousness of the host to the vector. In contrast to the observations of Carter *et al.* (1979c), I was particularly struck by the frequently abnormal exflagellation

(lethargic, early gamete death, and surface fixation) that could be observed in the blood of naturally infected Gambian patients, but nonetheless must still concur that there is no evidence that this activity correlates with a suppression of host infectivity.

Evidence for the presence of humoral agents other than antibodies capable of inhibiting infections of the vector is fragmentary and often circumstantial. Carter and Gwadz (1981) have suggested that the infectivity of gametocytes of *P. gallinaceum* falls significantly when the blood parasitaemia exceeds 5×10^4 mm^{-3}. Similar observations have been made by Gwadz (in Carter and Gwadz, 1981) on *P. knowlesi* infections, where parasitaemias of 10% or over were inhibitory to infection. This inhibition was transferable with the serum component of the blood. Other observations include those of Ponnudurai *et al.* (1982a) and Sinden and H. Carter (unpublished work) that some non-immune sera do not support (inhibited?) exflagellation of *P. falciparum in vitro*, and that such sera are highly variable in their ability to support infection of mosquitoes in membrane feeding experiments. Further, we have noted in our laboratories, particularly using rodent malarial parasites, that exflagellation can cease very abruptly (within minutes) in sequential blood samples taken from an infected host irrespective of the time of day or the parasitaemia. This we have associated with the stress reactions of the host (we have, however, been unable to inhibit exflagellation by the administration of adrenalin or noradrenalin (A. Knight, unpublished work)). Since it has been shown (Bishop and MacConnachie, 1956, 1960) that exflagellation *in vitro* can occur in a very basic medium with a requirement only for inorganic salts and glucose (for continued energy supply) it is unlikely that the above inhibitory effects occur because of a lack of nutrients, but more likely that substances are present which block the triggering, or action, of a secondary messenger molecule.

III. The Cell Biology of Gametocyte and Gamete Development

A. Cytology and Ultrastructure

1. *Gametocytogenesis*

Detailed descriptions of the morphological development of the sexual stages of *Plasmodium* are peculiarly complicated by the individual patterns of development of the parasites. In those species where maturation usually occurs in accessible tissues it is very difficult, if not impossible, to differentiate the young gametocyte from a young asexual parasite, whereas in those parasites where there is a recognizable sexual differentiation, development occurs in inaccessible tissues. Detailed studies of the sexual development of

the Haemoproteidae may therefore prove useful models for our understanding of this aspect of gametocyte biology.

The following description of gametocytogenesis will, very largely, be based on that of *P. falciparum* for it is in this species that sexual development is readily followed by light microscopy and it remains to date the only species where these changes have been reliably observed by electron microscopy or biochemical methods.

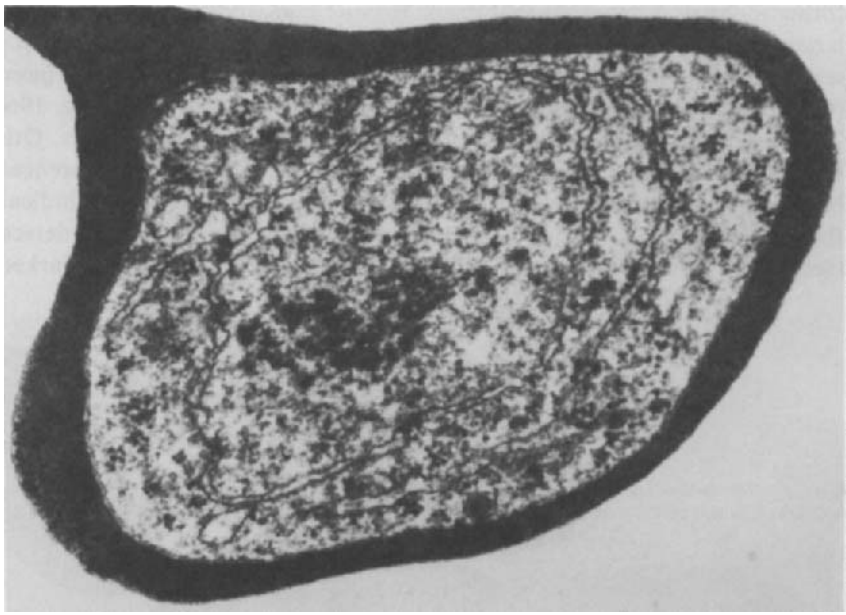

FIG. 3. Transmission electron micrograph (T.E.M.) of cultured stage II macrogametocyte of *P. falciparum* showing newly developed subpellicular microtubule and membrane vesicle complex, and intranuclear microtubules. Magnification ×19 000.

The merozoite having invaded the erythrocyte loses its apical complex and subpellicular structures, rounds up, and enlarges within the parasitophorous vacuole. However, it does not assume the amoeboid form of the asexual parasite and therefore does not have an apparent 'vacuole' within it. This solid parasite is rarely applied to the erythrocyte membrane but often assumes a juxtanuclear position in avian and reptilian species. In *P. falciparum*, but not other human parasites, the developing gametocyte fails to produce any visible structural alterations to the erythrocyte plasmalemma. This does not mean to say that the membrane is unaltered, but does pose the dilemma as to why erythrocytes containing both sexual ('knobless') and asexual ('knobbed') parasites are similarly sequestered in the deep tissues. The solid round parasite (stage I) is distinguishable at the ultrastructural level solely by

its near spherical shape and the absence of knobs on the host cell. However, before any change in shape is noticed a prominent subpellicular flattened membrane vesicle and microtubule array is seen to form *de novo* (Sinden, 1982a; Fig. 3). Some authors described the cytoplasm of these young gametocytes as being pale bluish-grey in Giemsa-stained smears (suggesting a less intense staining than the asexual parasites). There is, however, no evidence at the ultrastructural level for a reduced ribosome level in these cells. Pigment formation may differ from that in the asexual cells, individual crystals being larger and more dispersed throughout the cytoplasm. Some authors describe a sexual dimorphism in pigment distribution, for example in *P. falciparum* (Garnham, 1931) and in *P. gallinaceum* and *P. gonderi* (see Garnham, 1966). This dimorphism is most reliably detected in the more mature cells. Other Haemosporidia (e.g. *Haemocystidium*), show a most dramatic difference in both pigment distribution and food vacuole formation which reliably indicates the sex of a gametocyte long before other cytological changes can be detected (see Fig. 4). In *P. falciparum* pigment is ultimately found in a markedly

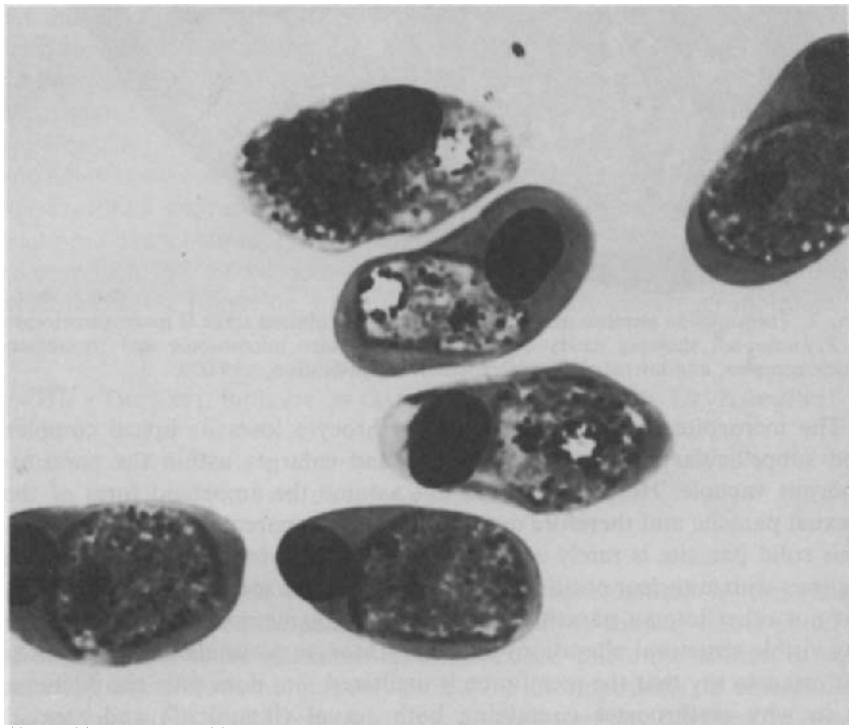

FIG. 4. Light micrograph of gametocytes of *Haemocystidium* sp. Microgametocytes are distinguishable by the large food vacuole with concentrated pigment and pale cytoplasm. Note double infection of macro- and microgametocyte (top). Magnification ×2200.

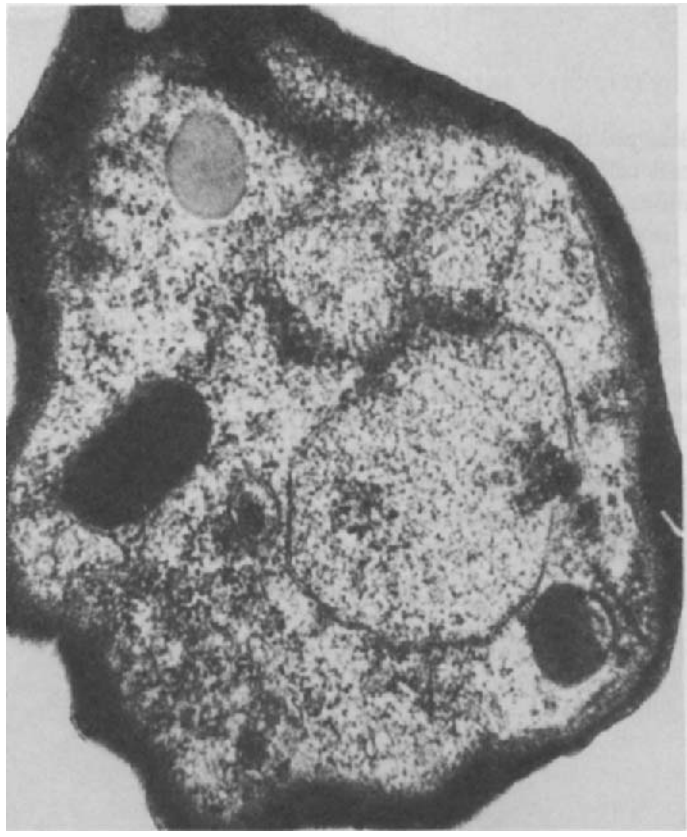

FIG. 5. T.E.M. showing T. S. of mature female gametocyte of *P. falciparum* which has a small nucleus in which lies the "frozen hemispindle" of the early mitotic nucleus. Magnification ×55 000.

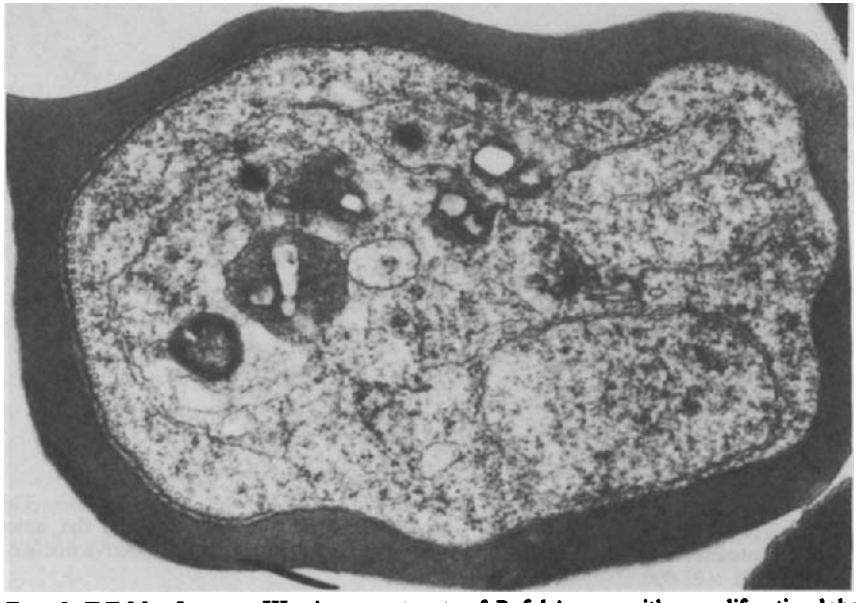

FIG. 6. T.E.M. of a stage III microgametocyte of *P. falciparum* with a proliferating lobed nucleus and expanding but asymmetrical subpellicular complex. Magnification ×35 000.

perinuclear position and, because of the difference in nuclear size in the male and female cells, its distribution may be used as an indicator of sex in living preparations.

With increasing age the gametocyte of *P. falciparum* becomes more elongate and spindle shaped (stage II) at which time the pigment is often found in linear arrays, caught between the lamellae of the endoplasmic reticulum. The nucleus occupies a terminal site or is elongated across the long axis of the cell. Within some nuclei, suspected as being those of the female cell (Sinden, 1982a), microtubules are found radiating from spindle plaques (Figs 3 and 5). This suggests most strongly that the female cell has become diploid and undergone mitosis reverting to a haploid condition (see page 183). Simultaneously the subpellicular membrane and microtubule complex is expanding rapidly both laterally around the cicumference of the cell, and in length distending the parasite into its typical segment or 'D' form (Fig. 6). The bilateral asym-

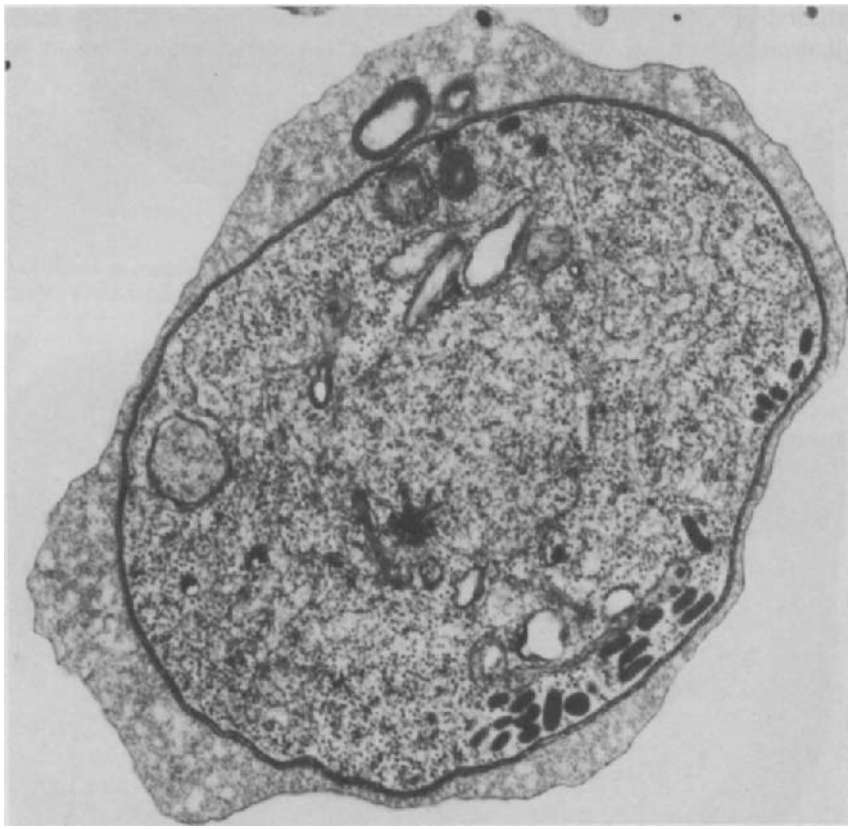

FIG. 7. Intracellular macrogametocyte of *P. falciparum* undergoing gametogenesis. The parasite is becoming spherical but has not yet ruptured the host cell and the inner pellicle membranes are still intact. Note the persistent hemispindle in the inactive nucleus. Magnification ×23 000.

metry of the cell reveals the position of development of the subpellicular complex, which is always found along the straight edge of the parasite.

Subsequent growth of the inflexible gametocyte distorts the erythrocytes and at this stage (stage III) sexual dimorphism is more pronounced and just detectable by light microscopy. The male nucleus is now markedly larger than that of the female and already is assuming the digitate form typical of the mature cell. This increase in nuclear size reflects the increased replication of the genome in the male cell compared to the female cell. This replication in the microgametocyte is *not* accompanied by nuclear division and leads to the formation of an octoploid nucleus. The cytoplasm of the female now contains slightly more ribosomes, a more extensive endoplasmic reticulum (e.r.) and more mitochondria than the male. Some authors can now detect, with difficulty, the typical pink/blue distinction of the male/female cells in Giemsa-stained films, a distinction caused by the different ribosome densities in the two cells. Food vacuoles, which although small were frequently found in the immature cell, are now less abundant.

With the complete envelopment of the gametocyte by the subpellicular complex the gametocyte again becomes bilaterally symmetrical and adopts a typical spindle shape (Stage IV). Now sexual dimorphism is readily apparent by all the criteria mentioned above (Figs 8, 10). Additionally, however, there appear dispersed throughout the cytoplasm numerous small membrane-bound electron-dense bodies (osmiophilic bodies) originally and perhaps prophetically identified as 'toxonemes' (Rudzinska and Trager, 1968). These bodies are synthesized in the e.r.–Golgi complex and are much more numerous in the macrogametocyte than the microgametocyte. Aikawa *et al.* (1969) have suggested that these organelles are more round in section in the avian parasites but elongate in the mammalian species. In *P. falciparum* a single 'frozen' hemispindle is invariably found in the nucleus of the female (Fig. 7) which may additionally contain a prominent nucleolus. By light microscopy the male nucleus has an extensive but reticular chromatin distribution; in contrast, the small female nucleus contains a tiny single intensely staining spot of 'chromatin'. In the electron microscope the digitate male nucleus contains an electron-dense intranuclear body (INB) adjacent to a nuclear pore. Around the periphery of the INB lie the kinetochores but their associated chromatin is not in a condensed form recognizable by electron microscopy. On the cytoplasmic face of the same nuclear pore is a very dense amorphous microtubule organizing centre (MTOC) (Fig. 11). In the avian and reptilian parasites some authors describe an atypical centriole in the MTOC (e.g. Sterling and Aikawa, 1973). Such a centriole is absent from *P. falciparum* which otherwise is typically avian in format. Sinden *et al.* (1978) have suggested that the exceptionally rapid formation of kinetosomes during gametogenesis (see page 174) may have allowed their development to

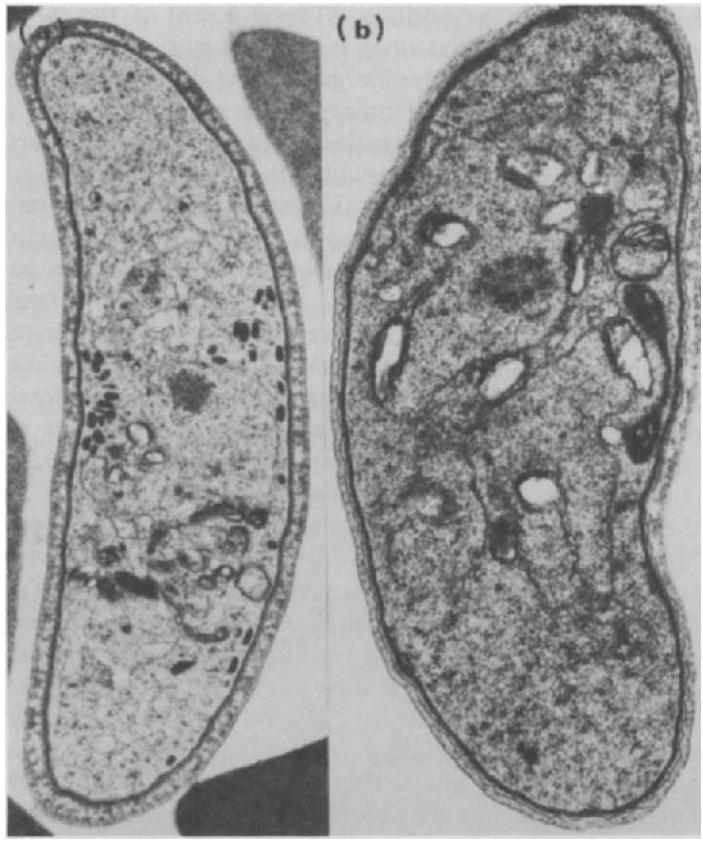

FIG. 8. Mature gametocytes of *P. falciparum*. (a) Microgametocyte, magnification ×19 000 and (b) macrogametocyte, magnification ×15 000.

have occurred in *P. gallinaceum* before fixation had occurred (compare, for example, Fig. 15 (Kass *et al.*, 1971) and Fig. 16 (Aikawa *et al.*, 1969)).

It remains to be proved whether this MTOC–INB–kinetochore complex is confined to the male cell throughout the Haemosporidia; to date it has been found only in the male cell in *P. yoelii* and *P. falciparum* (Sinden *et al.*, 1975, 1978), *P. knowlesi*, *P. cynomolgi* and five avian species (Aikawa *et al.*, 1969), but in both male and female cells of *P. gallinaceum* (Sterling and Aikawa, 1973). A bright crimson dot, termed the karyosome, has been described in Giemsa-stained smears in, or close to, the nucleus of both male and female gametocytes of various malarial parasites; this may be the MTOC–INB–kinetochore complex. With remarkable foresight Garnham (1966) considered this structure to be involved in kinetosome formation during microgametogenesis.

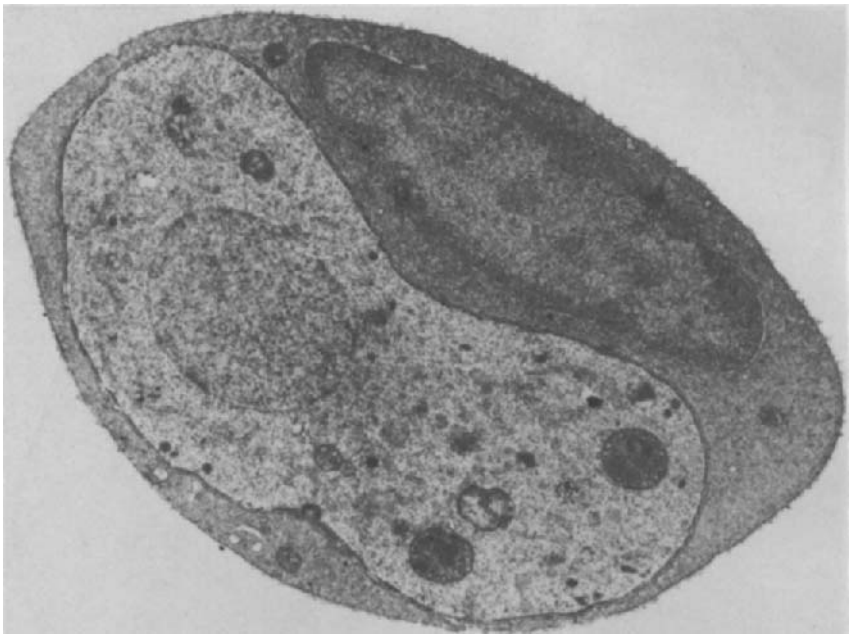

FIG. 9. Mature macrogametocyte of *P. mexicanum* showing small nucleus prominent mitochondria and enveloping trilaminar pellicle typical of the avian and saurian parasites. Magnification ×14 000.

The final transformation of the developing cell into the mature crescent involves the loss of the subpellicular microtubules; at this point the rigid spindle-shaped cell loses its pointed ends and becomes distorted by the enveloping erythrocyte into the typical crescentic form (Stage V), nevertheless retaining its tubular shape due to the persistence of the trilaminar pellicle (Fig. 8). Aikawa *et al.* (1969) and Sinden *et al.* (1975) have described subpellicular microtubules in gametocytes of both avian and rodent parasites; however, unlike *P. falciparum*, the absolute maturity of these cells cannot be assured. It is likely that these cells were not fully mature and that the microtubules observed are lost in the terminally differentiated adult cell. The description of intraspecific variation between the chloroquine-resistant (Smith) and sensitive (Camp-Sadun) lines of *P. falciparum*, based particularly upon the presence of subpellicular microtubules in the former (Aikawa and Ward, 1974), is now open to an alternative and simpler interpretation, that is that immature gametocytes were observed in one isolate but not the other.

The extensive comparison made between the morphology of gametocytes of avian/reptilian parasites and those of mammalian hosts (Aikawa *et al.*, 1969; Sterling and Aikawa, 1973) has highlighted notable differences in these groups, some of which have been mentioned above. Briefly summarized these are: a

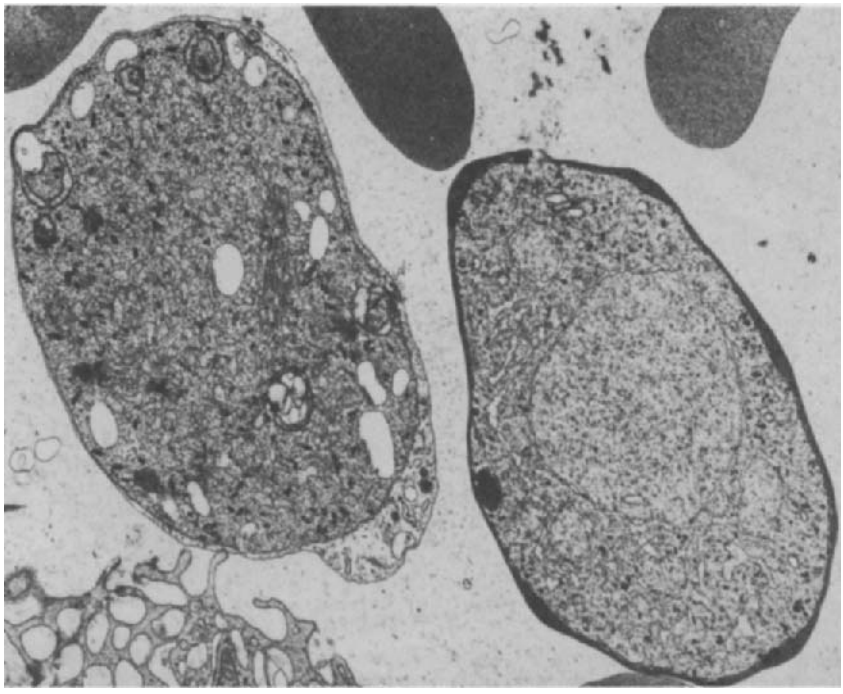

FIG. 10. Mature macrogametocyte (left) and microgametocyte of *P. yoelii nigeriensis* showing the marked sexual differences in cytoplasmic organization and the lobed male nucleus. Magnification ×11 000.

complete trilaminar pellicle in avian parasite (Fig. 9) versus a variable double/triple membrane layer in the mammalian species (Fig. 10) (the three individual components are more correctly described as the parasitophorous vacuole membrane, gametocyte plasmalemma, and underlying *double* membrane layer of the inner pellicular sacs); the occurrence of cytostomal feeding in the avian parasites, but not the mammalian species (this is possibly related to the avian parasites' relative inability to transfer nutrients across the complex pellicle); the presence of cristate mitochondria in avian, and acristate mitochondria in rodent parasites; round osmiophilic bodies in avian and elongate forms in rodent parasites; and a nucleolus in the macrogametocyte of avian but not rodent species (cf. Figs 8 and 10).

These differences are superimposed on the characteristic ultrastructural sexual dimorphism described above (Fig. 10). Needless to say, *P. falciparum* (and presumably other *Laverania* species) conforms precisely to the avian/reptilian morphology. This led Sinden *et al.* (1978) to concur with Landau *et al.* (1976) and suggest that this species is ancestral to both 'vivax' or 'malariae' groups. Alternatively it could be considered that *P. falciparum*

is more recently derived from an ancestral avian/reptilian species, a conclusion that would be supported by its lack of adaptation to the human host (expressed as a high level of pathogenicity).

The ultrasturucture of mature gametocytes reflects perfectly their strategic position in the life cycle (Fig. 10). They are the 'life boat' of parasite development, fully prepared (i.e. terminally differentiated) and poised at the edge of one environment ready to enter the hostile seas of the mosquito bloodmeal. Here it must achieve gamete formation, fertilization and zygote production before it can escape into relative tranquillity within the vector's haemocoele. The preparedness of the gametocytes must be enforced on it by the very hostility of the mosquito bloodmeal, where within 18 hours the ookinete (and presumably other stages) are susceptible to the mosquito trypsin-like enzymes (Gass, 1977, 1979; Gass and Yeates, 1979) and the rapid formation of the peritrophic membrane which prevents the escape of the ookinete.

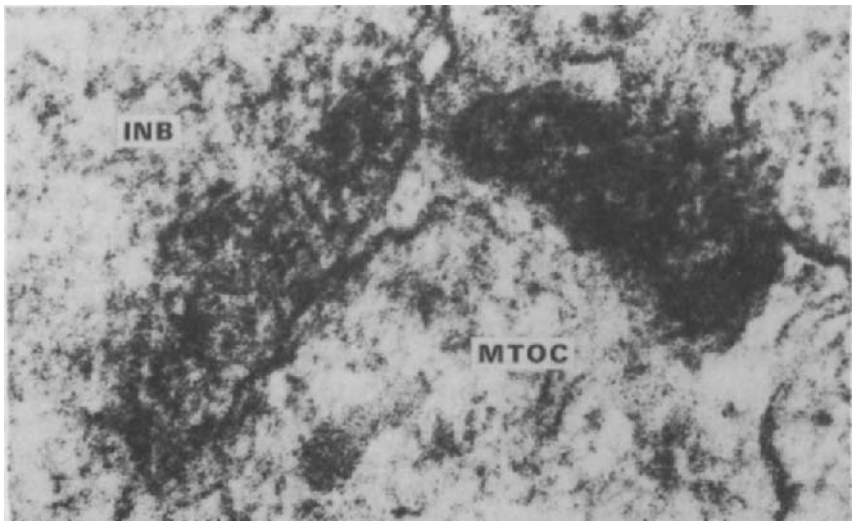

FIG. 11. The cytoplasmic microtubule organizing centre (MTOC) and intranuclear body (INB) of the microgametocyte of *P. yoelii nigeriensis* situated together on either side of the nuclear envelope. Magnification ×151 000.

It is the purpose of the male gametocyte to produce with great rapidity eight nucleated motile gametes. The genome is already replicated, being octoploid in the large nucleus of the mature cell (Canning and Sinden, 1975; Toyé *et al.*, 1977) and is localized to the INB where it is physically linked to the cytoplasmic MTOC which in turn governs axoneme assembly, thus irrevocably linking the genome to the motile apparatus. The speed of axoneme assembly (see below) precludes synthesis of large amounts of structural proteins which, like the genome, must be presynthesized. Thus the mature microgametocyte

does not require numerous ribosomes nor extensive endoplasmic reticulum. The microgametes do not possess mitochondria and there are therefore few in the microgametocyte. Both gametocytes must escape the host erythrocyte, but the female, being non-motile, cannot disrupt the erythrocyte mechanically. She does, however, have many more osmiophilic bodies than the male, these bodies like the rhoptries and micronemes appear to disrupt the erythrocyte plasmalemma (see below).

The macrogametocyte (which I consider to be haploid in *Plasmodium*) does not undergo nuclear division in the vector, hence the small nucleus. She will, however, rapidly be called on to support protein sythesis in the zygote. To this purpose there is a nucleolus within the mature cell from where newly synthesized ribosomal ribonucleic acid (RNA) will be distributed to the cytoplasm. The cytoplasm has retained or even expanded both its ribosome population to achieve maximal rates of synthesis in the macrogamete/zygote, and its endoplasmic reticulum in which to modify and secrete the newly synthesized polypeptides. Energy for parasite development within the vector is provided by cristate mitochondria (Howells, 1970), thus the macrogametocyte has invested in their production and consequently "allowed" an accelerated development in the vector.

Unless induced to undergo gametogenesis, mature gametocytes are terminally differentiated cells requiring little if any transcription of the parasite genome for their survival. Therefore it is not surprising that the deoxyribonucleic acid (DNA) of these cells was more difficult to stain by the Feulgen technique than the asexual parasites, the immature gametocyte or the microgamete (Canning and Sinden, 1975; Sinden *et al.*, 1978). Cornelissen (1982) has, however, shown that a positive reaction can be achieved with care. These results suggest a different (masked?) conformation of the DNA in the mature sexual cell. The longevity of the mature cell is possibly therefore determined by the half-life of pre-existing mRNA species and proteins within the cell, and in the case of *P. falciparum* this longevity is pronounced. Because all gametocytes have such similar structural organization it is difficult to justify in cellular terms the very brief periods of innate infectivity implicit in the "Hawking phenomenon". All gametocytes would appear at the ultrastructural level to be 'designed' to be infective for a proportionately extended period of the cell cycle, and for this reason I consider the period of infectivity to mosquitoes may significantly be determined by the influences of the vertebrate host on both their distribution in the vascular system and their development within the vector

2. *Gametogenesis*

In contrast to the relative quiescence of the mature gametocyte the events of gametogenesis are explosive. Light microscopic observations recognized

the vital role of gametogenesis from the outset (Laveran, 1881) and many beautiful descriptions have been given of the events in living and stained specimens. Notable are those of Ross (1897), McCallum (1897), Anderson and Cowdry (1923) and Raffaele (1939). More recently the events have been described in detail (Sinden and Croll, 1975; Sinden *et al.*, 1975, 1978).

When observed by phase- or interference-contrast microscopy the first observation is that all gametocytes rapidly assume a spherical shape, at the same time increasing significantly in volume. Simultaneously the plasmalemma of the host erythrocyte becomes progressively less distinct and may show intermittent blebbing of the surface (see, e. g.Nijhout and Carter, 1978), this being the exact converse of membrane behaviour during merozoite invasion. It is therefore not surprising to find that the microneme-like osmiophilic bodies have become attached to the parasite plasmalemma by small ducts at points where the inner membranes have become separated by the enlargement of the parasite surface area, and can be seen to disorganize the host cell membranes and cytoplasm (Sinden *et al.*, 1975). The weakened host cell membrane either "dissolves", or may be ruptured either by the activity of the exflagellating microgametocyte, or by the increasing size of parasites of either sex. It has been suggested that the plasmalemma is more readily disorganized by the macrogametocyte than by the microgametocyte of *P. gallinaceum* (R. Carter and M. Aikawa, personal communication).

Macrogametogenesis at the structural level involves little more than escape from the host cell. The pigment granules do, however, become noticeably more active following gamete formation. The enlarged cell contains a single nucleus which may (*P. falciparum*) or may not (*P. berghei, P. yoelii, P. gallinaceum*) contain a hemispindle inherited from the mature gametocyte. In *P. gallinaceum* the genome is suspected as being similarly localized to a "modified spindle plaque" represented in this case by a group of plugged nuclear pores (R. Carter and M. Aikawa, 1983). Despite good evidence to the contrary in *Haemoproteus* (Galucci, 1974a) and *Hepatocystis* (Canning *et al.*, 1975), there is no evidence for a nuclear division during macrogametogenesis in *Plasmodium*. This has already occurred in the immature gametocyte (Sinden, 1982a). Unfortunately the fate of the resulting unwanted haploid genome or nucleus has not been determined by light microscopy. Early descriptions of nuclear division in gametocytes of *P. falciparum* are unclear as to whether they applied to very immature female cells (which cannot be sexed!) or to the lobed/digitate nucleus of the more mature male cell; see, for example, Garnham, (1931). Thomson (1932) and Thomson and Robertson (1935). In view of the current ease of gametocyte culture techniques this dilemma may now be re-examined more diligently.

Microgametogenesis is deceptively uneventful by light microscopy until the moment of exflagellation; however, pigment granules may be seen to be

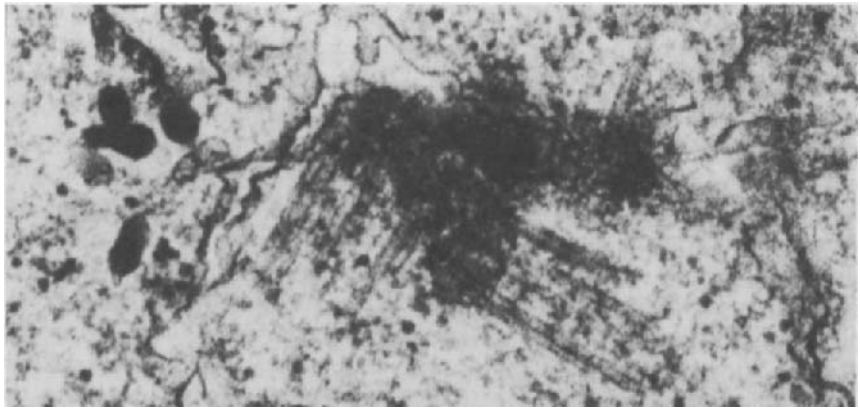

FIG. 12. The MTOC of the microgametocyte of *P. yoelii nigeriensis* 15 seconds after the induction of gametogenesis showing the rapid development of the planar orthogonal tetrads of kinetosomes. Magnification ×75 000.

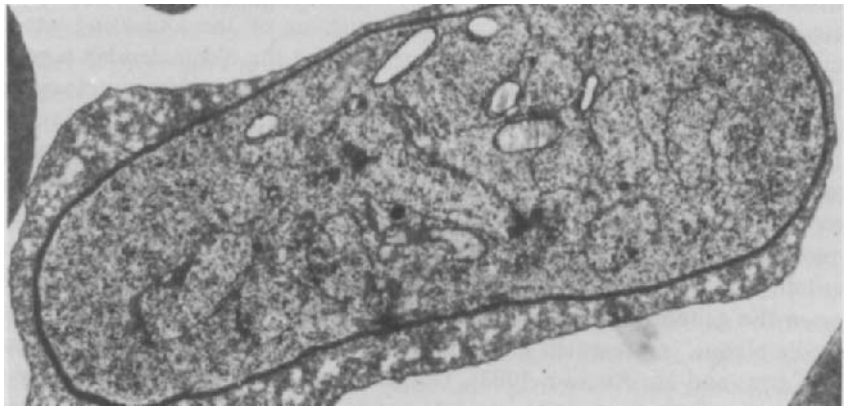

FIG. 13. Low-power transmission electron micrograph of *P. falciparum* microgametocyte undergoing gametogenesis showing the first mitotic spindle in the lobed nucleus. Magnification ×14 000.

displaced by the repeatedly dividing nucleus (Sinden and Croll, 1975). Ultrastructural changes are, however, rapid and stunning. Within *15 seconds* the cytoplasmic MTOC (Fig. 11) has transformed into two orthogonal planar tetrads of kinetosomes (Fig. 12) upon which axonemes immediately polymerize in the conventional manner. At two minutes the first intranuclear mitosis occurs splitting the spindle plaque (kinetic centre). The movement and opposition of the two daughter plaques inevitably divides the cytoplasmic MTOC (Fig. 13) and moves four kinetosomes to each pole (to which a tetraploid genome is attached). Separation of the spindle plaques and kinetosomes can

be inhibited by cytochalasin B suggesting that this is mediated by microfilamentous systems (R. Sinden and N. King, unpublished observations). Two further divisions at about 5 and 9 minutes further lower sequentially the kinetosome number at each pole to 1 and the associated genome to a haploid condition. The perverse parasite achieves these exceptional rates of chromosome segregation without condensation of the genome into recognizable compact structures! Hence it is impossible to count the number of chromosomes directly; however, the reduction in the genome is signalled by a stepwise fall in spindle microtubule number from 40 to 10 and the number of kinetochores on the final spindle, i.e. chromosome number, is similarly estimated at 10 (Sinden, 1978).

During this period of nuclear division each of the eight kinetosomes has acted as the template for the production of a typical flagellar axoneme some 20–22 μm in length, which lie coiled in the cytoplasm (Fig. 14). Despite the normal pattern of axoneme production the speed of assembly (2–10 μm min^{-1}) frequently results in errors, some doublets failing to associate along their length, others forming flagella with 0, 1 or 2 central microtubules, others with 0 to 9 outer doublets. This microtubule system if pursued could offer an insight into normal axoneme assembly (Sinden *et al.*, 1975). The assembled axonemes are totally quiescent until the moment of exflagellation. At the time the third mitotic division is taking place a third class of microtubule based organelle is being assembled, this is the perikinetosomal basket (Sinden *et al.*, 1975). As its name suggests it is found enveloping the kinetosome as a helical lattice work of tubules 1–3 layers deep. The microtubules are of variable diameter and slightly larger than either the spindle or axoneme microtubules (Fig. 15). These microtubules extend from the plasmalemma down to the eight spindle poles which now lie close to the cell surface. The very distinct form of these organelles would suggest that they derived at least in part from a different pool of tubulin to the cytoplasmic axonemes and nuclear spindle which are being assembled concurrently. Polymerization of all these microtubular organelles can be inhibited by colchicine and vinblastin sulphate (R. Sinden and N. King, unpublished observations).

Within the perikinetosomal basket lies an electron-dense granule 80 nm in diameter surrounded by a less dense sphere 150 nm in diameter, this is attached to the kinetosome at the opposite side to the axoneme and precedes the axoneme as the flagellate microgamete is driven from the cell surface.

Irrespective of whether the microgametocyte has successfully escaped the host cell or not (Fig. 16), immediately following the third division (about 10 minutes in *P. falciparum*) the microgametes are explosively expelled from the microgametocyte (exflagellation), the whole event normally lasting only some 15–45 seconds (Fig. 17). Some gametes often fail to be released from the parental cell and it is the movement of these attached gametes which

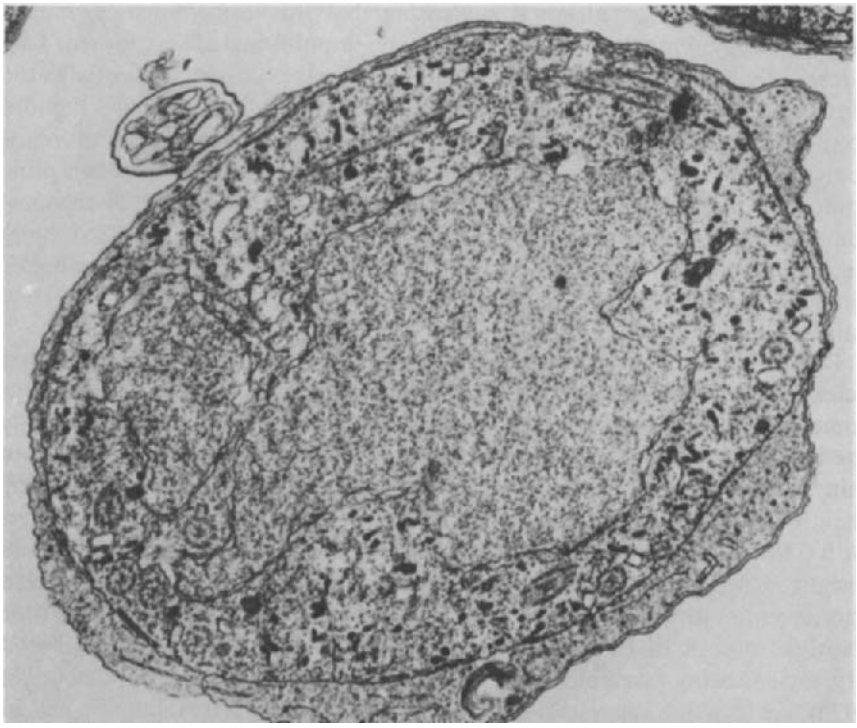

FIG. 14. Microgametocyte of *P. yoelii nigeriensis* at time of second mitotic division. Note numerous sections of developing intracytoplasmic axonemes. Magnification ×25 000.

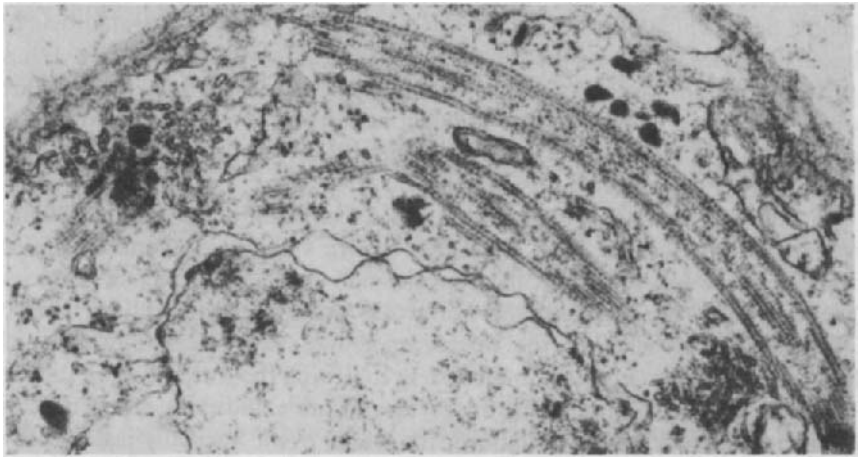

FIG. 15. Microgametocyte of *P. yoelii nigeriensis*, nucleus contains haploid spindle poles of final mitotic division. Single kinetosomes are now enveloped by microtubule basket and bear the kinetosomal sphere and granule. Magnification ×46 000.

is detected as exflagellation centres in physiological studies (see page 185). The activity of the axoneme propels the kinetosome and its associated sphere and granule toward the plasmalemma. Here the axoneme passes through the basket and the kinetosome drags the last hemispindle and its associated haploid genome out of the parental cell (Fig. 18). It is only at this final moment that the chromosomes condense and the budding nucleus is torn from the lobed parental organelle. In the normal microgamete the cigar-shaped nucleoplasm is totally occupied by condensed chromatin. Despite the cunning strategy of the parasite to ensure the production of flagellated and nucleated gametes, in many gametes the violence of exflagellation tears the kinetosome free of the spindle, with the result that as many as 60% of the microgametes are anucleate (Sinden, 1975; Raffaele, 1939). Scanning electron microscopy has shown clearly the location of the kinetosomal sphere and granule at the distal pole of the emerging gamete, and that the trailing pole is pointed where it is ripped unceremoniously from the gametocyte surface (Fig. 17). The production of the eight microgametes involves a massive assembly of new plasmalemma which may therefore contain new surface proteins specifically adapted to the requirements of macrogamete recognition and fertilization (equivalent to fertilizin/antifertilizin in other systems). High localized concentrations of a few such specialized proteins could explain the highly immunogenic nature of these gametes and also the efficacy of the resulting antigamete antibodies (see page 204).

The free male gamete normally, though not invariably, bears a single axoneme, a single small highly condensed nucleus (though this too may be abnormally large and not condensed) and a single kinetosome with its sphere

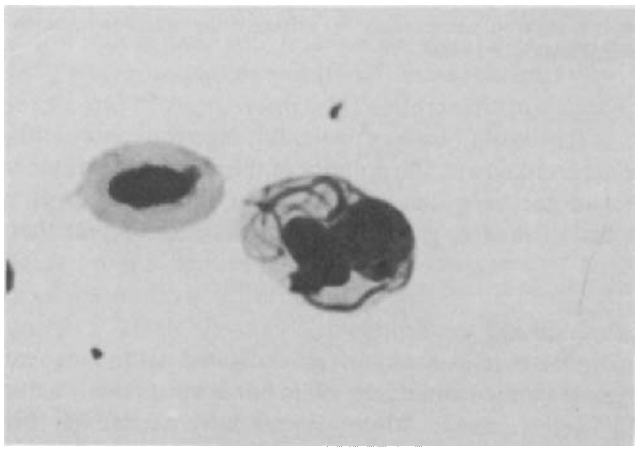

FIG. 16. Light micrograph of exflagellated *Plasmodium* sp. retained within intact erythrocyte. Magnification ×1100. (Micrograph kindly provided by Professor A. Gabaldon.)

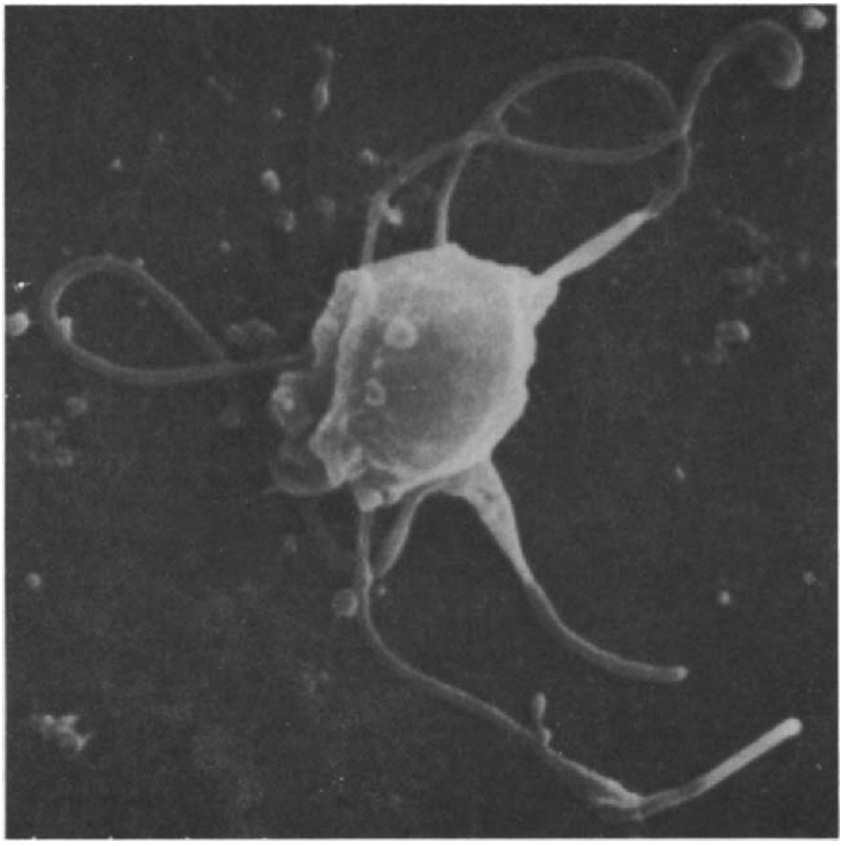

FIG. 17. Scanning electron micrograph of exflagellating microgametocyte of *P. yoelii nigeriensis*. Magnification ×18 000.

and granule at the distal (? leading) pole. Whether the sphere and granule act as an acrosome is unknown. The polarity of the microgamete at the moment of fertilization has not been determined, but if the direction of penetration is similar to that of the free gamete it is indeed the distal pole that penetrates the female.

3. *Gamete dispersal and fertilization*

Individual gamete release is not co-ordinated within an exflagellating microgametocyte (Sinden and Croll, 1975) but is most readily achieved by the very "young" active gamete. Microgametes have a relatively brief lifespan of 0·5–2 hours and fertilization is most efficiently achieved usually within the first 20 minutes (Carter *et al.*, 1979a). During this time the microgamete

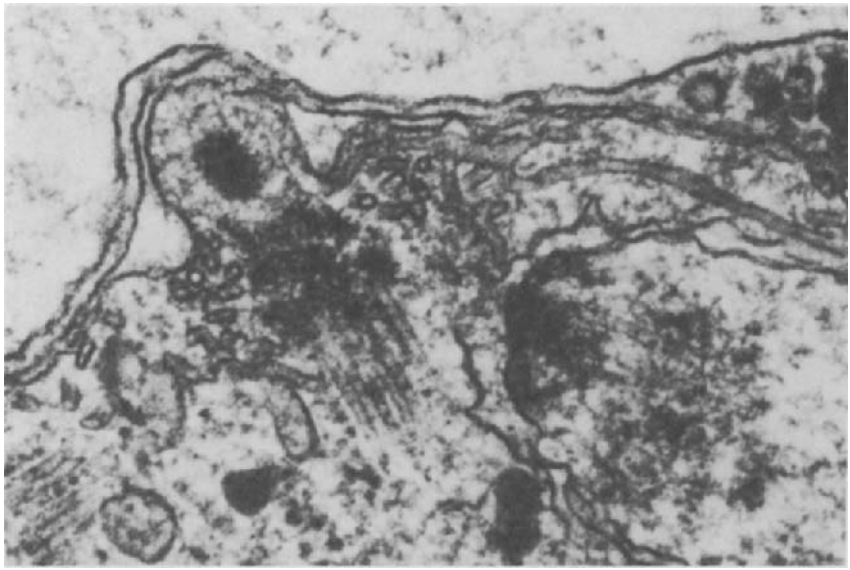

FIG. 18. Transmission electron micrograph of exflagellating microgametocyte of *P. yoelii nigeriensis* showing emergence of flagellum, kinetosome first, through the perikinetosomal basket, closely followed by the haploid nuclear spindle. Magnification ×100 000.

displays numerous distinctive behaviour patterns. Initially they are continually and highly active, but rapidly assume a motility pattern of alternate fast and slow waves which progresses toward alternate slow and stationary phases (Fig. 19) (Anderson and Cowdry, 1923; Sinden and Croll, 1975; Sinden *et al.*, 1978). The fast waves are of low amplitude and short wavelength, whereas the slow waves have a high amplitude and longer wavelength (fast, > 10 waves s^{-1}, 1·1 μm amplitude and 10–12 μm wavelength: slow, 1 wave s^{-1}, 2·5 μm amplitude and 15 μm wavelength). The fast waves propelled the gametes predominantly in one direction (kinetosome first?) at speeds of 100 μm s^{-1}. Slow waves result in the gamete girating on the spot and usually therefore changing in direction. No authors have been able to demonstrate a tactile response of a male gamete to a female gamete. Phagocytes clearly recognize tactile gradients around extracellular gametes (Sinden and Smalley, 1976) and the role of chemotaxis in fertilization should be examined diligently. Having contacted a female the male gamete "shunts" gently back and forth over the macrogamete surface whilst maintaining intimate contact (Fig. 20), after a period of some seconds the male gamete draws back and then with a vigorous burst of fast activity plunges into the female cytoplasm. At this point the plasmalemma of the male and female gametes have fused and it is the "denuded" cytoplasmic contents that enter the female cytoplasm. The

fertilized female plasmalemma is therefore a mosaic of female and male components. Within the female cytoplasm the naked axoneme reverts to its cyclic activity pattern (despite the immediate availability of energy from the female cytoplasm) so disturbing the cytoplasmic contents. However, this activity rapidly ceases, as it does in those microgametes that have failed to achieve their objective. The male nucleus becomes separated from the axoneme, and rapidly the chromatin becomes decondensed as the nucleus enlarges (Fig. 21; Sinden *et al.*, 1975), nuclear fusion follows, usually within minutes, and is achieved by the amalgamation of convoluted nuclear membranes (R. Carter and M. Aikawa, personal communication) resulting in zygote formation.

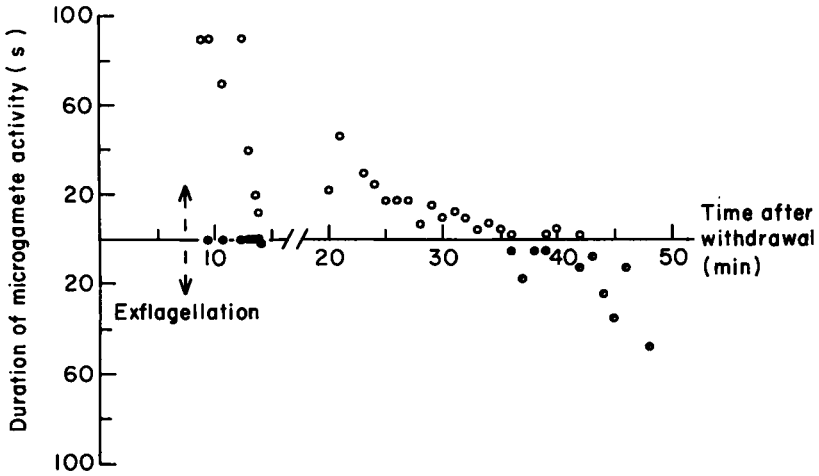

FIG. 19. Microgamete motility pattern, illustrating alternate periods of rapid (o), slow (•) and sessile (●) behaviour (from Sinden and Croll, 1975).

Multiple fertilization (polyspermy) does not usually occur in *Plasmodium*, indeed McCallum (1897) describes the tardy microgametes as "vainly beating their heads about the fertilized female", which suggests the fertilized cell is refractory to other male gametes. However, R. Carter and M. Aikawa (personal communication) have observed fertilized macrogametes which appear to contain more than one microgamete axoneme. This inhibition could be achieved either by a rapid loss, or substitution, of the female's binding molecule or the mosaic nature of the zygote plasmalemma.

4. *Zygote development*

Details of zygote-ookinete morphogenesis do not strictly lie within the span of sexual development but because many laboratories inevitably study both gametocyte and ookinete biology a brief outline is included here.

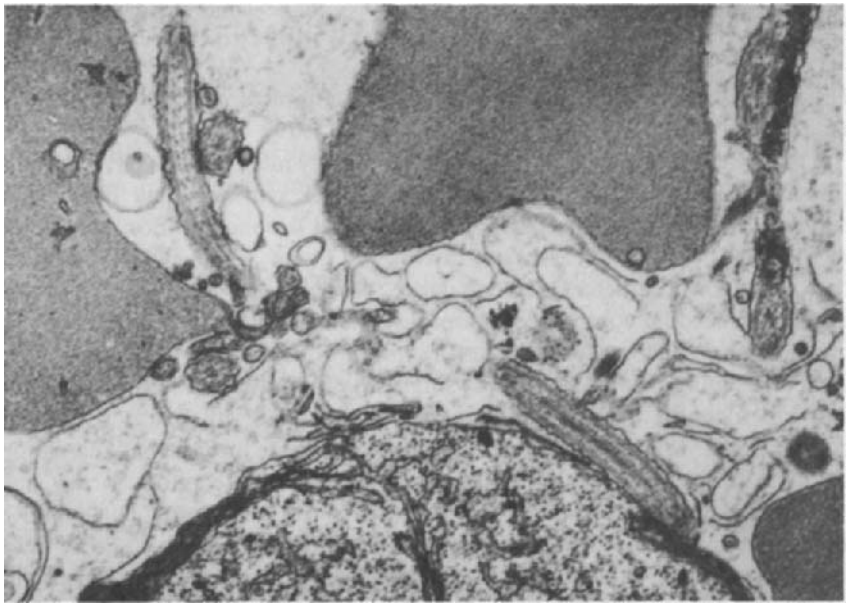

FIG. 20. Transmission electron micrograph showing the intimate contact of the microgamete and macrogamete prior to the fusion of the two plasmalemmas and fertilization. Magnification ×25 000.

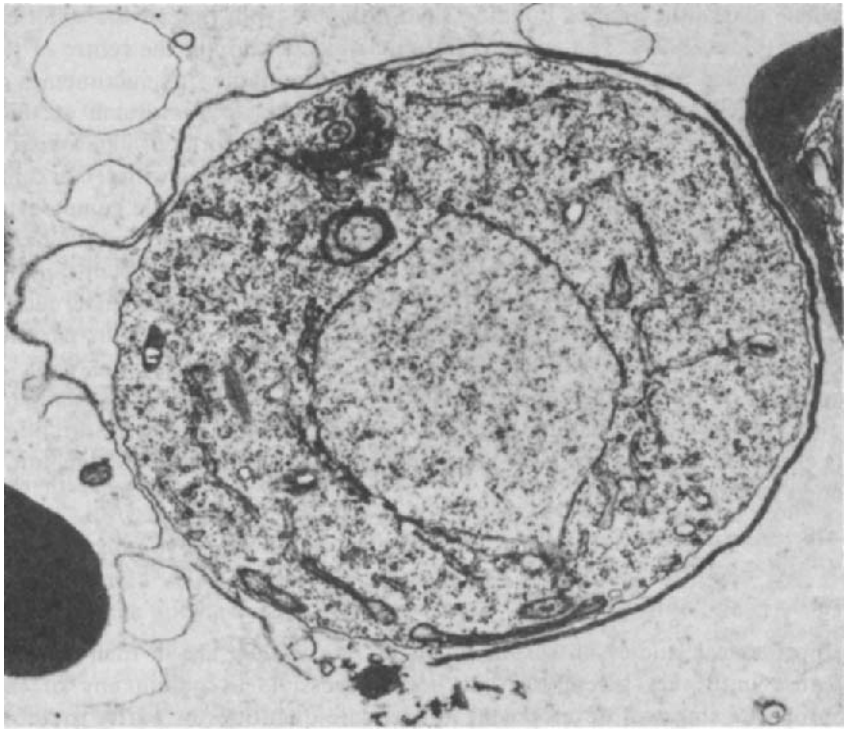

FIG. 21. Transmission electron micrograph of fertilized macrogamete of *P. yoelii nigeriensis* clearly illustrating the large pale female nucleus and the small condensed male nucleus associated with the long and convoluted microgamete axoneme. Magnification ×18 000.

However, authors are directed to the more complete descriptions given by Galucci (1974b) and Sinden (1978) and Gass (1979).

The nucleus and nucleolus of the zygote rapidly incorporates [^{3}H]adenosine (Sinden, 1978) indicating nucleic acid (RNA) synthesis has begun. The endoplasmic reticulum proliferates rapidly and a concentration of smooth-membraned vesicles is associated with it. These vesicles transform into the 20–35 nm diameter electron-dense particles of the crystalloid and eventually become compacted, adopting a hexagonal form. The nucleus has been shown to divide within the first 18 hours while the zygote is in the blood-meal or crossing the midgut wall. A single small spindle with few microtubules and kinetochores extends between spindle plaques which are situated in nuclear pores. Transformation of the spherical zygote into the banana-shaped ookinete is not well documented but proceeds via the "retort form" stage (Gass, 1979; Gao, 1981). The development of the trilaminar pellicle and its associated apical complex may *a priori* be expected to occur as in other dividing phases, with the subpellicular vesicles and microtubules developing adjacent to the zygote MTOC. In *P. gallinaceum* this MTOC is inherited from the femal gametocyte and acts as the focus of a conical nuclear extension around which cytoplasmic microtubules are localized. The assembly of the pellicle marks the area of budding rigid ookinete from that of the spherical zygote (Gass, 1979). The apical collar and rings develop at the centre of the newly formed inner pellicular membranes and the numerous micronemes of the complex aggregate beneath. The subpellicular vesicles attach at their lateral edges and a continuous trilaminar pellicle is formed. The massive elaboration of new organelles to form the ookinete is achieved with remarkable speed which reflects the "pre adaptation" of the mature female gametocyte.

Once the ookinete pellicle is formed, the parasite becomes motile. This is achieved by an actin-based unidirectional capping of substratum binding ligands (see Russell and Sinden, 1981) directed by the subpellicular microtubules. The appearance of the substratum binding molecules in the parasite glycocalyx suggests rapid changes in the zygote plasmalemma. This is confirmed by the observations of Carter *et al.* (1979a) and D. C. Kaushal and coworkers (personal communication) who found that antigamete antibodies are effective in transmission blocking only between the times of exflagellation and zygote formation.

B. BIOCHEMICAL ORGANIZATION

Biochemical studies on sexual stages of *Plasmodium* are in their infancy because until very recently it has been impossible to obtain any of the appropriate stages of development in adequate quantities or purity to allow

such work to be undertaken (see page 200). However the advent of *in vitro* culture techniques for *P. falciparum* on both the large and small scale, together with the revived interest in the biology and immunology of both gametocytes and gametes have stimulated much new work.

1. *The sexual cell cycle*

The development of the gametocyte of *P. falciparum* obviously differs from that of the asexual parasite in time, form, and in drug susceptibility; and electron microscope observations suggest that the mitotic cycle of the male and female gametocytes differ one from the other (Sinden, 1982b).

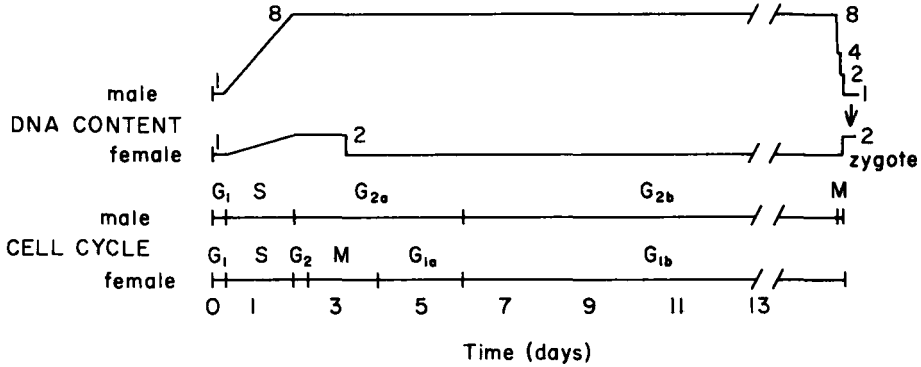

FIG. 22. Proposed cell cycles of macrogametocyte and microgametocytes of *P. falciparum* showing relative positions of phases of the cell cycle and the associated DNA content of the gametocyte nucleus (from Sinden, 1982).

Using a primitive microculture technique Sinden and Smalley (1979) examined the sensitivity of the developing gametocytes to a range of antimetabolites directed against DNA, RNA and protein synthesis. Whilst noting that the culture system used did not support the growth of gametocytes to full maturity these authors concluded that DNA synthesis (revealed as mitomycin C sensitivity of *all* gametocytes) was confined to the first 2–4 days of culture, i.e. when the stage I and II gametocytes are present. In contrast, sensitivity to actinomycin D killed the gametocytes readily for the first 5 days of culture, i.e. stages I–III and inhibitors of protein synthesis killed at all stages of growth although the stage IV and V parasites were apparently less sensitive than the immature forms. These observations correlate well with the observed morphological changes, i.e. DNA synthesis precedes the increase in microgamete nuclear volume, and macrogamete nuclear division. The decrease in RNA synthesis occurs at a time when the microgametocyte suffers a reduction in ribosome density and a nucleolus forms in the macrogametocyte.

Protein synthesis in the more mature parasites (stages IV and V) has been detected by the autoradiographic localization of [^{3}H]leucine (Phillips *et al.*, 1978), their results showed that the gametocytes incorporate less extensively than the asexual schizont. More recently in our laboratories gametocytes (stages III to V) have been shown to incorporate readily [^{35}S]methionine into macromolecular proteins, and thus it is now possible to re-examine the conclusions of inhibitor studies on protein synthesis by direct observation.

Correlating the ultrastructural observations on nuclear division with the antimetabolite studies it is possible to construct a hypothetical cell cycle for the gametocytes of *P. falciparum* (Sinden and Smalley, 1979; later modified by Sinden, 1982b; see Fig. 22). It remains to be seen if this cycle, suitably abbreviated in absolute time, applies to species other than *P. falciparum.*

Toyé *et al.* (1977) examined the effects of antimetabolites on exflagellation of the microgametocyte of *P. yoelii* and concluded that *de novo* DNA synthesis was not required, but that *de novo* protein synthesis was essential; they were, however, uncertain as to the essential nature of *de novo* RNA metabolism. These have been substantiated by the observation that specific proteins are synthesized *de novo* during gamete formation (R. Carter, personal communication).

2. *The induction and control of the sexual cycle*

In *Eimeria* it has been shown that the asexual schizont and invading merozoite may be distinguished morphologically into those producing sexual or asexual parasites in the subsequent generation (Klimes *et al.*, 1972). Thomson and Robertson (1935) suggested that in *Plasmodium* it is the sporozoite which is predetermined to produce either sexual or asexual progeny, there is no evidence for this nor for the notion of Boyd *et al.* (1935) that the asexual schizont or merozoite like that of *Eimeria* is precommitted to one or other developmental path.

The induction of sexual commitment *in vivo* is often predictable within an infection, but is not an inevitable occurrence. The factors correlated or incriminated as inducers are: a rising immune reponse to the asexual parasite; a rising asexual parasite burden *per se;* the presence of parasite debris; or the action of subcurative drug levels. *In vitro* culture techniques further indicate that a variety of conditions, all of which produce an environment detrimental to the growth of the asexual parasite, will enhance the conversion of asexual parasites into a sexual course of development. Carter and Miller (1979) clearly showed the environmental modulation of gametocyte induction was suppressed if the culture was diluted with fresh erythrocytes. Perhaps the most interesting observation, however, is that of Kaushal

et al. (1980) that the addition of 1 mM cAMP (adenosine cyclic 3′:5′-phosphate) to cultures at exactly the time when natural conversion was occurring could push all the asexual cells to a sexual path. Despite the inability of other laboratories to confirm precisely this finding, we find that addition of cAMP to the asexual ring stage may increase gametocytaemias suggesting it is this stage of growth which is susceptible to stimulation. The trophozoite, if similarly treated, is inhibited from entering schizogony. If sexual determination is mediated via cAMP it is possible, indeed likely, that a variety of stimuli within an infected host could trigger generation of the molecule by adenylate cyclase within the parasitized erythrocyte. There is at present no simple or acceptable hypothesis to explain this critical event initiating the sexual cycle either *in vitro* or *in vivo*.

The induction of gametogenesis (exflagellation) is, however, more fully understood mostly because it can be stimulated *in vitro* under very simple conditions (Bishop and MacConnachie, 1956; Nijhout and Carter, 1978). The early studies of Micks *et al.* (1948) showed that an extract of the mosquito gut would induce exflagellation in blood *in vitro*, and amongst others Bishop and MacConnachie (1956, 1960) showed that an atmosphere of 5% CO_2 inhibited the event, and that pH was also an important controlling factor. Roller and Desser (1973), Sinden and Croll (1975) and Yamaguchi and Nakabayashi (1976) further demonstrated that a temperature fall of about 5°C below that of the host body temperature was essential to induce microgametogenesis *in vitro*, although infections *in vivo* can occur even at temperatures above that of the host (Ross, 1897). The temperature profiles of exflagellation for *P. gallinaceum* and *P. yoelii* (A. Knight, personal communication; Fig. 23) illustrate the differing thermal responses of the avian and mammalian parasites.

Carter and Nijhout (1977) and Nijhout and Carter (1978) correlated the changes in the pCO_2 of the atmosphere with the presence of bicarbonate in the medium, and this, in conjunction with pH, controlled exflagellation in an otherwise simple medium. A. Knight (unpublished work) has confirmed these observations on *P. gallinaceum* but has detected a different pH–response curve in the rodent malaria parasite *P. yoelii* (Fig. 24). Martin *et al.* (1978) have shown that some inhibitors of phosphodiesterase activity, for example caffeine and Squibb 20009 together with 8-bromo-cyclic AMP, all stimulate exflagellation in *P. gallinaceum* in the presence of low concentrations (1%) of serum. The stimulation, however, never reached the same levels as that achieved by the addition of whole serum adjusted to pH 8·0 with sodium bicarbonate. A. Knight (unpublished work) has again confirmed these observations in part but has failed to achieve a similar response from the rodent parasite *P. yoelii*. The dilemma which is not yet resolved is whether the induced rise in intracellular cAMP levels is exerting its effect on the avian parasite directly or through the nucleated erythrocyte. Attempts to introduce

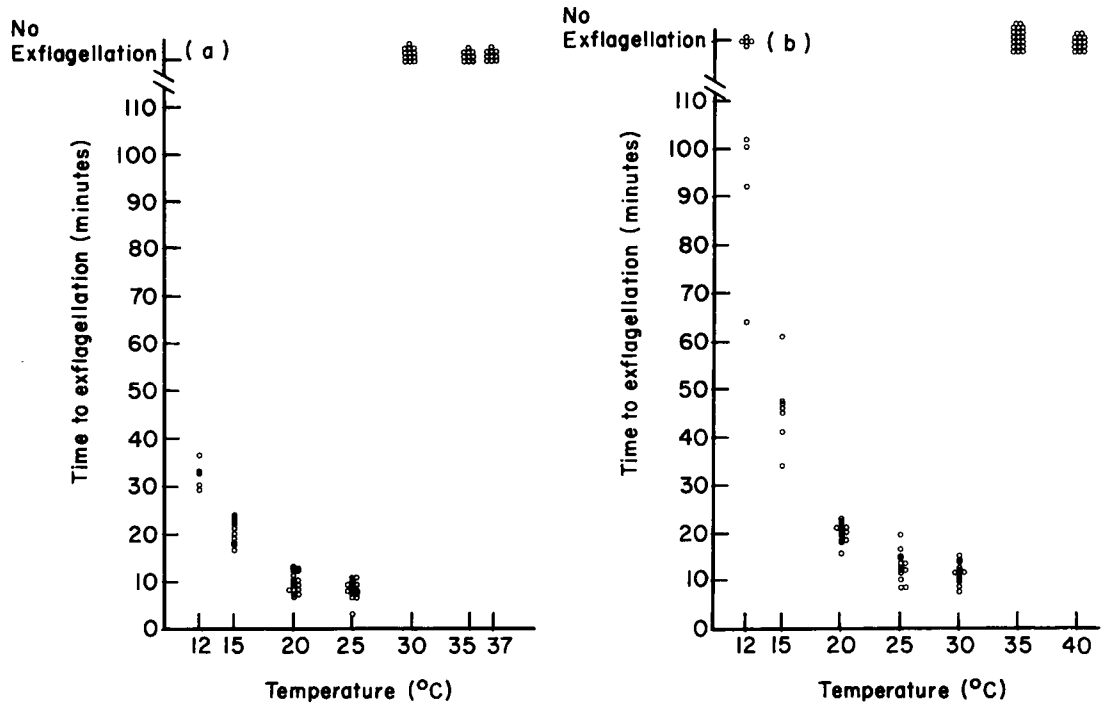

FIG. 23. The contrasting temperature profiles of exflagellation of the rodent parasite *P. yoelii nigeriensis* (a) and *P. gallinaceum* (b) (from A. Knight, unpublished observations).

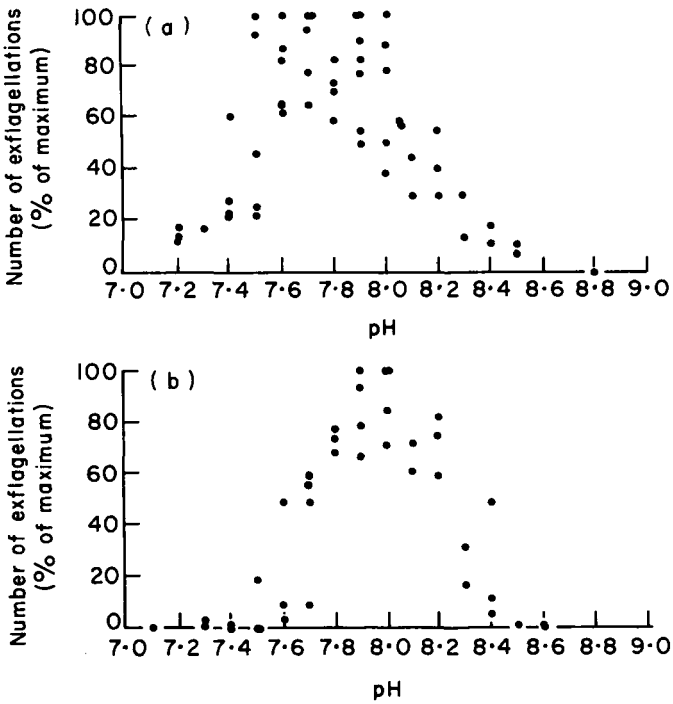

FIG. 24. The pH profiles of exflagellation of the rodent parasite *P. yoelii nigeriensis* (a) and *P. gallinaceum* (b) (from A. Knight, unpublished observations).

the avian parasite into erythrocytes of neonatal mice in an attempt to resolve the problem have, to date, proved unsuccessful.

Knight has also considered that the events of microgametogenesis and of escape from the host erythrocyte could be achieved by different pleomorphic effects of cAMP. Support for this idea stems from his ability to induce gametocytes to escape from host cells, but not exflagellate, by addition of the ionophore A 23187, in the presence of calcium or manganese (but not magnesium) ion, to the "suspended animation medium" of Nijhout and Carter (1978). However, it is relevant to note that the same ionophore has been shown to mediate the exit of *Toxoplasma* trophozoites from mouse macrophages (Endo *et al.*, 1982).

Nijhout (1979) has produced evidence to support the results of Micks *et al.* (1948) namely that a macromolecule (mosquito exflagellation factor, MEF) in the mosquito triggers exflagellation in the absence of bicarbonate. She has shown that extracts of the midgut or head of both vector and non-vector species will stimulate gametogenesis. The active agent is stable to boiling and decarbonization, is dialysable and is more effective at pH 8·0 than pH 7·4.

She has suggested that neurotransmitters, or neurohormones in the mosquito head, can mimic the action of MEF found in the mosquito midgut. However, an even more tempting interpretation would be that the neurohormones which control enzyme secretion and release following bloodmeal ingestion would themselves be the MEF *in vivo*.

Once again it appears as if a variety of stimuli (albeit well defined) may trigger a critical event in the sexual cycle and that this triggering is achieved via a common pathway involving a secondary messenger which may be a cyclic nucleotide. Studies on the biochemical control of exflagellation *in vivo* are now possible and should be pursued vigorously.

3. *Nucleic acid synthesis and organization*

Little is directly known of nucleic acid synthesis in malarial gametocytes; indirect observations, however, may be very briefly restated here. Following erythrocyte invasion by the haploid merozoite, DNA is synthesized in both male and female gametocytes for a brief period in the first 2 days of their development. Male gamete formation in the mosquito bloodmeal, which entails three mitotic divisions, is achieved without DNA replication. Macrogametogenesis does not require nuclear division, but division is seen in the immature (stage II–III) female gametocyte. Hence the mature microgametocyte is octoploid, and the macrogametocyte becomes diploid but reverts to a haploid state before reaching maturity.

Structural organization of the genome is poorly understood. In the young gametocyte the nucleus is Feulgen positive, but it is more difficult to stain in the mature cell. Changes in Feulgen sensitivity have been correlated with increased histone packaging which accompanies the terminal differentiation of cells (Ringertz, 1969). However, histones and other basic proteins could not be detected in gametocytes by cytochemical means (P. Toyé, unpublished work). Alternatively it may be that in the mature gametocyte the genome becomes very diffuse and does not stain intensely, or becomes highly susceptible to acid hydrolysis (Cornelissen, 1982). During microgametogenesis the DNA is, however, readily stained by the Feulgen technique and is simultaneously undergoing condensation prior to inclusion in the small microgamete nucleus. The macrogamete nucleus similarly becomes Feulgen positive but here the genome does not condense, it may be that this change signals the derepression or unmasking of the genome for the synthesis of new proteins in the macrogamete and zygote.

Perhaps the most interesting observation on gametocyte DNA is that of Birago *et al.* (1982) who have demonstrated that, in lines of *P. berghei* producing infective gametocytes up to 16% of the total DNA is composed of reiterated sequences, whereas this fraction is only 3–5% of the genome of lines that have lost their infectivity. The reiterated DNA is clearly not essential

for the growth of the sexual stages themselves, but is required for development within the mosquito. It is possible that this fraction represents the amplified ribosomal RNA genes which may be found in the nucleolus—a prominent feature of the macrogametocyte which becomes highly active in the fertilized zygote. If confirmed in *P. falciparum*, the interpretation of the effects of inhibitors of DNA synthesis on gametocytogenesis will require careful re-examination.

Very little is known of RNA metabolism in gametocytes. A molecular explanation is required for the control of the different cytoplasmic ribosome populations and nucleolar morphology observed in mature male and female gametocytes. Perhaps the reiterated DNA sequences observed by Birago *et al.* (1982) are associated with nucleolus formation? Probably the most significant question to be asked, however, is: "Does the *de novo* protein synthesis detected in the mature gametocyte, and more particularly in the activated gametocyte, require *de novo* RNA synthesis, i.e. are the relevant mRNA molecules long or short-lived?" Protein synthesis in the zygote is associated with the rapid labelling of the nucleus and nucleolus with [^{3}H]-adenosine suggesting that at least ribosomal RNA is being synthesized (and presumably therefore mRNA) at this stage of development (Sinden, 1978).

4. *Protein synthesis*

Phillips *et al.* (1978) examined the incorporation of [^{3}H]leucine into gametocytes of *P. falciparum* and found that its uptake was less rapid than in the erythrocytic schizont. A. Simm (unpublished work) has similarly demonstrated the uptake of [^{35}S]methionine into the stage III to V gametocyte, he has further revealed, using polyacrylamide-gel electrophoresis, that at least 26 individual proteins are synthesized at this stage of which six are unique to the sexual parasite. These have molecular weights of 380 000, 330 000, 290 000, 270 000, 230 000, and 92 000 $\pm$ 3%. The other proteins are common with those of the sexual schizont.

Protein synthesis *de novo* is essential to microgametogenesis (Toyé *et al.*, 1977). Radiolabelling techniques have shown that major proteins are synthesized at this stage, at least one of which is precipitable with a transmission blocking monoclonal antibody (R. Carter, personal communication).

Howard *et al.* (1982) have shown that the zygote (collected as a fraction of fertilized macrogametes) has at least 30 proteins detectable by polyacrylamide-gel electrophoresis molecular weights (M_r) ranging from 40 000 to 200 000 daltons of which 15 may be radioiodinated using the IODOGEN labelling technique, suggesting that these are exposed on the cell surface. All 15 of the labelled proteins were precipitable with anti-zygote antibodies.

Rener and coworkers (personal communication) have found that transmission-blocking antigamete monoclonal antibodies are capable of precipitating

proteins from microgamete preparations, which in the case of *P. gallinaceum* gametes has M_r 230 000. It will be interesting to see if this protein is identical to the 230 000 dalton protein found in the mature gametocyte (A. Simm, unpublished work). Gwadz (1976) has suggested that gametocyte immunization is capable of inducing a transmission-blocking immunity, but if this protein is exclusively synthesized *de novo* during microgametogenesis it would suggest that at least some gametocytes were activated in Gwadz's preparations.

IV. The Culture of the Sexual Stages of *Plasmodium* and *in vitro* Techniques

A. Gametocytogenesis

The first description of the culture of gametocytes of *Plasmodium falciparum* was given by Row (1929) who used the very simple culture method of Bass and Johns. Despite the clear growth of the immature gametocyte there was little evidence for the formation of morphologically mature gametocytes *in vitro*. Following the development of the technique for the long term culture of the asexual stages of *Plasmodium falciparum* (Trager and Jensen, 1976; Haynes *et al.*, 1976) the technique was applied to the culture of the gametocytes. The first significant success was achieved by Smalley in 1976, although brief observations had been reported by others showing the production of gametocytes in preparations designed to culture the asexual stages (Mitchell *et al.*, 1976; Haynes *et al.*, 1976; Phillips *et al.*, 1978; Trager and Jensen, 1976). Until very recently such culture success has been largely confined to *P. falciparum*, but now includes *P. inui, P. coatneyi and P. fieldi* (Collins and coworkers, personal communication).

Using a 200 µl culture version of the basic technique of Haynes *et al.* (1976), Smalley (1976) was able to confirm that *in vitro* the development of ring stage parasites into gametocytes followed the patterns observed *in vivo* both in man and more particularly in *Aotus* (Hawking *et al.*, 1971), both in terms of the morphological sequence of development and in the prolonged period of growth (about 10 days). Of the 27 primary cultures made, 22 produced gametocytes *in vitro*, but Smalley noted that while only 59% of bloods from patients with asexual infections produced gametocytes *in vitro* in the first few generations, that 100% of gametocyte carriers did so. The vagaries of induction of gametocytogenesis *in vitro* have been noted by all those intensively involved in the subject. Ponnudurai *et al.* (1982b) noted that all 22 isolates from European donors produced gametocytes *in vitro* but that each

isolate had a characteristic sexual capacity. Some cultures produced gametocytes in low numbers (<0·1%) for brief periods, others in high numbers (>3%) for many months. A few strains have produced gametocytes for years, e.g. Honduras 1 (Ifediba and Vanderberg, 1981), 'Z' (Carter and Beach, 1977) and Tanzania 1 (Campbell *et al.*, 1980). As with other laboratories we have found that some isolates fail to produce gametocytes at all (i.e. six of eight Gambian deep-frozen isolates) and that these isolates are often those that are the most difficult to establish in culture. Significantly Chin and Collins (1980) found that these poorly adaptable strains are similarly reluctant to develop sexual infections in *Aotus*. Fortunately the sexual capacity does not appear to be influenced by cryopreservation (H. Carter and R. Sinden, unpublished observations; Ponnudurai *et al.*, 1982b). Clearly, therefore, a prerequisite of any prolonged studies on gametocytes *in vitro* is an ensured constant supply of an identified isolate from the outset. Those studies requiring the use of cloned material will be markedly limited by the significant "loss" of generations incurred by the cloning procedure. Potential clones must therefore be selected from strains that have proven prolonged sexual capacity.

In attempting to review current methods in gametocyte culture, an inevitable problem arises. As with any new discipline in which a few independent laboratories, each with different objectives, are developing culture techniques, each laboratory has different practices with disconcertingly few common components, and numerous differences all regarded as absolutely essential by their users.

Taking the common factors, all cultures currently use RPMI 1640 medium supplemented with human serum at 10–15%, 0·2% sodium bicarbonate, and a zwiterionic buffer (either HEPES or TES) at 20–40 mM, with many laboratories preferring HEPES at the lower molarity. However, a note of caution, HEPES varies significantly from source to source and should be monitored before subject to significant use (T. Ponnudurai, personal communication). Gas mixtures also vary between laboratories, i.e. defined mixtures of O_2 (3–7%), CO_2 (2–5%) and N_2 (88–95%) or 5% CO_2 in air, or candle jars (CO_2 2–3%, O_2 14–17%). All these mixtures use a 0·2% bicarbonate buffer. Other gas mixtures give optimal growth if different bicarbonate concentrations are considered (Meuwissen *et al.*, 1980). These mixtures may be delivered continuously or solely at the time the medium is changed. All workers concur that fresh medium or that recently thawed from the deepfreeze is advantageous, although techniques differ as to the time of addition of serum and bicarbonate (i.e. prior to freezing or not). The frequency of medium change varies considerably, some consider that two virtually complete daily changes are essential (Ponnudurai *et al.*, 1982a), whilst others find a single medium change adequate (R. Sinden and H. Carter, unpublished observations), yet others happily use the continuous-flow method of Trager (1979), for

example Campbell *et al.* (1980, 1982). The only common factor appears to be a significant replacement of medium within a period of not more than 24 hours. In our hands, even using 40 mM TES does not allow medium change to be delayed to 48 hours without significant loss of gametocytes in the culture. Medium *must* be delivered to the cultures above 30°C, it has been shown that mature gametocytes of *Plasmodium* if exposed to temperature drops of 5°C or more will be induced to undergo gamete formation (Sinden and Croll, 1975), hence they will be lost from the culture on each medium change unless this simple precaution is taken.

Mechanical aspects of culture also vary widely. Static cultures include the usual range from 100 μl aliquots in microtitre trays through Petri dishes to sealed falcon flasks of 75 cm^2 or larger surface areas (all gassed intermittently). Automated and semi-automated static systems include the continuous flow vessel of Trager (1979; Campbell *et al.*, 1980, 1982), the "puffing-billy" vessel (Fig. 25) of Ponnudurai *et al.* (1982a), the falcon flask system of Scott-Finnegan (R. Sinden and H. Carter, unpublished observations) and the shaking Erlinmejer flask system of Butcher (1981) using either 50 or 100 ml inclined vessels (T. Ponnudurai and coworkers, personal communication). (All these have continuous gassing facilities.)

Cultures are initiated at erythrocyte concentrations varying from 2 to 12% at parasitaemia levels of 0·1 to 0·2%. Some authors suggest the high erythrocyte density is stimulatory. Taking note of the observations of Smalley (1976) and Ifediba and Vanderberg (1981) there may be advantage in initiating cultures taken from gametocyte producing sexual parasites rather than routine "asexual" cultures.

Once established, the erythrocytes are not diluted or replaced for the duration of the culture; however, the medium is changed as described above. The optimum frequency of medium change for gametocyte induction, particularly in static cultures, may be influenced by the type of culture vessel used (see Table 1); 200 μl cultures in our laboratory produce more gametocytes if the medium is changed only every 48 hours, whereas in 5 ml culture in 25 cm^2 falcon flasks changes at 24 hours are optimal.

As stated above, different isolates have markedly individual innate capacities for gametocyte production. However, it is also particularly clear to all workers that culture conditions can be modified to stimulate gametocytogenesis to a variable degree. This is not surprising in view of the clear suggestion from studies *in vivo* that sexual development is "stimulated" at identifiable times in natural infections (see above). At an early stage in the development of gametocyte culture techniques, the general observation was made that when a culture becomes less than ideal for the growth of asexual parasites, leading to the production of "crisis forms", gametocytogenesis is enhanced (e.g. Phillips *et al.*, 1978; Ponnudurai *et al.*, 1982b).

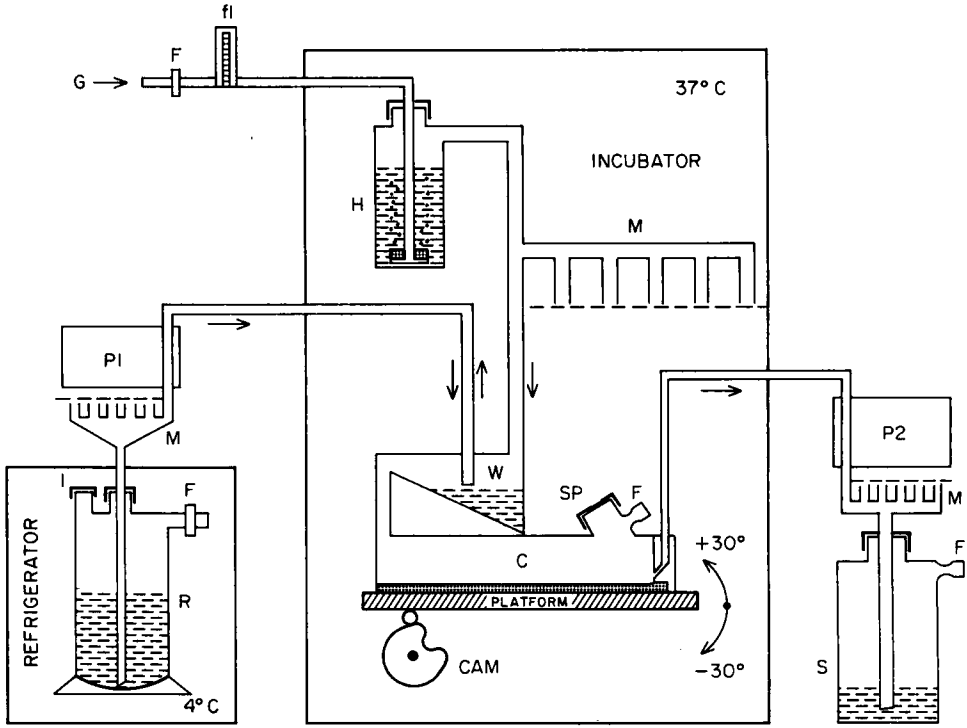

FIG. 25. Diagram of the semi-automated apparatus for the culture of mature capacitated gametocytes of *P. falciparum* (from Ponnudurai *et al.*, 1982a). The apparatus automatically replaces the medium whilst maintaining the cultures at the correct temperature and gas conditions. C, culture chamber; F, filter; fl, flow meter; G, gas (3% O_2, 4% CO_2, 93% N_2); H, humidifier; I, port for filling medium; M, manifold; P1, Watson-Marlow pump; P2, Cenco pump; R, (fresh) medium reservoir; S, (spent) medium reservoir; Sp, sampling port; W, warming chamber.

This problem was examined in depth by Carter and Miller (1979). Using a subjective, but nonetheless very useful, assay which estimates the change of ring stage parasites to stage II gametocytes 48 hours later, they showed clearly that upon dividing and diluting a culture ("subbing") gametocytogenesis was reduced below existing levels for approximately 6 days, and that this effect was not due to the addition of new (young) erythrocytes and must therefore be due to the "dilution" of the culture (note, however, that the daily change of medium does not have the same dramatic effect). This would suggest that it is the change in parasite density and not the addition of new medium which is the critical factor suppressing sexual development. This could be significant in the suggestion that old gametocyte cultures (with higher parasite burdens and more cell debris) are more effective at inducing gametocyte formation than younger (more dilute) cultures (Ifedeba and Vanderberg, 1981), and in

TABLE 1

The induction of gametocytes of P. falciparum (*CR18*) in vitro *by variation in frequency of medium exchange, and its modification by changes in culture size.*

Time before initial medium change, followed by daily replacement (hours)	Mean daily production of gametocytes per 10^5 erythrocytes	
	Microcultures (200 μl in microtitre plates (Days 6–15)	5 ml cultures in Falcon flasks (Days 6–12)
24	30 ± 35*	43·6 ± 31
48	74 ± 104	27·7 ± 19
72	65 ± 51	13·3 ± 12
96	100 ± 91	7·4 ± 4
120	52 ± 55	3·8 ± 3
144	—	0·4 ± 0·3

*The large standard errors reflect the significantly increasing gametocytaemia observed throughout the 9-day period of the experiment (see Figs 27 and 28).

the observation of Mons and Van der Kaay (1981) that the inoculation of lysed parasites into mice induced a higher gametocytaemia in blood-induced infection of *P. berghei*. None of these observations is clarified by the production of mature infective gametocytes in continuous-flow cultures where it might be argued that the effects of parasite metabolites or lysates are minimized compared to static cultures with only daily medium change!

Further observations on the induction of gametocytogenesis *in vitro* have followed other suggestions of Thomson (1914), Garnham (1931) and Thomson and Robertson (1935) that the mounting of the immune response by a host induces a switch to sexual development. Brockelman (1979) suggested that the addition of serum from patients "recently exposed to *P. falciparum*" induced gametocyte formation in cultures routinely diluted with fresh erythrocytes every 2 days. This effect was not produced by the addition of "ammonium sulphate precipitate (? IgG)" from the same sera and therefore required the effect of "additional factors in immune serum". Smalley and Brown (1981) have shown that the addition of serum from infected Gambian patients did *not* enhance the rate of gametocyte induction compared to non-immune European serum although a very significant increase was recorded when the Gambian serum was present at the same time as Gambian lymphocytes. This might suggest that some (inhibitory ?) secretion from the lymphocytes induces gametocytogenesis.

Recently it has been strongly argued that the addition of purine hypoxanthine at 50 μg ml^{-1} is essential to the growth of mature infective gametocytes

of *P. falciparum in vitro* (Ifedeba and Vanderberg, 1981). This observation is not without substance but requires careful justification. It is perfectly possible to produce mature gametocytes capable of exflagellation and reliable infection of mosquitoes without the addition of extra hypoxanthine (Campbell *et al.*, 1980; C. Campbell, personal communication) using the continuous-flow culture technique. In our own laboratories we have been producing mature gametocytes which exflagellate with great efficiency (60% plus) in the absence of added hypoxanthine. However, in controlled studies we note that the addition of hypoxanthine at 50 μg ml^{-1} does enhance the rate of growth of the asexual parasites and thus brings forward the appearance of gametocytes in a culture such that a subpopulation reaches a peak at day 12 of culture (5 days after appearance of gametocytes) prior to a second larger population which matures at the "normal" time of about 18 days culture (11 days after onset of gametocyte formation, Fig. 26). It is therefore a distinct possibility that the appearance of mature gametocytes in a younger and therefore healthier culture could contribute greatly to the eventual infectivity to mosquitoes, where the age of the erythrocytes currently appears to be of paramount

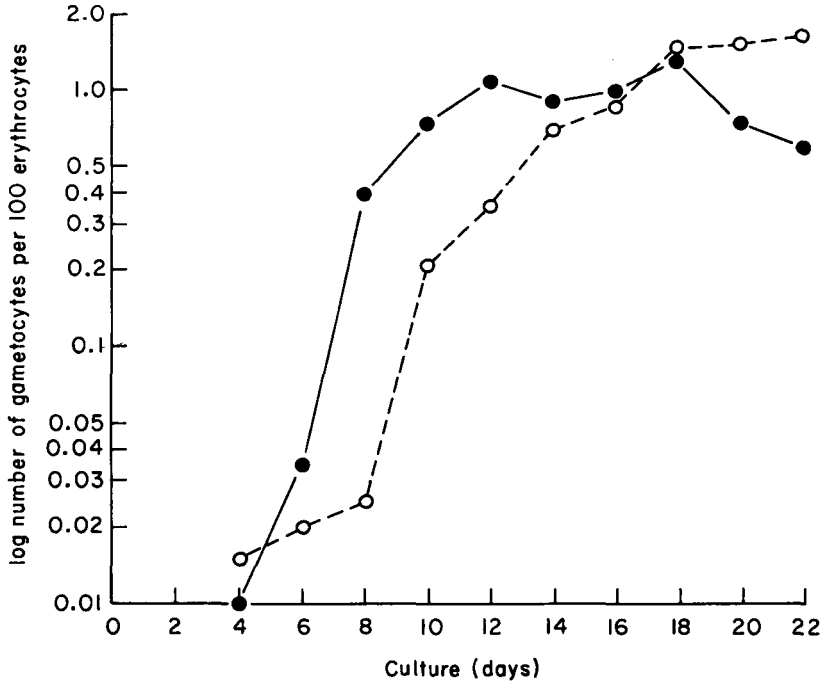

FIG. 26. The growth of gametocytes of *P. falciparum in vitro* in the presence (•) and absence (o) of added hypoxanthine at 50 μg ml^{-1}.

importance to transmission. At present it appears therefore that additional hypoxanthine is not essential but does enhance the production of mature infective gametocytes *in vitro*, and is therefore used routinely by some laboratories (Ponnudurai *et al.*, 1982a, b; R. Sinden and H. Carter, unpublished observations).

Gametocyte growth *in vitro* follows a predictable pattern of morphological development and change in gametocyte numbers. These changes are illustrated in Fig. 27 for a static culture of the "Ifedeba" type, initiated from a standard asexual stock culture. The appearance of exflagellating mature male gametocytes is in this system first detected at 12 days and they persist until day 23. Macrogametogenesis has not been examined in the same detail, but mosquito infectivity has been shown to occur from day 14 until day 23 (Ponnudurai *et al.*, 1982a, b; Campbell *et al.*, 1982).

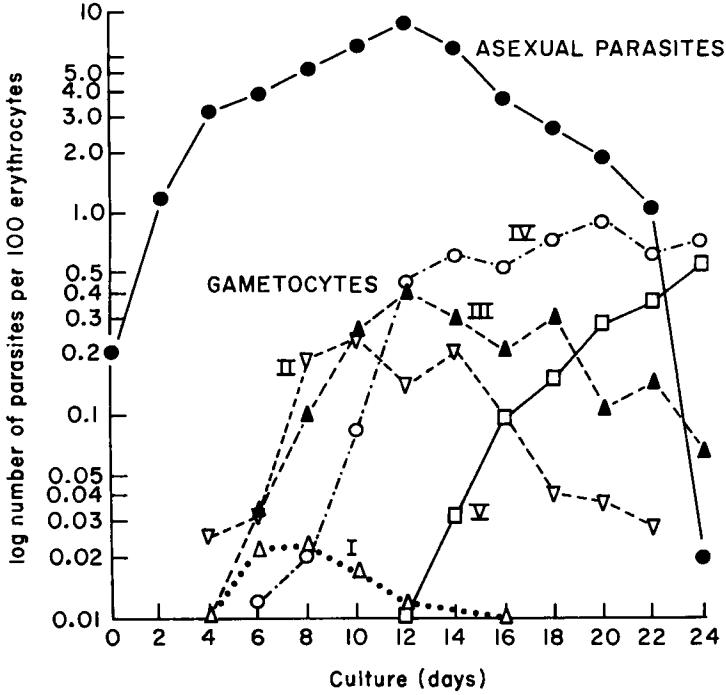

FIG. 27. The asexual parasitaemia and individual densities of gametocytes of different stages (I–V) of maturity (Hawking *et al.*, 1971) in a culture of *P. falciparum* showing the timing of parasite maturation.

Techniques for the continued maintenance of gametocyte cultures differ widely. In all cases the erythrocytes are not replaced for the life of the culture. In some laboratories cultures are initiated at a high erythrocyte concentration

(10–12%); this high cell density is reduced at day 6 or 7 to 5–6%, the proposed logic being that the high erythrocyte concentration (cell depth ?) induces high conversion rates, and the low concentration enhances cell survival and gametocyte maturation (Ifedeba and Vanderberg, 1981; Ponnudurai *et al.*, 1982a, b; R. Sinden and H. Carter, unpublished observations). We have compared the efficiency of culture at continuous or stepped cell densities (R. Sinden and H. Carter, unpublished observations; see Fig. 28). The high initial density suppresses the initial asexual infection and enhances the gametocyte conversion, subsequent dilution enhances the asexual multiplication rate and may therefore enhance gametocyte growth rate also, leading to a higher early gametocyte density (although the ultimate "late" gametocytaemia is higher in the cultures of uniform density). Automated systems for simplicity's sake do not use the dilution technique but nonetheless produce infective gametocytes, presumably because the medium change is as effective as the decrease in cell density at achieving effective feeding and washing of the

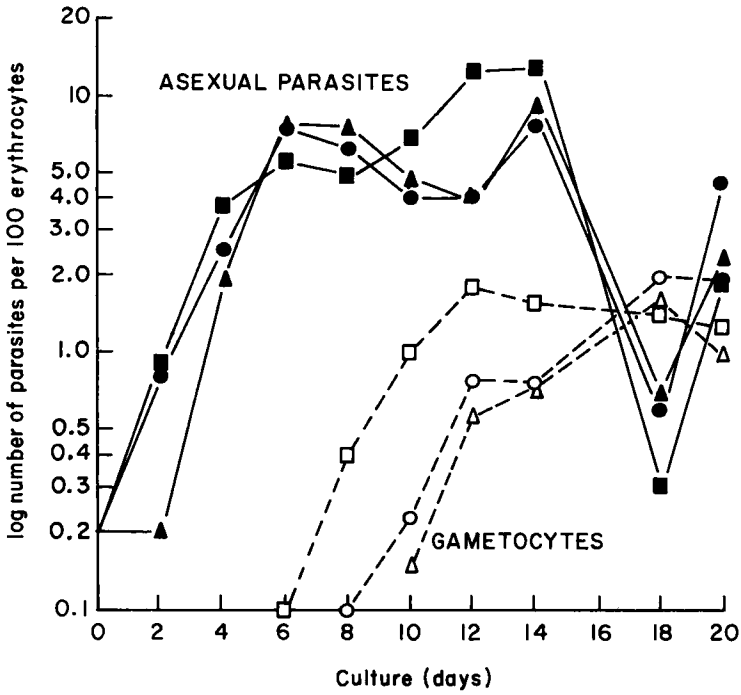

FIG. 28. The effect of erythrocyte density upon gametocyte (– – –) and asexual (———) parasite number in cultures of *P. falciparum*. □, Culture initiated at 12% erythrocytes but diluted to 6% on day 7; o, culture maintained at 10% erythrocytes throughout; Δ, culture maintained at 5% erythrocytes throughout. (Note erythrocyte densities after day 0 are nominal—erythrocyte lysis is significant after day 6 of culture.)

growing parasite. Early studies were restricted by the poor survival of the developing gametocytes and initial indications that this resulted from a poor nutritional supply was the apparent necessity for high (50%) serum concentrations late in the life of the culture, for example days 15–17. This enabled first exflagellation to be achieved (Carter and Beach, 1977) and later mosquito infection (Campbell *et al.*, 1980). Such elevated serum requirements are no longer necessary.

The growth of gametocytes in suspension cultures is less well documented but it has been suggested that maturation may be achieved more rapidly than in static systems, exflagellation being observed as early as 9 days in the culture (J. Meuwissen, personal communication). These figures are similar to those suggested by Mitchell *et al.* (1976) and Miller (1958), but in the opinion of the author if confirmed these must still be considered unnaturally short maturation periods.

Despite the plethora of unidentified and sometimes doubtful "stimuli" for gametocyte induction it would appear that once a strain has lost its ability to produce gametocytes *in vitro* it is unlikely to regain this potential (Ponnudurai *et al.*, 1982b; Jensen, 1979). Comparisons of data obtained *in vitro* and *in vivo* are inevitable (see Vanderberg and Gwadz, 1981). Repeated blood passage in the vertebrate host without intervening mosquito transmission has resulted in the loss of gametocyte production within seven blood passages in some isolates of *P. yoelii* (R. Sinden, unpublished observations) and even more rapidly in *P. gallinaceum* (Eyles, 1951). This loss of sexual potential is accompanied by a loss of moderately reiterated DNA in *P. berghei* suggesting that sexual development requires the expression of an amplified section of the genome (Birago *et al.*, 1982). The loss of the amplified section alone would readily account for a temporary loss of sexual expression, a permanent loss might require the inactivation of a "master" gene in the "sexual cascade" of expression. Kaushal *et al.* (1980) have shown that, in one instance at least, the addition of the ubiquitous "secondary messenger" cAMP at relatively high concentrations (1 mM) to cultures which have ceased (or markedly reduced) asexual multiplication would enhance the concurrent natural conversion of these parasites into gametocytes. This conversion was specific in that it was not achieved by cGMP, 5′: 3′-AMP or ADP, ATP, adenine or adenosine. An observation made by these and other authors is that cAMP is also a highly lethal compound to the growing asexual stages of *P. falciparum*. In contrast with the earlier suggestion of Carter and Miller (1979) that it is the merozoite of the preceding schizont that is determined to a sexual course, the time scale of action of 1 mM cAMP suggests that it is the invading, or recently invaded, merozoite that is affected, a conclusion also favoured by Ponnudurai *et al.* (1982a). Work in our own laboratories suggests that it is the transition of the ring stage to the trophozoite which is inhibited by cAMP, an effect which could support the supposition that it is the recently invaded parasite which is being diverted toward either a sexual or asexual pattern of development.

B. GAMETOGENESIS

The purposes of gametocyte production *in vitro* are threefold: first to study the biology of sexual development; second to produce gametocytes or gametes *en masse* for antigenic analysis; third to study the effects of antimalarials on sexual development and transmission. All studies require the demonstration that gametocytes cultured or grown *in vitro* are mature and will undergo either gamete- and zygote-formation *in vitro*, or mosquito transmission.

The standard technique for the induction of gamete formation *in vitro* is to take blood from an infected animal or culture, to allow (a) a fall in temperature at least 5°C below host body temperature, and (b) a rise in pH to 7·8–8·0 caused by the release of dissolved CO_2 by exposure of the blood to air in a moist chamber. Exflagellation ensues after a period characteristic for each species and temperature (see Fig. 23). It has been argued that the induction of gamete formation of *P. falciparum* in culture medium RPMI 1640 with 10% human serum and 20 mM HEPES is limited by the exceptional buffering capacity of the medium (Carter and Beach, 1977). In contrast, other laboratories have little if any difficulty observing exflagellation under these conditions (Ponnudurai *et al.*, 1982a, b) or indeed when using the more powerful buffer TES (R. Sinden, unpublished observations). Nevertheless it is convenient if a reliable exflagellation inducing medium is available. A minimal medium for this is 10 mM Tris containing 166 mM NaCl, 170 mM $NaHCO_3$ and 10 mM glucose (Carter and Nijhout, 1977; Nijhout and Carter, 1979); a more reliable, though less defined medium, is foetal bovine serum adjusted to pH 8·0 with 1·5% sodium bicarbonate (Carter and Beach, 1977). The latter medium in particular has the property that when exflagellation is observed under a coverslip the preparation behaves homogeneously thus allowing meaningful analysis of efficiencies of gamete release. It is, in the experience of the author, unwise to substitute foetal calf serum with serum from the natural host as this can produce highly variable exflagellation between serum batches. For large-scale preparation of gametes for vaccine studies a minimal medium similar to that indicated above is acceptable (A. Vermeulen, personal communication). A useful medium is "suspended animation medium" which will maintain mature gametocytes for a period of up to an hour without significant loss of activity, yet simultaneously inhibits gamete formation, thus gametocyte purification may be achieved, e.g. by Percoll gradient centrifugation, prior to any studies on gametogenesis and may considerably facilitate gamete purification. This is particularly important for biochemical studies (Carter and Nijhout, 1977; Nijhout and Carter, 1978). This "suspended animation medium" medium is 10 mM Tris, 166 mM NaCl and 10 mM glucose, pH 7·4.

It should be recalled that gametogenesis involves two distinct processess: the escape from the host erythrocyte and the simultaneous but independent

event of gamete formation (Sinden, 1978). These two events may be separated readily. Both events may be triggered physiologically, for example with foetal calf serum at pH 8·0, and one simultaneously inhibited, for example gamete formation is blocked by colchicine (Toyé *et al.*, 1977; R. Sinden and N. King, unpublished observations). Alternatively one event alone may be triggered, for example host cell release by ionophore A 23187 (A. Knight, unpublished observations). With the former technique there is the potential disadvantage that macrogametogenesis may or may not involve microtubule polymerization (e.g. reported in *Haemoproteus* (Galucci, 1974a) but excluded in *Plasmodium* (Sinden, 1982b)). In the absence of specific evidence the exclusive inhibitory effects of microtubule inhibitors upon *micro*gametogenesis should be questioned. Inhibitors of protein synthesis do, however, inhibit both macro- and microgametogenesis (Toyé *et al.*, 1977). Irrespective of such procedures, extracellular gametocyte preparations could be prepared using standard haemolysis methods (e.g. immune/saponin lysis) in conjunction with suspended animation medium.

C. GAMETOCYTES AND GAMETE PURIFICATION

To date techniques for the purification of intraerythrocytic gametocytes are few and of variable efficiency. Kass *et al.* (1971) described a column filtration technique for the partial purification of *P. falciparum* gametocytes. Williamson and Cover (1975) and Hommel *et al.* (1979) reported buoyant density techniques using a 0·25–0·77σ continuous sucrose gradient in Krebs glucose, and 20% bovine serum albumin in Krebs glucose to isolate *P. falciparum* and *P. chabaudi* gametocytes respectively. Of these authors only Hommel *et al.* (1979) reported any viable gametocytes in the enriched preparations. Recently Knight and Sinden (1982) have described an isotonic Percoll step gradient technique which purifies gametocytes of both *P. yoelii* and *P. falciparum* and may yield *pure* preparations of viable gametocytes of the human parasite (see Table 2). This method has been further advanced (A. Vermeulen, unpublished observations) whereby the rodent gametocytes may be obtained with improved efficiency. It is currently impossible to separate the intraerythrocytic male and female gametocytes, mainly because of the highly variable buoyant density of the female cell (Knight and Sinden, 1982). Such techniques, if developed, would be a significant advance.

Gamete purification has been achieved most readily in the case of microgamete which conveniently has a unique shape, consistent density and behaviour and has been obtained with absolute purity by differential centrifugation (Carter *et al.*, 1979a, b), and with Percoll gradient techniques (Vermeulen *et al.*, 1982). Such success has not, however, been achieved with macrogamete preparations which are invariably contaminated with gametocytes and unexflagellated microgametocytes and residual bodies, thus making

TABLE 2

The enrichment in microgametocyte density and exflagellation activity obtained after Percoll gradient centrifugation.
(Taken from Knight and Sinden, 1982)

Species	Interface density ($g\ ml^{-1}$)	Enrichment in microgametocyte concentration in giemsa-stained blood films	Enrichment in exflagellation activity
P. falciparum	1·064/1·083	106x (28–185)	82x (21–142)
P. yoelii	1·047/1·064	160 (82–284)	102 (53–223)
P. yoelii Non-isotonic gradient	1·040/1·064	219 (76–428)	74 (43–97)

the specific identification of female gamete proteins a difficult task unless monoclonal antibodies to them are made. Further, Howard *et al.* (1982) point out that up to 70% of the macrogametes are in fact fertilized zygotes. In this regard a specific difficulty must be considered. At present both macrogametes and microgametes must be prepared simultaneously, if as one may reasonably assume their respective surface proteins have specific and complementary binding sites one for the other it is possible that, for example, the purified microgamete may already carry some previously soluble macrogamete surface proteins bound to it and thus monoclonal antibodies prepared against either gamete may then react with both male and female cells (see Rener *et al.*, 1980).

D. ZYGOTE FORMATION AND ARTIFICIAL MEMBRANE FEEDING OF MOSQUITOES

Whilst gamete formation *in vitro* may be achieved readily by even the most inexperienced worker, and fertilization can be seen to occur with very high frequency under the microscope, observations on zygote development *in vitro* remain surprisingly rare. The technique of Yoeli and Upmanis (1968), like that of McCallum (1897), required that blood be ingested by a mosquito for successful ookinete formation. Two techniques for the repeatable large-scale production of zygotes *in vitro* without a requirement for other cultured cell types have been more recently given by Chen *et al.* (1977), Weiss and Vander-

berg (1977), Carter *et al.* (1979b), Chen *et al.* (1981) and Gao (1981). Both methods require that infected blood from either rodent or avian host be diluted substantially in a buffered salt solution (Eagles minimal essential medium (MEM) or Hanks balanced salt solution (BSS)) containing high concentrations of foetal bovine serum (15–50%). Weiss and Vanderberg (1977) simply incubated this mixture in tubes for 18–24 hours at 21–22°C (*P. berghei*) whereas Chen *et al.* (1977) initially incubated for 1–2 hours at 19°C to allow zygote formation, then placed the cell suspension on a (ρ = 1·08) Ficoll-Hypaque gradient for 15 minutes at 18 000 ***g***. The zygotes collect at the Ficoll/Overlay interface yielding 60–80% macrogametes (both fertilized and unfertilized). Parasites were washed in 50% foetal calf serum/Hanks BSS containing 50 i.u. of penicillin and streptomycin per ml, pH 8·2, then incubated in this medium at 19°C in 100 µl aliquots in microtitre plates. Yields of 3×10^6 ookinetes per ml of parasitized blood were obtained.

Ookinetes produced *in vitro* may be fed to mosquitoes via a membrane feeding apparatus, or delivered by enema and result in normal oocyst infections within the mosquito (R. Gwadz, personal communication). One might anticipate that the efficiency of transformation of ookinete to oocyst should be higher than in normal infections as migration of the parasite across the midgut epithelium is less likely to be inhibited by the formation of a peritrophic membrane, and enzyme attack on the ookinete will be minimized.

Production of zygotes *in vivo* by membrane feeding of mosquitoes from blood of patients (Graves, 1980) or cultured gametocytes has proved to be a surprisingly problematical process. Various laboratories have for a number of years produced gametocytes capable of exflagellation but it is only very recently that these apparently mature gametocytes have routinely been made to produce infections in mosquitoes. Preliminary observations reported infections of one or two oocysts in a very low percentage of the mosquito (*A. gambiae* and *A. freeborni*) population (Ponnudurai *et al.*, 1980; Campbell *et al.*, 1980). Some infections were shown to produce sporozoites. However, soon some laboratories began to report repeated infections with tens of oocysts per gut (Ponnudurai *et al.*, 1982*c*) and some suggested this was due to the addition of hypoxanthine to the culture medium (Ifedeba and Vanderberg, 1981). Currently infections are produced in a number of laboratories (Ponnudurai *et al.*, 1982a, b; Campbell *et al.*, 1982). Techniques again vary considerably between laboratories. Some differences in the success of mosquito infection may depend upon the strain of parasite cultured, for example Tanzania 1 infects *A. freeborni* with greater efficiency than *A. stephensi* (Campbell *et al.*, 1982); whereas NF 54 has been shown to infect both vectors equally well (Table 3 from Ponnudurai *et al.*, 1982a, c). *Anopheles gambiae* is the poorest successful vector of NF54 and NF7. Cultures have successfully been used from 14–23 days after their initiation-periods which correspond

TABLE 3

Mosquito infection with cultured Plasmodium falciparum *(NF 54) produced in a static semi-automated system*

Experiment no.	Culture material diluted with	No. positive/ no. mosquitoes dissected (%)	Range oocysts	Mean oocyst per pos. gut	Species of mosquito
1	Defibrin, blood C	11/47 (23)	1–8	2·3	*Anopheles freeborni*
2	Defibrin, blood D	16/23 (70)	1–19	6·9	*A. stephensi*
3	Washed cells plus serum X	0/31 (0)	—	—	*A. stephensi*
4		0/9 (0)	—	—	*A. stephensi*
5		3/15 (20)	1–4	2·0	*A. stephensi*
6		0/24 (0)	—	—	*A. stephensi*
7		0/13 (0)	—	—	*A. stephensi*
8		0/7 (0)	—	—	*A. stephensi*
9*	Washable cells plus serum Y	11/13 (85)	2–10	3·6	*A. freeborni*
10*	Washed cells plus foetel calf serum	14/16 (88)	2–73	29·3	*A. freeborni*
11		19/33 (58)	1–50	13·7	*A. freeborni*
12		16/22 (73)	4–40	6·7	*A. freeborni*
13		16/22 (73)	4–40	6·7	*A. freeborni*
14		5/10 (50)	7–20	12·9	*A. freeborni*
15		26/32 (81)	1–60	17·0	*A. freeborni*
16		47/50 (94)	1–68	17·7	*A. stephensi*
17		103/105 (98)	1–109	24·0	*A. stephensi*

Gametocyte counts were about 8 per 1000 erythrocytes.

* Material from the same culture vessel and harvested at the same time.

with the times at which exflagellation may be seen. It is suggested (T. Ponnudurai, personal communication) that the occurrence of exflagellation corresponds well with the success of the resulting infection.

The exact mechanisms for achieving infection differ dramatically between laboratories. Some workers (Ponnudurai *et al.*, 1982a, b; R. Sinden and coworkers, unpublished results) diligently maintain the temperature of the gametocyte preparation above 35°C to inhibit activation prior to loading the membrane feeder. Others carry out manipulations at room temperature (Campbell *et al.*, 1982). The latter laboratory also uses blood from a single human volunteer to "carry" the culture, others have found that human serum is too variable and may actually inhibit mosquito infection even when taken from a non-immune host (T. Ponnudurai, personal communication) and that foetal calf serum is as good as human serum in mediating infection. It must, on current evidence, be recommended that foetal calf serum be used as a standard medium between laboratories to ensure comparability of results. A point of consistency is that *fresh* carrier erythrocytes (either as defibrinated blood or as washed cells) are required to ensure infection. The mechanics of transfer of culture are reasonably consistent and involve a minimum of centrifugation of the culture whilst ensuring the addition of fresh erythrocytes and human or foetal calf serum. Defibrinated blood or washed cell suspension is spun to produce a cell pellet (of 2 volumes), to this is added the concentrated gametocyte suspension taken at the time of medium change such that 1 volume is spun at about 800 *g* for 2–3 minutes at (25 –) 37°C, the final pellet is resuspended in 3 volumes of foetal calf serum, mixed and transferred rapidly into a membrane feeder. The feeder which is maintained at 37°C may, but need not, be stirred and may use either Baudruche membrane or 2-way stretch parafilm. Attempts in our laboratory to use chick skin have failed to produce infections despite inducing a good feeding response in *A. stephensi*. Mosquitoes are thereafter maintained under strict security and examined for oocyst number, or salivary gland infections. To date the most efficient mosquito transmission has been reported by Ponnudurai *et al.* (1982a), who, using *A. freeborni* and *A. stephensi* as a vector of N.F. 54 produced in the tipper flask system, achieved 73–98% infection with a mean of 6·7–29·3 oocysts per gut (see Table 3).

V. Strategies For The Control of Sexual Development of *Plasmodium*

A. IMMUNOLOGICAL METHODS

Considerable interest has re-emerged on the use of sexual stages as a potential vaccine to control malaria. Inevitably the lack of an immediate

protective action has led to their description as the "altruistic" vaccine. A very full description of the subject has recently been provided by Carter and Gwadz (1981) and will therefore only be summarized here.

The serum of some individuals naturally infected with *P. falciparum* contain antibodies which react with gametocytes in the IFA test (Swanson-Beck *et al.*, 1970; Smalley and Sinden, 1977; Carter *et al.*, 1979c). These sera have no detectable transmission-blocking potential. However, Gwadz (1976) showed that formalin or X-ray treated gametocyte containing blood of *P. gallinaceum* induced a transmission-blocking immunity in the host. Carter and Chen (1976) and Carter *et al.* (1979a) demonstrated that this immunity was more effectively produced if extracellular gametes were used as immunogen, particularly when the antigen contained mixed preparations of male and female gametes. Pure micro- or macrogamete antigens were poorly immunogenic. Similar high levels of protection have been achieved using gamete immunogens of *P. yoelii* (Mendis and Targett, 1979, 1981, 1982), *P. knowlesi* (Gwadz and Green, 1978) and *P. berghei* (Vermeulen *et al.*, 1982). The results of Mendis and Targett (1979, 1981, 1982) are interesting in that an adjuvant was not required to induce effective immunity, which lasted for up to 6 months.

More recently Rener *et al.* (1979, 1980 and personal communication) have prepared monoclonal antibodies to gametes of *P. gallinaceum*. Two of 10 such antibodies acted synergistically to block transmission of the parasite to mosquitoes. Each antibody alone reacts with both male and female gametes and zygotes (J. Rener and coworkers, personal communication) but neither is capable of inhibiting gamete formation *in vitro*, whilst in combination they effectively agglutinate the microgametes as they emerge during exflagellation. Neither antibody reacts with ookinetes, suggesting the target antigen is strictly stage specific to the gametes; its temporary persistence in the zygote may result simply from the fusion of male and female gamete membranes.

Aikawa *et al.* (1981) have shown that each monoclonal antibody (one IgM the other IgG) reacts with foci of antigen on both macro- and microgamete surface, whereas the mixture gives a continuous reaction over the cell surface. They suggest it is the latter effect which achieves effective agglutination and transmission blockade. The IgG monoclonal antibody recognizes a protein M_r 230 000 (under reducing conditions) on the surface of the macrogamete (Rener *et al.*, 1979), microgamete and zygote (J. Rener and coworkers, personal communication). This protein is suggested to be a single polypeptide without subunits and with intrachain disulphide bridges.

All the above studies concur that the antigamete antibodies act by the agglutination and inactivation of the extracellular male gametes during exflagellation thus preventing their dispersal and hence the fertilization of the female cell, and that the antigamete antibodies do not inactivate the intracellular gametocyte within the vertebrate host, nor do they inhibit the

development of the zygote following fertilization. However, Carter *et al.* (1979b) suggest that not all antibodies produced are effective in blocking transmission. They distinguish AG (agglutinating) antibodies and SF (surface fixing) antibodies and found that while SF antibodies were invariably associated with transmission blocking immunity AG antibodies were not.

Gwadz and Green (1978) showed that gamete immunization with *P. knowlesi* was effective when challenged with parasites from a different antigenic variant, but did not cross protect against *P. cynomolgi.* Thus this antigen has a potentially useful "broad spectrum" protection within a species.

Polyspecific antibodies to zygotes of *P. gallinaceum* have been prepared which precipitate up to 15 proteins which can be labelled as surface proteins by the IODOGEN technique (Howard *et al.*, 1982). These antibodies also have a transmission blocking potential (Kaushal and coworkers, personal communication). The transmission blocking antibody recognizes bands with M_r of 240 000, 200 000, 180 000, 80 000, 55 000 and 50 000 of which M_r 180 000 appears to be an adsorbed host serum protein. Additionally a lipid or glycolipid is believed to be a target antigen.

At present it appears therefore that an effective gamete vaccine is a real possibility, the gametocyte *per se* is not easily attacked with its protective erythrocyte and is in any case not significantly pathogenic and is thus no candidate as a target for vaccine. The possiblity exists that the zygote (ookinete) too is a suitable target for immunological attack, it has numerous distinct immunogenic surface proteins and should therefore be examined in detail.

With the advent of routine culture of mature gametocytes of *P. falciparum* the way is now open for similar transmission blocking studies on this serious human pathogen.

B. CHEMOTHERAPY

Studies on the cell cycle of gametocytes has suggested that DNA replication is confined to the early phase of gametocytogenesis, i.e. days 1–2 in the 10 day cycle of *P. falciparum*, thereafter protein and RNA metabolism proceed at ever decreasing rates until the moment of gamete formation (Sinden and Smalley, 1979). This observation provides the rational explanation for early observations that the mature gametocytes of *P. falciparum* are not killed by quinine and the 4-aminoquinolines (Barber *et al.*, 1929; Mackerras and Ercole, 1949a, b; Terzian *et al.*, 1968), by dehydrofolate reductase inhibitors, for example pyrimethamine and proguanil (Mackerras and Ercole, 1947; Jeffrey *et al.*, 1956), or by inhibitors of PABA (*p*-aminobenzoic acid), for example sulphonamides (Terzian *et al.*, 1968; Powell *et al.*, 1967). With the advent of

gametocyte culture techniques it has been possible to show conclusively that chloroquine is exquisitely lethal to the stage 1 gametocyte of *P. falciparum*, i.e. during S phase (Smalley, 1977; Sinden, 1982b) and that pyrimethamine, proguanil and cycloguanil are also lethal at this time with the latter two compounds being effective possibly for a further 2 days (Sinden cited in Richards *et al.*, 1979).

The 8-aminoquinolines, for example primaquine and pamaquine, whose effects are suspected as being mediated through parasite energy metabolism kill all gametocytes irrespective of age or species. This effect is exerted rapidly, within 6–12 hours infectivity is lost and within 2–4 days *P. falciparum* gametocytes are effectively eliminated from the circulation (Mackerras and Ercole, 1949b; Jeffrey *et al.*, 1956; Rieckmann *et al.*, 1968, 1969). Surprisingly primaquine does not, however, inhibit exflagellation and fertilization (Mackerras and Ercole, 1949b) nor did it inhibit sporogonic development if administered to mosquitoes (Terzian *et al.*, 1968). The latter observation is not surprising if it is drug metabolites produced in the vertebrate which are the effective compounds (Strother *et al.*, 1981).

Our ability to culture gametocytes of *P. falciparum*, to manipulate gametogenesis *in vitro*, and to feed mosquitoes via a membrane feeding apparatus means now that careful studies can be made upon the drug susceptibility of gametocytes throughout their entire development. The unique events essential to the successful conclusion of sexual development will surely prove amenable to such an attack; particularly, if, instead of directing our attention to the metabolism of the parasite, we were to address the critical events triggering both gametocytogenesis and gametogenesis, the latter being apparently easily inhibited by external pressures (see page 184). Further, the action of the unique assemblage of organelles involved in exflagellation can be blocked with standard anti-cytoskeletal agents (Toyé *et al.*, 1977; R. Sinden and N. King, unpublished observations), it should prove possible to develop similar agents with selective atimalarial activity.

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The Genetic Basis of Diversity in Malaria Parasites

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I. Introduction

Malaria parasites have been traditionally differentiated by characters such as morphology, patterns of infection, host specificity, etc., and this has led to the creation of more than 80 species as well as numerous subspecies of these organisms (Garnham, 1966; Killick-Kendrick, 1978). In recent years biochemical characters such as electrophoretic forms of enzymes have provided more sensitive markers, enabling variation within as well as between species to be studied. Genetic studies have complemented this approach, demonstrating how variant forms are generated by mechanisms such as recombination and mutation (Beale *et al.*, 1978). This work has also provided information on the breeding structure of parasite populations, enabling groups that are reproductively isolated from one another to be identified unequivocally.

The purpose of this paper is to review briefly what is known of the genetics of the malaria parasite, and to show how this knowledge can be used to interpret the nature of the diversity of various characters seen in natural parasite populations. In the first part, some basic genetic studies on rodent malaria parasites are described, to illustrate the patterns of inheritance of characters such as enzymes, drug sensitivity and virulence. An account is then given of enzyme diversity in populations of the rodent malaria species *Plasmodium berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei*, followed by a discussion of the relationships between populations of these organisms in different parts of Africa. Diversity in the human malaria parasite *P. falciparum* is then considered, and variations in enzymes, antigens, drug sensitivity and other characters are described. In the final section, the findings from the rodent work are discussed in relation to those with *P. falciparum*.

II. Definitions

In this paper, the principal terms used to describe parasites derived from naturally infected animals or man are "isolate", "line" and "clone". These will be taken to have the following meanings.

Isolate. A sample of parasites, not necessarily genetically homogeneous, collected from a naturally infected host on a single occasion.

Line. Parasites which have undergone a particular passage in the laboratory, usually following a special treatment, such as selection for drug resistance.

Clone. Genetically identical organisms derived from a single cell by asexual division.

III. Genetic Studies

Few genetic studies have been made on malaria parasites mainly because of the difficulty of applying conventional techniques of genetic analysis to organisms with complex life cycles. The earliest attempts were those of Greenberg and Trembley (1954a, b), using the avian species *Plasmodium gallinaceum*. Their approach involved mixing two parasite lines differing in two characters (drug sensitivity and patterns of exo-erythrocytic schizogony), transmitting the mixture through mosquitoes to allow cross-fertilization of gametes to occur, and examining the resulting parasites for evidence of recombination between the parent-line characters. Some evidence of hybridization was claimed, although the work was inconclusive because of the relative instability of one character (exo-erythrocytic schizogony). Since then, all genetic work has been carried out with the rodent species of *Plasmodium*, especially *P. yoelii* and *P. chabaudi* (Beale *et al.*, 1978). These have proved especially suitable for this type of study, because they are easily maintained in the laboratory and because a large number of wild isolates is available which provide a good source of natural genetic variation.

A. METHODS

The main requirements for conventional genetic analysis of an organism are (1) a method for making clones, in order to obtain genetically pure lines, and (2) methods for making crosses between organisms differing in a number of clearly defined characters, and for analysing the progeny of such crosses.

1. *Cloning*

Two methods are available for cloning micro-organisms, micromanipulation and dilution. Both methods have been used to clone blood forms of malaria parasites. Demidowa (1934), Coulston and Manwell (1941), Downs (1947) and Bishop (1958) used micromanipulation to isolate single parasites of various avian species of *Plasmodium* from which clones were established. Diggens (1970) used this technique to make clones of the rodent species *P. berghei* in mice, and Trager *et al.* (1981) isolated single blood forms of *P. falciparum* to establish clones in cultures *in vitro*. Dilution methods were used by Coulston and Manwell (1941) for *P. circumflexum*, by Walliker *et al.* (1973, 1975), Rosario (1976), Knowles and Walliker (1980) and Padua (1981) for rodent malaria species, and by Rosario (1981) for *P. falciparum*. Micromanipulation is potentially a more accurate method than dilution, as cells containing single parasites can usually by isolated with a high degree of certainty. Dilution methods, which are less time-consuming than micro-

manipulation, have been employed in genetic work where large numbers of clones need to be analysed.

2. *Hybridization and analysis of progeny*

The technique for making crosses between two parasite lines is shown in Fig. 1. A mixture is made of blood forms of the two lines, containing gametocytes. Mosquitoes are fed on the mixture to allow cross-fertilization of gametes to occur. The resulting zygotes are permitted to develop to sporozoites which are used to infect new hosts. The parasites developing in these animals, the progeny of the cross, are then examined for the presence of recombinant forms exhibiting characteristics of both parent lines. These can be detected by direct examination of uncloned parasites if suitable markers (e.g. differences in drug sensitivity) distinguish the parent lines. Cloning is needed for a precise genetic analysis.

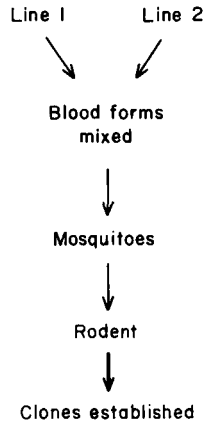

FIG. 1. Technique for making cross between rodent malaria parasites.

It should be noted that male and female gametocytes of each parent line are present in the initial blood mixture. Zygotes produced by both self- and cross-fertilization can thus be formed. Assuming that fertilization occurs randomly, equal numbers of parent-type and hybrid zygotes will be obtained. The resulting progeny therefore consist of three types of parasite, those of each parent line as well as hybrids. A more satisfactory way of making crosses would be to arrange fertilization between purified male gametes of one line and female gametes of the second line, so that all the progeny would be derived from hybrid zygotes. However, reliable methods for separating male and female gametes are not yet available. Some progress in this field with *P. gallinaceum* has been reported (Carter *et al.*, 1979).

The basic technique outlined above, first used for *P. gallinaceum* (Greenberg and Trembley, 1954a), was subsequently adapted for use with the rodent malaria species (Walliker *et al.*, 1971). No genetic cross has yet been made with any of the human malaria parasites, because of the difficulty of maintaining the complete life cycle of these organisms in the laboratory.

B. PATTERNS OF INHERITANCE

1. *Enzymes*

Extensive use has been made of electrophoresis of enzymes for basic genetic studies on the malaria parasite. Enzymes are direct products of genes, and thus variations in enzymes reflect gene differences. Studies on numerous organisms, including the free-living protozoa *Tetrahymena* (Allen, 1960) and *Paramecium* (Tait, 1968), have shown that the vast majority of enzyme forms are controlled by nuclear genes which undergo a Mendelian type of inheritance. Enzymes are also remarkable for their stability over many passages.

Six enzymes have proved of particular value in studies on malaria parasites, *viz.* glucose phosphate isomerase (GPI, EC 5.3.1.9), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.43), lactate dehydrogenase (LDH, EC 1.1.1.27), NADP-dependent glutamate dehydrogenase (GDH, EC 1.4.1.2), adenosine deaminase (ADA, EC 3.5.4.4.) and peptidase (PEP, EC 3.4.11 or 13).

The blood forms are the parasite stages most readily accessible for enzyme study. The parasites are grown in laboratory rodents and freed from their host cells by methods such as saponin lysis before being examined for enzymes. Starch gel electrophoresis has been used most widely (Carter, 1978; Sanderson *et al.*, 1981), although cellulose acetate electrophoresis (Kreutzer and Christensen, 1980; Lanham *et al.*, 1981) is now being employed in many laboratories. Parasite enzymes can be distinguished from those of the host by including samples of uninfected blood on each gel. Each electrophoretic form of a given enzyme is denoted by a number, for example GPI-1 or GPI-2, according to its position in the gel.

The species in which the inheritance of enzymes has been most studied is *P. chabaudi*. A large number of enzyme variants has been found among natural populations of this organism (see Section IV, p. 229). The results of a typical cross between two *P. chabaudi* lines differing in two enzymes are shown in Fig. 2 (Rosario, 1976). One line was characterized by forms of PGD and LDH denoted PGD-2 and LDH-3 respectively, and the second by PGD-3 and LDH-2. Following the cross, four classes of parasite were recovered in blood form clones, two of parental type (PGD-2, LDH-3 and PGD-3, LDH-2) and two of recombinant type (PGD-2, LDH-2 and PGD-3, LDH-3).

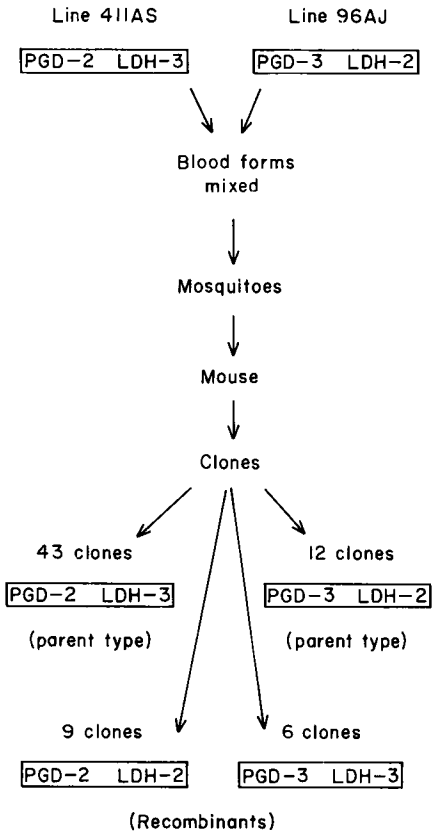

FIG. 2. Inheritance of enzymes (PGD and LDH) in *Plasmodium chabaudi*.

Two conclusions could be drawn from this result.

(*a*) *The blood forms were haploid.* This was shown by the presence of only one form of each enzyme in each clone. Assuming that the gametes were haploid, cross-fertilization would result in diploid zygotes which would exhibit both forms of both enzymes (Fig. 3). The two forms of each enzyme would segregate from each other following meiosis. The presence of only single forms of each enzyme in each blood form clone, and the occurrence of recombinant-type clones, show that meiosis had occurred before the emergence of parasites into the blood.

(*b*) *There was no preference for gametes to undergo self- rather than cross-fertilization.* This is shown by the numbers of each type of progeny clone obtained. Random mating would give rise to equal numbers of parent- and

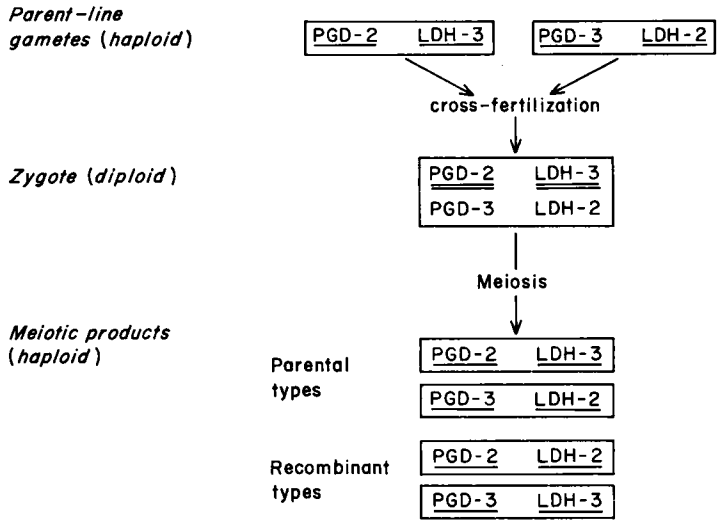

FIG. 3. Explanation of results of Fig. 2 assuming typical eukaryotic inheritance of chromosomal genes in *Plasmodium*.

hybrid-type zygotes. Each parental zygote would produce only parental-type meiotic products. Hybrid zygotes. however, could give rise to four types of meiotic products, two parental and two recombinant (see Fig. 3); these would occur in equal numbers assuming that the markers involved were unlinked and that no selection against any of the four types occurred. The proportions of each type of parasite expected among the progeny, therefore, are six parentals: two recombinants taking into account meiosis of parental as well as hybrid zygotes. In the example given, 15 out of 70 clones were recombinants for the two enzymes involved (Fig. 2), a proportion of approximately 3·7 parentals: 1 recombinant.

The inheritance patterns of enzymes are thus consistent with those expected for a eukaryotic type of life cycle with diploid and haploid phases. Similar results have been obtained in other *P. chabaudi* and *P. yoelii* crosses between lines differing in two or more enzymes (Beale *et al.*, 1978; Knowles *et al.*, 1981).

2. *Drug resistance*

Drug-resistant forms of human malaria parasites are now common in many parts of the world (Wernsdorfer and Kouznetsov, 1980). In the laboratory such resistant forms can often be readily obtained, either by "single-step" exposure of sensitive forms to high doses of drug, or by gradual increases in drug dose over several passages (Peters, 1970). Genetic studies have been made mainly on resistance to two drugs, pyrimethamine (an antifolate drug) and chloroquine (a 4-aminoquinoline drug) (for review see Beale, 1980).

The earliest experimental work on resistance to antifolates was by Bishop and Birkett (1947, 1948), Williamson and Lourie (1947), Knoppers (1947), Greenberg (1949) and Bishop and McConnachie (1950), who developed forms of the avian malaria parasite *P. gallinaceum* resistant to proguanil. Bishop (1962), after many studies on resistance to this and other antifolates, showed that resistant forms could be obtained from drug-sensitive clones, and once obtained the resistance was stable in the absence of drug pressure. The resistance often appeared suddenly, and different levels of resistance could be obtained after treatment with similar doses of drug. These observations suggested strongly that mutation events were involved in the appearance of the resistance. Bishop (1962) estimated that the frequency at which such mutant forms arose was less than 1 in 10^9 parasites, although it is probable that the methods used to select the mutants resulted in only a small proportion of those actually present being detected.

In contrast to antifolate resistance, chloroquine resistance has proved difficult to obtain in the laboratory. Early attempts to produce chloroquine-resistant forms of *P. gallinaceum* were unsuccessful. Subsequently, several workers reported production of resistant forms of the rodent species *P. berghei* (e.g. Ramakrishnan *et al.*, 1957; Sautet *et al.*, 1959; Sergent and Poncet, 1959). Peters (1965) produced a highly chloroquine-resistant line of this species following treatment of sensitive forms with gradually increasing doses of the drug over many passages; the resistance obtained was unstable once drug pressure was removed. In later work, Peters *et al.* (1970, 1978) found that an apparently stable chloroquine-resistant *P. berghei* produced by single-step selection was actually the innately chloroquine-resistant species *P. yoelii*, which had been selected by the drug from a mixed infection of the two species. Stable chloroquine resistance was obtained in *P. vinckei* by Powers *et al.* (1969)

TABLE 1

Inheritance of pyrimethamine resistance in Plasmodium yoelii *following cross between lines A and C*

	Characteristics	Number of clones isolated
Parents		
Line A	GPI-1 Resistant	
Line C	GPI-2 Sensitive	
Progeny		
Parental types	GPI-1 Resistant	21
	GPI-2 Sensitive	30
Recombinant types	GPI-1 Sensitive	13
	GPI-2 Resistant	7

and in *P. chabaudi* by Rosario (1976) and Padua (1981) using prolonged treatment with drug at low doses.

Genetic studies have confirmed that resistance to both pyrimethamine and chloroquine can be caused by mutation. In *P. yoelii* and *P. chabaudi* crosses have been made between pyrimethamine-resistant and sensitive lines and the inheritance of the resistance character studied. In *P. yoelii*, for example, a cross was made between a pyrimethamine-resistant line which possessed enzyme form GPI-1 and a drug-sensitive line characterized by GPI-2 (Table 1; Walliker *et al.*, 1973). Recombinant forms, both resistant/GPI-2 and sensitive/GPI-1, were detected among the progeny, the proportions obtained fitting well with the numbers expected for the inheritance of two nuclear genes. Similar results have been obtained in other *P. yoelii* and *P. chabaudi* crosses (Walliker *et al.*, 1975, 1976; Rosario, 1976; Knowles *et al.*, 1981).

TABLE 2

Inheritance of chloroquine resistance in cross between two P. chabaudi *lines highly resistant and sensitive to the drug*

	Chloroquine-response	Number of clones isolated
Parents		
Line AS(30CQ)	Resistant (30 mg kg^{-1})[a]	
Line AJ	Sensitive	
Progeny		
Parent-type	Resistant (30 mg kg^{-1})	3
"Intermediate"	Resistant (15 mg kg^{-1})	4
response	Resistant (3 mg kg^{-1})	7
Parent-type	Sensitive	7

[a]The resistance levels indicated (30 mg kg^{-1}, etc.) refer to six daily treatments with chloroquine at the dose indicated.

The inheritance of chloroquine resistance was studied in *P. chabaudi* by Rosario (1976). The chloroquine resistance character segregated in crosses independently of pyrimethamine resistance, showing that the two types of resistance were caused by mutation of different genes. High level resistance to chloroquine appears to be due to the presence in each parasite of several mutations, each conferring a low level of resistance. Padua (1981) crossed a line of *P. chabaudi* exhibiting stable resistance to high levels of chloroquine (30 mg kg^{-1}) with a sensitive line. Progeny clones characterized by intermediate levels of resistance were obtained (Table 2). This was interpreted as being due to the segregation of different resistance genes, each conferring a low level of resistance, following meiosis of hybrid zygotes. If the high level of resistance

had been caused by mutation at a single locus, no such intermediates would have been obtained.

Little genetic work has been carried out on resistance to other drugs. Several workers have shown that when parasites are made resistant to one drug, the response to others may be affected. Parasites resistant to antifolate drugs, for example, are often hypersensitive to sulphonamides (Peters, 1970). On the other hand, some sulphonamide-resistant lines are also resistant to antifolates (Bishop and McConnachie, 1948; Rollo, 1951; Thurston, 1953). Some limited genetic studies by MacLeod (1977) on *P. chabaudi* showed that independently obtained resistant mutants differed in their response to each type of drug, depending on the conditions used for selection.

A novel mechanism for the genetic transfer of pyrimethamine resistance was proposed by Yoeli *et al.* (1969). In this work, a pyrimethamine-resistant *P. vinckei* was mixed with a sensitive *P. berghei* line in mice. The mixture was then passaged into hamsters, from which pyrimethamine-resistant *P. berghei* was recovered. It was concluded that the *P. berghei* had acquired its resistance from the *P. vinckei* during simultaneous infection of erythrocytes with two species—a process called "synpholia" by these authors. No subsequent confirmation of this work has been reported. The most likely alternative explanation for the appearance of resistance in the *P. berghei* was that a mutation had occurred and had been selected by pyrimethamine treatment (Diggens *et al.*, 1970; Schoenfeld *et al.*, 1974).

3. *Virulence*

Variations in the "normal" pattern of infection with malaria parasites have been noted by numerous workers. Many of these variations are caused by non-genetic or environmental factors. For example, the quantity of *p*-aminobenzoic acid (PABA) in the host's diet affects the parasitaemia of *P. berghei* (Jacobs, 1964), because this substance is an essential growth requirement for the parasite. Concomitant infections with other organisms, for example *Eperythrozoon coccoides* or *Haemobartonella muris* in mice, can enhance or diminish the parasitaemia of rodent malaria species (Cox, 1978). In human malaria, genes such as sickle haemoglobin and glucose-6-phosphate dehydrogenase deficiency affect the development of *P. falciparum*, while the Duffy-negative erythrocyte genotype is involved in resistance to *P. vivax* (for review see Luzzatto, 1979). Studies on the behaviour of *P. berghei* in different mouse strains have shown that several host genes appear to influence the course of infection (Nadel *et al.*, 1955; Greenberg and Kendrick, 1957, 1958, 1959).

Changes in the parasite which affect its course of development have also been observed. Alger *et al.* (1971), for example, found variation between "demes" of *P. berghei* affecting their lethality to mice. McGhee and Weathers-

by (1979) showed that mosquito-transmitted lines of *P. gallinaceum* varied in virulence when grown in chick embryos. A line of *P. berghei* that became markedly less virulent to mice following a series of passages in culture was recorded by Weiss and de Giusti (1964). In contrast, an increase in virulence was noted following prolonged blood passage (Sergent and Poncet, 1959). Yoeli *et al.* (1975) observed a dramatic increase in virulence of the normally avirulent species *P. yoelii* during passage in mice. This species typically invades immature erythrocytes by preference, infections of mature cells occurring only fleetingly. In the virulent line, parasites were able to develop extensively in mature cells, resulting in the death of most mice within a week of infection.

Genetic studies have identified virulence genes in *P. yoelii*, in both the line obtained by Yoeli *et al.* (1975) and a second line obtained by Morgan (1972). The existence of such genes was first shown in crosses between the former line (denoted YM) and an avirulent *P. yoelii* line, the two lines differing in enzyme type as well as pyrimethamine-sensitivity (Walliker *et al.*, 1976). Following the cross, progeny clones were obtained which showed recombination between the virulence and the other markers; the virulence character had thus undergone a pattern of inheritance typical of that expected for a nuclear gene mutation. The virulence character was generally stable, although a few clones were "intermediate" in their virulence characteristics. When one of these clones was transmitted through mosquitoes, it exhibited full virulence typical of the parent line (Knowles and Walliker, 1980). Thus, the gene for virulence was present in this clone; the reason for the variability in its expression before mosquito transmission was not investigated.

In further work (Walliker, 1981), the virulent lines YM (Yoeli *et al.*, 1975) and 33X (Pr3) (Morgan, 1972) were crossed. Five out of 49 clones obtained from the progeny of this cross were avirulent, developing only in immature erythrocytes. This result was explained by assuming that the virulence of each line had been caused by mutation events at different genetic loci (Fig. 4). Recombination between the loci during the cross had resulted in the appearance of avirulent forms.

The mechanism of action of virulence genes is not understood at present, but appears to be related to the ability of the parasite to develop to maturity inside mature erythrocytes, rather than to an enhanced ability of the merozoites of virulent lines to enter this cell type. Parasites of avirulent *P. yoelii* lines are frequently found inside mature erythrocytes, but few schizonts are seen in the infected cells. Panton (1981) was able to identify, by crossed immuno-electrophoresis, an antigen which was present in greater amounts in parasites in mature cells than in those in immature cells. In a cross between a virulent and an avirulent line, mature cell invasion and high quantity of this antigen were found to segregate together, suggesting that these characteristics were different phenotypic effects of the same gene.

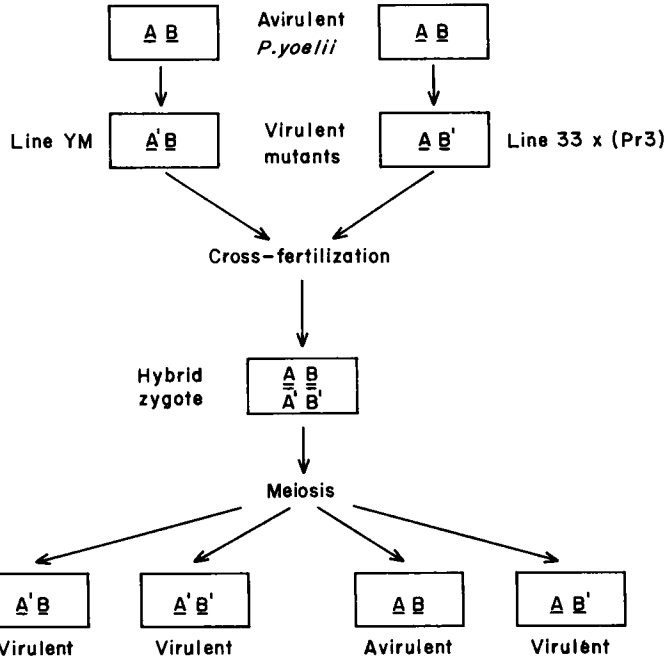

FIG. 4. Occurrence of avirulent parasites among progeny of cross between virulent *P. yoelii* lines. A′ and B′ represent virulence mutations of genes A and B.

C. CYTOLOGICAL AND MOLECULAR STUDIES

Many questions remain concerning the basic genetic organization of the parasite, for example the number of chromosomes, the precise stage at which meiosis occurs and the genetic basis of gametocyte formation. Cytological studies have provided a little information on some of these topics. For example, Sinden (1978) provides evidence that meiosis may occur during the early divisions of the oocyst, and suggests that the haploid chromosome number is at least eight. This question could be resolved by using more precise genetic methods than have been possible hitherto, for example by cloning zygotes and sporozoites, and by having more genetic markers available so that linkage groups could be identified.

With regard to gametocyte development, Bannister and Sinden (1982) claim that in *P. falciparum* the deoxyribonucleic acid (DNA) is replicated 8-fold in the male gametocyte, divisions then taking place to restore the haploid DNA amount in each microgamete. In *P. berghei*, Dore *et al.* (1980) and Birago *et al.* (1982) produce evidence for a relationship between the amount of repetitive DNA in the parasite and gametocyte infectivity; in a

parasite line which had lost its ability to infect mosquitoes, less repetitive DNA was present than in a normally transmissible line.

A detailed treatment of this subject is beyond the scope of this paper. For further information, see the paper by Sinden in this volume (pp. 153–216).

D. SUMMARY

The principal findings of the genetic work on rodent parasites are as follows.

(a) The parasite appears to undergo a normal eukaryotic type of life cycle, with haploid blood forms. The stage at which meiosis occurs is not yet known.
(b) Variant forms of enzymes and other characters are inherited in the manner expected for nuclear genes, recombination occurring frequently following cross-fertilization between parasite lines. The number of linkage groups has not been established.
(c) Resistance to antimalarial drugs can be caused by mutation followed by drug selection. High level resistance to pyrimethamine can arise as a result of single mutation events, while high resistance to chloroquine probably results from several mutations, each conferring low resistance.
(d) Virulent forms of *P. yoelii*, affecting the ability of parasites to develop in mature erythrocytes, can arise by mutations at more than one gene locus.

IV. Diversity Among Rodent Malaria Parasites

Malaria parasites have been isolated from rodents, principally thicket-rats (*Thamnomys rutilans* and *Grammomys surdaster*) in five African countries—Cameroun, the Central African Republic, Congo, Nigeria and Zaire (Killick-Kendrick, 1978; Bafort, 1977). Four species are recognized, *Plasmodium berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei*, different subspecies of which occur in each country. Characters such as blood form morphology, duration of exo-erythrocytic schizogony, oocyst and sporozoite sizes and temperature of mosquito transmission are used to distinguish the various species and, to a lesser extent, the subspecies. Enzymes have proved especially useful in this regard. Surveys of enzymes in cloned parasite lines derived from naturally infected rodents have shown that (a) mixed infections with more than one species are common; (b) considerable enzyme variation occurs within, as well as between, each species; (c) no interbreeding between species occurs; and (d) subspecies of a single species can be differentiated enzymically, but can mate under laboratory conditions.

This section concerns the diversity of parasites found in animals of a single region, as well as the differences between those of different regions.

A. PARASITES OF A SINGLE REGION

Numerous isolates of rodent malaria parasites have been obtained from each of the countries in which they occur. The population which has been studied most intensively is that of the Central African Republic.

The first descriptions of parasites of rodents of this region were by Landau and Chabaud (1965) who recognized two types of parasite, one inhabiting reticulocytes by preference and one inhabiting mature erythrocytes. The former was subsequently named first as a subspecies of *P. berghei*, *P. berghei yoelii*, by Landau and Killick-Kendrick (1966) and later as *P. yoelii yoelii* (Killick-Kendrick, 1974). The second parasite was named *P. chabaudi* (Landau, 1965) and later made a subspecies of *P. vinckei*, *P. v. chabaudi*, by Bafort (1968).

TABLE 3

Enzyme forms[a] *of clones of rodent malaria parasites from the Central African Republic*

Clone	Enzyme forms GPI	PGD	LDH	GDH	*Plasmodium* spp.
17X	1	4	1	4	*Plasmodium yoelii yoelii*
33X	2	4	1	4	
86X	1	4	1	4	
3AF	1	4	1	4	
6AL	4	2	2	5	*Plasmodium chabaudi chabaudi*
3AR	4	2	3	5	
10AS	4	2	3	5	
2BJ	4	2	4	5	
57AF	4	2	5	5	
9AJ	4	3	2	5	
14AQ	4	3	2	5	
54X	4	3	3	5	
20CE	4	3	4	5	
2CW	4	3	4	5	
3CQ	4	3	5	5	
1BS	9	5	7	6	*Plasmodium vinckei petteri*
4BZ	9	5	7	6	
11CE	5	5	7	6	
2CR	5	5	7	6	

[a]See p. 221 for explanation of abbreviations of names of enzymes.

Many parasites isolated from this region were examined for enzymes by Carter (1973) and Carter and Walliker (1975). When cloned infections were studied, it was realized that there were not two but three distinct species present in these animals (Table 3). Parasites identified as *P. y. yoelii* on the basis of their reticulocyte-preference and morphology possessed enzyme forms GPI-1 or -2, PGD-4, LDH-1 and GDH-4. Parasites identified as *P. v. chabaudi* because of their mature erythrocyte preference were found to comprise two distinct enzymic groups, one characterized by GPI-4, PGD-2 or -3, LDH-2, -3, -4 or -5, and GDH-5, and the other by GPI-5 or -9, PGD-5, LDH-7 and GDH-6. It was clear from the survey that these two groups of parasites were reproductively isolated, even though mixed infections in the same host animal were common. If mating were taking place between the groups, clones characterized by enzyme combinations such as GPI-4 with GDH-6, would have been found. The two groups could thus be classified as distinct species for which the names *P. chabaudi* (subspecies *chabaudi*) and *P. vinckei* (subspecies *petteri*) were chosen (Carter and Walliker, 1975). The two species also differed morphologically as well as enzymically; however, mixed infections were difficult to recognize by morphological characters alone, especially when only ring forms were present.

A further conclusion was that while the three species in these animals were reproductively isolated, considerable random mating was probably taking place *within* each species. This was particularly clear in *P. chabaudi*, where two forms of PGD (PGD-2 and -3) and four of LDH (LDH-2, -3, -4 and -5) were found. All possible combinations of these forms were seen among the clones (Table 3), providing evidence of extensive cross-fertilization between gametes of different lines.

Similar surveys have been carried out on rodent malaria populations of other African countries. The three species found in the Central African Republic, *P. yoelii*, *P. chabaudi* and *P. vinckei*, occur together in rodents of the Congo (Carter and Walliker, 1976), and Cameroun (Lainson, 1979). In Nigeria, only *P. yoelii* and *P. vinckei* have been found, and in Zaire *P. berghei*, *P. vinckei* and, possibly, *P. yoelii* (Killick-Kendrick, 1978; Peters *et al.*, 1978). Only a limited number of isolates is available from the latter two countries, however, so the existence of other species in these regions cannot be ruled out.

B. PARASITES OF DIFFERENT REGIONS

1. *Enzyme surveys*

Parasites of a single species occurring in different countries possess certain enzyme forms in common, and differ in others. Table 4 illustrates the enzyme

TABLE 4

Enzyme forms[a] *of* Plasmodium vinckei *and* P. yoelii *isolates from different African countries*

Species and subspecies	Country	Enzyme forms GPI	PGD	LDH	GDH	No. of isolates examined
P. vinckei vinckei	Zaire	7	6	6	6	2
P. vinckei petteri	Central African Republic	9	5	7	6	3
		5	5	7	6	2
P. vinckei lentum	Congo	6	5	7	6	3
		11	5	9	6	1
P. vinckei brucechwatti	Nigeria	6	6	9	6	2
P. vinckei subsp.	Cameroun	5	5	7	6	1
		6	5	11	6	1
		12	5	9	6	1
		13	5	7	6	1
		6	6	11	6	1
P. yoelii yoelii	Central African Republic	1	4	1	4	17
		2	4	1	4	6
		10	4	1	4	1
P. yoelii killicki	Congo	1	4	1	1	2
P. yoelii nigeriensis	Nigeria	2	4	1	2	1
P. yoelii subsp.	Cameroun	1	4	1	4	1

[a]See p. 221 for explanation of abbreviations of names of enzymes.

forms of isolates of *P. vinckei* and *P. yoelii* of each country in which they occur. Variation is particularly marked in *P. vinckei.* Only one enzyme (GDH) possesses a form common to all regions (GDH-6). In contrast, GPI occurs as seven different forms, of which some (e.g. GPI-6) occur in more than one region, while others (e.g. GPI-7) are unique to one region. The *P. vinckei* populations in the Central African Republic, Congo, Nigeria and Zaire are considered to be sufficiently different to be designated distinct subspecies. In Cameroun, one isolate possesses enzyme forms identical to those of two Central African Republic isolates (GPI-5, PGD-5, LDH-7, GDH-6), while the others are characterized by at least one enzyme form unique to Cameroun.

The taxonomic status of these parasites has not yet been resolved (Lainson, 1982).

Plasmodium yoelii and *P. chabaudi* populations are also divided into subspecies in different countries largely on the basis of their enzymes (Carter, 1978; Killick-Kendrick, 1978). In Cameroun, representatives of these species are indistinguishable enzymically from *P. y. yoelii* and *P. c. chabaudi* respectively of the Central African Republic (Lainson, 1979).

2. *Hybridization studies*

A question arising from the enzyme surveys is whether each subspecies of a given species is reproductively as well as geographically isolated. This subject has been investigated mainly in *P. yoelii* by attempting to make deliberate crosses in the laboratory between parasites from geographically remote areas.

Most work has been carried out using lines of *P. y. yoelii* (Central African Republic) and *P. y. nigeriensis* (Nigeria) differing in enzyme and drug-sensitivity markers. Five crosses were made by Knowles *et al.* (1981) between

TABLE 5

Analysis of clones derived from cross between P. y. yoelii (*line A*) *and* P. y. nigeriensis (*line D*)

	Enzyme forms[a]			Pyrimethamine response	Number of clones isolated
	GPI	GDH	ADA		
Parents					
Line A	1	4	2	Resistant	
Line D	2	2	1	Sensitive	
Progeny					
Parent A-type	1	4	2	Resistant	8
Recombinant types	2	4	2	Resistant	3
	1	2	2	Resistant	3
	1	4	1	Resistant	2
	2	4	1	Resistant	1
	1	4	2	Sensitive	7
	2	2	2	Sensitive	1
	1	2	1	Sensitive	1
	1	4	1	Sensitive	2
Parent D-type	2	2	1	Sensitive	9
				Total	37

[a]See p. 221 for explanation of abbreviations of names of enzymes.

line A of *P. y. yoelii* (GPI-1, GDH-4, ADA-2, pyrimethamine resistant) and line D of *P. y. nigeriensis* (GPI-2, GDH-2, ADA-1, pyrimethamine sensitive). The uncloned progeny were treated with pyrimethamine. Those of the first four crosses were examined only for GPI, and in each instance both GPI-1 and -2 were present showing that hybridization had occurred. In the fifth cross, the pyrimethamine-treated progeny were examined for all three enzymes GPI, GDH and ADA, and for each enzyme both forms distinguishing the parent lines were present. The undrugged progeny of this cross were examined further by cloning, and each clone obtained examined for enzyme-type and drug-response (Table 5). Of 37 clones examined, 17 were of parental types, and the remainder were recombinants.

It could be concluded that these two lines readily hybridized, and that from the numbers of recombinants recovered there was no preference for self- rather than cross-fertilization. The result was also of interest in demonstrating that each marker underwent independent assortment following the cross, indicating that they were probably unlinked.

Similar crosses have been successfully made between *P. yoelii* lines from Cameroun and the other *P. yoelii* subspecies, and between certain *P. chabaudi* subspecies (Lainson, 1982 and unpublished work).

C. SUMMARY

Surveys of enzyme variation in natural populations of rodent malaria parasites enable the following conclusions to be drawn.

(a) The four species *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei* can be clearly differentiated by their enzymes, and are reproductively isolated from one another.
(b) Considerable enzyme variation occurs within as well as between each species.
(c) Mixed infections with more than one species, and with more than one genetically distinct form of each species, commonly occur.
(d) Random mating is common among parasites of each species in a given region.
(e) Subspecies of each species occurring in different parts of Africa can be differentiated by their enzymes. Crossing experiments in the laboratory show that the subspecies are not reproductively isolated.

V. DIVERSITY IN *Plasmodium falciparum*

Plasmodium falciparum is generally regarded as a single species the world over, although a number of "strains" or "varieties" has been recorded.

Many of these reports have been considered by Garnham (1966) to be of doubtful validity and based on inadequate observations, but there seems little doubt that some variant forms of this species do occur. Parasites with abnormal blood form morphology were noted by earlier workers; for example, Ziemann (1918) described a variety named "*perniciosa*" in some African countries, and Raffaele and Lega (1937) recorded a variety "*aethiopicum*" in East Africa, as well as other regions. The duration of exo-erythrocytic schizogony may vary from one isolate to another; Garnham (1966) gives examples of a Rumanian form which takes 5½–6 days to complete the cycle, and a Roman strain which takes only 4 days. Another character which has been much studied is the infectivity of different *P. falciparum* strains to mosquitoes. For example, Shute and Maryon (1951) found that *P. falciparum* from Nigeria was able to infect *Anopheles stephensi* readily, but *A. labranchiae atroparvus* only poorly; the latter mosquito, however, proved to be a good vector of European strains of *P. falciparum*. Similar results were obtained by Ramsdale and Coluzzi (1975). Warren and Collins (1981) found that the New World mosquito *A. albimanus* was able to transmit parasites from Central and South America easily, but was poorly susceptible to parasites from Africa.

Recent technical advances have made it possible to study the diversity of intrinsic *P. falciparum* characters in more detail than has been possible hitherto. The development of methods for culturing blood forms *in vitro* (Trager and Jensen, 1976) has enabled numerous isolates to be established in the laboratory without the need for primate hosts. Cloning techniques (Rosario, 1981; Trager *et al.*, 1981) have made it possible to examine the genetic composition of some isolates in detail. This section is concerned with the diversity of characters such as enzymes, antigens and drug-sensitivity among uncloned and cloned *P. falciparum* isolates from several countries. The main objective of these studies is to determine whether the species comprises a single interbreeding population, or whether it consists of genetically distinct populations in different regions.

A. UNCLONED ISOLATES

1. *Enzymes*

The first work on enzyme variation in *P. falciparum* made use of short-term culture material, or parasites extracted from placentae of infected patients (Carter and McGregor, 1973; Carter and Voller, 1975). Subsequently, long-term cultures were used, enabling repeated checks to be made on the enzymes of some isolates (Sanderson *et al.*, 1981; Thaithong *et al.*, 1981; Hempelmann *et al.*, 1981).

The majority of isolates examined has been from West Africa (mainly The Gambia), East Africa (mainly Tanzania) and South-East Asia (mainly

TABLE 6

Enzyme forms[a] *of* Plasmodium falciparum *from The Gambia, Tanzania and Thailand*

	GPI				ADA				LDH				PEP			
	Number examined	1	2	1 + 2	Number examined	1	2	1 + 2	Number examined	1	2	1 + 2	Number examined	1	2	3
Gambia	170	64%	10%	26%	53	92%	4%	4%	164	40%	17%	43%	52	100%	—	—
Tanzania	21	43%	29%	29%	8	13%	13%	74%	26	73%	8%	19%	8	—	75%	25%
Thailand	176	62%	22%	16%	135	94%	3%	3%	143	100%	—	—	64	100%	—	—

[a] The enzyme forms (GPI-1, -2, etc.) are the electrophoretic variants found in *P. falciparum*; they are not the same forms as those of the rodent malaria species. See p. 221 for explanation of abbreviations of names of enzymes.

Thailand). Table 6 illustrates the proportions of isolates in each of these countries exhibiting each form of each enzyme found. Many isolates showed more than one form of a given enzyme, for example 26% of Gambian isolates possessed GPI-1 and -2. From the results obtained with the rodent species, such isolates could be presumed to be mixed infections of two types of parasite, some possessing GPI-1 and some GPI-2. For one isolate, this has been confirmed by cloning (Rosario, 1981; and Section B 1, p. 243).

Most enzymes exhibit similar forms in each country. PGD and GDH occur as type 1 in almost all isolates, the exceptions being rare forms (types 2 and 3) in some African samples (Sanderson *et al.*, 1981; Hempelmann *et al.*, 1981). GPI and ADA each possess two forms, and it is noteworthy that these forms occur at similar frequencies in The Gambia and Thailand, from where most isolates have been examined. LDH and PEP provide some evidence for a regional variation in the frequencies of particular enzyme forms. For LDH, parasites characterized by types 1 and 2 are seen in African isolates, but no LDH-2-type parasite has been found in South-East Asia. PEP appears to show some regional variation in Africa, parasites from The Gambia being characterized by PEP-1 and those from Tanzania by PEP-2 and -3. Only a small number of Tanzanian isolates has been examined for peptidase, however, so it is not excluded that PEP-1 may occur here also; PEP-1 has been found in a few isolates from other East African countries, for example Malawi (D. Walliker and A. Sanderson, unpublished work).

A small number of isolates from other countries has been examined for enzymes, including some from Indonesia, China, Vietnam, Papua New Guinea, Senegal, Ghana, Zaire, Colombia and Honduras (Sanderson *et al.*, 1981; and unpublished work). The enzyme forms found so far in these countries are the same as those found elsewhere.

2. *Other proteins*

O'Farrell (1975) developed a two-dimensional system of electrophoresis by which proteins could be separated by their iso-electric points and molecular weights. The system has been used by Tait (1981), Brown *et al.* (1982) and A. Walker (personal communication) to examine proteins of *P. falciparum*. The procedure involves growing the parasite in culture in the presence of radio-labelled amino acids, extracting the labelled proteins from the parasites and subjecting them to electrophoresis, first in an iso-electric focussing gel and then in a sodium dodecyl sulphate–polyacrylamide gel. The gels are then examined by autoradiography, the labelled proteins appearing as discrete spots.

Tait (1981) detected more than 100 parasite proteins by this method, of which seven exhibited variant forms in different isolates. This work has been extended considerably by A. Walker (personal communication), who

examined the protein composition of 13 isolates from Thailand and one from The Gambia. Approximately 90 proteins were invariant in all isolates while a further 11 exhibited variant forms in different isolates. The positions on gels at which the variable proteins occurred were denoted by letters (A, B, C, etc.) and each variant form by a number (e.g. A1, A2, B1, B2, etc.). Table 7 shows the forms of each variable protein found in the isolates examined.

TABLE 7

Variant forms of proteins at 11 positions on two-dimensional gels of P. falciparum *isolates*[a]

		Protein positions										
Area	Isolates	A	B	C	D	E	G	H	I	J	K	L
Thailand												
	SK15	1	2	2	1	1	2	2	2	1	2	2
	SK16	1	2	2	1	1	2	3	2	1	3	2
Songkhla	SK17	1	1	1	1	1	1	1	1	1	1	1
	SK18	2	3	2	2	1	2	1	2	1	1	1
	SK19	2	3	2	2	1	2	1;4	2	1	1	1
	K1	4	–	2	3	1	1	3	2	1	3	2
Kanchan-	K28	2	4	2	2	2	1	1;5	2	1	1;4	2
aburi	K29	2	–	2	2	1	2	3	2	1	3;4	1;2
	K36	2	5	2	3	1	2	1;3	2	1	1	2
	T17	3	–	2	2	1	1	1;3	2;3	2	3;4	2
Tak	T19	3	–	1	6	1	1	6	1	1	6	2
	T20	3	–	1	4	3	1	3	1;2	1	4	2
	T22	4	2	3	3;4	1	2	2	1	1	2	1
	T9	2;4	7;8	2	2;5	1;3	2	6;7	1;3	1	3;6	1
The Gambia	G1	2	6	4	4	3	1;3	7	1	2	5	1

[a] Positions on gel indicated by capital letters; protein forms by numerals (A. Walker, personal communication.)

Each isolate could be distinguished by at least one protein. There was also evidence for a regional distribution of some forms. The Gambian isolate, for example, differed from Thai isolates in its possession of four characteristic variant proteins (B6, C4, G3 and K5). Within Thailand, protein A occurred as forms 1 and 2 in Songkhla, while types 3 and 4 predominated among isolates from Tak. The latter isolates were especially variable, perhaps because these parasites had originated from migrant workers who had come to this region from other parts of South-East Asia.

3. *Antigens*

Evidence for the existence of antigenically different forms of *P. falciparum* came first from cross-immunity studies. For example Sadun *et al.* (1966) and Voller and Richards (1970), using experimental *P. falciparum* infections in primates, claimed that isolates from West Africa and South-East Asia did not cross-protect against each other. Subsequently, Voller *et al.* (1973) showed that monkeys which were immune to challenge with a West African isolate were partially susceptible to one from East Africa. Cadigan and Chaicumpa (1969) produced evidence that isolates from a single region of Thailand were antigenically diverse; using gibbons as experimental hosts good protection was obtained when immunized animals were challenged with homologous parasites, but not when heterologous parasites were used.

More direct evidence for diversity among *P. falciparum* antigens has come from the studies of Wilson and his coworkers (Wilson *et al.*, 1969, 1975; Wilson, 1980) and McBride *et al.* (1982).

TABLE 8

Distribution of S-antigens in isolates of P. falciparum[a]

P. falciparum isolates	*S-antigen specificities*[b]					
	a	b	c	d	e	f
Malayan (Camp)	+	+	−	−	−	−
Uganda (Palo Alto)	+	+	−	−	−	−
Salvador (St Lucia)	+	+	−	−	−	−
West Africa I	+	+	−	−	−	−
West Africa (Lagos)	−	−	+	+	(+)	−
Nigeria I	−	−	−	(+)	+	−
Haitian III	−	−	−	−	(+)	+

[a] Wilson (1980).
[b] + = presence; (+) = trace; − = absence.

Wilson *et al.* (1969) used gel diffusion to demonstrate the presence of soluble parasite antigens in the sera of infected patients in The Gambia. The antigens were classified as L-, R- and S-antigens according to their heat-stability and other characteristics. Diversity was especially marked among the S-antigens, 18 distinct forms being detected in samples taken from 50 patients. Individual patients contained up to five S-antigens in their serum at any one time. The same combinations of antigens occurred only occasionally in different individuals. The diversity of antigens seen in single patients was considered to be due to mixed infections of more than one antigenically distinct organism, although it was not excluded that a single parasite could contain several antigens. In subsequent work (Wilson, 1980), it was found that common S-antigens occurred in parasites from different countries (Table 8),

and that the S-antigens of one isolate were stable during numerous passages in monkeys and *in vitro*. The origin of the antigens was unclear, although they were shown to be released from parasites into the serum at the time of schizont breakdown (Wilson *et al.*, 1975).

McBride *et al.* (1982) used monoclonal antibodies and immunofluorescence to demonstrate antigenic diversity among *P. falciparum* isolates. Monoclonal antibodies were raised against two Thai isolates, K1 and PB1, by immunizing mice with cultured blood forms. Thirty-two antibodies were obtained, and their reaction with parasites tested using immunofluorescence. Some of the antigens recognized were common to all blood stages (rings, trophozoites, schizonts, merozoites, gametocytes), while others were specific to certain stages only (e.g. schizonts, merozoites).

The antibodies were tested against *P. falciparum* isolates from various countries. Eighteen antibodies gave positive reactions with all isolates, indicating that the antigens recognized were common to all. The remaining 14 antibodies, which appeared to react mainly with the surface of schizonts and merozoites, gave positive reactions with some isolates and negative reactions with others. In some isolates, some parasites gave positive reactions with a given antibody while others were negative. These were interpreted as mixed infections, some organisms possessing the respective antigenic deter-

TABLE 9

Antigenic diversity in P. falciparum *detected by monoclonal antibodies using immunofluorescence*[a]

		Monoclonal antibodies to isolate K1					Monoclonal antibodies to isolate PB1			
Isolate	Area	5·1	7·5	6·1	7·3	7·6	9·2	9·7	10·3	9·5
K1	Thailand	+	+	+	+	+	−	−	−	−
SL3	Sri Lanka	+	+	+	+	+	−	−	−	−
M23	Honduras	+	+	+	+	+	−	−	−	−
R-FCR-3	Gambia	+	+	+	+	+	−	−	−	−
PB1	Thailand	−	±	−	−	−	+	+	+	+
K28	Thailand	+	±	−	−	−	+	+	+	+
G1	Gambia	+	+	−	−	−	+	+	+	+
MAD20	Papua New Guinea	+	+	−	−	−	+	+	+	+
Palo Alto	Uganda	−	+	−	−	−	+	+	+	−
S145	Thailand	+	±	−	−	−	+	+	+	−
T9	Thailand			+	+	+	+	+	+	+

[a] + = bright fluorescence; − = no fluorescence; ± = intermediate fluorescence.

minants and others lacking them. Examples of some of the reactions found are given in Table 9. There was no obvious regional distribution of antigens, isolates from different parts of the world sometimes possessing a similar antigenic composition; for example, isolates K1 from Thailand, SL3 from Sri Lanka and M23 from Honduras possessed similar antigens.

4. *Drug sensitivity*

Drug-resistant forms of *P. falciparum* have now arisen in many parts of the world, presenting serious obstacles to control of the disease (Peters, 1970; Wernsdorfer and Kouznetsov, 1980). In the past, such resistant forms were detected by the failure of parasites to respond to treatment in man, or by testing parasites for their drug response in experimental monkeys (Schmidt, 1978a, b). More recently, *in vitro* methods have been developed, enabling tests to be made directly on the parasites. Rieckmann *et al.* (1968, 1978) and Rieckmann (1982) devised techniques of short-term culture to test the response of parasites taken directly from patients, while other workers (e.g. Richards and Maples, 1979; Nguyen-Dinh and Trager, 1980; Nguyen-Dinh and Payne, 1980; Thaithong and Beale, 1981) used long-term cultures; these methods enabled repeated tests to be made on the same isolates. Most work of this type has been carried out with pyrimethamine and chloroquine.

Forms of *P. falciparum* resistant to antifolate drugs have arisen soon after the drugs have been introduced for therapy. Early reports of resistance to proguanil, for example, are those of Field and Edeson (1949) in Malaysia, and McGregor and Smith (1952) in The Gambia. Clyde (1967) showed that in Tanzania pyrimethamine-resistant forms could persist in the parasite population for many years after use of the drug was discontinued. More recently, *in vitro* tests have revealed the existence of parasites highly resistant to pyrimethamine in some countries; examples are given in Table 10. In Thailand, where the drug has been widely used, the majority of isolates tested have proved resistant (Thaithong and Beale, 1981). In The Gambia, Smalley and Brown (1982) found that the majority of isolates tested were sensitive, although a few were slightly resistant (see Table 10); in that country, pyrimethamine had been used less widely than in Thailand.

Chloroquine resistance in *P. falciparum* was slower to emerge than pyrimethamine resistance, the first reports coming from South America (e.g. Moore and Lanier, 1961) and South-East Asia (e.g. Young *et al.*, 1963). Once arisen, chloroquine resistance spread rapidly, although it has only recently been detected in Africa (e.g. Kean, 1979; Fogh *et al.*, 1979). Three levels of resistance, denoted RI (low), RII (medium) and RIII (high), are generally recognized, according to the response of parasites in patients undergoing a standard course of drug treatment (World Health Organization, 1973). Rieckmann (1971) showed that parasites exhibiting different levels of resist-

TABLE 10

In vitro *responses of some* P. falciparum *isolates to pyrimethamine and chloroquine*

Country	Number of isolates tested	Drug concentration for inhibition of growth (μM)
		Pyrimethamine
The Gambia[a]	2	0·001
	5	0·005
	50	0·05
	3	1·0
Thailand[b]	1	0·1
	5	50·00
	2	100·00
		Chloroquine
"West Africa"[c]	1	0·03
Nigeria[c]	1	0·03
The Gambia[b]	1	0·1
Kenya[c]	1	0·3
Thailand[b]	9	1·3
	1	1·3

[a] Smalley and Brown (1982).
[b] Thaithong and Beale (1981).
[c] Nguyen-Dinh and Trager (1980).

ance *in vivo* showed similar differences *in vitro*. Numerous workers have recorded the existence of chloroquine-resistant parasites in field surveys, and in long-term cultures; some examples are given in Table 10.

5. *Genome structure*

The techniques of molecular genetics are now being used to investigate the organization of the genome of some parasitic protozoa. In trypanosomes, genes coding for specific antigens have been isolated and cloned in plasmids; hybridization of some of these clones to nuclear DNA extracted from different trypanosome stocks has revealed the presence or absence of the genes in these stocks, and shown that considerable variations occur in the DNA sequences flanking the genes (for review see Borst and Cross, 1982). In work on malaria parasites, Goman *et al.* (1982) have prepared clones of repetitive DNA of *P. falciparum* in bacteriophage λ, and studied the hybridization patterns of one clone with nuclear DNA from two parasite isolates. Markedly different patterns were seen with each isolate, showing that there were different DNA arrangements in the genome of each isolate.

B. CLONES

The studies on enzyme diversity in populations of rodent malaria parasites, reviewed in Section IV, showed that mixed infections with more than one genetically distinct organism occurred commonly. The *P. falciparum* studies outlined above also provide evidence for similar parasite mixtures in isolates of this species. The extent of the diversity found in single isolates has been investigated in two instances by cloning. Rosario (1981) established clones of an isolate from Thailand using a dilution method, and showed that enzymically distinct parasites could be obtained. Some of the clones obtained have now been examined for variation in other characters. Trager *et al.* (1981) used micromanipulation to obtain single parasites of a Gambian isolate which were used to initiate cloned lines; these clones were found to differ in their possession of "knobs" on infected host erythrocytes. These two pieces of work are described separately here.

1. *Clones of Thai isolate T9*

This isolate originated in Tak, Thailand. It was characterized by two forms of GPI, GPI-1 and -2, and two of ADA, ADA-1 and -2. After 2 weeks in culture, ADA-2 could no longer be detected, and after 5 more weeks GPI-2 became fainter and disappeared. These observations suggested that several genetically distinct parasites differing in enzyme type and growth rate were present in the original isolate; parasites characterized by GPI-1 and ADA-1 appeared to outgrow other forms during culture.

Clones of T9 were made soon after the isolate was established in culture (Rosario, 1981). Parasites were diluted into wells of microtitre plates so that each well contained an estimated 1 or 0·5 parasite. After 3 weeks, some wells contained growing parasites. The majority were characterized by either GPI-1 or GPI-2, while the remainder exhibited both forms. The numbers of each type obtained fitted in well with the expectations of the Poisson distribution, especially those originating from an estimated 0·5 parasite. It was concluded that the vast majority of cultures showing single enzyme forms were pure clones, and that those exhibiting both GPI-1 and -2 had originated from two or more parasitized cells, or from cells containing more than one parasite.

Four of the diluted cultures characterized by single GPI-forms and presumed to be pure clones have been subsequently examined for variation in other characters, including the enzymes ADA and PEP, other proteins detected by two-dimensional electrophoresis, antigens and drug-sensitivity. The results of this work are given below.

(a) *Enzymes* (*Table 11*). Two clones (numbers 32 and 94) were characterized by GPI-1, PEP-1 and ADA-1, and the other two (numbers 19 and 57) by GPI-2, PEP-2 and ADA-1. As mentioned above, a second form of ADA, ADA-2, was detected in the uncloned material immediately after isolation, but no clone possessed this enzyme form. Parasites possessing it had presumably become much reduced in numbers at the time of cloning.

TABLE 11

Enzyme characteristics[a] *of* P. falciparum *isolate T9 and four clones*

	GPI	PEP	ADA
Uncloned T9	1; 2	1; 2	1; 2
Clones 32, 94	1	1	1
Clones 19, 57	2	2	1

[a]See p. 221 for explanation of abbreviations of names of enzymes.

(b) *Other proteins* (*Table 12*). Table 12 shows the forms of eight proteins detected by two-dimensional gel electrophoresis in the uncloned isolates and the four clones (A. Walker, personal communication). The uncloned parasites showed two forms of each protein while the four clones exhibited only one form each. Most of the variant proteins of the clones were one or other of those seen in the uncloned material. Exceptions were forms C1 and D8 found in clones 19 and 57 only and not in the uncloned parasites. The most likely explanation of this finding is that parasites characterized by these proteins formed a large proportion of the original isolate at the time of cloning, but had become reduced in numbers at the time the isolate was examined by two-dimensional electrophoresis several weeks after cloning had been carried out.

TABLE 12

Variant forms of proteins detected by two-dimensional electrophoresis in isolate T9 of P. falciparum *and four clones*[a]

	Protein positions							
	A	B	C	D	E	F	H	K
Uncloned T9	2; 4	7; 8	2; 5	2; 5	2; 3	1; 3	6; 7	3; 6
Clones 32, 94	4	7	2	5	2	3	6	6
Clones 19,57	2	8	1	8	3	1	7	3

[a] See Table 7 for explanation. (A. Walker, personal communication.)

(c) *Antigens* (*Table 13*). J. S. McBride (personal communication) has examined isolate T9 and the four clones derived from it for antigens detected by monoclonal antibodies. Some of the reactions found are given in Table 13.

TABLE 13

Antigens of P. falciparum, *isolate T9 and four clones, detected by monoclonal antibodies*[a]

	Monoclonal antibodies				
	6·1 7·1 7·3 7·6	9·2 9·3 9·7 10·3	9·5 9·11	9·21	10·4
Uncloned T9	+	+	+	+	±
Clones 32, 94	+	−	−	−	−
Clones 19, 57	−	+	−	+	±

[a] J. S. McBride, personal communication.

The uncloned material contained parasites that reacted with each of the antibodies used, while the clones reacted with some but not others. Two types of antigenic specificities were seen, those represented by clones 32 and 94 and those by 19 and 57. A third antigenically distinct parasite could also be detected among the uncloned parasites; antibodies 9.5 and 9.11 reacted with some of these parasites, but not with any of the clones. Cloning had thus succeeded in purifying two out of at least three antigenically different forms in the original isolate.

TABLE 14

Drug-sensitivity of isolate T9 and four clones, and two standard isolates (K1 and G1), of P. falciparum

Parasites	Drug concentration for inhibition of growth (μM)	
	Pyrimethamine	Chloroquine
T9 uncloned	0·1	0·3
clones 32, 94	0·1	0·3
clones 19, 57	0·1	0·1
K1 (Thailand)	50·00	1·3
G1 (The Gambia)	0·001	0·1

(d) *Drug sensitivity* (*Table 14*). The uncloned isolate and the four clones exhibited a similar response to pyrimethamine, the minimum inhibitory dose being 0·1 μM. Some variations in chloroquine sensitivity were noted, however. Clones 19 and 57 were sensitive to 0·1 μM chloroquine, while the uncloned isolate and clones 32 and 94 survived this dose.

2. *Clones of Gambian isolate FCR-3*

This isolate was maintained in continuous culture for about 4 years before being cloned. The technique of cloning (Trager *et al.*, 1981) was to dilute parasitized cells into small droplets of culture medium, and to examine the droplets microscopically for the presence of erythrocytes containing a single parasite. The droplets were then transferred to the wells of a microculture plate, to which culture medium was added daily, and new uninfected erythrocytes every 6 days. Nine clones were successfully grown, the first parasites becoming visible on slides after 21 days. The clones were found to vary in two characters, the presence or absence of knobs on infected erythrocytes, and chloroquine sensitivity.

(a) *Knobs.* Knobs are observed on the plasma membrane of erythrocytes infected with late stages of *P. falciparum* blood forms, i.e. trophozoites and schizonts. They have been observed in the electron microscope on infected cells taken from man (Trager *et al.*, 1966; Miller, 1972) and monkeys (Luse and Miller, 1971), and subsequently in human cells in cultures *in vitro* (Langreth *et al.*, 1979). The function of the knobs is not clear, although they are known to be antigenically distinct from the normal erythrocyte membrane (Kilejian *et al.*, 1977) and appear to be involved in the attachment of infected cells to vascular endothelial cells (Miller, 1969; Luse and Miller, 1971). In long term cultures, Langreth *et al.* (1979) showed that parasites appeared to lose their ability to form knobs; after 18 months, one isolate (FCR-4) had become entirely knobless.

At the time of cloning of FCR-3, about 40–50% of the erythrocytes infected with late stages of the parasite exhibited knobs. When clones were

TABLE 15

Characteristics of clones derived from P. falciparum *isolate FCR-3*

Parasites	Knobby (K+) or Knobless (K−)	Chloroquine response (μM)
Uncloned	approx. 50% K+	0·10
Clone 1	K+	0·15
Clone A-2	K+	0·10–0·15
Clone A-3	K−	0·18
Clone B-3	K−	0·06
Clone B-4	K−	0·12
Clone C-1	K−	0·12
Clone D-1	K−	0·15
Clone D-3	K−	0·06–0·10
Clone D-4	K−	0·06–0·12

[a] From Trager *et al.* (1981).

examined, two proved to be knobby while the remaining seven were knobless (Table 15). One of the knobless clones was followed for 5 months in culture, and retained this character.

The genetic basis of the knobby character has not been investigated. It is possible that the knobless condition might be due to a genetic alteration in the parasite, allowing it to grow well in culture (Trager *et al.*, 1981).

(b) *Drug sensitivity.* Tests for sensitivity to chloroquine revealed slight variations between the clones; the chloroquine dose causing 50% inhibition of growth varied from 0·06 to 0·18 μM (Table 15).

C. GENETIC BASIS OF DIVERSITY

For technical reasons, a conventional genetic analysis of human malaria parasites is not yet possible. It can be assumed, however, that many of the characters described in this section are controlled by genes that are inherited in a similar manner to that found among the rodent parasites. Mating between different parasites is likely to generate considerable reassortment of the genes involved, and there is no expectation that a given form of one character will be correlated with that of another. For example, the S-antigens of isolates and clones of *P. falciparum* were found to be unrelated to enzyme (GDH-type) (Hempelmann *et al.*, 1981). In the studies described above, the different forms of enzymes found are not correlated with drug sensitivity or antigenic type.

Recent advances in the maintenance of *P. falciparum* in the laboratory, especially in obtaining mosquito infections from cultured gametocytes (Ponnudurai *et al.*, 1982; Campbell *et al.*, 1982), may make genetic studies possible soon.

D. SUMMARY

The principal findings of the *P. falciparum* surveys are given below.

(a) Considerable diversity of enzymes, antigens, drug sensitivity and other characters is seen among *P. falciparum* isolates.
(b) Cloning studies show that certain isolates contain mixtures of parasites which may be diverse in one or more of these characters.
(c) No obvious regional distribution is seen in the enzymic and antigenic characters examined, although differences in the frequencies of certain enzymes appear to exist.

(d) Variations in drug sensitivity are seen among parasites from different regions, the occurrence of resistant forms usually being correlated with the extent of use of the drug concerned.

VI. Conclusions

The studies outlined in this review show that considerable diversity occurs among characters such as enzymes, antigens and drug response in malaria parasite populations. The rodent work has demonstrated that many of these characters are under the control of genes which are inherited in a straightforward Mendelian fashion. This knowledge has considerable practical as well as theoretical value, which can be illustrated by considering the breeding structure of parasite populations, the origin and spread of drug resistance, antigenic diversity, and possible future developments, including the role of molecular genetics.

A. POPULATION STRUCTURE

One of the principal aims of studying diversity in a population of organisms is to determine whether they comprise a single interbreeding population, or whether they occur as discrete, reproductively isolated populations. This has important implications when considering, for example, the spread of drug resistance. Drug-resistant forms of *P. falciparum* are now common in many parts of the world, such as South-East Asia and South America, and in planning new control measures based on chemotherapy it is important to be able to predict whether a resistant parasite imported into a region of drug-sensitive organisms can transmit its resistance gene(s) to local parasite populations.

Most information on the breeding structure of parasite populations has come from the rodent malaria studies. Surveys of variation among parasites of a single region have shown that mixed infections with more than one species are common, and that no genetic exchange between the species occurs. This was especially clear in the example of the parasites of the Central African Republic, where *P. yoelii*, *P. chabaudi* and *P. vinckei* may infect the same host at any given time. A further important finding has been that considerable diversity occurs within as well as between each species. A principal cause of the diversity appears to be genetic recombination following mating between parasites of different genotypes. Parasites of a single species show regional differences in certain characters, notably enzymes. This is probably due to the geographical isolation of the rodent hosts, which may be leading to the

accumulation of genetic differences between their parasites, for example in the frequencies of certain alleles. For *P. yoelii* and *P. chabaudi*, however, these differences are not sufficient to prevent interbreeding, at least under laboratory conditions.

The human malaria species *P. falciparum* exhibits considerable diversity in characters such as enzymes and antigens, and mixed infections with more than one genetically distinct organism are common. The genetic basis of the variation has not been studied in this species, but can be assumed to be similar to that found in the rodent species. The diversity seen is generally less than that found among the rodent parasites, similar forms of enzymes and other characters occurring in isolates from different parts of the world. Many more populations need to be examined, however, to obtain a full picture of the variation existing in this species. For the present, *P. falciparum* can probably be regarded as a single, potentially interbreeding population, at least in the countries examined so far.

B. DRUG RESISTANCE

Work on the malaria species *P. gallinaceum*, *P. berghei*, *P. yoelii* and *P. chabaudi* has shown that resistance to antifolate drugs such as pyrimethamine and 4-aminoquinolines such as chloroquine can arise following drug treatment of sensitive forms. Genetic studies have demonstrated that mutation is an important cause of resistance, the mutations involved being stable and capable of undergoing recombination with genes determining other characters.

In vitro surveys of drug resistance in *P. falciparum* have shown that forms exhibiting stable resistance to pyrimethamine and to chloroquine are common in natural parasite populations. Such forms seem likely to have arisen because of widespread use of these drugs in certain regions. The genetic basis of drug resistance in *P. falciparum* has not been studied yet, but there are similarities in the nature of the resistance seen in *P. falciparum* and in the rodent species (especially *P. chabaudi*) suggesting that similar genetic mechanisms are involved.

Parasites resistant to antifolate drugs can arise in rodent species after a single course of treatment. The level of resistance obtained may be higher than the dose used for selection (Bishop, 1962). The early appearance of forms of *P. falciparum* resistant to these drugs following mass chemotherapy suggests strongly that single mutations may be a cause. The persistence and spread of pyrimethamine resistance in Tanzania after widespread use of the drug was terminated, documented by Clyde (1967), was probably due to spread of the resistance genes through the parasite population; the genes appeared to be at little or no disadvantage in the absence of the drug. In

South-East Asia, the widespread occurrence of pyrimethamine resistance may have assisted in the recent appearance of resistance to Fansidar, a pyrimethamine–sulphadoxine combination used extensively in this part of the world (e.g. Hurwitz *et al.*, 1981).

The development of resistance to chloroquine shows similarities in *P. chabaudi* and *P. falciparum*. In *P. chabaudi* stable chloroquine-resistant mutations are found only after prolonged treatment with the drug; high level resistance appears to be due to the presence in each parasite of several genes, each conferring a low level of resistance. In *P. falciparum*, chloroquine resistance took many years to appear after the drug was introduced for chemotherapy. The vast majority of cases of chloroquine resistance are of the low (RI) type (World Health Organization, 1973; Wernsdorfer and Kouznetsov, 1980), high levels of resistance (RIII) occurring mainly in regions such as South-East Asia where there has been very extensive use of the drug. In such conditions, hybridization between parasites containing independently acquired resistance genes is likely to occur, resulting in the production of parasites containing several genes having an additive effect. The spread of resistance genes into regions of sensitive parasites may also be aided by a selective advantage of some mutants. Such an advantage was found in a chloroquine-resistant mutant of *P. chabaudi*, which was found to outgrow its parent sensitive form in a mixed infection in the absence of the drug (Rosario *et al.*, 1978).

C. ANTIGENIC DIVERSITY

The genetic control of parasite antigens is a subject of particular importance to the current interest in antimalaria vaccines. An understanding of the capacity of single parasites to express a variety of antigens would enable predictions to be made concerning the effectiveness of a given vaccine. Little information is yet available on this subject although in the monkey species *P. knowlesi* it seems very likely that a single parasite can give rise to a succession of antigenically different forms (Brown, 1973). The speed with which new antigens appeared suggested that this was due to the differential activation of different genes, rather than to mutations of a single gene.

The studies outlined in this paper have established that diversity occurs among soluble S-antigens and antigens detected by monoclonal antibodies in *P. falciparum* populations. The genetic basis of this diversity has not been studied. However, it is likely that at least some of the variant antigens studied by McBride *et al.* (1982) were due to allelic variation of a single gene rather than to genes at different loci. This was suggested by the reactions of two sets of monoclonal antibodies illustrated in Table 9. Anti-K1 antibodies 6.1, 7.3

and 7.6 reacted identically to one another and in a mutually exclusive manner with anti-PB1 antibodies 9.2, 9.7 and 10.3. These two sets of antibodies may recognize alternative forms of the same antigen, some parasites expressing one form and others the second form. Isolate T9, reacting with both sets of antibodies, was shown in the cloning experiment to comprise a mixed infection of the two types (Table 13). It remains to be established whether any of the antigens detected in these studies are of any importance in stimulating protective immune responses; in this regard it is of interest that many of the variable antigens appeared to be associated with the surface of merozoites, the blood stage thought by some workers to be of most importance in protective immunity (Cohen, 1979).

D. FUTURE PROSPECTS

The studies reviewed in this paper have provided an understanding of how processes such as recombination and mutation give rise to variant forms of malaria parasites, and how the variation is inherited in parasite populations. In future it can be expected that molecular genetics will play an increasingly important part in such work. This approach complements conventional genetic studies by providing information on the detailed organization of the genetic material and on mechanisms of gene action. Considerable advances have been made in recent years in understanding the molecular genetics of trypanosomes, especially with regard to their antigen genes (see review by Borst and Cross, 1982), and similar studies are now under way on malaria parasites in some laboratories. Goman *et al.* (1982) have cloned fragments of *P. falciparum* DNA into bacteriophage λ, and have identified among the clones genes for ribosomal RNA and actin, as well as sequences of repetitive DNA. It is hoped that antigen genes will be isolated in this way, so that studies can then be made on their diversity and mechanisms of expression. Such studies will be of fundamental importance to the design of antimalaria vaccines.

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Hydatidosis and Cysticercosis: The Dynamics of Transmission

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I. Introduction

The infective pattern describes the numbers and distribution of parasites within a host population at a particular time. It is a measure of the success, or otherwise, of parasite transmission and is the outcome of interactions between the level of availability of infective stages (infection pressure) and the host response. The host response is largely determined by its physiological state, innate resistance and level of acquired resistance.

Among the Taeniidae the parasite-related factors include all those events that effect the density, infectivity and distribution of eggs in the environment (Gemmell and Lawson, 1982a). In this review, particular attention has been given to environmental factors that modify the survival and infectivity of eggs and to the mechanisms and agents that are responsible for their dispersal.

The host-related factors include natural and acquired resistance as well as such factors as feeding behaviour patterns. Host-related factors that modify the infective pattern of Taeniidae have recently been reviewed (Rickard and Williams, 1982) and will, therefore, be summarized only briefly.

The principal species discussed are those of economic importance and/or public health significance and include *Echinococcus granulosus*, *Taenia hydatigena*, *T. ovis*, *T. saginata* and *T. solium*. The first three have dogs and sheep, respectively, as principal definitive and intermediate hosts. The last two use man as the definitive host and either cattle or pigs respectively as the intermediate host. Man is also an intermediate host for *Echinococcus* spp. and *T. solium*.

II. Parasite-related Factors

Parasite-related factors influencing the infective pattern include the number of eggs and their distribution in the environment. The number of parasite eggs available is a function of the rate at which eggs are deposited (immigration rate) and the rate at which they are lost (emigration rate) (Anderson *et al.*, 1977). The immigration rate of taeniid eggs is determined, largely, by the number of infected definitive hosts and their defaecation habits, the number of patent worms harboured by each and their egg production. The rate at which eggs are lost from pasture is determined by four main factors: natural mortality, removal by the natural host and other animals, removal by wind or other mechanical means, and biological transfer into the soil profile. The resulting spatial distribution of the eggs in the environment also influences the pattern of egg-acquisition by the host.

The build-up and loss of eggs has been measured for *T. hydatigena* by grazing sentinel lambs for specific periods on pasture that had been contamin-

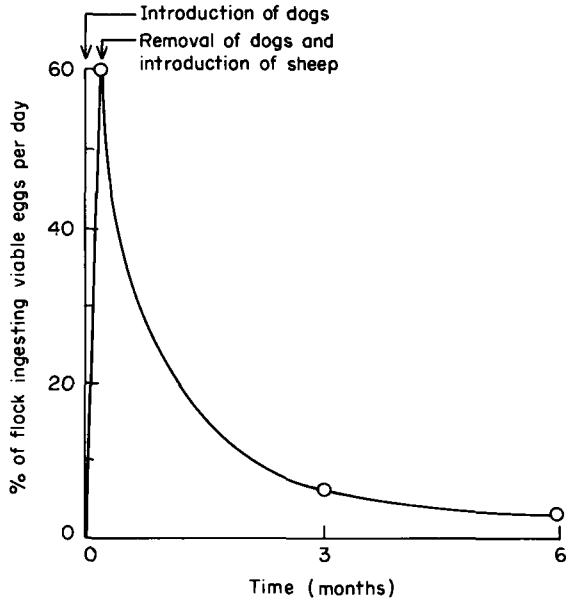

FIG. 1. The rapid build-up and slow decline in availability of eggs associated with the introduction and removal of dogs infected with *Taenia hydatigena*. (Data from Gemmell, 1976a; Gemmell and Macnamara, 1976.)

ated with eggs from experimentally infected dogs (Gemmell, 1976a; Gemmell and Macnamara, 1976). These studies showed that the build-up of taeniid eggs on pasture can be rapid and their loss can be slow if conditions are ideal for survival (Fig. 1). It was found that within 10 days of introducing infected dogs, the build-up of eggs on the pasture was such that 60% of the flock ingested eggs daily within 10 days of the removal of the dogs. This was reduced to 6·5% and 3·5% 3 and 6 months later, respectively.

A. FACTORS AFFECTING THE NUMBERS OF INFECTIVE EGGS IN THE ENVIRONMENT

1. *Immigration of eggs into the environment*

In areas endemic for tapeworms the number of infected definitive hosts varies within wide limits. For example, the prevalence of *E. granulosus* in dogs has been reported to vary between 18% and 60% in South America, between 2% and 63% in Africa and between 2% and 70% in Europe (reviewed by Gemmell, 1959; Schantz and Schwabe, 1969; Williams *et al.*, 1971; Matossian *et al.*, 1977). A similar wide range of prevalence has been recorded for *T. saginata* in man (reviewed by Pawlowski and Schultz, 1972).

In many countries there is very little information on the numbers of tapeworms in definitive hosts. In man this is because accurate estimations can only be obtained at autopsy. In human taeniid infections the prevalence of multiple infections is generally less than 1 % (Pawlowski and Schultz, 1972). In the case of dogs, the purgative arecoline hydrobromide has been used to ascertain natural infection rates. From the various surveys it seems that there are rarely more than 15 of the large taeniid tapeworms such as *T. hydatigena* and *T. ovis* present (Gemmell, 1968; Jackson and Arundel, 1971). High worm counts frequently occur in definitive hosts with organisms such as *T. multiceps*, which reproduce asexually in the intermediate host. Very high tapeworm burdens are not infrequently observed in *Echinococcus* spp. infections, particularly *E. multilocularis*, in definitive hosts.

TABLE 1

The number of eggs in proglottids and the numbers of proglottids released per day by taeniid tapeworms

Species	No. of eggs per proglottid	No. of proglottids shed each day	Reference
T. ovis	78 000	–	Arundel (1972)
	95 000	–	Coman and Rickard (1975)
	88 000	3	Gregory (1976)
T. hydatigena	27 000–63 000	–	Sweatman and Plummer (1975)
	28 000	2	Featherston (1969)
	31 075	–	Coman and Rickard (1975)
	53 000	2	Gregory (1976)
T. pisiformis	41 200	–	Coman and Rickard (1975)
T. saginata	80 000	9	Penfold *et al.* (1937a)
	100 000	–	Mönnig (1956)
	200–82 430	16	Rijpstra *et al.* (1961)
T. solium	40 000	–	Mönnig (1956)
	30 000–90 000	5	Webbe (1967)

–, Number not stated.

The biotic potential of the large taeniids is high and some estimates of the number of segments and eggs shed daily are recorded in Table 1. These studies show that many thousands of eggs may be released. In contrast, the biotic potential of *Echinococcus* spp. is low and individual segments contain 200–800 eggs (Rausch and Schiller, 1956; Arundel, 1972). On the basis of the maturation rate, it has been suggested that one segment is shed every 14 days (Gemmell, 1962). The low rate of egg production by *Echinococcus* spp. may also be compensated for by high worm burdens in individual hosts. For example, Rausch and Schiller (1956) found 450 000 *E. sibiricensis* (*E. multilocularis*) in a natural infection in a fox in Alaska. There may be

seasonal fluctuations in the excretion of proglottids of *E. granulosus*. Sokolov *et al.* (1975) have reported that none were excreted during the winter months but that production resumed in warm weather.

Under favourable laboratory conditions, detached proglottids can remain active for a few days after being expelled in the faeces. They perform rhythmic contractions and relaxations, which assist in egg expulsion (Rijpstra *et al.*, 1961; Fay, 1973). Nosik (1939, 1952a) observed segments of *E. granulosus* migrating "considerable distances" from faecal masses and reported that the segments climbed vertically on plants and thereby distributed eggs along the stalks. Eggs were expelled from the uterus together with a fluid that dried rapidly and left the eggs fixed to the vegetation. Matoff and Kolev (1964) observed that proglottids of *E. granulosus* could move over 25 cm, and Sweatman and Plummer (1957) reported that proglottids of *T. hydatigena* migrate horizontally up to 90 cm from faeces. This movement may provide a mechanism for egg dispersal. However, as shown by Rijpstra *et al.* (1961) with *T. saginata* and by Coman and Rickard (1975) with *T. pisiformis*, the majority of eggs are usually distributed in the faeces at the time of excretion. Webbe (1967) has pointed out that "terminal proglottids of both *T. solium* and *T. saginata* frequently become separated from the strobila and are passed in the faeces or migrate out of the bowel, and large numbers of eggs may escape from the uterus either before or after these ripe proglottids become free." It seems that the contribution made by proglottid movement to egg dispersal has not been determined.

2. *Survival of eggs*

Eggs of all taeniid species are morphologically similar. When mature they measure from 29 to 50 μm by 20 to 35 μm (Mönnig, 1956). They consist of an oncosphere surrounded by two thin oncospheral membranes, a tough layer of keratin blocks called the embryophore and a vitelline layer contained within a vitelline membrane (Silverman, 1954a, b; Smyth, 1963).

(a) *Measuring survival.* Natural mortality of eggs due to ageing is an important factor influencing the rate of loss of eggs from pasture. Investigation of the ageing of eggs suggests that the loss of the ability to infect occurs some time before actual death. In epidemiological terms, the rate at which infectivity decreases is more important than death. The infectivity of taeniid eggs can only by measured by administering known numbers to susceptible animals and determining the proportion that develop fully into the larval form. However, this *in vivo* method is difficult to apply in endemic areas and can be expensive.

A simple *in vitro* method has been developed using artificial gastric and intestinal fluids to assess viability (Silverman, 1954a). This has been used

TABLE 2

The maximum survival time of taeniid eggs stored under various conditions in the laboratory and in the field

Parasite	Where aged	Viability or infectivity assay used	Storage conditions	Maximum survival time (days)	Reference
T. saginata	Laboratory	"Survived"	"Room temperature" dry	30	Isobé (1922)
T. saginata	Laboratory	Infectivity *in vivo*	2–5°C	95	Penfold *et al.* (1937b)
T. saginata	Laboratory	"Survived"	"Room temperature"	270–300	Shestakova (1955)
T. saginata	Laboratory	*In vivo* and *in vitro*	4–5°C	168	Froyd (1962)
T. saginata	Laboratory	Using Kamalova's *in vitro* technique	Various:		
			0–15°C, in proglottids	13–14	Suvorov (1965)
			0–15°C, in free eggs	28–30	
			10°C, in proglottids	35	
			10°C, in free eggs	50–54	
T. saginata	Laboratory	*In vitro*	4°C	335	Silverman (1956)
			"Room temperature"	60	
T. pisiformis	Laboratory	*In vitro*	4°C	187	Silverman (1956)
T. ovis	Laboratory	*In vitro*	7°C in water	151–300	Gemmell (1977)
			16°C	91–150	
			21°C	51–90	
			27°C	41–50	
			32°C	21–30	
			38°C	2–10	
T. hydatigena	Laboratory	*In vitro*	7°C } surface moisture	210–294	Gemmell (1977)
			21°C } present	56	
			38°C	7	
T. multiceps and *E. granulosus*	Laboratory	*In vitro*	7°C } surface moisture	210–294	Gemmell (1977)
			21°C } present	28	
T. pisiformis	Laboratory	*In vivo*	5–10°C, moist chamber	100–150	Enigk *et al.* (1969)
T. pisiformis	Laboratory	*In vivo*	3–5°C, RH 32–33%	56	Coman (1975)
			3–5°C, RH 89–94%	300	
			37–39°C, RH 89–94%	7	

E. granulosus	Laboratory	*In vivo*	up to 30°C, in water or moist sand	21	Ross (1929)
E. granulosus	Laboratory	*In vivo*, intraperitoneal injection	10–21°C, wet	32	Batham (1957)
E. granulosus	Laboratory	*In vivo*	6°C, in water	225	Sweatman and Williams (1963)
E. multilocularis	Laboratory	*In vivo*	"Room temperature" in dry fox faeces	730	Thomas and Babero (1956)
T. saginata and *T. pisiformis*	Laboratory	*In vivo*	In silage—10°C —20°C	60–80 30–40	Enigk *et al.* (1969)
T. saginata	In field	*In vivo*	On pasture	101	Penfold *et al.* (1937b)
T. saginata	In field	*In vivo*	On pasture, Kenya	413	Duthy and van Someren (1948)
T. saginata	In field	*In vivo*	On pasture—winter —summer	159 58	Jepsen and Roth (1949)
T. saginata	In field	"Survived"	On soil surface in Azerbaidzhan, SSR —winter —summer	 51 5	Abasov (according to Suvorov, 1965)
T. saginata	In field	"Survived"	On soil, winter	105–225	Nadzhafov and Chobanov (1973)
T. saginata	In field	"Survived"	In soil, winter (Nov/Dec) In soil, spring (March)	90–165 45	Shekekov (1976)
T. pisiformis	In field	*In vivo*	Eggs sprayed on pasture —winter —summer	 125 0	Coman (1975)
T. hydatigena and *E. granulosus*	In field	*In vivo*	Eggs sprayed on pasture —autumn	 365	Sweatman and Williams (1963)
Echinococcus sp.	In field	*In vivo*	In sachets on ground	630	Vibe (1968)
T. saginata	In field	*In vivo*	In stored hay	21	Lucker and Douvres (1960)

extensively to study the effect of various factors modifying viability (Parnell, 1965; Mackie and Parnell, 1967; Laws, 1967, 1968; Gemmell, 1977). There are, however, limitations with this technique. For example, Penfold *et al.* (1937b, c) found that the percentage activation of the same batch of eggs could vary considerably from one experiment to the next. Parnell (1965) used *in vitro* activation to test chemicals for ovicidal activity and reported that "activity tends to be sporadic and, therefore, the percentages frequently are erratic and at best approximate". Coman and Rickard (1977) compared the infectivity of eggs of *T. pisiformis* to rabbits with their viability as shown by *in vitro* hatching and activation. The *in vitro* method consistently overestimated the infectivity of the eggs. Similarly, Gemmell (1977) found that the *in vitro* activity of eggs of *T. hydatigena* was considerably higher than the infectivity to lambs.

The question arises as to whether or not there is any relationship between *in vitro* activity and *in vivo* infectivity. Huffman and Jones (1962) showed that there was a linear correlation between the hatchability and infectivity of different batches of eggs of *T. taeniaeformis*. The standard error was large and *in vivo* infectivity ranged between 10% and 70% of the *in vitro* estimate. Coman and Rickard (1977) compared *in vitro* activity and *in vivo* infectivity of eggs of *T. pisiformis* under controlled conditions. They showed that a general decrease in both the percentage activation *in vitro* and infectivity *in vivo* occurred as the eggs aged. The initial *in vitro* estimate was almost double that of the *in vivo* estimate although both declined at similar rates. These results, therefore, suggest that as taeniid eggs age, they first lose their ability to infect and only subsequently their ability to hatch and activate *in vitro*. It seems likely that there is a theoretical basis for the assumption that *in vitro* activation rates and *in vivo* infectivity of taeniids are related. However, the low statistical correlation between the two estimates suggests that *in vitro* activation can only be a poor substitute for *in vivo* infectivity estimations.

The maximum longevity of viable infective taeniid eggs has been investigated by several workers (Table 2), but the variety of different parasites and the differences in the experimental techniques used and results achieved, make it difficult to construct a comprehensive picture. For example, the recorded longevity of eggs of *T. saginata* varied from 21 to 413 days and that of eggs of *T. pisiformis* from 0 to 300 days.

(b) *Lethal factors.* (i) *Temperature extremes.* Above and below certain temperatures irreversible damage to an organism occurs. The level at which this happens depends upon the duration of exposure. For instance, Meymerian and Schwabe (1962) were unable to activate the eggs of *E. granulosus* after exposing them to moist heat at 60°C for 10 minutes, at 70°C for 6 minutes, or at 100°C for 1 minute. Williams and Colli (1970) were unable to activate the

eggs of *T. hydatigena* after 5 minutes at 55°C, 2 minutes at 60°C, 1 minute at 65°C or 0·5 minute at 85°C. Nosik (1952b) reported that exposure of eggs of *E. granulosus* to 50°C for 1 hour or to 100°C for 20 seconds rendered them uninfective to cattle. Similarly, Williams (1963) demonstrated that exposure of the eggs of *T. pisiformis* to boiling water for as little as 5 seconds destroyed their infectivity. Gemmell (1977) showed that storage at 38°C for 2–10 days prevented activation of the embryos of *T. ovis* and *T. hydatigena*. Although high temperature was apparently an independent lethal factor, soil surface temperatures above 38°C rarely occur and may not be important in transmission in the natural environment.

The tolerance of eggs to cold conditions seems to be high, although there are limits beyond which temperatures are lethal. Suvorov (1965) recorded the survival of eggs of *T. saginata* for 77 days at —4°C and for 16–19 days at —30°C. Gemmell (1977) demonstrated that some embryos of *T. ovis* and *T. hydatigena* could still be activated after storage at —9°C for 170 days. After storage for 54 days at —27°C, eggs of *E. multilocularis* were still infective to voles (Schiller, 1955). In contrast, storage of eggs of *E. granulosus* for 24 hours at —50°C severely reduced infectivity, and storage at —70°C for 24 hours completely inhibited it (Colli and Williams, 1972). Low environmental temperatures seem to be unlikely to have a major effect on the transmission process.

(ii) *Desiccation.* Desiccation seems to be a much more important lethal factor than either extreme high or low temperatures. Penfold *et al.* (1937b) showed that storing eggs of *T. saginata* in dry conditions for as little as a day prevented them from infecting calves. Suvorov (1965) considered that the viability of the eggs of *T. saginata* was directly proportional to the relative humidity (RH) of the environment. Silverman (1956) found that the eggs of *T. pisiformis* and *T. saginata* did not survive longer than 14 days in the absence of surface moisture irrespective of relative humidity. Laws (1968) demonstrated an "unsuspected susceptibility of the taeniid egg to desiccation" and that this was "likely to dominate all other natural restrictions on the survival of taeniid eggs in nature". Eggs of *T. pisiformis*, *T. ovis* and *E. granulosus* were reported to have a greater susceptibility to desiccation than those of *T. hydatigena*.

(iii) *Combined factors.* In the natural environment, temperature, humidity and micro-organisms interact to determine the longevity of eggs. Gemmell (1977) demonstrated that, *in vitro*, the absence of surface moisture at temperatures between 7 and 38°C significantly reduced the time that the embryos of several taeniid tapeworms showed activation. Coman (1975) examined the combined effect of temperature and RH on eggs of *T. pisiformis*. He stored the eggs under the following conditions:

(i) low temperatures (3–5°C) and low RH (32–33%),
(ii) low temperatures (3–5°C) and high RH (89–94%),
(iii) high temperatures (37–39°C) and low RH (32–33%),
(iv) high temperatures (37–39°C) and high RH (89–94%).

At low temperatures and high humidities, the eggs remained infective to rabbits for over 300 days. However, a low humidity and the same low temperature caused loss of infectivity within 84 days. At the higher temperature, few eggs remained infective for more than a week at either humidity level. It would appear that the high temperature selected was independently lethal to the organisms and thus obscured any effect of desiccation. The effect of weather on the survival of taeniid eggs has been reviewed by Gemmell (1978c).

(c) *Ageing of eggs.* (i) *Effect of temperature.* It seems that environmental temperature is one of the factors that has a considerable influence on the longevity of eggs. Within certain limits, as the environmental temperature rises, the rate of ageing of the taeniid eggs increases and the longevity decreases. Silverman (1956) showed that by increasing the temperature from 4°C to "room temperature" the life span of the eggs of *T. saginata, in vitro*, was reduced from 335 days to 60 days and those of *T. pisiformis* from 187 to 60 days. Coman (1975) demonstrated that by raising the temperature from 3–5°C to 37–39°C (RH 89–94%) the longevity of eggs of *T. pisiformis, in vivo*, was reduced from about 300 days to 14 days. Similarly, Gemmell (1977) reported that the longevity of eggs of *T. ovis, in vitro*, was reduced from 150–300 days to 2–10 days by raising the temperature from 7°C to 38°C (Fig. 2).

(ii) *Pattern of ageing.* Although knowledge on maximum survival times is relevant, the patterns of loss of infectivity during the ageing process within egg populations are probably of greater importance in determining infection rates. In contrast to the large number of maximum and minimum longevity studies, few workers have studied changes with time in the infectivity within egg populations. Batham (1957) used an intraperitoneal injection of eggs into mice to determine the infectivity of eggs of *E. granulosus* stored between 10°C and 21°C. Eggs stored up to 12 days produced multiple infections, but after 12 days the proportion of mice infected declined rapidly. As part of an experiment to determine the effect of low humidity on the survival of eggs of *T. pisiformis*, Coman (1975) stored eggs in the laboratory at low temperature (3–5°C) and high humidity (89–94%) (see above). The initial infectivity of 9·7% fell to 0·2% by day 300. However, the rate of decrease in infectivity was irregular. After an initial decrease, infectivity increased before finally declining towards zero. Coman and Rickard (1977) stored eggs of *T. pisiformis* at

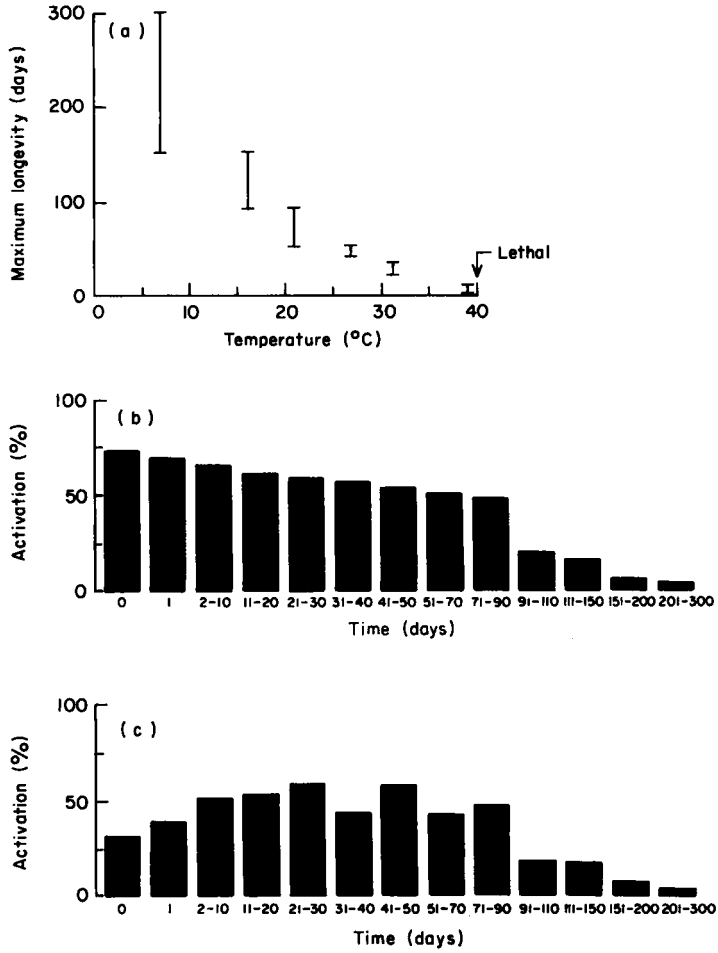

FIG. 2. The effect of temperature on the hatching characteristics of taeniid eggs. (Data from Gemmell, 1977.) (a) Range of longevity of *Taenia ovis* eggs after storage in water at specified temperatures. (b) The gradual decline in activity of *T. hydatigena* eggs after storage at 7°C for specified periods. (c) The increase followed by a decrease in activity of *T. hydatigena* eggs after storage at 7°C for specified periods. The difference between the two tapeworms (b) and (c) may be due to maturation of immature eggs in the bottom example during storage.

36–38°C and 87–93% RH and measured their infectivity. The infectivity declined more or less linearly with storage time. However, in an almost identical experiment reported in the same paper, the infectivity of the eggs remained roughly constant for 4 days before declining rapidly. From these limited data, it seems that the loss of infectivity during the ageing of taeniid eggs is variable.

More information on the variability of the shapes of the curves representing the decline of infectivity can be deduced from the *in vitro* study of Gemmell (1977). Eggs of *T. hydatigena*, *T. ovis*, *T. multiceps* and *E. granulosus* were stored at various temperatures in distilled water and measurements made of their *in vitro* hatching characteristics at regular intervals. These results showed that the decline in activation was variable (Figs 2b and 2c). Within certain temperature ranges, the initial decline in the percentage activation of individual tapeworms was followed by an increase, before declining to zero (Fig. 2c).

Gemmell (1977) examined the *in vitro* activity in replicate samples of eggs of *T. hydatigena* from chains of apparently "ripe" segments from different sectors and different segments of the same worm and from different worms and found that activity varied from 5% to 80%. He suggested that only fully developed eggs hatched and activated successfully *in vitro* and that these differences were due to variations in the proportion of mature and immature eggs between different segments. This was similar to the conclusions drawn by Silverman (1954b) for *T. saginata*. Here, only the last 30–50 segments contained fully developed ova and the proportion of these varied considerably from one segment to the next. By storing eggs with a low percentage activity in saline at room temperature their activity could be increased significantly (Silverman, 1954b).

From these studies it seems very likely that the shape of the curves for both infectivity and percentage activation during storage will be a consequence of the relative proportions of mature and immature ova and their rates of development and ageing respectively. Their form will depend on these relative proportions and on environmental factors. Thus, it is considered that heterogeneity within the egg population and environmental factors together determine the shape of the curves representing the ageing process.

3. *Emigration of eggs due to causes other than natural mortality*

Although mortality probably accounts for the major part of the loss of infective eggs from the environment, there are several other mechanisms that contribute to the emigration rate. The natural host removes some eggs and once immunity is established most are destroyed. Vertebrates that do not act as hosts may destroy eggs (Penfold *et al.*, 1936) and invertebrates that pick up eggs may destroy or remove them to environments where they are no longer available to the host. For example, ants, earthworms and dung beetles may take eggs down into the soil. Miller (1961) and Miller *et al.* (1961) have shown that the grinding mandibles of beetles crush many helminth eggs and it is probable that the mouth-parts and/or the digestive processes of many insects destroy eggs.

Wind and water may also transport eggs to environments where the host cannot contact them. Heavy rainfall, for example, can wash eggs

down into the soil. Sweatman and Williams (1963) reported that eggs of *T. hydatigena* were carried below the depth of normal tilling although Coman (1975) found that heavy rainfall did not dislodge all the eggs of *T. pisiformis* and rabbits continued to become infected after a precipitation of 300 mm. Gemmell *et al.* (1978) reported that neither heavy rain nor the height of the grass affected the infective pattern of *T. hydatigena.*

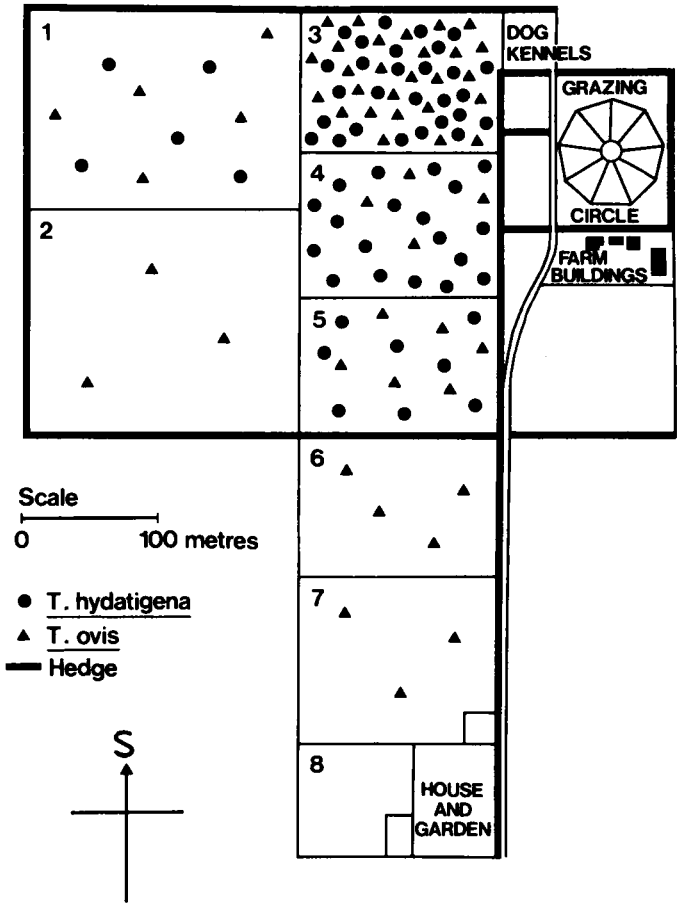

FIG. 3. Results of an investigation of an accidental epidemic of *Taenia ovis* emanating from the research farm of the Hydatid Research Unit onto a neighbouring farm. The epicentre was identified as the Unit's dog kennels. Each symbol represents a cyst in the 'sentinel' lambs used to investigate the outbreak. The grazing circle is an experimental plot for studying the egg dispersal of *T. hydatigena* which also caused cysticercosis in the neighbour's lambs (Anon, 1979.)

B. FACTORS AFFECTING THE DISTRIBUTION OF EGGS IN THE ENVIRONMENT

1. *Evidence of dispersal*

Little is known of the relevance of the various ways by which man may become parasitized with *Echinococcus* spp. and *T. solium*. Man, sheep and cattle, unlike pigs, do not normally eat dog or human faeces. However, natural infection of sheep and cattle with larval taeniids should usually result from grazing pasture that has been contaminated with eggs. If this is so, it must be assumed that eggs are dispersed from the faecal mass. Indeed, evidence is now accumulating that the extent of this dispersion and its implications to the dynamics of transmission are much greater than has previously been suspected. Gemmell and Johnstone (1976) reported the results of an experiment in which sheep were grazed at different distances from dogs infected with *T. hydatigena*. Some of the sheep that grazed up to 80 m from these dogs acquired heavy infections within 10 days.

Field evidence of egg dispersion comes from an investigation of 'epidemic-type' outbreak of *T. ovis* in New Zealand (Anon., 1979). Lambs grazed on several adjacent paddocks were found to be heavily infected. 'Sentinel' sheep were grazed on these paddocks for 2 months and careful cyst counts were then made by dissecting the carcases. Sheep grazed in the paddock nearest to the dog kennels (Fig. 3) showed the highest cyst counts and the level of infection in the animals decreased with increasing distance from this location. If it is assumed that an infected dog in the kennels was the source, the distance of egg dispersal was at least 175 m.

The maximum distance that eggs are transported is probably much greater than indicated by the observations described above. Circumstantial evidence for this comes from two main sources. The first comes from the Styx Field-Trial in New Zealand, where an outbreak of *T. hydatigena* occurred on one farm and in the same year a low prevalence of the parasite was found on farms as far away as 10 km (Gemmell, 1978b; Fig. 4). It is possible that the dog responsible wandered onto some of the farms and so distributed eggs, but another explanation is that eggs were dispersed by some other means. If this was the case, it implies that a single infected dog can be the source of infection over an area of 30 000 ha.

The second observation comes from an abattoir survey of *T. ovis* on 10 000 farms in New Zealand (Anon., 1979, 1980, 1981). When the positions of infected farms were plotted on a map it was found that areas with high infection rates were surrounded by areas of decreasing infection (Fig. 5). It has been suggested that eggs from an infected dog at an epicentre could have accounted for the pattern of infection in the surrounding areas.

The directional pattern of the spread of taeniid eggs has been studied by Gemmell *et al.* (1978). Sheep were grazed in a 'grazing circle' divided into nine

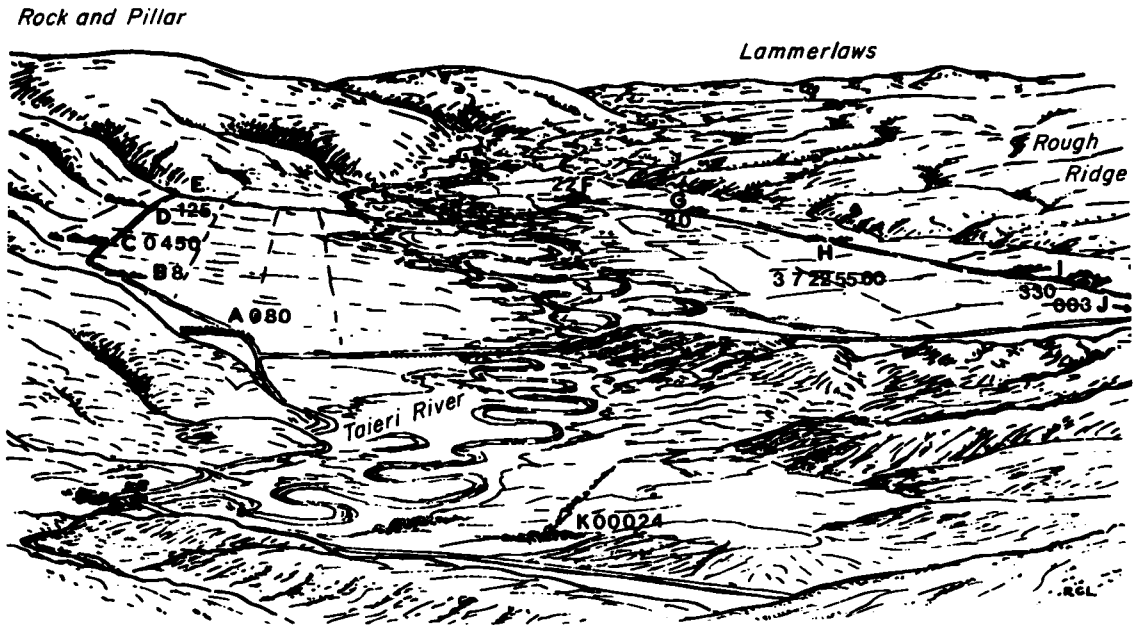

FIG. 4. The Styx Valley showing the prevalence of *Taenia hydatigena* in lambs in 1977 when the dogs in the valley were treated with nitroscanate at 100 mg per kg every month. (From Gemmell, 1978b.) The letters A to E refer to farms lying below the Rock and Pillar Range, the letters F to K to farms below the Rough Ridge Range. These two ranges of hills are contiguous with the Lammerlaw Range and thus there is a barrier to stock movement at the head of the valley. The entrance to the Styx Valley is between farms A and K. The numbers 0 to 60 refer to the prevalence of *T. hydatigena* in lambs at the times they were drafted from each farm. On farm H, the epicentre of the cysticercosis storm, there were five drafts of lambs and the prevalence of *T. hydatigena* in these was 3, 7, 22, 55, and 60% respectively. The distance between the homesteads of farms H to B and C is approximately 10 km.

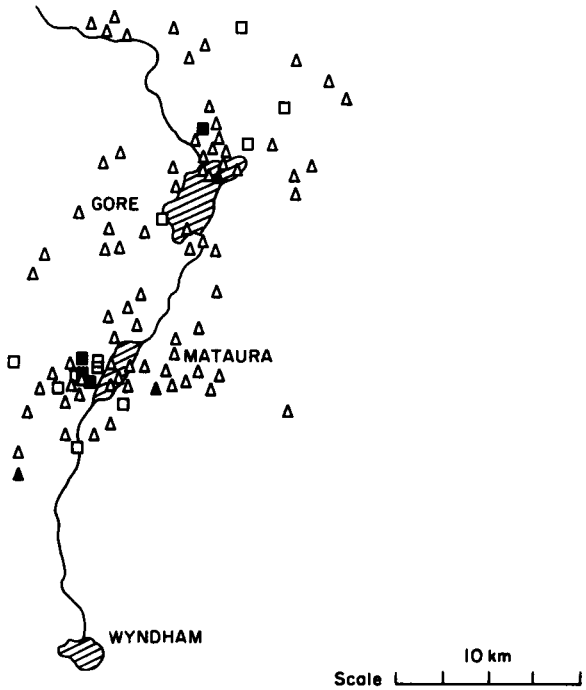

FIG. 5. Percentage infection of *Taenia ovis* in lambs on farms in a closely settled region of the South Island of New Zealand. The pattern of infection possibly results from the spread of eggs from epicentres (> 15%) involving infected dogs (Anon, 1980). Percentage lamb infection: △, 1–4·9%; □, 5–9·9%; ▲, 10–14·9%; ■, > 15%.

equal sub-plots radiating from a central ungrazed kennel area. This housed four dogs infected with *T. hydatigena*. Analysis of the cyst counts in the sheep showed no significant difference between the plots, thus indicating that the eggs were dispersed equally in all directions despite a prevailing south-west wind and slope.

The identifiable characteristics of this egg dispersion can be summarized as follows: (i) eggs disperse up to 80 m in 10 days; (ii) radial dispersion is uniform, and (iii) small numbers of eggs probably travel further. The question arises as to how this dispersion is brought about. Several possible agents have been suggested and these will be examined.

2. *Potential dispersal agents*

(a) *Wind.* The dispersal of taeniid eggs by wind lacks substantial experimental evidence. The circumstantial evidence comes from two main sources. The first is the occurrence of hydatid cysts in the lung. It has been suggested

that some pulmonary cysts could be derived from inhalation of eggs (Bird, 1877). Borrie *et al.* (1965) reported that eggs introduced experimentally into the lungs of lambs were capable of hatching and developing into hydatid cysts. However, Morseth (1963, in an unpublished report for the New Zealand Medical Research Council) argued that particles the size of taeniid eggs are more likely to be coughed up and swallowed before reaching the lungs.

The other evidence for wind transmission is the acquisition of *T. hydatigena* and *E. granulosus* cysts by lambs grazed on apparently uninfected pasture located down-wind of a plot sprayed with eggs (Sweatman and Williams, 1963). Although these workers considered the possibility that these eggs might have been carried by insects or birds, they concluded that prevailing strong winds, typical of the region, were more likely to have transported them. However, these workers did not determine whether or not these infections were due to 'wild' eggs deposited on the pasture before or during the experiment. Also, unfortunately, they did not test for dispersion of eggs in other directions.

Eggs trapped in moist faecal material and/or in proglottids are unlikely to be transported by wind until the faecal mass or proglottid has dried up and disintegrated. Taeniid eggs are intolerant of desiccation (see Section IIA2, p. 269). Thus, it seems likely that the majority of wind-borne eggs would lose their infectivity. In addition, eggs readily lose their outer vitelline membrane and stick to each other and to other surfaces. Furthermore, Laws (1968) found that dried eggs become electrically charged and stick to surfaces with which they come in contact.

In conclusion, it would appear that direct evidence for the dissemination of taeniid eggs by wind is lacking. The adhesive qualities of the outer surface of the eggs make it unlikely that they can become airborne, and if they do become so, they are likely to be non-infective. In addition, the evidence of Gemmell *et al.* (1978) for the uniform radial dispersion of the eggs of *T. hydatigena* provides an argument against the involvement of wind.

(b) *Rainfall.* Heavy rainfall may break up faeces and Beaver (1975) suggested that raindrops could splash eggs several metres over a flat surface and vertically to heights of 30 cm. Running water on the soil surface could transport eggs longer distances, but there seems to be no way in which water, except under flood conditions, could bring about the observed dispersion pattern of eggs.

(c) *Sewage disposal.* Epizootics of *T. saginata* have frequently been attributed to poor sanitary practices (Miller, 1956; Schultz *et al.*, 1969). The use of raw sewage to fertilize cattle pastures is an important means of spreading *T. saginata* infections to cattle (Profé, 1934; and reviewed by Silverman and

Griffiths, 1955; Greenberg and Dean, 1958; Pawlowski and Schultz, 1972; Owen, 1980). Conventional sewage treatment does not seem to eliminate taeniid eggs completely (Newton *et al.*, 1949; Wang and Dunlop, 1954) and some epizootics of *T. saginata* infection have been attributed to this (Sinnecker, 1955; Denecke, 1966). For example, over 50% of 10- to 11-month-old cattle grazed on pasture irrigated with sewage effluent from the city of Melbourne, Australia, were shown to be infected with *T. saginata* cysticerci (Rickard and Adolph, 1977). Since only the heart, masseter muscles, tongues and laryngeal muscles were examined, the actual infection rate was probably much higher.

(d) *Herbivores.* Jones *et al.* (1960) have suggested that immature eggs of *T. saginata* may pass through cattle and be redeposited on the pasture. Just what role other herbivores play in egg-dispersion of other species is not known.

(e) *Birds.* Seagulls, starlings, rooks and other scavenging birds have all been implicated in the transfer of eggs of *T. saginata* from sewage works to pasture. Silverman and Griffiths (1955) showed that immature taeniid eggs passed unharmed through the gut of seagulls and young chicks. Eggs of *T. saginata* and *T. pisiformis* that had passed through the gut of seagulls produced infections when fed to cattle and rabbits respectively. Guildal (1956) found that black-headed gulls (*Larus ridibundus*) and common gulls (*L. canus*) transported the eggs of *T. saginata*, while Crewe (1967) and Crewe and Crewe (1969) reported hymenolepid, trichiurid and taeniid eggs in the faeces of gulls that had fed on settling beds of a sewage works. Götzsche (1951) showed that calves became infected with *T. saginata* when fed seagull faeces. Gladkov (1969) reported that taeniid eggs could pass undamaged through the guts of crows, sparrows and young chicks. Zenkov (1978) stated that pigeons could disseminate the eggs of *T. solium* and that they remained viable for 24 hours. However, Silverman and Griffiths (1955) failed to recover infective eggs of *T. saginata* from pigeons.

The epidemiological significance of birds in the dispersal of tapeworm eggs will depend on the frequency with which they ingest faeces, tapeworm segments, insects and other invertebrates. Apart from the literature on the scavenging by birds at sewage works, no reference could be found to the feeding behaviour of birds with respect to faeces. However, white-backed magpies (*Gymnorhina hypoleuca*) have been observed to scavenge whole tapeworms expelled from dogs treated with arecoline hydrobromide (M. A. Gemmell, unpublished observation). Chickens are known to peck at faeces and proglottids and in addition to breaking up the faecal mass may disseminate eggs. Laurence (1954) reported that jackdaws (*Corvus monedula*) broke up cow pats and that in spring the emergence of beetles from the pats attracted

birds. Sometimes the pats were completely destroyed. Scavenging birds with different feeding ranges may be responsible for both short and long-range dispersal patterns. Whether or not by feeding on egg-carrying invertebrates birds can contribute to the further dissemination of taeniid eggs, also remains to be investigated. The pattern of dispersal of eggs would be determined by the home range and migratory behaviour of the birds involved.

(f) *Insects.* The role of insects in the transmission of viral, bacterial and protozoan infections is well documented and there is also evidence that insects are capable of transporting the much larger helminth eggs. Many groups have been implicated in egg transmission (reviewed by Goddeeris, 1980a). Their role is discussed below.

(i) *Diptera—Muscidae, Calliphoridae and Sarcophagidae.* There are several mechanisms by which flies could transport taeniid eggs from the faecal mass to the surrounding area. These include: (i) externally on their legs, bodies and wings; (ii) internally by ingesting and then vomiting or excreting them; (iii) internally ingesting them as larvae in the faeces, retaining them during pupation and then excreting them; and (iv) in the case of biting flies, picking up eggs while visiting faeces and transmitting them parenterally.

Flies from several families are attracted to human and animal faeces for both feeding and breeding. Blowflies (Calliphoridae) require protein for the development of eggs (Harlow, 1956; Hughes and Walker, 1970) and Webber (1958) has shown that animal faeces are an important source of protein for females of *Lucilia sericata.* Fresh moist faeces attract flies more readily than old dry material (J. R. Lawson and M. A. Gemmell, unpublished observation). Papp (1976) examined the fly fauna of cow pats and found that the families Sepsidae, Sphaerocerodae and Muscidae were the most common and that as much as 2% of the dry weight of the pat could consist of fly larvae. Schiller (1954) noted that the Calliphoridae fed readily on the excrement of canine animals. Poorbaugh and Linsdale (1971) reared 15 species of flies from 90 dog stools collected in California. Wilton (1963) observed that two different fly populations were associated with dog faeces; one visits faeces to feed only, whereas the other feeds and reproduces within them. Disney (1972, 1975) made a similar observation and noted that the species associated with dog faeces in an urban locality (Bath, England) were totally different from those associated with them in a rural area (Malham Tarn, England). For example, in Bath, *Paregle radicum* was the most common fly reared in faeces, and *Musca autumnalis, Fannia canicularis, Copromyza hirtipes* and *Piophila vulgaris* were among those recorded as visiting them only. At Malham Tarn, the most common flies visiting faeces were *Scatophaga squalida, S. stercoraria, Polietes lardarius* and *Azelia zetterstedti.* Lawson (1982) trapped flies attracted to

canine faeces in sheep pasture in the South Island of New Zealand. The four main species caught in descending order of occurrence were *Hybopygia varia*, *Calliphora quadrimaculata*, *C. hortona* and *C. stygia*.

Helminth eggs have been found attached externally under both experimental and natural conditions. Schiller (1954) demonstrated that *Phormia regina* can transmit eggs of *E. multilocularis* from the faeces of arctic foxes (*Alopex lagopus*) to red-backed voles (*Clethrionomys rutilis dawsoni*). Gupta *et al.* (1972) found large numbers of parasite cysts and eggs (e.g. *Ascaris lumbricoides*, *Hymenolepis nana*, *Entamoeba histolytica*, *Escherichia coli* and *Endolimax nana*) attached to the outer surfaces of *M. domestica* that had been exposed to highly infected faecal material. Heinz and Brauns (1955) recovered a few eggs of *E. granulosus* from the bodies of *Sarcophaga tibialis*.

Zmeev (1936) found eggs, which he identified as *T. saginata*, strongly attached to the legs of *Musca vicina* trapped in toilets. Pokrovskii and Zima (1938) found the eggs of several species of helminths attached to the legs and wings of 'wild' flies. Nadzhafov (1967) found eggs of *T. saginata* attached externally to a variety of flies including *M. domestica*, *Calliphora erythrocephala*, *Coprosarcophaga haemorrhoidalis* and *Sarcophagidae* sp., and Bakiev (1973) noted that 9% of synanthropic flies caught on state farms carried helminth ova on their bodies. Sarcophaginae and *M. domestica* were most heavily contaminated.

The body and legs of flies are invested with numerous bristles and are well adapted for carrying particulate material. Flies usually clean themselves shortly after feeding so that material attached to the outer surfaces should not remain there long. Pipkin (1943) experimentally contaminated the outer surfaces of *M. domestica* with the eggs of various nematodes. He found that the flies remained contaminated for a mean maximum time of 3·5 hours. Gupta *et al.* (1972) concluded that flies removed most of their external contaminants within 24 hours.

The ability of flies to transmit eggs internally depends upon the construction of the proboscis and size of the egg. The interbifid spaces of the pseudotrachea exclude eggs above a certain size (i.e. 50 μm) although they can still be swallowed if the fly spreads its labellar lobes and directly applies its prestomum (Greenberg, 1973). It follows that the main factor determining the size of the largest egg that can be accommodated by the proboscis appears to be the size of the prestomum. Nicoll (1911) suggested that the house fly (*M. domestica*) cannot ingest particles larger than 45 μm, but Pipkin (1943) has shown that larger flies can ingest larger particles. The eggs of most cestode species are smaller than 45 μm.

There have been many laboratory investigations of the ability of flies to carry cestode eggs internally. These are summarized in Table 3. All studies, except that of Podiapolskaia and Gnedina (1934), found that *M. domestica*

TABLE 3

Laboratory experiments on the internal carriage of cestode eggs by flies

Species of fly	Species of cestode egg	Positive or negative	Reference
Musca domestica	*Hymenolepis nana*	+	Calandruccio (1906)
M. domestica	*Hymenolepis diminuta*	−	Nicoll (1911)
	Dipylidium caninum	+	
	Bothriocephalus marginatus	+	
	Taenia pisiformis	+	
"flies"	*Echinococcus granulosus*	+	Ross (1929)
Calliphora vicina	*Diphyllobothrium latum*	+	Podiapolskaia and Gnedina (1934)
M. domestica	*D. latum*	−	
M. domestica	*D. latum*	+	Aleksander and Dansker (1935)
	H. nana	+	
Sarcophaga tibialis	*E. granulosus*	+	Heinz and Brauns (1955)
M. domestica	*E. granulosus*	+	
genera *Sarcophaga*	*Taenia saginata*	+	Round (1961)
Chrysomyia		+	
M. domestica	*T. saginata*	+	Nadzhafov (1967)
Muscina stabulans		+	
Lucilia sericata		+	
Lucilia caesar		+	
Calliphora erythrocephala		+	
Coprosarcophaga haemorrhoidalis		+	
M. domestica	*T. saginata*	+	Khan (1979)
M. domestica	*T. saginata*	+	Goddeeris (1980b)
Calliphora quadrimaculata	*Taenia hydatigena*	+	Lawson (1982)
Calliphora hortona		+	
Calliphora stygia		+	
Calliphora erythrocephala		+	

was able to carry a variety of cestode eggs. Other fly genera in which internal carriage was observed included *Calliphora*, *Sarcophaga* and *Chrysomyia*. Lawson (1982) found that from 50% to 95% of flies ingested eggs when exposed for 2 hours to canine faeces contaminated with *T. hydatigena* (Table 3). The maximum number of eggs recovered from an individual fly was over 5000. Several workers have observed cestode eggs in 'wild' flies. These reports are summarized in Table 4. The fly genera that appear to be important in the field include *Musca*, *Chrysomyia*, *Calliphora* and *Sarcophaga*. The greater part of this work has involved the examination for eggs of large numbers of flies trapped in the field. However, Lawson (1982) has

TABLE 4

Field observations on the internal carriage of cestode eggs by wild flies

Species of fly	Species of cestode egg found	Geographic location of observation	Reference
Musca domestica	*Taenia saginata*	Mombassa	Shircore (1916)
A variety of flies including *Chrysomyia megacephala* and *Musca sorbens*	*H. diminuta*	Guam	Harris and Down (1946)
Cristalis aeneus	Taeniid eggs	Bukhara Uzbekistan U.S.S R.	Sychevskaya and Petrova (1958)
M. domestica			
M. sorbens			
Musca stabulans			
Dasyphora asiatica			
Calliphora erythrocephala			
Chrysomyia albiceps			
Pollenia rudis			
Sarcophaga haemorrhoidalis			
20 spp. of synanthropic flies including:	*T. saginata*	Azerbaidzhan, U.S.S.R.	Nadzhafov (1967)
M. domestica			
M. stabulans			
C. erythrocephala			
Calliphora vomitoria			
Ch. albiceps			
Lucilia sericata			
C. haemorrhoidalis			
Sarcophagidae sp.			
Bercaea haemorrhoidalis	*Hymenolepis nana*		Markariants and Gadzhei (1968)
L. sericata	*Taeniidae* sp.		
Ravinia striata			
M. domestica vicina			

demonstrated by another method the potential of 'wild' flies to transfer eggs to the intermediate host. 'Wild' flies which had fed in the field on canine faeces contaminated with *T. hydatigena*, were administered to lambs grazed in a known taeniid tapeworm-free area. Control lambs were given flies which had been trapped after feeding on liver. It was found that only those sheep that had received flies that had contacted contaminated faeces were infected.

Nicoll (1911) made an important observation about the behaviour of flies with respect to tapeworm segments. He found that they selected proglottids in preference to moderately fresh faeces. By sucking at the surface of the proglottid the flies appeared to be able to pierce the tegument and extract eggs. These were later found in the intestine. No other reference to this behaviour

was reported in the literature studied, although the present authors have sometimes observed calliphorids apparently feeding on proglottids. If this behaviour occurs it could provide an efficient mechanism for the dissemination of clusters of eggs.

Estimates of the time that eggs remain in the gut of the fly differ considerably. Nadzhafov (1967) estimated that those of *T. saginata* remained there for 20 to 48 hours. Aleksander and Dansker (1935) reported that *M. domestica* excreted viable eggs for up to 27 hours. Round (1961) fed eggs of *T. saginata* to three species of fly. He found that *Chrysomyia chloropyga* and *Sarcophaga* sp. continued to excrete eggs for 11 days. *Chrysomyia albiceps* contained viable eggs for 18 hours and *C. chloropyga* for 24 hours. Nicoll (1911) found that flies retained tapeworm eggs from 2 to 3 days but in one case continued to excrete eggs of *T. pisiformis* for 14 days. Lawson (1982) found that eggs of *T. hydatigena* were excreted by *Calliphora* spp. between 5 and 48 hours after they had been ingested. A. C. G. Heath (personal communication) found the eggs of *T. hydatigena* were excreted over a 96 hour period.

The above evidence suggests that the relationship between the fly and taeniid eggs is mainly mechanical. However, the embryos of a few species of cestode hatch and develop in the gut or body cavity of insects that act as intermediate hosts. Species of tapeworm in which this occurs include *Choanotaenia infundibulum*, *Raillietina cesticillus* and *R. (Davainea) tetragona*, all of which are poultry tapeworms and use *M. domestica* as the intermediate host (Ackert, 1920; Reid and Ackert, 1937; Mönnig, 1956; Wardle and McLeod, 1952). *Hymenolepis carioca*, another poultry tapeworm uses *Stomoxys calcitrans*, the stable fly, as an intermediate host (Gutberlet, 1920).

Maggots generally feed on liquids, but various workers have shown that they can ingest particulate matter including helminth eggs. For example, Fontana and Severino-Brea (1961) found the eggs of *Ascaris lumbricoides*, *Trichuris* sp. and *E. granulosus* undamaged in the gut of maggots of *Lucilia* sp. and *Calliphora* sp. that had fed on contaminated faeces. Roberts (1934) reported that maggots of *M. domestica* and *Phaenicia (Lucilia) cuprina* can ingest *A. lumbricoides* eggs. Nicoll (1911) found that maggots of *M. domestica* could also ingest eggs of *T. pisiformis* although all the eggs inside the larvae appeared damaged. He also observed that pupae, which developed from larvae that had ingested eggs, contained no eggs. These observations were not confirmed by Roberts (1934). He fed eggs of *A. lumbricoides* to maggots and found that they persisted in a viable state through metamorphosis and were excreted in the faeces of the adult flies. It has yet to be determined whether or not tapeworm eggs ingested by maggots are disseminated in the guts of flies following metamorphosis.

It has been suggested that biting flies might transmit taeniids parenterally to the intermediate host (Urquhart, 1965). Injections of hatched oncospheres

of *T. pisiformis* and *T. saginata* have been shown to produce infections in rabbits and cattle, respectively (Leonard and Leonard, 1941; Froyd and Round, 1960). However, unhatched eggs were shown to have a very low infectivity to cattle when injected intravenously (Urquhart, 1965). The same results were observed for *E. granulosus*, *T. hydatigena*, *T. ovis* and *T. pisiformis* in sheep and rabbits, appropriately, following intramuscular injection of eggs (reviewed in Gemmell and Johnstone, 1977). It seems, therefore, that parenteral infection is not important in the natural environment.

Because flies carry so many pathogenic organisms, their dispersal behaviour has been considered to be important to public health and several extensive studies have been made. This work, which has generally involved mark–release–recapture experiments, has been reviewed by Schoof (1959) and Norris (1965). In the earlier studies, the flies were marked with coloured chalk or dye but in later studies, flies have been given radioactive tags by feeding them on solutions containing radioactive phosphorus (^{32}P).

One of the earliest investigations on the dispersion of flies was made by Copeman *et al.* (1911). They found that flies travelled between 1·3 km and 1·5 km in 48 hours or less and that the in-flight direction was associated with that of the prevailing wind. Parker (1916), investigating the dispersion of flies in a city in Montana, U.S.A., captured marked flies over 1·6 km and in one case 3·2 km from the release point, there being no evidence that wind direction was involved. In another series of experiments by Bishop and Laake (1921), in which a variety of species of marked flies were released in a rural area, the maximum distances travelled (in an unspecified time) were: *M. domestica*, 21 km; *Chrysomyia macellaria*, 24 km; *Phormia regina*, 18 km; *Lucilia sericata*, 1·6 km and *Sarcophaga* sp., 5 km.

Lindquist *et al.* (1951) tagged three species of flies with ^{32}P and recorded that the maximum distances travelled by these flies were *M. domestica* 19 km, *L.* (*Phaenicia*) *sericata* 6 km and *P. regina* 13 km. They observed that the majority were caught within 0·8 km of the release point within the first 24 hours. They could find no association between prevailing wind and dispersal. The above observations were substantially confirmed by Schoof *et al.* (1952) and Yates *et al.* (1952). They found that the majority of flies were captured between 0·8 km and 1.6 km from the release point within the first 24 hours. They also could find no evidence that wind direction and dispersal were associated. Macleod and Donnelly (1960) demonstrated that such barriers as 200 yards of water and a 90 yard wide wood did not impede the dispersal of *Lucilia caesar* and *C. erythrocephala*.

Schoof and Siverly (1954) pointed out that the distance that flies migrate is only one facet of their dispersal behaviour. They suggested that a second and perhaps more important factor was the manner in which the flies reached their destination. In order to gain some insight into this aspect, they released

^{32}P tagged flies and recaptured them, marked them with coloured dyes, released and recaptured them. Analysis of the results showed that the flies displayed a random dispersion from each secondary liberation site, with many reversals in the direction of movement. They concluded that "dispersion involves a random movement from the point of origin until the insect reaches a site that provides the necessary stimuli for a temporary cessation of migration" and that "the same sequence . . . occurs at each succeeding site until terminated by the death of the individual". Aggregation of flies at particularly favourable sites occurred because more flies were stimulated to stop there.

Macleod and Donnelly (1963) studied the dispersal and interspersal of a blowfly (*C. erythrocephala*) population. They released differently marked groups of flies from a number of points. The relative numbers recovered at the "home" release points and the other release points were used to assess the change in distribution. From their analyses, the authors concluded that flies exhibited two types of flight. Firstly, a unidirectional fast or sustained flight with an apparent length of under 15 minutes which was termed the dispersal or exodus flight. Secondly, there was a random type of flight (interspersal flight) involving little or no further dispersal. This type was open to interruption and its direction was independent of the preceding flight.

The above results suggest that if flies do transport taeniid eggs, the pattern of dispersion should conform closely to that which has been observed. Based on the time taken for eggs to be ejected from flies and the distances likely to be travelled prior to ejection, it seems probable that the majority will be deposited within 1·6 km of their point of origin. Indeed, the observed dispersal patterns and feeding habits of flies could account for both the radial uniformity and aggregation of cysts in the intermediate host reported by Gemmell and Johnstone (1976) and Gemmell *et al.* (1978). Individual flies retain eggs for long periods and perhaps fly long distances. Further studies may show that these long-distance flights are responsible for the 'wild' infections observed to occur on farms in the absence of an infected definitive host. In temperate and cold climates fly abundance and activity are seasonal (Oldroyd, 1964; Norris, 1965). It has yet to be determined whether or not egg dispersion also shows seasonal patterns and, if so, how this affects the dynamics of transmission.

(ii) *Diptera—other than Muscidae, Calliphoridae and Sacophagidae.* Ross (1929) reported that mosquito larvae were able to ingest and secrete undamaged the eggs of *Echinococcus* sp. He was unable to determine whether they were also present in the nymphal or imago stages. Supryaga (1972) found that the first to fourth larval stages of *Culex pipiens molestus* ingested oncospheres of *T. saginata*, but that the oncospheral membranes of many were destroyed. It is possible that intermediate hosts become infected with larval taeniids by drinking water containing mosquito larvae.

(iii) *Coleoptera*. After flies, beetles are the most popular candidates in the literature for disseminating eggs. Bílý *et al.* (1978) found that, apart from dung beetles (Scarabaeidae), the most common beetles involved are predatory groups (Carabidae and Histeridae). These latter are attracted to the faeces by moisture requirements and to feed on insect larvae. Fincher *et al.* (1970) measured the attraction of faeces of various animals for coprophagous beetles in three different habitats. The species within the three habitats (pasture near a swine farm, woodland habitat and dairy pasture) differed somewhat, but the most common species caught were *Phanaeus vindex*, *P. igneus*, *Onthophagus* spp. and beetles of the family Histeridae. Generally, the order of preference was for: pig, opossum, fox, cow, human, rat, racoon, horse, sheep, rabbit, dog and chicken faeces.

There are three possible ways in which beetles could transport helminth eggs from the faeces onto the pasture. They are (i) as an external contaminant, (ii) in the faecal balls and (iii) by ingestion and excretion. Bílý and Prokopic (1977) reported that beetles were involved in the passive transfer of helminth eggs. The coprophagous beetles, in particular, can carry them attached to their bodies for relatively long distances. They become attached to the beetle either while it is feeding or when emerging from dung. In some situations, dung pusher beetles may aid the dispersal of helminth eggs. Round (1961) suggested that when they remove and bury faeces they may spread eggs. Eggs may also be dispersed by dung-eating and carnivorous beetles. Bílý *et al.* (1978) showed that scarabid beetles ingest proglottids although any eggs ingested by beetles have to pass the mandibles. Miller (1961) examined these in detail and described the microscopic ridges that occur on the molar lobe of the mandibles. These act as the grinding instrument. Food is squeezed into a thin film and then ground between the two closely opposed molars. The largest particles in the gut were between 6 and 16 μm. Despite these findings, the evidence discussed below indicates that helminth eggs can survive passage through beetle mouth parts. Several workers have fed helminth eggs to beetles in order to determine whether or not they can survive the action of the mandibles. Miller *et al.* (1961) fed human faeces containing protozoan cysts and nematode eggs to beetles. Virtually no eggs and few cysts survived ingestation by *Canthon laevis* or *C. vigilans*. Only in the largest species, *Pinotus carolinus*, were helminth eggs regularly excreted intact and although many ascarid eggs were decorticated and crushed they retained their viability.

Mutinga and Madel (1981) have shown that coprophagous beetles of the genera *Onitis* and *Heliocopris* ingested *T. saginata* eggs which remained viable over a 10-day expulsion period. Silverman's (1954a) method was used to demonstrate that the majority of these expelled eggs were viable. Bílý *et al.* (1978) fed four beetle species with eggs of *T. saginata* in proglottids or mixed in humans faeces. Only a small number of viable eggs were subsequently found.

Lonc (1980b) found that as little as 1 hour exposure to excreta contaminated with eggs of *T. saginata* was sufficient to enable all the *Aphodius fimetarius* used in the experiment to acquire eggs both externally and internally. The maximum number of eggs in one beetle was 66. Viable eggs of *T. solium* have been observed over a period of 48 hours in coprophagous beetles (Zenkov, 1978), and leather beetles (*Dermestes* spp.) and their grubs have been found to contain viable *T. saginata* (Archipova, 1975).

The eggs of other tapeworm species are ingested and survive in beetles which act as intermediate hosts for many genera of cestodes of carnivorous and insectivorous vertebrates (Joyeux, 1920; Cram and Jones, 1929; Jones, 1930; Jones and Alicata, 1935; Joyeux and Baer, 1936; Horsfall and Jones, 1937).

Unfortunately, there appears to be little known about the dispersal behaviour of beetles. Bílý *et al.* (1978) state that *Aphodius* "may carry eggs of *T. saginata* to distant sites because they are known to cover long distances in their flight". Mutinga and Madel (1981) demonstrated that *Onitis* spp. and *Heliocopris anterior* fly to food sources at a maximum distance of 25 m. They pointed out, however, that they considered the flight range of these beetles under normal conditions to be far greater than indicated by their results. They therefore concluded that coprophagous beetles could act as efficient dispersal agents of *T. saginata* and could have an important role in the epidemiology of cysticercosis.

(iv) *Lepidoptera. Hymenolepis diminuta* can develop in the larvae of several moths including *Pyralis farinalis*, *Aglossa dimidiata*, *Aphornia gularis* and *Tinea granella* (Joyeux and Baer, 1936). Infection of the definitive host (rodents) occurs when infected larvae or imagos are eaten. Most lepidopteran larvae are herbivorous and must ingest eggs by accident when feeding. Disney (1975) noted the hatching of *Monopis rusticella* from dog faeces. This moth is also known to breed in bird nests, carrion, owl pellets, dried excrement and other refuse. Studies are required to determine whether lepidopteran larvae can transport taeniid eggs.

(v) *Hymenoptera.* Ants of the genera *Tetramorium* and *Pheidole* act as intermediate hosts of several species of *Raillietina*, the poultry tapeworm (Mönnig, 1956). Brian (1977) reported that *Lasius flavus* feeds on many soil animals including soft-bodied mites, beetle larvae and woodlice. *Lasius niger*, a larger and more aggressive ant, forages above and below ground and preys on several species of ant, beetle larvae, caterpillars, earwigs and woodlice. *Lasius alterius* and *Tetramorium caespitum* eat soil invertebrates, especially centipedes and wireworms, and *Myrmica* spp. eat aphids, fly larvae, adult flies and spiders. Laurence (1954) reports the presence on cow pats of ants of the genus Myrmica carrying fly larvae and adults. Elton (1932) observed that the trackways of wood ants (*Formica rufa*) varied from 7·3 to 73 m in length.

Dispersal of eggs by ants could, therefore, conform to the pattern that has been observed and they may contribute to the dispersal of cestode eggs not only by direct ingestion, but also by preying on insects that are themselves carrying cestode eggs.

(vi) *Siphonaptera and Phthiraptera.* Fleas and lice act as the intermediate hosts of several common species of tapeworms. Eggs of *Dipylidium caninum*, a common tapeworm of dogs, are swallowed by the non-parasitic, maggot-like larvae of several species of flea including *Ctenocephalides canis* and *Pulex irritans*. Also the biting louse, *Trichodectes canis*, can serve as an intermediate host of this tapeworm. The embryos hatch and penetrate the tissues where they develop into cysticercoids. The definitive host is infected by ingesting parasitized fleas and lice. Several hymenolepid tapeworms also utilize fleas. Joyeux and Baer (1936) record the development of *H. diminuta* in *Ceratophyllus fasciatus*, *C. wickharmi*, *Ctenocephalides canis* and *P. irritans*.

(vii) *Other insects.* Round (1961) demonstrated that some eggs of *T. saginata* ingested by the cockroach (*Blatta germanica*) were ejected intact within 24 hours in faecal pellets. The possible role of this synanthropic insect in *T. solium* cysticercosis of man requires investigation.

Hymenolepis diminuta and *H. microstoma* can develop in the earwig *Anisolabis annulipes* (Joyeux and Baer, 1936). Grasshoppers also act as intermediate hosts to several species of cestode. Cysticercoids of *Metroliasthes lucida* have been found in *Melanoplus* spp., *Chorthippus curtipennis* and *Paroxya clavuliger* (Jones, 1936). *Choanotaenia infundibulum* can develop in *Melanoplus femur-rubrum* (Wardle and McLeod, 1952). It seems unlikely that these insects would distinguish taeniid eggs from those of other cestodes and they may pass through them to be dispersed on the pasture.

(g) *Other arthropods.* The woodlouse *Oniscus* sp. acts as an intermediate host for *Taenia rotunda* larvae (Joyeux and Baer, 1936). Two species of cestode have been shown to develop in myriapods. These are *Anomotaenia constricta* in *Glomeris limbata* and *H. diminuta* in *Fontaria virginiensis* miriapods (Joyeux and Baer, 1936). It seems likely that isopods and myriapods also pick up eggs of cestodes for which they are not hosts. These may pass through the gut unharmed to be dispersed on the pasture.

Oribatid mites are well known as the intermediate hosts of a range of anoplocephalid cestodes of mammals including sheep and cattle. At least 30 species have been implicated. Zyromska-Rudzka (1974) summarized the experimental work of others and recorded that the cestodes *Moniezia benedeni* and *M. expansa*, tapeworms of sheep, goats and cattle, can infect five different species of mite. He also recorded the potential oribatid intermediate hosts of the cestodes *Thysaniezia giardi*, *Paranoplocephala mamillana*, *Anoplocephala magna*, *Anoplocephala perfoliata*, *Ctenotaenia ctenoides*, *Cittotaenia denticulata* and *Mesocestoides lineatus*.

Oribatid mites are common inhabitants of litter and soil. Wallwork and Rodriguez (1961), using Tullgren funnels, estimated their density on sheep and cattle pasture. The numbers of oribatids exceeded those of other animal groups. They were most dense in the organic matter on the pasture floor and in the grass above, comprising 85 and 51% of the animals extracted from sheep and cattle pastures, respectively. Zyromska-Rudzka (1974) found that pastures which had not been recently grazed by sheep showed the most abundant species composition.

There are no good reasons to suppose that oribatids would differentiate between different species of tapeworm eggs. Thus, they must be taken into consideration in the dispersal of taeniid eggs, although no dispersal behaviour has been cited in the literature.

(h) *Annelida.* Earthworms act as intermediate hosts of several species of cestode (Joyeux and Baer, 1936). *Amoebotaenia sphenoides* has been found in several oligochaetes including *Allolobophora foetida*, *A. chlorotica* and *Pheritima* sp. *Lumbricus variegatus* has been shown to act as a host for *Anomotaenia pyriformis*, *Haploparaxis crassirostris*, *H. furcigera*, and *Dilepis undula* have shown to utilize *Lumbricus terrestris* and *L. variegatus*.

Earthworms have been shown experimentally to ingest eggs of *T. saginata* when exposed to contaminated sludge (Jones, 1978) and soil (Lonc, 1980a). They excreted these eggs over a period of 72 hours. Two Russian workers have reported that taeniid eggs remain viable during passage through the earthworm gut. E. Szymacha (personal communication to Z. S. Pawlowski) observed that earthworms may effectively transport eggs of *T. saginata* and Zenkov (1978) showed that eggs of *T. solium* remained viable in earthworms for 22 hours. Archipova (1975) reported that 8 out of 44 earthworms gathered from natural habitats in the Moscow region contained eggs of *T. saginata*. Further studies are required to determine their role in the dispersal of taeniid eggs, particularly as they are a common food of some birds.

(i) *Mollusca.* The snail genera *Helix*, *Helicella* and *Cepaea* act as intermediate hosts for *Raillietina echinobothrida*, *R. tetragona* and *Davainea proglottina*. Slugs also act as intermediate hosts for several species of cestode. For example, *D. proglottina* will develop in several species of *Limax* and *Arion*, *Raillietina bonihi* can utilize *Malacolimax* sp., *Lehmannia* sp., *Arion* sp., *Arianta* sp. and *Helicigona* sp., and *Anomotaenia arionis* will develop in *Arion ater* slugs (Joyeux and Baer, 1936). Molluscs also appear to pick up the eggs of taeniids for which they are not intermediate hosts. For example, Archipova (1975) reported that slugs gathered in a natural habitat harboured large numbers of the eggs of *T. saginata*. Terrestrial molluscs are slow-moving herbivores. They probably ingest taeniid eggs only by accident and unless transported by birds, travel only short distances with them.

III. Host-related Factors

In the previous sections, emphasis was placed on the enormous egg output, rapid egg dispersion and the agents involved. Several host-related factors are involved in determining the infective pattern. Among the more important are innate and acquired resistance to infection as well as host-feeding patterns associated with livestock management. These are reviewed here in summary only as they have recently been the subject of extensive reviews.

A. INNATE RESISTANCE TO INFECTION

Little is known of the factors involved in innate resistance to larval tapeworm infections among sheep, cattle, pig and human populations. Factors that have been demonstrated to be important in *Taenia taeniaeformis* infections in rodents include strain, sex and age (see reviews by Smyth, 1969; Weinmann, 1970; Wakelin, 1978; Rickard, 1983; Rickard and Williams, 1982).

B. ACQUIRED RESISTANCE

It is now generally agreed that acquired resistance plays a central role in determining the infective pattern (see reviews by Gemmell and Soulsby, 1968; Gemmell and Macnamara, 1972; Gemmell, 1976c, 1978a; Gemmell and Johnstone, 1977; Flisser *et al.*, 1979; Williams, 1979, 1983; Gemmell and Lawson, 1982b; Rickard, 1983; Rickard and Williams, 1982).

Aspects of host immunity which have been identified as being important in determining the infective pattern, particularly of the sheep metacestodes, are summarized below.

(i) A variable degree of immunity to *T. hydatigena*, *T. ovis* and *T. saginata* is transferred maternally via colostrum (Gemmell *et al.*, 1969; Rickard and Arundel, 1974; Heath *et al.*, 1979a, b; Lloyd, 1979; Sutton, 1979).
(ii) The gradual development of full immune competence within the first few months of birth (Urquhart, 1961; Froyd, 1964; Gemmell *et al.*, 1968a).
(iii) The development of strong immunity within 14 days of the ingestion of the first eggs by immunologically competent animals (Gemmell *et al.*, 1968b) (Fig. 6).
(iv) The induction of resistance by as few as 10 to 50 eggs (Sweatman, 1957; Gemmell, 1969).
(v) The loss of acquired immunity within 9–12 months in the absence of eggs (Gemmell and Johnstone, 1981) (Fig. 6).

(vi) The possible induction of a "sterile" immunity from the ingestion of aged eggs (Gemmell, 1977).

(vii) The species-specific acquired immunity being considered of greater epidemiological significance than inter-specific interactions (Ermalova *et al.*, 1969; Varela-Díaz *et al.*, 1972; Gemmell and Johnstone, 1977).

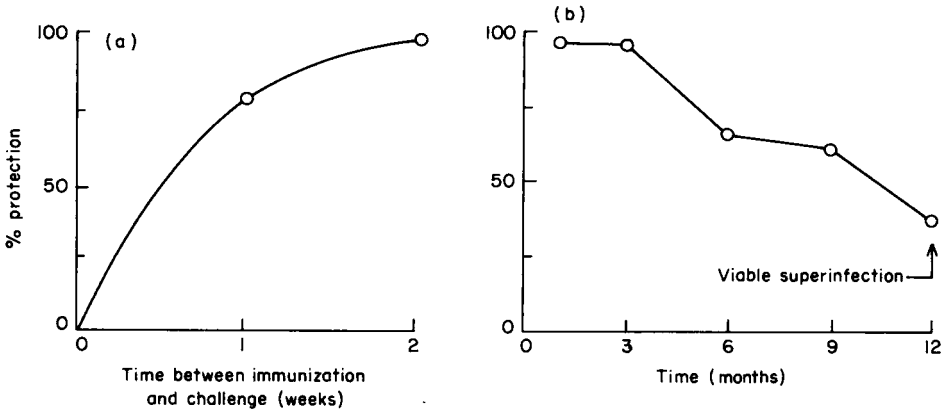

FIG. 6. Immune responses by sheep to eggs of *Taenia hydatigena*. (a) Time interval for the induction of immunity. (Data from Gemmell *et al.*, 1968b.) (b) Duration of immunity in the absence of impinging egg infections. (Data from Gemmell and Johnstone, 1981.)

C. FEEDING BEHAVIOUR

The behaviour of man and animals with respect to the faecal material both of their own and other species may influence their acquisition of parasites. For example, cattle normally avoid grazing around faecal material (Taylor, 1954, 1957), but under some conditions (e.g. drought) they have been observed to ingest human faeces. It has been suggested that this behaviour accounts for the increased prevalence of *T. saginata* infections observed in cattle in South Africa after periods of drought (Viljoen, 1937). Another explanation might be that immunity to superinfection was lost during the drought period. The pig is a natural scavenger and will readily ingest faeces and Viljoen (1937) reported cases in which pigs were actively encouraged to dispose of human faeces. He suggested that this activity may be linked with an observed high incidence of *T. solium* in these situations. With regard to man, Flisser *et al.* (1979) have suggested that the low infectivity of taeniid eggs (1 %) means that large egg doses may be required to cause *T. solium* cysticercosis. They considered that coprophagy may be important in transmission.

With regard to grazing behaviour and stock management, young animals such as sheep gradually learn to feed during the transition from sucking to

grazing behaviour. Thus, sheep that are reared from birth in egg-contaminated pasture usually ingest only small numbers of eggs before the onset of acquired immunity to superinfection (Gemmell, 1976b). However, if uninfected animals, which have reached grazing competence, are transferred onto egg-contaminated pasture, they frequently acquire heavy infections. If the pasture is very heavily contaminated and the animals ingest eggs in clusters, a 'cysticercosis storm' may ensue (Gemmell, 1976a). A similar phenomenon occurs if humans infected with *T. saginata* defaecate in cattle feed lots (Schultz *et al.*, 1969).

IV. The Infective Pattern

The infective pattern of a parasite within a host population is described by both the mean level of infection and its distribution within the population. Almost all the factors previously discussed in this review contribute towards the mean level of infection within the host population.

The distribution of parasites within their host populations is generally over-dispersed with the majority of hosts being lightly infected and only a few harbouring many organisms. The negative bionomial has been found to provide a good fit for the distribution of metazoan parasites within their hosts (Crofton, 1971a, b; Anderson, 1974, 1978b). Over-dispersion is generated by heterogeneity in the host and parasite populations as well as by spatial aggregation of the free-living infective stages in the environment. It occurs even under tightly controlled experimental conditions when host and parasite populations are as uniform as possible. In an experimental model of the infection of fish by the trematode *Transversotrema patialense* and a theoretical model of snail infection by miracidia, Anderson (1978a) and Anderson *et al.* (1978a, b) have demonstrated that the degree of over-dispersion (as measured by the variance to mean ratio) increases as the density of the parasite population increases.

Heterogeneity seems particularly well marked in the host/taeniid egg system. The variability in maturity within the egg-population results in considerable variation in infectivity. Eggs are distributed in clusters from the primary site of deposition by a number of mechanisms and agents. This results in enormous variations in the number of eggs that individual hosts ingest before the onset of immunity. The host population shows variation in such factors as genetic constitution, age, weight, sex, physiological condition and grazing behaviour. These give rise to heterogeneity in behaviour patterns, natural resistance and/or acquired immune responses.

Measurements from experimental situations have confirmed a significant degree of over-dispersion within the host/taeniid system. For example,

the values for the index of dispersion (Southwood, 1966) calculated from data for the infection of rabbits with *T. pisiformis* larvae (Coman and Rickard, 1977) showed significant aggregation. Similarly, Gemmell and Macnamara (1976) grazed lambs in the presence and absence of dogs infected with *T. hydatigena*. They determined the dispersion pattern by Fisher's (1954) and Morisita's (1962) indices of dispersion. These showed that the cyst counts were highly aggregated when the dogs were present, but when they were removed, the distributions began to conform more closely to the Poisson distribution.

V. Stability and Implications for Control

The stability of a biological system describes its ability to return to equilibrium after wide fluctuations in its population components. Although the response of ecosystems to external perturbations may be modelled theoretically (Silvert and Smith, 1981), Bradley (1972) suggests that stability can only be determined "by giving the system a push and seeing what happens". He argues that stability is an essential part of the description of host/parasite systems and that epidemiological studies should include the effects of attempting to disturb the population. The most practical way to do this is to measure the effects of trial control programmes on the system. The evidence from the few control projects against taeniid tapeworms appears to indicate that the stability of *E. granulosus* is much lower than that of the large taeniid tapeworms. Control programmes against the ovine strain of *E. granulosus*, such as those in Cyprus, Tasmania, Iceland, New Zealand and the Falkland Islands, have produced substantial reductions in prevalence, within relatively short time periods (reviewed in Gemmell, 1978a, 1979; Gemmell and Varela-Díaz, 1980). In contrast, similar dog-dosing programmes directed against *T. hydatigena* and *T. ovis* have been much less successful. A 4-year trial in Western Australia (White and de Chaneet, 1976) resulted in limited interruption of transmission with 'epidemic-type' outbreaks of *T. ovis* and *T. hydatigena* occurring throughout the trial. A similar result for *T. hydatigena* was recorded in New Zealand in the 35-year-old Styx Field-Trial (Gemmell, 1968, 1978b). During the same trial the prevalence of *T. ovis* actually increased.

The overall stability of a host/parasite system appears to be a product of the complex interaction of stabilizing and destabilizing forces (Anderson, 1978b). Several theoretical studies have implicated a variety of factors influencing stability (Crofton, 1971b; Bradley, 1972; Anderson, 1978b; Anderson and May, 1978; May and Anderson, 1978). These include, *inter alia*, overdispersion of parasites within the host population, immunological response of the host, time delays in reproduction and transmission, and reproduction of the parasite within the host.

All the above studies involved systems where the parasite induces mortality in the host population. This is not usually the case with host/taeniid systems where the adult parasite is well tolerated by the definitive host and the powerful immune response of the intermediate host prevents lethal levels of larval stages maturing. The details of the life cycles of the larger taeniid tapeworms and the hydatid organisms differ in several important respects and at present it is only possible to speculate as to the reason(s) for the differences in their stability. Gemmell and Lawson (1982a, b) have suggested that the high biotic potential in the definitive host, the short maturation time of the metacestodes and the rapid onset of immunity in sheep which persists under a high and wanes under a low infection pressure may all contribute towards stability in the larger taeniids. *Echinococcus multilocularis* also has some of the above characteristics, but little is known as yet as to how it would respond to control measures.

VI. Summary and Conclusions

The infective pattern of taeniids in their intermediate hosts is determined by the complex interaction of parasite- and host-related factors. Many of these have been examined in this review, but particular emphasis has been placed on environmental factors that affect the free-living egg. While number, infectivity and distribution of eggs are among the important factors determining the infective pattern, the dynamics of this aspect of transmission have been previously neglected.

The biotic potential of the large taeniid tapeworms is enormous. Eggs deposited on pasture are subjected to the microclimatic effects of the environment. They seem to tolerate a relatively wide temperature range. Heat damage does not appear to occur until about 38°C and they withstand freezing down to —30°C. However, they are rapidly killed by low humidity at all temperatures. Under more favourable conditions, they have finite life spans largely determined by the environmental temperature. At deposition, the egg population seems to be at various stages of maturity. Immature eggs appear to be able to mature under suitable environmental conditions and to become infective. They then age, and their infectivity to the intermediate host declines. Finally they lose the ability to be activated *in vitro* before eventually dying. At the population level, the changes in infectivity with time are determined by the relative stages of maturity within the egg population and the temperatures that it experiences. Under certain circumstances the infectivity of a given population may increase temporarily.

The movements and defaecation habits of the definitive host determine the primary site of egg deposition. However, evidence is accumulating

that considerable dispersion occurs almost immediately afterwards. Eggs have been shown to disperse up to 80 m within 10 days and there are indications that small numbers travel much further. Intermediate hosts, such as cattle and sheep, generally avoid grazing areas contaminated with faeces. Thus, dispersal enhances the chance of the eggs being ingested. The combination of high biotic potential and long-range dispersal means that individual infected definitive hosts can be responsible for infecting intermediate hosts over a very wide area.

Although various possible agents have been suggested, the mechanisms responsible for disseminating eggs are still uncertain. The movement of freshly deposited proglottids leads to some dispersal, but this cannot account for the patterns that have been observed. It also seems unlikely that wind or water (except sewage systems) are important. It must therefore be assumed that animals are involved. The list of possible animal agents is extensive, but the evidence suggests that some groups are likely to be more important than others. Of the vertebrates, birds should be seriously considered. Their role in the dissemination of *T. saginata* is well documented. Of the invertebrate groups implicated in egg dispersion, arthropods seem to be important candidates. One good reason for seriously considering them is the fact that they appear to have had a close association with cestodes during evolution. Most members of the orders Proteocephala and Pseudophyllidea use arthropods as intermediate hosts. Within the Cyclophyllidea, almost all families for which the life cycle is known, except Taeniidae, parasitize arthropods. In this context, it would seem probable that taeniids also developed an association with arthropods using them as transport hosts. The taeniid egg is morphologically similar to that of many other cyclophyllideans and there seems good reason to suggest that arthropods could not distinguish between them.

The habitat and habits of many terrestrial insects ensures close contact with taeniid eggs. The three most likely groups include flies, beetles and ants. Many fly species use faeces for both feeding and breeding, and can ingest and excrete taeniid eggs. Behavioural studies suggest that they can generate the observed egg-dispersal pattern. The possibility that beetles disperse taeniid eggs must also be considered. This is because several species of carnivorous and dung beetles contact faeces, and beetles act as intermediate hosts for some cestodes. There appears to have been no reports of ants carrying taeniid eggs, but there is a strong possibility that they pick them up during foraging, directly or indirectly from other arthropods. Other invertebrate groups that almost certainly ingest taeniid eggs include oribatid mites, gastropod molluscs and earthworms. These animals are all slow moving and, unless they are themselves carried by birds, it seems improbable that they could generate the observed pattern of egg dispersal.

Although the high biotic potential of taeniid tapeworms often results in the rapid accumulation of large numbers of eggs on the pasture, efficient host-regulatory mechanisms operate to prevent hyperinfection. All mammalian hosts studied have shown various degrees of innate resistance and strong acquired immunity.

There is considerable heterogeneity within both the egg and the intermediate host populations. Eggs vary widely in their infectivity and intermediate host populations show a wide range of resistance to infection. There is also spatial aggregation of eggs within the environment. The combination of these factors gives rise to over dispersion of the larval population within its host population.

In the large taeniids the combination of such factors as high biotic potential, short maturation time for the larval phase, and strong host immunity may ensure their considerable stability. The apparent resistance of the equilibria of these systems to external perturbations has meant that most control programmes have been unsuccessful. In contrast, control programmes against *E. granulosus* in sheep have shown marked reductions in transmission. As yet it is not possible to predict how a particular transmission situation will respond to perturbation but we agree with D. J. Bradley that the only way to determine how a parasite will respond to control is to implement field trials as a prerequisite to the introduction of a formal control programme.

This review has attempted to show that the transmission process among Taeniidae is highly complex and involves a number of potential agents. The relative importance of these in the 'on-farm' situation has yet to be ascertained.

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Fig. 4 is reproduced from the *Bulletin of the World Health Organization*, Vol. 56, No. 3, with kind permission of the editor.

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Lipid Metabolism in Parasitic Helminths

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I. Lipid Composition

Data on lipid composition of parasitic helminths have been continuously changing due to the evolution of advanced elaborate methods of incubation, saponification, extraction, qualitative and quantitative determinations using column, thin-layer and gas–liquid chromatography, autoradiography and infra-red, ultraviolet and mass spectrometry. Most of the data reported in this review are the result of modern techniques and derive from studies published within the last 15 years. However, some old data which are of historical value and still considered valid are also cited.

The nomenclature of lipids has been inconsistent. Numerous classifications have been reported from organic chemistry and biochemistry textbooks and applied in studies of lipid chemistry and biochemistry of parasitic helminths. In this review, and for the sake of minimizing confusion, we have denoted each lipid by its name without ascribing it to any particular classification.

A. ADULT CESTODES

In the first study on the lipid composition of cestodes, Faust and Tallquist (1907) detected palmitic (C_{16}), stearic (C_{18}) and oleic ($C_{18:1}$) acids as well as cholesterol in the pseudophyllidean cestode *Diphyllobothrium latum*. About three decades later Totterman and Kirk (1939) identified phosphatidylethanolamine (cephalin) and phosphatidylcholine (lecithin) in the same parasite. In another pseudophyllidean, *Spirometra mansonoides*, Meyer *et al.* (1966) determined fatty acids, cholesterol, cholesterol esters and phospholipids as being the most common lipids in both adult and larval stages. A large amount of polyunsaturated fatty acids were identified in the free as well as bound acids. von Brand (1933) demonstrated in *Moniezia expansa* the presence of stearic (C_{18}) and oleic ($C_{18:1}$) acids and some higher hydroxy fatty acids. Another peculiar finding with *Moniezia* was the unique detection of small amounts of ketocholesterol by Thompson *et al.* (1960). In addition, substantial amounts of phospholipids and glycolipids (23 and 28·5% respectively) were found in the lipids of *Moniezia expansa* (Shorb and Shorb, 1966) and also a high cholesterol concentration (85% of unsaponifiable lipids; Thompson *et al.*, 1960). The glycolipid of *M. expansa* is a galactose-containing compound rather than the more common glucose-containing compound (Kent *et al.*, 1948).

Smorodinstrev and Bebeshin (1939) observed significant unsaturation in the fatty acids of *Taenia saginata* because their iodine number was higher than that of oleic acid ($C_{18:1}$). Another study on the same parasite was conducted by Čmelik and Bartl (1956) who fractionated, by paper chromatography,

the phospholipids of *T. saginata* and found them to consist chiefly of phosphatidylcholine and phosphatidylinositol. They also identified saturated fatty acids (C_{16} and C_{18}) as well as unsaturated fatty acids with one, two, three, four and five double bonds in the free fatty acid and neutral lipid fractions. Reid (1942) examined the ether-extractable lipids of *Raillietina cesticillus* and found them to be 15·5% of the dry weight of the worm, but later, and probably due to more sensitive techniques, Botero and Reid (1969) reported a higher level in the same species—20·1–21·6% lipids. They also observed that linoleic acid ($C_{18:2}$) was the most abundant fatty acid in the tissues of the parasite, a result probably related to the fact that the host fowls ingested high quantity of this acid in their diet.

The lipids of *Taenia taeniaeformis* have been extensively studied. Thompson *et al.* (1960), for instance, demonstrated in this parasite that cholesterol accounted for 98% of the unsaponifiable lipids. The sterol predominantly exists as free sterol rather than sterol ester (von Brand *et al.*, 1965). McMahon (1961) observed that the total phospholipids in both adults and larvae of *T. taeniaeformis* were quantitatively made up of ethanolamine, serine, choline, glycerol and sphingosine.

Probably because of the ease in raising it in the laboratory, adult *Hymenolepis* spp. and particularly *H. diminuta* were used extensively as a model for lipid analyses and metabolism in the 1960s. Contemporary techniques of thin-layer chromatography (TLC), gas–liquid chromatography (GLC) and spectrophotometry were applied to this parasite and a number of important findings were reported. For example, Roberts (1961) found that 34·6% of dry tissues of *H. diminuta* were lipids (see Table 1). These lipids, according to Fairbairn *et al.* (1961), were 9·2% unsaponifiable, mainly cholesterol (6·8% in total lipids), and 70% free fatty acids; seven phospholipids were identified by Fairbairn *et al.* (1961) and Cain *et al.* (1977) and nine by Webb and Mettrick (1975) (see Table 3). Cardiolipins and glycolipids were also detected (Fairbairn *et al.*, 1961; Harrington, 1965; Ginger and Fairbairn, 1966a). From the latter, two glucose cerebrosides were separated (Ginger and Fairbairn, 1966a). Harrington (1965) and Ginger and Fairbairn (1966a) found that most of the bound fatty acids of *Hymenolepis citelli* and *H. diminuta* were unsaturated despite the fact that their habitat, the rat intestine, was deficient in oxygen which is a prerequisite for the biosynthesis of unsaturated fatty acids in other animals (Goldfine and Bloch, 1963). More than twenty fatty acids were detected in the phospholipids and neutral lipids of *H. citelli* and *H. diminuta* (Harrington, 1965; Ginger and Fairbairn, 1966a) of which the C_{18} acids were predominant. For example, the linoleic ($C_{18:2}$) acid of *H. citelli* constituted 9% of the fatty acids in the phospholipids and 40% in the neutral lipids. The neutral lipids of *H. diminuta* accounted for 75% of the total fat, thus emphasizing the predominance of unsaturated fatty acids in

TABLE 1

Total lipid content of mature stages of parasitic helminths

	Fresh tissue (%)	Dry tissue (%)	Reference
Cestodes			
Taenia saginata	3·8	31·1	Čmelik and Bartl (1956)
Taenia saginata	3·3		Cicchini *et al.* (1976a)
Taenia taeniaeformis	3·8	10·6	McMahon (1961)
Hymenolepis diminuta		34·6	Roberts (1961)
Hymenolepis diminuta	5·8	30·9	Ginger and Fairbairn (1966a)
Hymenolepis diminuta	5·3		Mettrick and Canon (1970); Webb and Mettrick (1975)
Hymenolepis diminuta	5·1	21·2	Harrington (1965)
Hymenolepis citelli	3·9	16·1	Harrington (1965)
Spirometra mansonoides		24	Meyer *et al.* (1966)
Thysaniezia giardi		15·8–41·3	Singh *et al.* (1977)
Calliobothrium verticillatum		25	Beach *et al.* (1973)
Trematodes			
Fasciola hepatica	1·9	12·2	Weinland and von Brand (1926)
Fasciola hepatica	2·4		Hrzenjak and Ehrlich (1975)
Fasciola gigantica	2·8	12·9	Goil (1958)
Paramphistomum explanatum	1·2	4·5	Goil (1958)
Gastrothylax crumenifer	0·4	1·4	Goil (1958)
Gastrothylax crumenifer	3·3	10·4	Yusufi and Siddiqi (1976)
Schistosoma mansoni		34·2	Smith and Brooks (1969)
Diclidophora merlangi	4·9		Arme (1975)
Paramphistomum microbothrium	2·5		Hrzenjak and Ehrlich (1975)
Eurytrema pancreaticum		15·7	Vykhrestyuk and Yarygina (1975)
Paramphistomum cervi		4·8	Vykhrestyuk and Yarygina (1975)
Gigantocotyle explanatum	7·1	34·4	Yusufi and Siddiqi (1976)
Cotolyphoron cotolyphorum	8·6	27·3	Yusufi and Siddiqi (1976)
Echinostoma malayanum	9·7	39·0	Yusufi and Siddiqi (1976)
Fasciolopsis buski	8·9	50·4	Yusufi and Siddiqi (1976)
Isoparorchis hypselobagri	2·4	29·5	Yusufi and Siddiqi (1976)
Gastrodiscoides hominis	5·2	14·5	Yusufi and Siddiqi (1977)
Echinostoma revolutum	2·5	15·0	Fried and Boddorff (1978)
Lobatostoma ringens	6·4		Halton and Hendrix (1978)
Haematoloechus medioplexus		12·8	Cain and French (1975)

TABLE 1 (*continued*)

	Fresh tissue (%)	Dry tissue (%)	Reference
Nematodes			
Porrocaecum decipiens		3·5	Fairbairn (1958)
Ascaris lumbricoides	1·3–1·6		Cavier *et al.* (1958); Monteoliva (1960)
Ascaris lumbricoides		5·9	Richard and Dodin (1968)
Ascaris lumbricoides	1·8		Ehrlich and Hrzenjak (1975)
Ascaridia galli	1·2–2·5		Monteoliva (1960); Shorb and Shorb (1966); Fulk and Shorb (1971)
Nippostrongylus brasiliensis		11·9	Roberts and Fairbairn (1965)
Strongyloides ratti		10·0	Barrett (1968)
Mecistocirrus digitatus		10·4	Vykhrestyuk and Yarygina (1975)
Dirofilaria immitis	2·1		Hutchison *et al.* (1976)
Dioctophyma renale		1·0	von Brand (1957)
Heterakis gallinae	1·5–1·6		Shorb and Shorb (1966)
Stephanurus dentatus	2·4–4·4		Shorb and Shorb (1966)
Tylenchorhynchus claytoni		39·7	Krusberg (1967)
Aphelenchoides ritzemabosi		10·6	Krusberg (1967)
Ditylenchus spp.		23–38	Tracey (1958); Krusberg (1967)
Tylenchus semipenetrans		30	Van Gundy *et al.* (1967)
Meloidogyne spp.		40–46	Krusberg *et al.* (1973)
Acanthocephalans			
Macracanthorhynchus hirudinaceus	0·9–1·7		Beames and Fisher (1964)
Moniliformis dubius	4·2–7·2		Beames and Fisher (1964)

Hymenolepis spp. and confirming earlier reports (Warren and Daugherty, 1957) which had revealed that such acids were very abundant in *H. diminuta*. With the cerebrosides, the most commonly found fatty acids, and in equal amounts, were $C_{16:0}$, $C_{18:0}$ and $C_{24:1}$. On the other hand, the free fatty acids of *Hymenolepis* spp. displayed predominant saturation in contrast to the prominent unsaturation in the bound acids (Harrington, 1965; Ginger and Fairbairn, 1966a); and, according to Ginger and Fairbairn (1966a), triacylglycerols were the most abundant component of the neutral lipids, followed by free fatty acids, then mono- and diacylglycerols. Cholesterol is the only sterol to be found in *Hymenolepis* (Fairbairn *et al.* 1961; Frayha and Fairbairn, 1968).

Ginger and Fairbairn (1966b) and also Kilejian *et al.* (1968) concluded that the lipids of *H. diminuta* were very similar to those of the host environment, but remarked that some degree of control of lipid concentration by the worm was evident. Mukhin (1973) found that chemical constituents of *Hymenolepis* antigens contained, among other chemicals, lipids and glycolipids.

Buteau *et al.* (1969) determined the fatty acid composition of *Poecilancistrium caryophyllum* and *Dasyrynchus giganteus* from the bull shark, *Thysanocephalum thysanocephalum* from the tiger shark and *Grillotia simmonsi* from the nurse shark. They found that $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{22:6}$ were the most common acids. Both sharks and cestodes contained identical polyunsaturated C_{20} and C_{22} fatty acids. On the other hand, distinct differences existed in C_{16} and C_{18} acids.

B. METACESTODES

Most of the work on the lipid composition of larval cestodes is confined to *Echinococcus granulosus*. Early studies by Flössner (1924, 1925) and Coutelen (1931a) revealed the presence of acetic, propionic, valeric, succinic and higher fatty acids in the hydatid cyst fluid. Later, Čmelik (1952) reported a low lipid content (1·3% of dry tissue) of the hydatid cyst membranes consisting chiefly of cholesterol. In recent studies, cholesterol was also demonstrated to be the only sterol present in the larval stages of *E. granulosus*, *E. multilocularis* and *Taenia hydatigena* constituting 3–6 mg per g wet weight of these parasites (Frayha, 1971). This cholesterol was completely derived from the hosts pool of sterol (Frayha, 1968). Digenis *et al.* (1970a) identified 17 fatty acids from the protoscoleces (of hydatid cysts) of *E. granulosus* and found $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:0}$, $C_{20:4}$ the most abundant. More extensive analysis of the lipid composition of the protoscoleces and hydatid cyst fluid of *E. granulosus* were the studies of Frayha *et al.* (1980) and Frayha and Haddad (1980) which revealed the presence of the seven major classes of lipids, namely phospholipids, fatty acids, cholesterol, cholesterol esters, mono-, di- and triacylglycerols. The phospholipids were further fractionated and the following were present in substantial amounts in the protoscoleces: phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine, phospatidylinositol, phosphatidylserine and sphingomyelin (see Table 4). McManus and Smyth (1978) showed that the amount of lipids varied between different species of *Echinococcus* and even between different strains of the same species. For example, the protoscoleces of *E. multilocularis* had significantly more lipids than those of *E. granulosus*, and the protoscoleces of the horse strain of *E. granulosus* had more lipids than those of the sheep strain (Table

2). The lipid contents of the antigens extracted from *E. granulosus* cyst fluid constituted 27·5% of the dry weight (Senutaite and Senutaite, 1977a).

TABLE 2

Total lipid content of immature stages of parasitic helminths

	Fresh tissue (%)	Dry tissue (%)	Reference
Cestodes			
Echinococcus granulosus			
cyst membrane		1·3	Čmelik (1952)
cyst fluid	0·02		Frayha and Haddad (1980)
protoscoleces	2·0	13·6	Agosin *et al.* (1957)
protoscoleces (horse strain)		10·8	McManus and Smyth (1978)
protoscoleces (sheep strain)		8·8	McManus and Smyth (1978)
protoscoleces	1·05	10·5	Frayha and Haddad (1980); Frayha *et al.* (1980)
Echinococcus multilocularis protoscoleces		16·1	McManus and Smyth (1978)
Taenia hydatigena			
cysticerci membrane		18·2	Kassis and Frayha (1973)
cysticerci fluid		4·7	Kassis and Frayha (1973)
Taenia taeniaeformis (*Cysticercus fasciolaris*)	2·3	6·9	McMahon (1961)
Spirometra mansonoides		16	Meyer *et al.* (1966)
Nematodes			
Ancylostoma caninum larva		39	Clark (1969)
Cooperia punctata larva		19	Eckert (1967)
Porrocaecum decipiens larva		3·5	Fairbairn (1958)
Trichinella spiralis larva		5·5–9·1	von Brand *et al.* (1952) Castro and Fairbairn (1969)
Eustrongylides ignotus	1·1	4·4	von Brand (1938)
Ascaris lumbricoides ova		36	Fairbairn (1955b)
Nippostrongylus brasiliensis larva		15–20	Wilson (1965)
Meloidogyne javanica larva		30	Van Gundy *et al.* (1967)
Meloidogyne arenaria larva		48	Krusberg *et al.* (1973)
Acanthocephalan			
Polymorphus minutus cystacanth		28·2	Barrett and Butterworth (1971)

The lipids of the larvae (*Cysticercus fasciolaris*) of *Taenia taeniaeformis* were also extensively investigated. Salisbury and Anderson (1939), by applying various solubility and extraction procedures, found in this parasite that the lipids consisted mainly of phospholipids, cholesterol and cerebrosides. They also identified the water soluble hydrolytic products of phosphatides which constituted 30% of the lipids and represented a mixture of phosphatidylcholine and phosphatidylethanolamine. In addition they isolated palmitic (C_{16}), stearic (C_{18}) and arachidic (C_{20}) fatty acids. From the same parasite, Lesuk and Anderson (1941) separated cerebrosides from phosphatides and identified dihydrosphingosine in the former and hydrolecithin in the latter. The nature of the carbohydrate moiety of the cerebroside was galactose instead of the commonly found glucose. McMahon (1961) reported that the index ratio of the phosphatidylcholine to phosphatidylethanolamine in this cestode was one-third the value found by Salisbury and Anderson (1939).

In their study on the lipids of the cysticerci of *T. hydatigena*, Kassis and Frayha (1973) separated the cysticerci into tissue and fluid fractions and determined the lipids of each fraction. The phospholipids identified were lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine. Mono-, di- and triacylglerols were also present and the bound fatty acids were identified and quantified by GLC. The even carbon and saturated fatty acids were the most common although a considerable degree of unsaturation of free and bound acids was observed. Palmitic (C_{16}) and stearic (C_{18}) acids were always found in the highest concentrations. 11, 14-Eicosadienoic acid ($C_{20:2}$) was present in high concentrations in the triacylglycerols of the fluid fraction, but for unknown reasons was totally absent from the tissue fraction. It was postulated that the unsaturated acids were the result of chain-lengthening of absorbed unsaturated fatty acids.

C. ADULT TREMATODES

The lipids of some trematodes have also been thoroughly studied. The early work of von Brand (1928) revealed that phospholipids of adult *Fasciola hepatica* constituted 30% of its total lipids. These phospholipids were later identified by Hrzenjak and Ehrlich (1975) as phosphatidylserine, lysophosphatidylcholine, sphinogomyelin, lysophosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine. In addition, two cerebroside fractions, cerebroside esters, ceramides, two sulphatide fractions and four globosides were determined in the polar lipids. Cholesterol and cholesterol esters were also identified. This was the first report of globosides in helminths and they were thought to be involved in immunological

reactions. As a matter of fact, Senutaite and Senutaite (1977b) found that the lipid contents of the antigens extracted from *F. hepatica* were 1·3% of the dry weights. El-Hehyawi (1969) analysed the lipid fractions of *F. gigantica* and found them to be 23% unsaponifiable lipids, 4% saturated fatty acids, 15% unsaturated fatty acids, 3% glycerol and 20% unidentified lipids. Goil (1964) analysed the lipids of the buffalo trematode *Gastrothylax crumenifer* and reported that a strikingly high phospholipid percentage (16%) was determined from the total lipids. The total lipids were, however, extremely low accounting for 1·4% of dry tissue compared with a high lipid content of 34·2% dry weight in adult *S. mansoni* (Smith and Brooks, 1969), and 34·4% in adult *Gigantocotyle explanatum* (Yusufi and Siddiqi, 1976). In *S. mansoni*, Smith and Brooks (1969) identified only cholesterol in the unsaponifiable lipid fraction and found it to be 90% of the total sterol fraction. On the other hand, only small amounts of cholesterol esters were detected in this parasite, and no cerebrosides (Meyer *et al.*, 1970). In the fatty acids of *S. mansoni*, arachidonic ($C_{20:4}$) acid was detected in substantial amounts (Smith *et al.*, 1969); polyenoic acids were also detected by Fripp *et al.* (1976) and found to be different from those of the host series. These findings are in contrast to that of Buteau *et al.* (1969) on sharks' cestodes. Alving *et al.* (1974) extracted from adult *S. mansoni* protein-free lipids which were mainly composed of glycolipids, small amounts of phospholipids and an unidentified pigment. These lipids were capable of inducing a complement-dependent immune response against adult worms during the course of schistosomiasis in monkeys. On the other hand, Carlier *et al.* (1978) detected no lipids in the circulating IgM antigen in human and experimental *S. mansoni* infections. Shaw and Erasmus (1977) found that lipid bodies appeared in adult *S. mansoni* at 10–12 days of culture, and Cesari and Marhiani (1978) concluded in a series of experiments that the agglutinating activity associated with the membranes of *S. mansoni* was mediated by phospholipids rather than by proteins.

Arme (1975) found that the total lipids of *Diclidophora merlangi* were 4·9% of fresh weight. The major lipid components were phospholipids, followed by triacylglycerols, cholesterol and cholesterol esters. Vykhrestyuk and Yarygina (1975) reported that the total lipids of the cattle trematodes *Eurytrema pancreaticum* and *Paramphistomum cervi* were 15·73 and 4·7% of the dry weight respectively. The total lipids of the two worms consisted of 24·72 and 49·67% phospholipids and 75·28 and 50·33% neutral lipids respectively. The neutral lipids were identified as cholesterol, triacylglycerols, pigment, di- and monoacylglycerols, ascarosides and fatty acids. The total lipids of *Paramphistomum microbothrium* were found by Hrzenjak and Ehrlich (1975) to be 2·5% wet weight. The polar lipids consisted of three globoside fractions, phosphatidylserine, lysophosphatidylcholine, sphingomyelin, lysophosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, three sulpha-

TABLE 3

Phospholipid and glycolipid of mature stages of parasitic helminths

	% Phospholipids of total lipids	Phosphatidic acid	Lysophosphatidic acid	Phosphatidylethanolamine (Cephalin)	Phosphatidylserine	Lysophosphatidylethanolamine	Phosphatidylcholine (Lecithin)	Lysophosphatidylcholine	Phosphatidylinositol	Sphingomyelin	Cardiolipin	Plasmalogen	Glycolipids (Cerebrosides)	Reference
Cestodes														
Hymenolepis diminuta	26			+	+		+	+	+		+	+	+	Fairbairn *et al.* (1961); Harrington (1965); Ginger and Fairbairn (1966a)
Hymenolepis diminuta	22–37			+	+	+	+		+	+	+			Cain *et al.* (1977)
Hymenolepis diminuta		+	+	+	+		+	+	+		+		+	Webb and Mettrick (1971, 1973, 1975)
Taenia taeniaeformis	50			+	+		+				+		+	McMahon (1961)
Spirometra mansonoides	53·4			+	+		+		+		+		+	Meyer *et al.* (1966)
Calliobothrium verticillatum				+	+		+				+			Beach *et al.* (1973)
Taenia saginata	10·1													Cicchini *et al.* (1976a)
Moniezia expansa	23												+	Kent *et al.* (1948); Shorb and Shorb (1966)

Trematodes													
Fasciola hepatica			+	+	+	+	+	+	+	+	+	+	Hrzenjak and Ehrlich (1975); Chappell (1980)
Paramphistomum microbothrium			+	+	+	+	+	+	+	+		+	Hrzenjak and Ehrlich (1975)
Cotolyphoron cotolyphorum			+	+		+	+	+	+	+			Yusufi and Siddiqi (1976)
Gastrothylax crumenifer			+	+		+	+	+	+	+			Yusufi and Siddiqi (1976)
Gygantocotyle explanatum			+	+		+	+	+	+	+			Yusufi and Siddiqi (1976)
Paramphistomum cervi	49·7												Vykhrestyuk and Yarygina (1975)
Eurytrema pancreaticum	24·7												Vykhrestyuk and Yarygina (1975)
Echinostoma malayanum			+	+		+	+	+	+	+			Yusufi and Siddiqi (1976)
Fasciolopsis buski			+	+		+	+	+	+	+			Yusufi and Siddiqi (1976)
Isoparorchis hypselobagri			+	+		+	+	+	+	+			Yusufi and Siddiqi (1976)
Clonorchis sinensis		+	+	+		+	+	+	+				Lee *et al.* (1977a)
Gastrodiscoides hominis	12·6		+	+		+	+	+	+	+			Yusufi and Siddiqi (1977)
Schistosoma mansoni	36–37		+	+	+	+	+	+	+	+	+		Smith and Brooks (1969); DiConza and Basch (1976); Meyer *et al.* (1970); Rumjanek and Simpson (1980)
Nematodes													
Nippostrongylus brasiliensis	20												Barrett (1968)
Mecistocirrus digitatus	23·7												Vykhrestyuk and Yarygina (1975)
Ascaris lumbricoides	38·5		+	+	+	+	+	+	+	+	+		Rogers and Lazarus (1949); Fairbairn (1955a, 1956); Beames (1964); Ehrlich and Hrzenjak (1975)

TABLE 3 (*continued*)

	% Phospholipids of total lipids	Phosphatidic acid	Lysophosphatidic acid	Phosphatidylethanolamine (Cephalin)	Phosphatidylserine	Lysophosphatidylethanolamine	Phosphatidylcholine (Lecithin)	Lysophosphatidylcholine	Phosphatidylinositol	Sphingomyelin	Cardiolipin	Plasmalogen	Glycolipids (Cerebrosides)	Reference
Ascaris suum					+				+	+	+		+	Matausic *et al.* (1976)
Ascaridia galli	40–72			+		+	+						+	Shorb and Shorb (1966); Parshad and Guraya (1977a)
Dirofilaria immitis				+	+		+		+	+		+		Hack *et al.* (1962); Hutchison *et al.* (1976)
Setaria cervi		+		+			+	+		+	+			Ansari *et al.* (1973)
Acanthocephalans														
Centrorhynchus corvi	11–14													Parshad and Guraya (1977b)
Macracanthorhynchus hirudinaceus	46–47			+	+		+		+	+		+		Beames and Fisher (1964)
Moniliformis dubius	9–19			+	+		+		+	+		+		Beames and Fisher (1964)

tide fractions, phosphatidylethanolamine, two cerebrosides, cerebroside esters and ceramides. Cholesterol and cholesterol esters were also identified, so was cardiolipin. This is the second report of globosides in helminths parasites, the first being in *F. hepatica* by the same authors.

Yusufi and Siddiqi (1976) detected high triacylglycerol concentrations (13·2–52·6%) in the total lipids of the six trematodes they examined. Kanwar and Agrawal (1977) studied the chemical composition of the vitelline glands of *Diplodiscus amphicrus*. Vitelline globules developed in the cytoplasm and had a duplex nature, with an outer rim consisting of lipids, carbohydrates, proteins and some traces of ribonucleic acid (RNA), and medullary region rich in proteins with a small amount of RNA. Four sterols, namely cholesterol, cholestane and two unidentified sterols were detected in *Echinostoma revolutum* (Barrett *et al.*, 1970a).

D. LARVAL TREMATODES

Except for *S. mansoni* larvae, little work has been done on the lipid composition of immature stages of trematodes. Ginetsinskaya (1961) observed the appearance of fat droplets in the early development of cercariae of *Cotylurus brevis*. When the cercariae were completely formed the fat droplets displayed their highest concentration and then gradually disappeared from some parts of the cercariae at the time of their emergence from the snail. Cheng and Snyder (1962) and Cheng (1963) reported that free fatty acids were the major lipid component in cercariae of *Glypthelmins pennsylvaniensis*. McManus *et al.* (1975) detected glycolipids of undetermined nature in the larval trematode *Microphallus similis*.

The remaining reports on the lipids of immature trematodes are confined to *S. mansoni* and *S. japonicum*. Kent (1963) lyophilized the cercariae of *S. mansoni* and found that 19% of their dry weight was lipids. Smith *et al.* (1966) identified four major lipid classes in this parasite, namely fatty acids, cholesterol, cholesterol esters and phospholipids, and, when DiConza and Basch (1976) grew *S. mansoni* sporocysts *in vitro*, they observed some phospholipid accumulation in their tissues. The phospholipids were identified by Smith *et al.* (1971) in the eggs of *S. mansoni* as phosphatidylethanolamine, phosphatidylserine, lysophosphatidylethanolamine, phosphatidylcholine and sphingomyelin. The same phospholipids, except for phosphatidylserine and lysophosphatidylethanolamine, were identified in *S. japonicum* eggs (Smith *et al.*, 1977). In addition, cardiolipin which was found in *S. mansoni* eggs was not detected in those of *S. japonicum*. The fatty acid composition of *S. japonicum* eggs varied from C_{12} to C_{26} carbons and contained 0 to 6 double bonds (Smith *et al.*, 1977; Brown *et al.*, 1977). Brown *et al.* (1977) reported

TABLE 4

Phospholipids and glycolipids of immature stages of parasitic helminths

	% Phospholipids of total lipids	Phosphatidic acid	Lysophosphatidic acid	Phosphatidylethanolamine (Cephalin)	Phosphatidylserine	Lysophosphatidylethanolamine	Phosphatidylcholine (Lecithin)	Lysophosphatidylcholine	Phosphatidylinositol	Sphingomyelin	Cardiolipin	Plasmalogen	Glycolipids (Cerebrosides)	Reference
Cestodes														
Taenia taeniaeformis larvae	47·0			+	+		+			+			+	McMahon (1961); Lesuk and Anderson (1941)
Spirometra mansonoides larvae	56·0			+	+		+		+		+		+	Meyer *et al.* (1966)
Taenia hydatigena														
cysticerci membrane	37·4			+	+		+	+	+	+				Kassis and Frayha (1973)
cysticerci fluid	38·7			+	+		+	+	+	+				Kassis and Frayha (1973)
Echinococcus granulosus														
cyst protoscoleces	56·8			+	+		+	+	+	+				Vessal *et al.* (1972); Frayha *et al.* (1980); Frayha and Haddad, (1980)
cyst fluid	0·1													
Trematodes														
Schistosoma mansoni														
sporocysts	traces													DiConza and Basch (1976)
Schistosoma mansoni ova				+	+	+	+	+		+	+			Smith *et al.* (1971)
Schistosoma japonicum ova				+			+	+		+				Smith *et al.* (1977)
Microphallus similis sporocysts				+	+		+	+	+	+	+		+	McManus *et al.* (1975)
Nematodes														
Trichinella spiralis larvae				+	+	+	+			+			+	Castro and Fairbairn (1969)
Ascaris suum ova		+		+	+	+	+	+	+	+				Lee *et al.* (1977b)

that the lipids of *S. japonicum* eggs were in general similar to those of *S. mansoni* eggs and cercariae.

E. ADULT NEMATODES

Lipids in nematodes fluctuate from host to host. Usually parasites of animals contain less lipids than those of plants (Table 1). The extremes of amounts were 3·5% dry weight for *Porrocaecum decipiens* and 39·7% for the plant nematode *Tylenchorhynchus claytoni* (von Brand, 1979). The first reliable estimate of total lipids in parasitic nematodes was that of Weinland (1901) who found in two determinations, using ether extraction techniques, that fat constituted 1·46% and 1·5% of the total fresh body weight of *Ascaris lumbricoides*. These quantities are still valid when compared to those recently obtained by modern techniques (Table 1, p. 313). By improving the extraction procedures Flury (1912) was able to identify in fresh *A. lumbricoides* many compounds including phosphatidylcholine, oleic ($C_{18:1}$), stearic (C_{18}) and palmitic (C_{16}) acids, and other low molecular weight acids namely valeric, butyric, caproic, propionic and acrylic acids. Glycerol was isolated in small quantities but surprisingly cholesterol was not detected. Simultaneously, von Kemnitz (1912) demonstrated the presence of lipids in the tissues of *A. lumbricoides*. In a series of experiments von Brand (1934, 1941) and Hobson (1948) took *A. lumbricoides* as an experimental model to improve the extraction techniques of lipids; they found lipid concentration to vary between 1·0 and 1·8% of the fresh body weight. A new era of detailed analytical lipid identification in *A. lumbricoides* began with the works of Rogers and Lazarus (1949), Moyle and Baldwin (1952), Fairbairn (1955a, b, 1956) and Beames (1964, 1965). Rogers and Lazarus (1949) identified phosphatidylcholine, phosphatidylethanolamine and sphingomyelins in the pseudocoelomic fluid and intestine of *A. lumbricoides*, and phosphatidylcholine and phosphatidylethanolamine, but no sphingomyelins in the ovaries. On the other hand, Shorb and Shorb (1966) found the phospholipids of adult *A. lumbricoides* to be 23% of total lipids (glycolipids, 38·5%) and Beames (1964) fractionated *Ascaris* phospholipids into phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine, phosphatidylserine, lysophosphatidylethanolamine, phosphatidylinositol, sphingolipids and cardiolipins. The phospholipids contained numerous saturated and unsaturated fatty acids and aldehydes varying in carbon chain from C_{12} to C_{18}; these chains were sometimes branched. Out of 30 different acids, Beames (1964) was unable to identify 14. He observed a high percentage of unsaturation in the acids (60–80%) of which the most common were $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$. Subrahmanyam (1967) and Subrahmanyam and Venkatesan (1968) identified the rare type of glyceryl-

ether phospholipids in *A. lumbricoides* as well as in the filarial nematode *Litomosoides carinii* and found them to be of the butylalcohol type. The choline containing lipids and the ethanolamine lipids were quantitatively the most important fractions. In addition, the authors found that the plasmalogen content of *Ascaris* was significantly higher than those found in any other animal. Moyle and Baldwin (1952) analysed the steam volatile fatty acids of the pseudocoelomic fluid of *A. lumbricoides* by using buffered silica partition columns. The acids were acetic, propionic and n-butyric. Fairbairn (1955a) and later Beames (1964, 1965) studied the lipid composition of the body wall and the entire female reproductive system of *A. lumbricoides*. They observed a pattern similar to that described earlier by Rogers and Lazarus (1949). Plasmalogens were highly concentrated in the body wall and weakly so in the reproductive organs. Nematodes usually contained large amounts of volatile fatty acids (C_2 to C_6) and these acids were mainly located in the tissues of female and male reproductive organs (Fairbairn, 1955a; Cavier *et al.*, 1958) where they formed constituents of acylglycerols and ascarosides. The fatty acids acetic, pentanoic (C_5) and hexanoic (C_6), were most abundant and were considered to be esterified fermentation acids. They occurred also in the acylglycerol fraction of muscle, cuticle and fertilized eggs but not in the phospholipid fraction (Fairbairn, 1955a, 1956). Beames (1964, 1965) established that the volatile fatty acids of *A. lumbricoides*, namely acetic, propionic, valeric, 2-methylbutyric and 2-methylvaleric, were also fermention acids. He also postulated that the dominant acids which were found in significant amounts in *Ascaris* had odd carbon numbers with straight or branched chains. However, analysis of the C_{20} and higher fatty acids, which are quite important in phospholipids, was incomplete. Volatile fatty acids have been detected in *Ascaris* as well as in *Parascaris*, mainly in the form of acylglycerols (Fairbairn, 1955a) and ascarosides (Jezyk and Fairbairn, 1967a) in the reproductive organs of females and males (Cavier *et al.*, 1958). Sterols and sterol esters were detected in *A. lumbricoides* (Cole and Krusberg, 1967a; Barrett *et al.*, 1970a). Along with cholesterol which constituted 39–40% of sterols, five other sterols namely campesterol, β-sitosterol, cholestanol, campestanol and stigmastanol have been determined in *A. lumbricoides* (Barrett *et al.*, 1970a; see Table 5). Sterol esters seem to be highly concentrated in *Ascaris* tissues constituting 40% of total esters in females and 60% in males (Cole and Krusberg, 1967a). The fatty acid moieties of the sterol esters had qualitatively the same composition as those in phospholipids, namely from $C_{10:0}$ to $C_{24:1}$ acids. They differed, however, in quantitative distribution (von Brand, 1973).

The major components of the unsaponifiable lipids in *Ascaris* (and the related genus *Parascaris*) were not sterols but rather ascarosides (Fouquey *et al.*, 1957; Jezyk and Fairbairn, 1967a; Tarr, 1973; Tarr and Fairbairn,

TABLE 5

Sterols of parasitic helminths

	Cholesterol	Sitosterol	Lathosterol	Spinasterol	Campesterol	Cholestanol	Campestanol	Stigmastanol	Cholestane	Reference
Echinostoma revolutum	+								+	Barrett *et al.* (1970a)
Schistosoma mansoni	+								+	Smith and Brooks (1969)
Ascaris lumbricoides	+	+			+	+	+	+		Fairbairn and Jones (1956); Cole and Krusberg (1967a); Barrett *et al.* (1970a)
Ditylenchus dipsaci	+		+	+						Cole and Krusberg (1967b)
Ditylenchus triformis	+		+	+						Cole and Krusberg (1967b)
Macracanthorhynchus hirudinaceus	+	+			+	+	+	+		Barrett *et al.* (1970a)
Moniliformis dubius	+	+			+	+	+	+		Barrett *et al.* (1970a)
	and all other species studied									

1973) which were previously called ascaryl alcohol (Flury, 1912; Fauré-Fremiet, 1913; Schultz and Becker, 1933; von Brand and Winkeljohn, 1945). Ascarosides were classified into three types, A, B, and C. They had a unique hexose sugar component ascarylose (3,6-dideoxy-L-arabino hexose) linked to an aglycone. Ascarosides A and B contained one molecule of ascarylose while ascaroside C contained two. The differences in the aglycone moiety were also distinct. The aglycone of ascaroside A consisted of secondary monols which varied in carbon number between 23 and 27, thus displaying the presence of several homologous substances. In ascarosides B and C, on the other hand, the aglycone has recently been shown to be a symmetrical molecule with the hydroxyl groups at the 2 and (ω-2) positions (Tarr and Schnoes, 1973), rather than a 2,6 diol as originally thought. Ascarosides of some nematodes exhibited strong resemblances in configuration when chemically analysed. For example, Jezyk and Fairbairn (1967a) found that the ascarosides of *Ascaris* were basically similar to those of *Parascaris* and contained aglycones with 11 monols having a chain length between C_{31} and C_{37} (C_{29} predominating), four diols with chain length between C_{31} and C_{37} (C_{29} predominating) and four diols with chain length between C_{31} and C_{37} (C_{31} and C_{33} being most abundant). They also found that the aglycones of ascarosides B and C consisted of diols and those of ascaroside A consisted of a mixture of monols and diols. Similarly, López-Gorgé (1964) noticed a resemblance between the ascarosides of *Ascaridia galli* and those of *Ascaris* and *Parascaris*. Ascarosides have been reported from many organs such as muscle, cuticle, intestine and reproductive organs; but the female reproductive organs and the eggs contained the highest amount.

Tarr (1973) compared the ascarosides of four species of ascarids and of oxyurids. He found that the proportions of monol ascaroside, diol ascaroside and diol diascaroside were roughly the same in all species. Aglycones averaged 29·2 to 30·4 carbons in monol ascaroside, 31·2–34·4 in diol ascaroside and 31·7–35·3 in diol diascaroside. A variable percentage of ascarid monol aglycones were branched. Ascaroside esters of all species were primarily acetates, although some were propionates. Tarr (1972, 1973) also observed that ascarosides in ascarid worms were restricted to the female reproductive tract, with free ascarosides occurring in uterine material containing eggs and ascaroside esters predominating in the ovary. Similarly, male oxyurids contained no ascarosides. It seems that ascarosides eventually deposit in the fertilized eggs as a tough protective layer, the vitelline membrane, to protect the eggs from unfavourable extraneous conditions (Fairbairn and Passey, 1955; Fairbairn, 1957; Fouquey *et al.*, 1957; Jezyk and Fairbairn, 1967b; Foor, 1967). More chemical analyses must be conducted on the egg membranes of various parasitic helminths to determine their ascaroside nature.

In a recent study, Ehrlich and Hrzenjak (1975) fractionated the 'polar lipids' of *A. lumbricoides* into phosphatidylserine, lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, two cerebrosides, two ceramides, ascarosides, cardiolipin, cholesterol, cholesterol esters and two globosides. This is another report of globosides in helminths.

In *Ascaridia galli* there was a sex variation in the phospholipid content. Male worms contained 71·6% phospholipids in their total lipids, while females had only 40·3% (Shorb and Shorb, 1966).

In some plant nematodes, for example *Ditylenchus triformis* and *D. dipasci*, Cole and Krusberg (1967b) detected lathosterol in addition to cholesterol. They identified some free and bound fatty acids but many acids having C_{20} and higher carbon numbers remained unidentified.

Vykhrestyuk *et al.* (1972) and Vykhrestyuk and Yarigina (1975) studied the lipids of the cattle nematode *Mecistocirrus digitatus* and found that total lipids were 10·38% of the dry weight of which 23·73% were phospholipids and 76·27% neutral lipids. The neutral lipids comprised cholesterol, tri-, di- and monoacylglycerols, ascarosides, free fatty acids and pigments; cerebrosides, and small quantities of phosphatidylethanolamine were also identified.

It is important to mention the characteristic finding of uneven carbon atoms in the fatty acids of certain nematode parasites. For example, Hutchison *et al.* (1976) found in three filariid worms *Dirofilaria immitis*, *Dipetalonema viteae* and *Litomosoides carinii* some odd carbon acids ($C_{15:0}$) constituting 1·1–4·0% of total fatty acids.

Roberts and Thorson (1977) demonstrated the presence of a lipid fraction of excretory and secretory products of both male and female *N. brasiliensis* which appeared to act as a pheromone to female worms. The nature of this lipid was unknown, although it was suspected to be a sterol.

A lipid complex corresponding to cytolipin P was obtained from *Metastrongylus apri* and found to be immunologically active (Hrzenjak *et al.*, 1979).

Study of carotenes in nematodes has been limited, although Frank and Fetzer (1968) detected several carotenoid fractions in the filariid *Foleyella furcata*.

F. LARVAL NEMATODES

Usually, the free living larval forms of parasitic nematodes contain high amounts of lipids. In the earliest studies on the lipid composition of larval nematodes, Busch (1905) observed that the third-stage larvae of *Necator americanus* contained lipid-staining granules but no glycogen. He postulated, therefore, that lipids were the primary food reserve of the larvae. A few

decades later, this hypothesis was confirmed by the detection of lipid granules by Payne (1923) and Giovannola (1936) in the larvae of *N. americanus*, *Ancylostoma caninum* and *Strongyloides fuelleborni;* the gradual depletion of these granules in older larvae thus indicating the consumption of lipids with time (Cort, 1925; Rogers, 1939). Recently Clark (1969) gave significant quantitative evidence of lipids being the primary food reserve in the larvae of *A. caninum*. One-day-old larvae contained 30% total lipid of the dry weight, while *Cooperia punctata* larvae had half this amount of lipid content (Eckert, 1967). By identifying the lipids of the free-living larval stages, Barrett (1968) found that triacylglycerols occurred in larger amounts than mono- and diacylglycerols. For example, the lipids of *Nippostrongylus brasiliensis* contained 55% triacylglycerol, but less than 1% mono- and diacylglycerol while the free fatty acids of the free living larvae of *N. brasiliensis* and *Strongyloides ratti* constituted more than 50% of the total lipids thus indicating the important role of free fatty acids as a source of energy in the metabolism of the free-living larvae. This could also be correlated with the penetration ability of the larvae because Nwosu (1977) observed that with time (over 2 to 8 weeks) the lipid content of the third-stage larvae of the cat hookworm *Ancylostoma tubaeforme* decreased, thus leading to a drop in activity and penetration rate of the parasite. On the other hand, Nwosu (1978a) did not find any significant difference in the size and lipid content of the larvae that had developed at different temperatures. However, the lipid content decreased with time at a rate related to storage temperature (Nwosu and Croll, 1978). Thus larvae at 5°C, although they became inactive first, had the highest lipid levels. Smales (1977), in describing the life history of *Labiostrongylus engenii*, a nematode parasite of the Kangaroo Island wallaby, found that the sheathed second-stage larvae were rich in neutral lipids which gradually disappeared after 3–4 days when the larvae moulted to third-stage infective larvae.

In *Ascaris suum* larvae lipid droplets were detected in the intestine which disappeared between the second and fourth day of development (Grineva and Chebyshev, 1975); and in the posterior half of these larvae Rubin (1977) identified the lipid droplets as triacylglycerols. Dubinsky and Rybos (1978) found that in post-invasive stages of *A. galli* the content of lipids decreased with the age of the worm and was not essentially influenced by its nutrition.

Most of the studies on the lipids of parasitic nematode larvae have been with *Trichinella spiralis*. Castro and Fairbairn (1969) demonstrated that the lipids of *T. spiralis* larvae contained 72·2% phospholipids and 8·5% glycolipids. The latter consisted mainly of phosphatidylinositol rather than cerebrosides. The former were composed of phosphatidylethanolamine, phosphatidylserine, lysophosphatidylethanolamine, phosphatidylcholine and sphingomyelin. These authors also found that the fatty acids of the worm

were mainly even in carbon number, saturated and unsaturated, and varied between $C_{14:0}$ and $C_{23:3}$.

G. ACANTHOCEPHALA

A few studies have been made on the lipid composition of acanthocephalan worms. The early works of von Brand (1939, 1940) gave partial characterization of the lipids of *Macracanthorhynchus hirudinaceus*. Beames and Fisher (1964) separated by column chromatography the phospho- and neutral lipids of *M. hirudinaceus* and *Moniliformis dubius*. In both species the male contained about double the quantity of lipids in the female. The phospholipids of the two worms were identified as phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, phosphatidylinositol and plasmalogens. On the other hand, the fatty acid methyl esters of the neutral lipids were fractionated by GLC into a spectrum of acids from C_{10} to C_{20}. The C_{18} acids constituted over 70% of the total acids (Beames and Fisher, 1964). Barrett *et al.* (1970a) identified in the same two acanthocephalans six sterols namely cholesterol, campesterol, β-sitosterol, cholestanol, campestanol and stigmastanol. Bullock (1949) demonstrated the presence of acylglycerols, fatty acids, cholesterol and phospholipids in *Echinorhynchus coregoni*, *E. gadi*, *Pomphorhynchus bulbocolli*, *Neoechinorhynchus cylindratus* and *N. emydis*.

Yellow and red pigments, which are carotenes of isoprenoid units have been detected in *Arhythmorhynchus* sp. (Van Cleave and Rausch, 1950), *Polymorphus minutus* (Barrett and Butterworth, 1968) and *Pallisentis nagpurensis* (Ravindranathan and Nadakal, 1971). The pigments of *Polymorphus* were mainly composed of esterified astaxanthin, whereas those of *Pallisentis* were of mutachrome or oxide of β-carotene and flavochrome or oxide of α-carotene. Barrett and Butterworth (1973) determined the carotenoid pigments of six species of adult acanthocephala. They found lutein (an α-carotene derivative) in *M. hirudinaceous*, *Pseudoacanthocephaloides galaxis* and *N. pseudemydis*; β-carotene in *P. laevis* and *Filicollis anatis*, and esterified astaxanthin in *Nipporhynchus ornatus*.

Parshad and Guraya (1977b) found that males of *Centrorhynchus corvi* contained more lipids than females but there were no qualitative differences as to phospholipids, triacylglycerols, unsaponifiable lipids and free fatty acids.

Barrett and Butterworth (1971) found that wax and sterol esters constituted 90% of the total lipids of the cystacanth of *P. minutus*. Cholesterol was the only steroid indentified, constituting about 10% of total sterol esters. The remainder of the total esters were long-chain alcohols, largely saturated and esterified, with predominantly C_{18} mostly unsaturated fatty acids. Corresponding quantities of wax esters were not found in the adults.

II. Histochemical Studies on Lipid Distribution in Parasitic Helminths

A. ADULT CESTODES

Histochemical studies, which depend primarily on stains such as osmic acid, Sudan III and others, have been used to demonstrate the distribution of lipid in the tissues or cells of parasitic helminths. They are, however, less sensitive and therefore less reliable than chemical methods in depicting small amounts of lipids in cells and tissues. Only modern chemical techniques (such as GLC, spectrophotometry and isotope studies) are capable of detecting traces of lipids in cells. On the other hand, histochemistry has been valuable in cases where the organism as a whole, or its organs or tissues or cells, were too small to yield a substantial quantity for chemical analysis.

In cestodes it has proved practically impossible to separate the different organs or tissues for lipid extraction and identification. Histochemical procedures have therefore been the main practices for such identification. Chemical techniques have only been applied in cestodes when a lipid gradient was to be determined in the whole strobila or collectively in various regions of the strobila.

It seems that the parenchyma of cestodes is in general the most important organ in the storage of lipids (Smyth, 1947, 1949; von Brand, 1966). In contrast, the tegument appears to be devoid of lipids except for that of *Hydatigena taeniaeformis* in which Waitz (1963) detected some lipids. Whether these lipids are unexcreted waste products as von Brand (1952) and Fairbairn *et al.* (1961) assumed, in view of their total loss after detachment of proglottides, or useful products for development and growth of larval stages as Vercelli-Retta *et al.* (1975) and Reissenweber *et al.* (1975) suggested, is still a matter of dispute and needs more elucidation.

The calcareous corpuscles which are a universal anatomical inclusion in the tissues of cestodes have also been shown to contain lipids (von Brand *et al.*, 1960; Waitz, 1963). Lipids have also been universally found in the eggs of cestodes usually located between the embryo and the shell. Tubular organs, such as the uterus of *Dipylidium caninum* (von Brand, 1966) and excretory canals of *Moniezia expansa* (von Brand, 1933) and *H. diminuta* (Hedrick, 1958) and the canals, ducts and pouches of many organs of *H. microstoma* (Chowdhury and De Rycke, 1976) and *E. granulosus* (Coutelen, 1931a; Reissenweber *et al.*, 1975; Vercelli-Retta *et al.*, 1975), also contain various amounts of lipids.

Smorodinstrev and Bebeshin (1935) determined the lipid concentration in the head and neck regions of *T. saginata* to be 3·05% of fresh tissues; it was,

however, 1·55% in the middle region and 1·25% in the gravid region of the strobila. In contrast, Fairbairn *et al.* (1961) and Mettrick and Cannon (1970) detected more lipids in the immature proglottides of *H. diminuta* (15% dry weight) than in the gravid proglottides (31%). Similar results were obtained by Hedrick (1958) on *Raillietina cesticillus*. Mettrick and Cannon (1970) also observed that the lipid content of *H. diminuta* varied with age. A 5-day-old immature proglottis contained 23·63% lipid per gram of dry tissue while 16-day-old same proglottides contained only 0·4%. The lipid distribution in *Hymenolepis* covered almost all organs and canals. Lipid was detected around the excretory canals of *H. diminuta* by Hedrick (1958). Pence (1970) detected lipids by histochemical techniques and electron microscopy in the outer envelope of the egg. In addition, Rybicka (1972) found that the inner envelope of the three embryonic envelopes surrounding the embryo of *H. diminuta* was formed by three macromeres covering the embryo as a thick syncitial layer. This envelope was filled with β-glycogen particles and also contained swollen mitochondria and lipid droplets. Rybicka (1973) observed that the changes occurring in these macromeres, at the time of the inner embryonic envelope formation, involved an increase in the diameter of the endoplasmic reticulum cisternae leading to the formation of the lipid bodies. Chowdhury and De Rycke (1976) determined the lipid distribution in stages of *Hymenolepis microstoma*. In the cysticercoid, neutral lipids were detected in the outer membrane, the lining of the cavity, the tegument and the calcareous corpuscles. Phospholipids were found, in decreasing concentrations, in the acellular layer through the circular fibrous layer, the longitudinal fibrous layer and the adjacent dense zone to the lining of the cysticercoid cavity; they were also detected in the calcareous corpuscles and the tegument of the larva. In the young adult (3 days after mouse infection), lipid globules were seen first in the last two to three proglottides. Mature proglottides contained fat granules and globules in the folds of the uterus, sperm ducts, cirrus pouch, tegument, vitellaria, ovary and testes. Pregravid and gravid proglottides contained the largest fat globules. The neutral lipids and phospholipids displayed continuous variation in form, intensity and location during the metamorphosis of the cleaving embryo to the fully developed oncosphere. Neutral lipids and phospholipids were constantly observed throughout the tegument and in calcareous corpuscles in all strobilar stages of the cestode. The authors believed that the lipids in the proglottides helped the maturation of gonads and the transformation of an ovum to an oncosphere.

During vitellogenesis in *Bothriocephalus clavibothrium*, maturing vitelline cells were reported to increase three times in size and contain many inclusions and organelles among which were lipid droplets (Swiderski and Mokhtar-Maamouri, 1974). Similarly, Mokhtar-Maamouri and Swiderski (1976) observed lipid droplets accumulating in the cytoplasm of maturing vitelline cells of *Echeneibothrium beauchampi*.

B. LARVAL CESTODES

Hydatid cysts of *E. granulosus* have been the basis for several histochemical studies on lipids. Coutelen (1931b) observed lipid droplets in the lumen of the excretory canals of *E. granulosus* protoscoleces. Moreover the excretory pores of the protoscoleces and brood capsules of *E. granulosus* hydatid cysts were rich in lipids and lipase in addition to other enzymes (Reissenweber *et al.*, 1975). Histochemical and histoenzymological studies of the germinal membrane of hydatid cysts by Vercelli-Retta *et al.* (1975) revealed that there were highly differentiated metabolic areas in the germinal membrane topographically related to the origin and insertion of brood capsules. The accumulation of lipids and enzymes in these areas suggested that lipids were not simple excretory products. They also speculated that the lipid metabolism or its resultant products were important in the development and growth of the protoscoleces.

In the parenchyma of the developing cysticerci of *T. saginata* (*Cysticercus bovis*), small amounts of hydrophobic lipids were detected by Zdarska (1975) in younger cysticerci as compared with older ones. Krasnoshchekov and Bondarenko (1976) detected large amounts of lipids in the outer parenchymal layer of the cysticercoids of the hymenolepid *Aploparaksis*. In the cultured plerocercoids of *Ligula intestinalis*, Smyth (1949) observed great quantities of fat present intercellularly in the parenchymal spaces but this may indicate abnormal development.

C. ADULT TREMATODES

Histochemically speaking, *Fasciola hepatica* has been the most studied adult trematode. As in cestodes, the excretory canals of this trematode contain and are surrounded by lipid droplets (see von Brand, 1966, 1973). In addition, Vogel and von Brand (1933) demonstrated the expulsion of lipid droplets through the excretory pores of *Fasciola*. These lipid droplets were found by microchemical tests (Stephenson, 1947) to consist mainly of triacylglycerols containing some unsaturated fatty acids. Cholesterol and cholesterol esters were not detected. Similar results of histochemical studies were reported later on the excretory lipids by Burren *et al.* (1967), Erasmus (1967) and Moss (1970). On the other hand, lipids of *F. hepatica* maintained in incubation media gave inconsistent results. For example Burren *et al.* (1967) detected cholesterol, cholesterol esters, triacylglycerols, free fatty acids and phospholipids, and Moss (1970), undertaking the same kind of experiment, published rather different results. He found di- and triacylglycerols and

phospholipids but no free fatty acids and cholesterol. The fact that cholesterol was not excreted and that it was abundant in the food of *F. hepatica* and in its environment, suggests that the excreted lipids are a product of catabolism of the worm or probably a by-product of carbohydrate metabolism, rather than a component of exogenous lipids which had been ingested. At this stage it is still difficult to ascertain exclusively that the excretory pore is the only route of lipid excretion. There might be other routes *via* body openings or via the tegument which need to be examined further. It is worth noting that some trematodes, similar to *Fasciola* in their habitats, such as *Dicrocoelium dendriticum* and *Chlonorchis sinensis* did not display the excretory lipids described for *F. hepatica* (von Brand, 1966).

The Mehlis' gland and vitellaria have also been studied for lipid content and distribution. Phospholipids were identified in the Mehlis' gland of *F. hepatica* (Rao, 1959) and the lipids were detected in the vitellaria of *Gorgodera cygnoides*, *Paragonimus ohirai* and *S. japonicum* (for references see von Brand, 1966). Hanumantha-Rao (1960) found that the Mehlis' gland secretion in trematodes and pseudophyllidean cestodes may be a phosphatidylcholine-like phospholipid. In *Penetrocephalus ganapatii*, after enzymic degradation of the phosphatidylcholine into lysophosphatidylcholine, the release of egg-shell precursors from the vitelline cells was effected; soon after the completion of the egg-shell the lysophosphatidylcholine may be reconverted into phosphatidylcholine in the vitelline cells. More recently, Erasmus (1973) reported that the vitelline cells of adult *S. mansoni* contained much lipid and little glycogen. In addition Halton *et al.* (1974) found that, with the onset of maturity, the vitelline cells of the monogenean parasites *Diplozoon paradoxum*, *Diclidophora merlangi*, *D. denticulata* and *Calicotyle kröyeri* accumulated food reserves in the form of yolk bodies, glycogen and lipids. Lipids have also been detected in the vitelline follicles and excretory ducts of *Fibricola cratera* (Jansma, 1972). Kanwar and Agrawal (1977) demonstrated the presence of lipids (with carbohydrates, proteins and traces of RNA) in the outer rim of the vitelline globules which were located in the cytoplasm of the vitelline cells of *Diplodiscus amphicrus*. Irwin and Maguire (1979) using transmission electron microscopy (TEM) detected lipid bodies in the vitelline cell cytoplasm of *Gorgoderina vitelliloba*. These authors suggested that nurse cells may have a role in the selection and transport of nutrient material for vitelline cells and that the latter manufactured precursors of lipid which was subsequently stored as a food reserve in mature vitelline cells.

During their cytochemical and morphological studies on the mitochondria in the spermatogenesis of the amphistome *Gastrothylax crumenifer*, Taneja and Nath (1972) observed that the mitochondria in the tail and head of the mature sperm contained lipid, protein and carbohydrate.

The digestive system also has been studied for lipid contents. Halton (1972) noticed that some caecal epithelial cells of *Aspidogaster conchicola* contained lipid droplets. These also accumulated in the large apical vacuole of the caecal epithelial cell of *Calicotyle kröyeri* (Halton and Stranock, 1976) and were periodically released to the lumen by exocytosis. Fried and Butler (1977) detected neutral lipids in the intestinal lumina as well as eggs and vitellaria of adults, and the excretory system of metacercariae of *Cotylurus* sp. Thin-layer chromatography (TLC) analysis of these lipids revealed free sterols, free fatty acids, sterol esters and triacylglycerols in adults. The metacercariae had all these lipids except triacylglycerols. The same kind of neutral lipids were identified in adult *Echinostoma revolutum* grown in culture (Butler and Fried, 1977). However, free sterols were the main component of neutral lipids in adult *E. revolutum* grown in chicks. Halton and Hendrix (1978) found that neutral lipids were localized in the gastrodermis and excretory system of *Lobatostoma ringens*. Taft (1979) detected lipids in the apical gland as well as the flame cells of *Cyclocoelum oculeum* and in addition, lipase activity was detected in eggs containing miracidia.

In adult trematodes, several other organs have been histochemically studied for lipid contents. For example, the syncytial layer of the epithelium of the lymph channels of *Megalodiscus temperatus* contained lipid droplets in its cytoplasm (Strong and Bogitsh, 1973). Small amounts of lipids have been detected in the adhesive disc of *Lissemysia macrorchis* (Gupta and Randev, 1974). Histochemical and TLC analyses of neutral lipids of the adult *Leucochloridiomorpha constantiae* by Fried and Pucci (1976) revealed that triacylglycerols and free sterols were the major fractions found in the suckers, parenchyma and eggs with trace amounts in the caecal epithelium and lumina of the gut. Lesser amounts of free fatty acids, cholesterol esters, mono- and diacylglycerols were detected in these organs. Prasad and Shyamasundari (1977) found that the neurosecretory cells of *Cymatocerpus undulatus* were rich in lipids and phospholipids.

D. LARVAL TREMATODES

In general, the larval stages of trematodes, i.e. miracidia, sporocysts, rediae and cercariae, do not excrete as much lipid as adults. A good example is *F. hepatica* whose miracidia, rediae and cercariae, as well as *Cercariae limnaea ovata* had some lipids in their tissues which were never found in the excretory canals (Vogel and von Brand, 1933; Palm, 1962). Contrary to these results, Parshad and Guraya (1976) observed that the excretory ducts of immature stages of *Cotylophoron cotylophorum* were common sites for the accumulation of neutral lipids (mainly triacylglycerols) and phospholipids;

these were very sparse at the corresponding sites in the adult. Fried and Boddorff (1978) detected sterols, free fatty acids and sterol esters in the excretory system of the miracidia of *Cotylurus* sp.; the same workers identified free fatty acids as major neutral lipids in the excretory system of excysted metacercariae of *Echinostoma revolutum*. On the other hand, Køie (1973) detected lipid-like bodies, which were suspended in a granular substance, in the lumen of the intestinal caeca of the intraredial cercariae of *Neophasis lageniformis*. More often, lipids are found in the tissues of larval trematodes. Asanji and Williams (1973) found that the inner layer of the metacercarial cysts of five trematodes contained lipids, among other substances and, based on TEM and histochemical tests, four layers were described in the metacercarial cyst of *Spetotrema nicolli* (Alderson, 1975). The second layer from outside was lipoidal. Hoskin (1975), using TEM, showed that the outermost surface of the redial wall of *Himasthla quissetensis* consisted of closely spaced microvilli, the plasmalemma of which was composed of lipids and proteins. Previously, the same author (Hoskin, 1973) had found lipid droplets in the epithelium as well as in the cytons of *H. quissetensis* rediae, whose respiratory quotient suggested metabolism of lipids.

Parshad and Guraya (1976) compared histochemically the lipid distribution in the immature and mature stages of *Cotylophoron cotylophorum*. As mentioned above, lipids accumulated more in the excretory ducts of immature stages than of mature. However, phospholipids and lipoproteins were observed in the tegument of the adult but not in that of immature stages. Intestinal caecae of both forms showed the presence of diffused and granular lipids but were more marked in the mature than the immature forms. The authors suggested that the differences in lipid composition may be related to the habitat of the different developmental stages.

E. ADULT NEMATODES

The lipid distribution in ascarid worms (adults and larvae) has been most extensively studied. In male and female *A. lumbricoides* the percentage of lipids in fresh tissues was 0·32–0·35 in pseudocoelomic fluid, 0·61 in cuticle, 0·75–0·98 in muscle and 3·2–6·0 in reproductive organs (Fairbairn, 1957; Cavier *et al.*, 1958); histochemical fat distribution in *A. lumbricoides* was similar to that in *Parascaris equorum*. For example, fat droplets were detected in chords, plasma bulbs of the body wall muscles, ganglion cells, intestinal cells, and various structures of the reproductive tract (see von Brand, 1966; Lee and Atkinson, 1976).

Leštan *et al.* (1974) found that potential energy reserves in the form of glycogen and lipids occurred in the subcuticular region of *A. suum*. From the

same species Matausic *et al.* (1976) isolated the polar lipids from the organs and eggs and found sphingomyelin in the cuticle, muscles, gut and uterus; phosphatidylinositol in the cuticle, gut and pseudocoelomic fluid; phosphatidylserine in the cuticle, gut and uterus; glycolipids (cerebrosides and their esters) and ascarosides in all organs except muscles. In the seminal vesicles of male *A. suum*, the spermatozoa contained lipid-like droplets randomly scattered throughout the cytoplasm which eventually coalesced to form a single large inclusion (Foor and McMahon, 1973). Studies on *Ascaridia galli* by Zmoray and Guttekova (1974) and on *Heterakis gallinarum* by Guttekova and Zmoray (1975) revealed the presence of lipid droplets as inclusions in the ectoplasm of the columnar epithelial cells of the intestine of *A. galli* and in the syncytial hypodermis of the body wall of *H. gallinarum*. Similarly, Parshad and Guraya (1977a) found that the outermost surface layer of *A. galli* contained bound lipids and the outer cortex contained both free and bound lipids. The polar lipids in the surface layer were phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine, phosphatidic acid and glycolipids. The non polar lipids were acylglycerols, sterol and sterol esters. By studying the lipids of the intestinal epithelium of *A. galli*, Parshad and Guraya (1978) found that the intestinal cells stored considerable amounts of lipids and glycogen. The lipids were mainly phospholipids and lipoproteins, distributed throughout the cytoplasm. The brush borders showed the activities of many enzymes including lipase.

The lipids of the cockroach nematode, *Thelastoma bulhöesi*, were located by Lee (1960) in the chords, intestine, oocytes, oogonia and ova. The same author also reported that lipid granules were detected in the lumen of *Hammerschmidtiella diesingi*, especially after 24 hours starvation. He concluded that these lipids were potentially broken by lipase into smaller products which were easily absorbed to be distributed to other regions of the body.

Zmoray and Guttekova (1970) observed a distinct lipid zone among the various zones which constituted the intestinal wall of *Dictyocaulus filaria*. Inclusions of lipids and glycogen were seen by Jenkins (1972) in the hypodermal cells of the body wall of *Trichuris suis;* they may serve as storage centres for the worm. In addition, Jenkins (1973) observed the presence of numerous lipid droplets and glycogen granules in the intestinal epithelium of *T. suis*.

The body wall of nematodes, including the cuticle, seems consistently to contain a significant amount of lipids. Mittal and Lal (1977) reported the presence of lipids in the matrix layer of the cuticle of *Protospirura*, *Syphacia*, *Aspiculuris* and *Procamallanus* spp. Sood and Kalra (1977) detected lipids in the cortical layers of the cuticle of *Haemonchus contortus*. The lipids that were found in the hypodermis as well as the phospholipids in the muscle cells were considered to be, with glycogen, energy reserve compounds. Sood and Sehajpal

(1978) also detected lipids (probably of phospholipid type) in the intestinal epithelium of *H. contortus*. Gupta and Kalia (1978) demonstrated in the body wall of the filarioid nematode *Setaria cervi* phospholipids in the matrix and fibrillar layer, neutral lipids in the basal lamella and hypodermis, neutral lipids in the epithelial layer and neutral and phospholipids in the bacillary layer. The authors also observed lipoproteins in the eggs and developing larvae. The fat body of the everted organs (the tube) of the bumble-bee nematode *Sphaerularia bombi* was considered to be a reservoir of lipids and glycogen (Madel and Scholtyseck, 1976). The lipid components, cholesterol, cholesterol esters, triacylglycerols and phospholipids constituted the predominant storage metabolites in the trophosome of *Romanomermis culicivorax*, the nematode parasite of larval mosquitoes of *Aedes aegypti* (Ittycheriah *et al.*, 1977).

F. LARVAL NEMATODES

Engelbrecht and Palm (1964) considered that the long-lived larvae of parasitic nematodes, particularly the ascarids, depended mainly on stored lipids as a reserve food, in contrast to short-lived larvae such as those of *Enterobius vermicularis*, as well as the miracidia, cercariae, or coracidia of other helminths, which depended mainly on stored glycogen as a reserve food. It was also suggested by von Brand (1973) that the concentration of lipids in the tissues is inversely proportional to the larval activity and growth implying that larvae do consume lipids during their growth. This could also mean that the concentration of fat in larvae is a good indication of physiological age of the worm (Rogers, 1939; Rogers and Sommerville, 1968, 1969). There seems to be contradictory views concerning the correlation between fat concentration in the larvae and their infectivity. Elliot (1954) found that the depletion of histochemically detected fat in *A. galli* coincided with loss of infectivity. On the other hand, Durie (1957) demonstrated that the larvae of *Haemonchus placei* kept their infectivity even after the total exhaustion of their lipids. There also seems to be inconsistent length of time for lipid consumption in immature stages of nematodes. For example, in the embryonated eggs of *Ascaris* sp. the lipids were totally consumed after 4 years storage at room temperature (Münnich, 1965) whereas total fat consumption in *A. galli* only took 10 months (Elliot, 1954). In *A. suum* larvae lipid droplets disappeared from the intestine of growing larvae between the second and fourth day of development (Grineva and Chebyshev, 1975).

In nematode larvae, lipids appear to be widely distributed in the tissues. In a number of different species, they have been detected in the intestinal region (Zeletzki, 1965; Kozar and Seniuta, 1974) and in other tissues (Kozar and

Seniuta 1974; Rubin, 1977; Rubin and Trelease, 1975). For example, Kozar and Seniuta (1974) detected in old muscle larvae of *Trichinella spiralis* lipids at the level of the stichocytes. They also found lipids in blastulating embryos in trichinae; whereas lipid droplets were distributed throughout the body of post-embryonic, migrating and young muscle larvae. Rubin and Trelease (1975) and Rubin (1977) identified triacylglycerol lipid droplets at the posterior half of the larvae of *A. suum*.

Popham and Webster (1979) detected lipids in the lateral cord cells of the dauer larva of *Caenorhabditis elegans*. Lipids were also found in oesophageal and body wall muscles and in neurones.

The egg of nematodes, from the time it is an oogonium until it is fertilized and embryonated, and particularly when it has acquired an egg shell, has been examined for lipid content and distribution (see Lee, 1975; Bird, 1976). Lee (1975) observed lipid droplets in the cytoplasm of the oogonia of *Dirofilaria immitis*. In the fertilized eggs of *A. galli*, Cruthers (1974) found a triple-layered plasma-membrane-like structure, the innermost layer of which is a homogeneous lipid layer. This innermost lipid layer was later universally found in almost all nematodes studied (Bird, 1976). For example, it was consistently detected in the three-layered egg shell of *E. vermicularis* (Hulinska and Hulinsky, 1973), of *Trichuris suis* (Wharton and Jenkins, 1978) and of the ascarid *Porrocaecum ensicaudatum* (Wharton, 1979c). On the other hand, Wharton (1979b) demonstrated that the egg shell of *Aspiculuris tetraptera* consisted of five layers: the external uterine layer, internal uterine layer, vitelline layer, chitinous layer and lipid layer. The material of the lipid layer was secreted at the surface of the egg cytoplasm and adhered to the inner surface of the chitinous layer (Wharton, 1979a).

G. ADULT ACANTHOCEPHALA

It seems that the lacunar system and subcuticular layer of acanthocephalans, in general, contain lipids (von Brand, 1939, 1966; Bullock, 1949). Early studies on *Macracanthorhynchus*, *Echinorhynchus*, *Neoechinorhynchus* and *Pomphorhynchus* showed lipids in muscles, tissues of reproductive organs, germ balls, vaginal glands and pseudocoelomic fluid. Phospholipids, cholesterol and cholesterol esters were among the lipids detected (von Brand, 1939, 1966; Bullock, 1949). von Brand (1939) quantitatively measured the lipids in the female of *M. hirudinaceous*, located in the pseudocoelomic fluid, muscle and reproductive organs, and found them to be 0·2, 1·3 and 1·9% of fresh tissues respectively.

During the spermatogenesis of *Prosthenorchis*, Guraya (1971) demonstrated that the dictyosomal granules and rods consisted of phospholipids and some

lipoproteins. Mitochondria which were in their early stage in the form of granules eventually fused with the dictyosome to form large deeply sudanophilic bodies with relatively more lipids. Byram and Fisher (1973) observed inclusions of lipid droplets in the syncytial epidermis of *Moniliformis dubius*; and Bogoyavlenskii *et al.* (1975) detected lipids in the cuticle and muscle of *Polymorphus phippsi.*

H. LARVAL ACANTHOCEPHALA

Most of the work has been done on the lipids of the ovum, from its oogonial stage to maturity. Guraya (1969) observed in the growing oocyte of *Prosthenorchis* diffusely distributed lipid inclusion bodies, mainly phospholipids, which proliferated during growth with other organelles and inclusions. The author also found lipoproteins in the yolk nucleus and mitochondria. Parshad and Guraya (1977c) found nuclear resemblances and cytoplasmic similarities of the oogonia and oogonial syncytium of *Centrorhynchus corvi* with regards to lipids, RNA and proteins; and Chu *et al.* (1977) identified lipids histochemically in the fertilization membrane of the ovum of *Echinorhynchus gadi.* They also detected lipids in the central nuclear mass of the acanthor and the lemnisci and hypodermis of the cystacanth. Anantaraman and Ravindranath (1976) found that the second layer of the embryonic envelopes enclosing the acanthors of *Acanthosentis* sp. was composed of glycolipoprotein of which the lipid moiety consisted of esters of unsaturated fatty acids.

III. ABSORPTION OF LIPIDS

A. ADULT CESTODES

Lipid absorption and metabolism has been thoroughly studied in cestodes, particularly in adult *H. diminuta.* As indicated below, many synthetic lipid pathways requiring energy expenditure were probably abandoned by parasitic helminths during their evolution. This is undoubtedly related to the fact that absorption of these lipids, which are constantly present in the small intestine, blood and tissues of the host, is more economical. For example, *H. diminuta* is able to absorb and incorporate into its lipids various saturated and unsaturated fatty acids such as acetate (C_2), palmitate (C_{16}), stearate (C_{18}), oleate ($C_{18:1}$) and linoleate ($C_{18:2}$) (Ginger and Fairbairn, 1966b; Lumsden and Harrington, 1966; Jacobsen and Fairbairn, 1967; Bailey and Fairbairn,

1968), cholesterol (Frayha and Fairbairn, 1968), monoacylglycerols (Bailey and Fairbairn, 1968; Buteau and Fairbairn, 1969) and lysophosphatidylcholine (Buteau and Fairbairn, 1969). The worms seem to display selective absorption of fatty acids from the environment and incorporate them into various lipid fractions thus establishing a species specific lipid pattern (Jacobsen and Fairbairn, 1967). In addition, two distinct sites for fatty acid absorption in *H. diminuta* were detected; one specific for short-chain acids (C_2–C_8) and the other for longer chain acids (C_{14}–C_{24}), known as the 'acetate site' and the 'palmitate site' (Pappas and Read, 1975; Chappell, 1980). It was shown that there was no need for bile salt in the absorption of the short-chain acids; it was only required for the long-chain acids. In fact, acetate was four times more absorbed than palmitate.

The origins of fatty acids available for absorption by *H. diminuta* appear to be from two main sources. The first is the food of the host together with host bile in which a high concentration of phosphatidylcholine exists (Spitzer *et al.*, 1964). The second is through the ingestion of faeces by the rat host (Barnes *et al.*, 1959). From the host's diet triacylglycerols are hydrolysed in the small intestine by pancreatic lipase yielding free fatty acids and 2-monoacylglycerols (Mattson *et al.*, 1952) both of which form micellar solution with bile salts (Hoffman and Borgstrom, 1962). Fatty acids and monoacylglycerols are then absorbed from the micellar solution by *H. diminuta* and incorporated into the lipids of the parasite (Bailey and Fairbairn, 1968), which absorbed these lipids less readily when they were emulsified or sonicated than when they were dissolved in sodium taurocholate micelles. Unsaturated fatty acids (e.g. oleic ($C_{18:1}$) or linoleic ($C_{18:2}$)) and monoacylglycerols (monoolein) were absorbed at a higher rate than saturated ones, even in micellar solutions; for that reason unsaturated acids and monoolein predominated in *H. diminuta* over palmitic (C_{16}) and monostearin (Bailey and Fairbairn, 1968). On the other hand, Chappell *et al.* (1969) found that *H. diminuta* could absorb some palmitate by active mechanisms without it first being dissolved in micellar solution. This mechanism was mediated by a carrier which was probably specific for both saturated and unsaturated long-chain fatty acids. The sites of entry of these long-chain acids seemed to be different from those for short-chain acids. Apparently, this active transport mechanism does not apply to the long-chain fatty acids in micelles, nor does diffusion, since Surgan and Roberts (1976a, b) showed that bile salts were first adsorbed on to the tegument of *Hymenolepis* worms prior to their absorption. On the other hand, the volatile fatty acids were assimilated by *H. diminuta* by active absorption or facilitated diffusion when they were at low external concentration; at higher concentrations (above 2mM) simple diffusion apparently prevailed (Arme and Read, 1968). In this species, acetate absorption was found to be competitive and could be inhibited

by other fatty acids, particularly propionate (Arme and Read, 1968). Apparently, the absorbed fatty acids underwent some modification inside the tissue of the cestode because the endogenous acids of *H. diminuta* were to a certain extent different from the exogenous acids in the diet. This is probably due to the ability of the worm to modify the length of the fatty acid chain, as discussed further below (Jacobsen and Fairbairn, 1967), or to the ability of the host to modify the fatty acids of their diet by the addition of rat bile lipids, chiefly phosphatidylcholine, just prior to their absorption by the worm (Ginger and Fairbairn, 1966b), or to the contribution of unknown sources of fatty acids to modify the lipid composition of the intestinal contents. In support of this last view are the results of Kilejian *et al.* (1968), who ligated the bile ducts of the rat and still found fatty acids transferred to the worm, but at a reduced rate. Similarly, *H. diminuta* adults, which were known not to synthesize cholesterol, efficiently absorbed cholesterol from a mixed micellar solution of sodium taurocholate and monoolein (Frayha and Fairbairn, 1968). Cholesterol, emulsified by ultrasonication, was also absorbed, but at a much lower rate. It seems that the worm competed with its host for micellar solutions of cholesterol, just as it did for micellar solutions of fatty acids and monoolein because a significant quanitity, about 10% of the micellar cholesterol, was absorbed by the parasite. Monoolein was also absorbed from micelles by *H. diminuta* (Bailey and Fairbairn, 1968; Buteau and Fairbairn, 1969). This monoacylglycerol was readily hydrolysed by the parasite and apparently did not become incorporated into triacylglycerols via the monoacylglycerol pathway which is common to the intestinal mucosa (Buteau and Fairbairn, 1969).

Biliary phosphatidylcholine is hydrolysed to form free fatty acids and lysophosphatidylcholine by the action of the phospholipase A present in pancreatic secretions (Van Deenen *et al.*, 1963). Conversely α-acyl-labelled lysophosphatidylcholine was taken up intact *in vitro* by intestinal slices and converted to phosphatidylcholine (Nilsson and Borgstrom, 1967); however, a considerable amount of the labelled fatty acid from lysophosphatidylcholine was also recovered from the triacylglycerols of the tissue. Studies on *H. diminuta* by Kilejian *et al.* (1968) suggested that lysophosphatidylcholine and other digestion products of biliary phosphatidylcholines could be absorbed by *H. diminuta* because when they injected intravenously [^{14}C]palmitate and [^{14}C]linoleate into rats, the label was detected in bile and then incorporated into the lipids of *H. diminuta*, and 70% of the label appearing in the bile was present in phosphatidylcholine, thus suggesting that lysophosphatidylcholine as well as fatty acids from phosphatidylcholine digestion could be absorbed by the parasite. However, these authors did not study the uptake of phosphatidylcholine by *H. diminuta*. Buteau and Fairbairn (1969) proved the incorporation of singly and doubly-labelled lysophosphatidylcholine into the

lipids of *H. diminuta* by incubating the worm in a micellar solution of sodium taurocholate containing [1-^{14}C]palmitic acid labelled lysophosphatidylcholine, or lysophosphatidylcholine labelled with [2-^{3}H]glycerol and [1-^{14}C]palmitic acid. Most of the label was detected in the acylglycerols of the worm.

It seems that bile salts may commonly be beneficial in the absorption of various components of lipids by *H. diminuta* and also in the general metabolism of the worm. Recently Fioravanti and MacInnis (1976) found that the addition of sodium taurocholate to the maintenance media appeared beneficial to *H. diminuta* by prolonging the retention of normal signs. They also found that a combination of additives, taurocholate–nucleosides–lipids, improved the maintenance of the worm for periods exceeding 24 hours as determined by observational criteria and metabolic indices. Of the other adult cestodes which have been examined in relation to lipid absorption is *Spirometra mansonoides* (Meyer *et al.*, 1966). This worm took up acetate *in vitro* as well as long-chain saturated and unsaturated fatty acids. It was also capable of absorbing intact triacylglycerols and phosphatidylcholine. *Raillietina cesticillus* also absorbed significant amounts of lipids from the intestine of chickens (Botero and Reid, 1969). In hosts on low fat diets, the worm absorbed about 10% of the lipid compared to about 30% in hosts on a high fat diet.

Beach *et al.* (1973) showed that *Calliobothrium verticillatum*, a cestode from the spiral intestine of the dogfish, was able to absorb ^{14}C-labelled docosahexaenoic acid ($C_{22:6}$) from *in vitro* preparations and incorporate it into triacylglycerols (50%) and unesterified fatty acids (28%), the rest of the label was also detected in all other lipid components.

During vitellogenesis, the tetraphyllidean, *Echeneibothrium beauchampi* has been reported to accumulate significant amounts of lipid droplets across the membrane system and into the cytoplasm of the vitelline cells (Mokhtar-Maamouri and Swiderski, 1976).

B. LARVAL CESTODES

Very little is known about the absorption of lipids in larval cestodes. Studies appear to be limited to cholesterol uptake by hydatid cysts (Frayha, 1968; Bahr *et al.*, 1979) and palmitic acid uptake by the plerocercoids of *Schistocephalus solidus* and *Ligula intestinalis* (Barrett and Körting, 1977; Körting and Barrett, 1978). *In vivo* experiments by Frayha (1968) on cholesterol absorption by hydatid cysts of *E. granulosus* showed that cholesterol passed from the host tissues into the cysts by a mechanism not yet defined. When albino mice previously infected with hydatid cysts were given radioactive cholesterol in their food pellets, about 10% of the label appeared

within 2 days in the cholesterol pool of the secondary hydatid cysts. This suggested that the parasites entirely derived their sterols from the host and were unable to synthesize it (Frayha, 1968, 1974). The differences in the specific activity of the cholesterol isolated from hosts and parasites probably reflected differences in the size of the respective pools or slow equilibration rates across the cyst wall. In an attempt to define the exact mechanism of action of cholesterol absorption by hydatid cysts, Bahr *et al.* (1979) incubated *in vitro* secondary hydatid cysts in a micellar solution of sodium taurocholate, monoolein and [^{14}C]cholesterol. The mechanism was studied as a function of time of incubation, size of cysts, temperature of incubation, concentration of cholesterol substrate and presence of metabolic inhibitor. The results indicated that cholesterol uptake continuously increased with time of incubation, that small cysts absorbed cholesterol better than large ones, that the optimum temperature for uptake was 25°C and that uptake increased with increase in concentration of substrate. The addition of the metabolic inhibitor 2,4-dinitrophenol did not affect the process of absorption thus eliminating the participation of the mechanisms of active transport or facilitated diffusion. It was suggested that mechanisms of simple diffusion and possibly exchange diffusion were operating in cholesterol absorption by hydatid cysts (Bahr *et al.*, 1979). By stydying the β-oxidation mechanism in the plerocercoids of the pseudophyllidean cestodes *S. solidus* and *L. intestinalis*, Barrett and Körting (1977) and Körting and Barrett (1978) reported that both larvae were able to absorb [^{14}C]palmitate and incorporate it into their neutral and phospholipid fractions.

C. TREMATODES

Little is known about lipid absorption in trematodes. Smith *et al.* (1970) demonstrated the uptake of cholesterol in *S. mansoni*. Isseroff and Walczak (1971) found that *F. hepatica* absorbed acetate, citrate, malate, pyruvate and succinate only by simple diffusion; and Wright and Isseroff (1973) postulated that acetate was absorbed by facilitated diffusion rather than by active transport. Large molecules of lipids such as glycolipids were not absorbed by the schistosomula of *S. mansoni* (Goldring *et al.*, 1977). Recently Rumjanek and Simpson (1980) studied the incorporation and utilization of radioactive lipids by adult *S. mansoni in vitro*. They found that the worm was able to absorb and utilize arachidonic acid ($C_{20:4}$), linolenic acid ($C_{18:4}$), phosphatidylcholine, tripalmitylglycerol (triacylglycerols) and cholesterol. The arachidonic and linolenic acids and fatty acids of tripalmitylglycerol and phosphatidylcholine were present largely as triacylglycerol with smaller amounts of labelled diacylglycerol, phospholipids and fatty acids. The

labelled polar head group of phosphatidylcholine was cleaved from the molecule during incorporation, which suggested that hydrolysis of complex lipids was an integral part of the absorption mechanism. On the other hand, cholesterol was not altered during absorption or metabolized. The authors also reported that arachidonic acid was better absorbed than any other lipid, but did not contribute in the biosynthesis of prostaglandin.

D. NEMATODES

Beams and King (1972) observed that fatty acids could enter the intestinal epithelium of *Ascaris* passively. Their experiments dealt with the rate of uptake of [^{14}C]palmitate as a function of the concentration of the fatty acid, the presence of O_2, CO_2, glucose and bile salts. The results indicated that *in vitro* absorption required exogenous glucose and CO_2; it was also facilitated by bile salts and enhanced by increasing concentration of the acid. On the other hand, it was inhibited by a high partial pressure of oxygen. Transport of the fatty acid into the nematode required energy, and thus was not considered as a simple passive diffusion. Beames *et al.* (1974) found that sterols could also pass across the *Ascaris* intestine by diffusion rather than by active transport. *Ascaris* eggs seem to absorb long-chain fatty acids and triacylglycerols during embryogenesis and convert them into carbohydrate (Jezyk and Fairbairn, 1969; Barrett *et al.*, 1970b).

Digenis *et al.* (1970b) reported the uptake of cholesterol by *T. spiralis* larvae.

E. ACANTHOCEPHALA

Results from studies on lipid absorption in Acanthocephala are somewhat controversial. Pflugfelder (1949) postulated that the lemnisci and praesoma were the sites of lipid absorption. He used lipid dyes rather than radioactive material in his work. After starving *Acanthocephalus ranae* until no lipids could be seen microscopically, Pflugfelder (1949) transplanted the worms into new hosts fed with fat-rich diet, the fat being dyed. After 4 days the lemnisci and praesoma were filled with lipids. Using radioactive substrates and autoradiography, however, Hammond (1968) refuted Pflugfelder's work and postulated that lipid uptake was through the tegument rather than through the lemnisci.

IV. Lipid Synthesis

Certain patterns of lipid metabolism are common to all phyla of parasitic helminths. For this reason we are describing them first and then, in order to avoid unnecessary repetition, discussing their occurrence in the various groups of worms.

However, many studies have reported general lipid synthesis from radioactive precursors without any specification to the nature of lipids. For example Vykhrestyuk *et al.* (1977) found that the cestode *Bothriocephalus scorpii* was capable of manufacturing lipids from host fatty acids. In trematodes, Pappas (1971) observed that *Haematoloechus medioplexus* absorbed 94% of radioactively tritiated arginine into protein and the rest into lipids and carbohydrates. McManus and James (1975) demonstrated that the daughter sporocysts of *Microphallus similis* and *P. pygmaeus* utilized under aerobic conditions ^{14}C-labelled, glucose, galactose, fructose and maltose and incorporated the label into lipids and other compounds. Similarly lipids in *Eurytrema pancreaticum* and *Paramphistomum* sp. were manufactured from host fatty acids (Vykhrestyuk *et al.*, 1977). In nematodes, [^{14}C]glucose was absorbed by *Cooperia punctata* (Slonka *et al.*, 1973), *Setaria cervi* (Anwar and Ghatak, 1976) and *Litomosoides carinii* (Anwar *et al.*, 1977) and the label appeared in lipids and other compounds. Ridley *et al.* (1977) found that the L_4 and adult stages of *Cooperia punctata* were able to incorporate carbon derived from sodium [^{14}C]propionate into their free pool and tissues and converted a portion of it into lipids and proteins. Croll (1972) found that the rates of growth and lipid synthesis in L_2 preinfective larvae of *Ancylostoma tubaeforme* were logarithmic with time thus indicating a predominant feeding phase.

A. Fatty Acids

As mentioned above, the lipids of cestodes are rich in unsaturated fatty acids. In higher metazoa, the synthesis of unsaturated fatty acids appears to be accomplished by desaturating existing fatty acids (Goldfine and Bloch, 1963). The reaction was shown by Erwin and Bloch (1964) to require molecular oxygen as an electron acceptor. The existing saturated fatty acids were synthesized *de novo* from aceytl-CoA which continuously supplied the system with C_2 units by way of malonyl-CoA, thus elongating the chain. In general, parasitic helminths are unable to synthesize fatty acids *de novo*, nor can they synthesize unsaturated acids (see Vykhrestyuk, 1972). For example, *H. diminuta* could not synthesize long-chain saturated fatty acids *de novo* from [^{14}C]acetate (Jacobsen and Fairbairn, 1967); however, the label that was incorporated from radioactive acetate into the fatty acids of *H. diminuta*

was entirely due to the chain elongation of existing fatty acids (Jacobsen and Fairbairn, 1967) which were absorbed from the host pool of acids with which they were similar (Ginger and Fairbairn, 1966b). Therefore *H. diminuta* can lengthen palmitic (C_{16}) and stearic (C_{18}) acids into saturated C_{20}, C_{22}, C_{24} and C_{26} acids. Similarly, the worms are probably capable of absorbing unsaturated fatty acids and transforming them into other acids, because, as anaerobes, they are probably not adapted to dehydrogenate the saturated acids, a process which requires molecular oxygen (Ginger and Fairbairn 1966b). The same pattern of fatty acid absorption and chain-lengthening applies to other cestodes such as *Spirometra mansonoides* (Meyer *et al.*, 1966; Meyer and Meyer, 1972), *Raillietina cesticillus* (Botero and Reid, 1969), *Calliobothrium verticillatum* (Beach *et al.*, 1973) and *T. saginata* (Cicchini *et al.*, 1976a). Only in larval *Spirometra mansonoides* did a very weak mechanism of *de novo* fatty acid synthesis occur, because Meyer *et al.* (1966) reported the incorporation of traces of malonyl-CoA into the higher fatty acids of this worm. Although, as mentioned earlier, cestodes seem to have lost their capability to synthesize fatty acids, they have retained their ability to synthesize all other lipid components from absorbed acids. These components include phospho- and neutral lipids in *S. mansonoides* (Meyer *et al.*, 1966; Meyer and Meyer, 1972), phospholipids, fatty acids and acylglycerols (from absorbed labelled acetate, palmitate, stearate, oleate and linoleate) in *H. diminuta* (Jacobsen and Fairbairn, 1967); triacylglycerols, mono- and diacylglycerols and polar lipids (Buteau and Fairbairn, 1969; Webb, 1974) as well as phospho- and glycolipids in the same parasite (Webb and Mettrick, 1973). Recently Körting (1976) reported that incubated *Schistocephalus* plerocercoids and *Bothriocephalus* and *Khawia* adults with [^{14}C]palmitate did not yield labelled CO_2, but label was incorporated into various lipid fractions. Similarly Körting and Barrett (1978) found that the plerocercoids of *Ligula intestinalis* were unable to oxidize exogenous labelled palmitate, but instead, they readily incorporated it into the phospho- and neutral lipid fractions. Sterol ester, wax ester, mono-, di- and triacylglycerols, cardiolipin, phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine were formed by *C. verticillatum* from absorbed radioactive fatty acids (Beach *et al.*, 1973); and also from absorbed acids, phospholipids and neutral lipids were formed by *Bothriocephalus scorpii* and *Raillietina tetragona* (Vykhrestyuk *et al.*, 1977).

Similarly, trematodes have lost the ability of *de novo* synthesis of fatty acids. This seems not to be only due to the anaerobic status of the parasites but is also an adaptation to the parasitic mode of life, because even trematodes in aerobic environments, such as *S. mansoni*, were found to be unable to use the available oxygen in the blood to synthesize fatty acids (Meyer *et al.*, 1970; Meyer and Meyer, 1972). Trematodes are apparently capable of assimilating

fatty acids by absorbing the already existing acids of the host environment, either without structural modification (Beach *et al.*, 1973; Rumjanek and Simpson, 1980) or, most probably, later elongating them through acetate incorporation. In support of the latter mechanism, Fripp *et al.* (1976) was able to demonstrate that *S. mansoni* can elongate $C_{18:2}$ into longer acids. Similarly *Fasciola hepatica* was unable to synthesize fatty acids *de novo* from acetate but was able to chain-elongate the absorbed fatty acids from the host by addition of acetate units (Coles, 1975; Chappell, 1980) but apparently at a low rate (Oldenborg *et al.*, 1975). Like cestodes, some trematodes namely *S. mansoni* (Meyer *et al.*, 1970; Meyer and Meyer, 1972), *Eurytrema pancreaticum*, *Paramphistomum* sp. (Vykhrestyuk *et al.*, 1977), *F. hepatica* (Coles, 1975; Chappell, 1980) have been shown to be capable of absorbing host fatty acids and incorporating them in almost all components of lipids.

In nematodes, Beames *et al.* (1967) reported that *A. lumbricoides* muscle incorporated the label from acetate into non-volatile fatty acids without establishing whether this was a *de novo* fatty acid synthesis or chain-elongation. The synthesis was weak in comparison to the formation of other lipid components and was carried out by the supernatant soluble mitochondrial fraction of the cell and enhanced by the addition of the cofactors pyridine nucleotide (reduced form), adenosine triphosphate (ATP), CoA, malonate, bicarbonate and manganese ions. It was assumed that the chain-elongation process via acetate incorporation is a more significant one. Greichus and Greichus (1970) were able to demonstrate in *Ascaris* sp. a transformation shift of label from the carboxylic end of one fatty acid into others. For example, the carboxyl group of linoleic acid ($C_{18:2}$) was incorporated into $C_{18:0}$, $C_{18:1}$ $C_{20:2}$, and also into $C_{18:3}$ and $C_{20:4}$ fatty acids. It was not known through which mechanism *Ascaris* sp. desaturated the fatty acids because the last two acids required molecular oxygen in order to be formed from $C_{18:2}$. More recently Turner and Hutchison (1979) showed that the dog heart nematode, *Dirofilaria immitis*, can incorporate [^{14}C]acetate as well as [^{14}C]oleate into its tissue and modify them to other acids, but not through a *de novo* synthesis.

B. ACYLGLYCEROLS

Of all acylglycerols in helminths, triacylglycerols always displayed the highest concentration, never dropping below 75%. Apparently the mono- and diacylglycerols are precursors of triacylglycerols. They are therefore always detected only in small amounts.

It is possible that in gutless helminths and particularly in cestodes, pathways of triacylglycerol synthesis could be similar to those functioning in the small intestine of hosts because the structure of the tegument of these worms

resembles the microtriches of the villi of vertebrate intestines (Rothman, 1963; Lumsden, 1965) in which triacylglycerol synthesis takes place. The mucosa of the vertebrate small intestine resynthesizes triacylglycerols from absorbed products of intestinal fat digestion (Senior, 1964). For this synthesis, intestinal mucosal cells employ two major pathways, the α-glycerophosphate–phosphatidic acid pathway, and the monoacylglycerol pathway. The former also operates in other tissues and more significantly in adipose tissue (White *et al.*, 1973) and the latter exhibits limited monoacylglycerol acyltransferase activity in liver, adipose tissue (Senior and Isselbacher, 1962), mammary gland (McBride and Korn, 1964) and kidney (Hubscher, 1961). In the first pathway, α-glycerophosphate is the metabolic product of endogenous glucose by way of glycolysis and becomes the precursor of acylglycerols. In the mucosal cells some glycerol comes from the hydrolysis of dietary fat or from the hydrolysis of absorbed monoglycerols and within these cells it becomes eventually incorporated into triacylglycerols (Saunders and Dawson, 1962; Haessler and Isselbacher, 1963). The α-glycerophosphate is esterified to absorbed fatty acids to form lysophosphatidic acid. This is a very unstable intermediate, difficult to isolate because it is converted upon the addition of another fatty acid into phosphatidic acid much faster than its formation from α-glycerophosphate (Lands, 1965). Phosphate is then hydrolysed from phosphatidic acid by phosphatidate phosphatase, yielding 1,2-diacylglycerol, and the diacylglycerol is finally acylated to form triacylglycerol (Coleman and Hubscher, 1962; Johnston and Bearden, 1962). In the second pathway, 2-monoacylglycerols are metabolic products of intestinal fat hydrolysis. They form micellar solution with bile salts, as described above, from which they are easily absorbed and directly converted by acylation into diacylglycerols and then triacylglycerols in the intestinal mucosa. The α-glycerophosphate and monoacylglycerol pathways operate independently in the mucosa and both give rise to 1,2-diacylglycerols which enter the same pool and are acylated to form triacylglycerols (Johnston *et al.*, 1967). Triacylglycerols from a given animal or plant species tend to have constant fatty acid patterns. This has been observed to be the case with the triacylglycerols of *H. diminuta* from rats fed a diet of Purine Rat Chow (Harrington, 1965; Ginger and Fairbairn, 1966a). Triacylglycerol synthesized via the monoacylglycerol pathway were characterized by the retention of the 2-monoacylglycerol structure (Brockerhoff, 1966) and, unlike α-glycerophosphate, a 2-monoacylglycerol is symmetrical and on acylation will most likely form a triacylglycerol with a symmetrical distribution of fatty acids (Brockerhoff and Hoyle, 1967).

In an attempt to determine triacylglycerol synthesis in *H. diminuta*, Buteau and Fairbairn (1969) found that the α-glycerophosphate–phosphatidic acid pathway was the only one operating in the cestode because

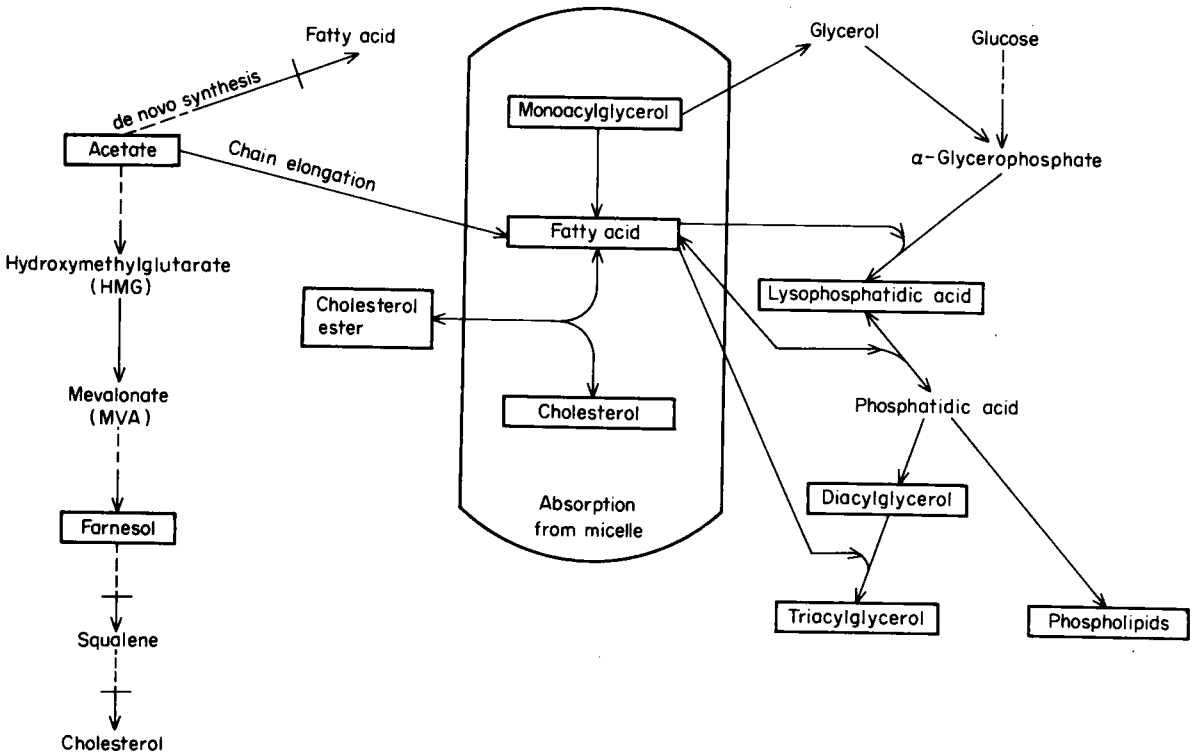


FIG. 1. Predominant metabolic pathways of the major classes of lipids in parasitic helminths.

when the worm was incubated with radioactive glycerol, fatty acids and lysophosphatidylcholine, the label eventually appeared in the triacylglycerols in a selective way and not by random distribution; in addition, the important intermediates of this pathway, namely phosphatidic acid and diacylglycerols, were extensively labelled. On the other hand, when monoacylglycerols were absorbed by the worm they were rapidly hydrolysed and did not retain their fatty acids before they were converted into di- and triacylglycerols via the monoacylglycerol pathway; their fatty acids were rather randomly and unsymmetrically distributed in other acylglycerols, instead of being specifically esterified with regularity if direct acylation had prevailed (see Fig. 1).

Buteau and Fairbairn (1969) also observed that *H. diminuta* had a significant capacity to synthesize phospholipids because when it was incubated with lysophosphatidylcholine it quickly absorbed this substrate and transformed it—not only to triacylglycerols—but also to phosphatidic acid, phosphatidylethanolamine and phosphatidylcholine.

More recently Webb (1974) and Webb and Mettrick (1975) confirmed that the triacylglycerol synthesis of *H. diminuta* was through the α-glycerophosphate–phosphatidic acid–diacylglycerol pathway.

Following the same pattern of triacylglycerol synthesis, adult and larval *Spirometra mansonoides* (Meyer *et al.*, 1966; Meyer and Meyer, 1972), *Schistosoma mansoni* (Meyer *et al.*, 1970; Meyer and Meyer, 1972), and homogenates of *Ascaris* ovaries (Jezyk, 1968) formed triacylglycerols provided that an exogenous supply of fatty acids was available (see also Vykhrestyuk, 1972). Recently Turner and Hutchison (1979) demonstrated that the dog heart nematode, *D. immitis*, was able to synthesize all classes of complex lipids including free cholesterol from [^{14}C]glycerol, [^{14}C]acetate and [^{14}C]oleic acid. In acylglycerols, the diacylglycerols and phosphoglycerols exhibited the greatest proportion of label regardless of the substrate employed, but was higher with oleic acid than with glycerol as a precursor. A very small fraction of label appeared in triacylglycerols as well as cholesterol esters.

C. PHOSPHOLIPIDS AND GLYCOLIPIDS

Little is known about the synthesis of these lipids by parasitic helminths. The first occasion on which ^{32}P was used in studies on cestode phospholipid metabolism was by Meyer *et al.* (1966) who found that *Spirometra mansonoides* was capable of synthesizing its own complex lipids, including phospholipids, provided that exogenous fatty acids and sterols were present. Adult and larval *S. mansonoides* incorporated [^{14}C]oleate at various degrees into the components of phospholipids. Phosphatidylcholine displayed the highest activity (65·5–67·6%) followed by phosphatidylethanolamine (14·2–24·8%)

and then minimal activity in cardiolipin, phosphatidylserine and phosphatidylinositol. On the other hand, incubation with labelled glucose yielded labelled glycerol which was almost totally (91%) transformed into phospholipids and acylglycerols. From these results Meyer and Meyer (1972) concluded that this cestode possessed the enzymes necessary for synthesizing their own complex lipids *de novo*. In *H. diminuta*, the phospholipids could originate from the lipids in the bile of the host (Ginger and Fairbairn, 1966b; Kilejian *et al.*, 1968; Bailey and Fairbairn, 1968) or by *de novo* synthesis (Webb and Mettrick, 1971). Labelled phospholipids as well as fatty acids and acylglycerols originated in this cestode from radioactive precursors such as acetate, palmitate, stearate, oleate and linoleate (Jacobsen and Fairbairn, 1967) but no *de novo* synthesis was assumed. Ginger and Fairbairn (1966b) suggested that lipids from the bile of the host contributed to the phospholipid metabolism of the tapeworm because when they fed ^{14}C-labelled starch to rats infected with *H. diminuta* they observed that label was incorporated in the water-soluble components of the parasite's polar lipids such as glycerol and inositol but not in higher fatty acids. Kilejian *et al.* (1968) provided evidence for the above suggestion by demonstrating an incorporation of label in the bile of the host when [U-^{14}C]palmitate and [U-^{14}C]linoleate were injected intravenously: 72% of the label was recovered as phosphatidylcholine and significant radioactivity was detected in the lipids of the worm. Bailey and Fairbairn (1968) suggested that lysophosphatidylcholine resulting from hydrolysis of radioactive phosphatidylcholine in bile may be absorbed by the parasite. Buteau and Fairbairn (1969) demonstrated that there was extensive labelling of diacylglycerols and phosphatidic acid in *H. diminuta* tissues after exposing the worm to ^{14}C- and ^{3}H-labelled glycerol and fatty acid. A complete *de novo* synthesis of phospholipids by *H. diminuta* was undertaken by Webb and Mettrick (1971). They followed the incorporation of [^{32}P]orthophosphate into the phospholipids of *H. diminuta* for 4 hours *in vitro*. Uptake began after 15 minutes incubation and the rates and pattern of incorporation of ^{32}P into phosphatidic acid, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, lysophosphatidylcholine and phosphatidylethanolamine plus cardiolipin indicated that *H. diminuta* possessed mechanisms for the *de novo* synthesis of these lipids. The label was most rapidly incorporated into phosphatidic acid and in the rest of phospholipids as a function of time. The authors concluded that the major pathway utilized by *H. diminuta* in fatty acid incorporation was phosphatidic acid, and this supported the conclusion of Buteau and Fairbairn (1969) that the major pathway of fatty acid incorporation in *H. diminuta* was via the α-glycerophosphate–phosphatidic acid–diacylglycerol pathway. Later, Webb and Mettrick (1975) found that when *H. diminuta* was incubated with labelled glucose the label appeared in the glycerol moiety of the acylglycerols and

phosphoglycerides, and also in the inositol moiety of phosphatidylinositol and galactose moiety of glycolipids. They observed that only traces of the label were incorporated into the phospholipids which were in the following order of abundance: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, lysophosphatidylcholine, cardiolipin, phosphatidic acid, lysophosphatidic acid and phosphatidylglycerol. The interrelationship between serine and the metabolism of phospholipids, glycolipids, and other lipids by *H. diminuta* was studied *in vitro* by Webb and Mettrick (1973). The label from [^{14}C]serine, [^{14}C]glucose and [1-^{14}C]oleic acid was rapidly incorporated by the worm into phospho- and glycolipids, the latter illustrating the synthesis of cerebrosides by *H. diminuta*. The important phospholipids were phosphatidylserine and phosphatidylethanolamine; the majority of the label in the two compounds was associated with serine and ethanolamine moieties. In the cerebrosides the label was associated with the sphingosine moiety and the sugar moiety was galactose instead of glucose. The absorption of serine and its conversion into phosphatidylethanolamine was not effected by exogenous ethanolamine. Moreover, incubation of *H. diminuta* homogenates with phosphatidyl[^{14}C]serine resulted in the recovery of considerable activity in phosphatidylethanolamine. The authors postulated that the major pathway of phosphatidylethanolamine synthesis is via the decarboxylation of phosphatidylserine. In their study on *in vitro* incorporation of [^{14}C]docosahexaenoic acid by *C. verticillatum*, Beach *et al.* (1973) recovered the label in the phospholipids of the worm tissues namely in phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cardiolipin. The major phospholipid detected was phosphatidylcholine. Phospholipids were also formed in the plerocercoids of *Ligula intestinalis* from labelled palmitate (Körting and Barrett, 1978).

Fasciola hepatica and *S. mansoni*, when incubated with [^{32}P]phosphate, [^{14}C]glycerol and ^{14}C-labelled long-chain fatty acids, were capable of forming phospholipids (Meyer *et al.*, 1970; Meyer and Meyer, 1972; Rumjanek and Simpson, 1980; Chappell, 1980). The label from the first two precursors appeared in the glycerol moiety of the phospholipids while that of the fatty acid appeared in the fatty acid moiety; the most important phospholipids synthesized were lysophosphatidylcholine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, ethanolamine plasmogen and cardiolipin.

Goldring *et al.* (1977) found that the schistosomula of *S. mansoni* was able to synthesize a lipid which chromographed with bovine cerebroside (ceramide monohexoside) but failed to elaborate larger glycolipids.

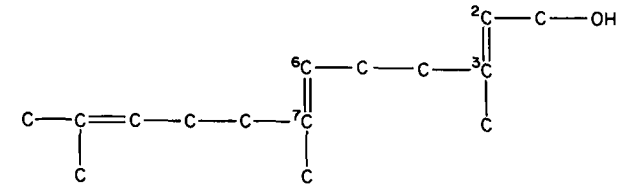
McManus *et al.* (1975) demonstrated that the sporocysts of *Microphallus similis* incorporated the label from [1-^{14}C]acetate and [1-^{14}C]palmitate into glycolipids (cerebrosides) and phospholipids of which phosphatidylserine,

lysophosphatidylcholine, sphingomyelin, phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine and cardiolipin were identified.

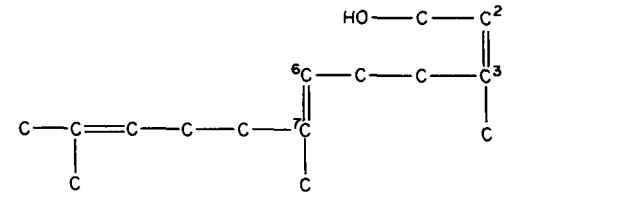
In nematodes, *Ascaris* homogenates were found to incorporate labelled fatty acid into phosphatidic acid prior to its conversion into triacylglycerols (Jezyk, 1968). Turner and Hutchison (1979) found that *D. immitis* can rapidly take up [^{14}C]acetate and [^{14}C]oleate and incorporate them into phosphoglycerides.

D. STEROLS AND STEROL ESTERS

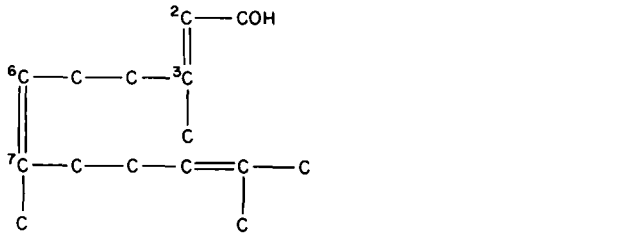
Since cholesterol was found to be the major and universal sterol of parasitic helminths, its assimilation and metabolism by the parasites has received considerable attention. Its *de novo* synthesis, absorption and metabolism has been studied in several parasites and certain patterns have been elucidated. In higher animals, the *de novo* synthesis of cholesterol from acetate has been established through a series of steps and in the presence of molecular oxygen (Bloch, 1965). The important intermediates in the pathway which were used as key compounds in cholesterol biosynthetic studies were the 6-carbon 3-hydroxy-3-methylglutaric acid (HMG), the 5-carbon mevalonic acid (MVA), the C-15 farnesylpyrophosphate or farnesol, the C-30 squalene and C-27 cholesterol. In the series of reactions leading to squalene, molecular oxygen is not needed. The conversion of squalene into cholesterol depends on four molecules of oxygen. The first is in the cyclization of squalene to lanosterol and the other three in the hydroxylation of each of the three methyl groups in lanosterol prior to their decarboxylation to form cholesterol. Any interference with oxygen availability will therefore block the synthesis. Another limiting factor in cholesterol biosynthesis is the operational three sites of inhibition during the conversion of acetate, or HMG, or MVA into cholesterol, for Gould and Swyryd (1966) found that when cholesterol becomes highly concentrated in the tissue its synthesis is inhibited by any or all of the following sites: one allosteric type preceding MVA, a second between MVA and farnesyl pyrophosphate and a third between farnesyl pyrophosphate and squalene. A third important limiting factor is the high specificity and isomerism occurring between the precursors and their enzymes. Most cholesterol intermediates, and particularly farnesol, possess many isomeric configurations due to the isoprene units in their structures. Four farnesol isomers are known, namely *cis–cis*, *trans–cis*, *cis–trans* and *trans–trans*; the last two occur in nature, and only *trans–trans* farnesol could serve as precursor for cholesterol synthesis (Fig. 2). Probably because of their adaptation to an anaerobic life or restricted oxygen, or because of the operational inhibitory sites or isomeric hindrance mentioned above, almost all parasitic



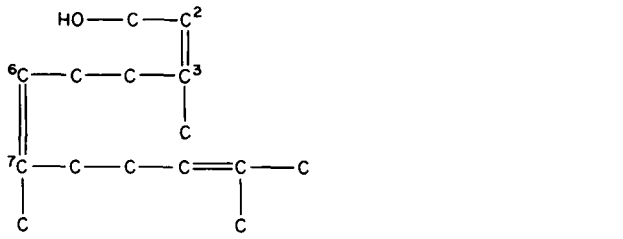
2-*trans*, 6-*trans*



2-*cis*, 6-*trans*



2-*trans*, 6-*cis*



2-*cis*, 6-*cis*

FIG. 2. Farnesol isomers.

helminths—with two exceptions noted so far (McManus *et al.*, 1975; Turner and Hutchison, 1979)—were not able to synthesize cholesterol *de novo* from the common precursors acetate or hydroxymethylglutarate (HMG) or mevalonate (MVA). This deficiency was reported from studies conducted on *S. mansonoides* (Meyer *et al.*, 1966; Meyer and Meyer, 1972), *E. granulosus*, *E. multilocularis* and *T. hydatigena* (Frayha, 1968, 1971, 1974), *H. diminuta* (Frayha and Fairbairn, 1969), *S. mansoni* (Meyer *et al.*, 1970; Meyer and Meyer, 1972; Rumjanek and Simpson, 1980), *E. revolutum*, *A. lumbricoides*, *M. hirudinaceus* and *M. dubius* (Barrett *et al.*, 1970a). However, once cholesterol was assimilated by absorption, it was readily esterified by the parasites

into cholesterol esters. This was demonstrated in experiments which used exogenous radioactively labelled fatty acids in the incubation media; the worms, on all occasions, displayed the label in cholesterol esters (Meyer *et al.*, 1966, 1970; Meyer and Meyer, 1972; Beach *et al.*, 1973; Turner and Hutchison, 1979; Rumjanek and Simpson, 1980). If the inability of parasitic helminths to undertake the *de novo* synthesis of cholesterol from acetate is an adaptation to anaerobic life, one would assume that the intermediates which do not need oxygen for their formation, such as HMG, MVA and farnesol, might be synthesized by the worms. In an attempt to clarify this point, Frayha (1968, 1974) and Frayha and Fairbairn (1969) conducted a series of experiments on the larval cysts of *E. granulosus* and *T. hydatigena* and on adults of *H. diminuta*. In these experiments, the label of a particular substrate was trapped in the subsequent intermediate compound after making a pool of this intermediate from exogenous authentic unlabelled compounds in order to trap the label from the precursor. In this way the authors demonstrated that the worms were able to synthesize HMG from acetate, MVA from acetate and HMG and farnesol from acetate, HMG and MVA. Farnesol was also found to be a naturally existing intermediate in both parasites (Thorson *et al.*, 1968; Fioravanti and MacInnis, 1977). However, the worms were unable to go beyond farnesol in the biosynthetic pathway to cholesterol. In addition, the farnesol synthesized seemed to show most of its activity in the *2-cis,6-trans* isomer and little in the *2-trans,6-trans* isomer. As it is known, in higher animals only the *2-trans,6-trans* isomer is used for squalene and cholesterol synthesis. It was therefore assumed that, due to steric hindrance in the precursor configuration, the worms were unable to form squalene and cholesterol. Raj and Ranjanathan (1972) found that *A. lumbricoides* was able to incorporate the label from [^{14}C]mevalonate into a non-saponifiable lipid fraction which had the characteristics of a polyprenol; this could be related to the results of Frayha on farnesol since this compound is classified as C-15 prenol. More recently, Fioravanti and MacInnis (1977) examined the farnesol in the tissue of *H. diminuta* and found it to be of the *2-trans, 6-trans* type. No *2-cis,6-trans* isomer was isolated, but instead a nonprenoid compound was co-chromatographed with *cis–trans* farnesol on GLC. In view of the above findings, it would be assumed that the inhibition of *de novo* cholesterol synthesis in parasitic helminths is not of the allosteric type, although there is always a high quantity of cholesterol in the tissues of worms which would have led to allosteric inhibition at the level of HMG. Nor is the inhibition due to the functional inhibitory sites since two of the three sites, namely the one between HMG and MVA and the one between MVA and farnesol, were not affected; in all instances HMG, MVA and farnesol intermediates were synthesized. Consequently, the absence of *de novo* synthesis of cholesterol by the worms may be due to the steric hindrance in the shift from

2-cis,6-trans farnesol to squalene and also to the adaptation to the anaerobic life (Fairbairn, 1970) because oxygen is needed in the cyclization process of squalene to lanosterol and in the oxygenase-type reactions which transform lanosterol to cholesterol firstly by hydroxylation and then by decarboxylation. In contrast to the usual pattern of lack of *de novo* cholesterol synthesis in parasitic helminths, McManus *et al.* (1975) found that the sporocysts of *Microphallus similis* were able to incorporate 26% of the label from [1-^{14}C]-acetate into sterols and sterol esters. Similarly, Turner and Hutchison (1979) reported that adult *D. immitis* could incorporate significant quantities of labelled acetate into free cholesterol.

Barrett *et al.* (1970a) showed the inability of *A. lumbricoides* and *M. hirudinaceus* to transform [^{14}C]β-sitosterol into cholesterol by dealkylating the side chain at C_{24} and thus the worms could not convert the phytosterol into cholesterol. In addition, the authors found that these two worms plus *M. dubius* and *E. revolutum* were not able to convert acetate or mevalonate into farnesols or cholesterol.

E. ASCAROSIDES

The origin of free and esterified ascarosides were studied in ovarian homogenates (Jezyk and Fairbairn, 1967b) and uterus sections (Tarr and Fairbairn, 1973) of *Ascaris*. Jezyk and Fairbairn (1967b) found that the precursor of the carbohydrate moiety of the ascarosides, ascarylose, was glucose which, after forming glucose 1-phosphate, was converted into 3,6-dideoxyhexose nucleotide phosphate. This carbohydrate intermediate was transformed into ascarosides by reacting with the aglycone. The aglycone itself originated from the combination of two fatty acids between the carboxyl group of palmitic acid or higher acids and the α-methylene carbon of a second acid. One mole of CO_2 was liberated from this reaction which in a way resembled the *de novo* mechanism of fatty acid synthesis via the malonyl-CoA pathway, because it required the same co-factors, ATP, CoA and reduced pyridine nucleotide. Another pathway, although less significant, was also probably operating in the synthesis of the aglycone, through chain lengthening by means of C-2 units since [^{14}C]acetate was incorporated into ascarosides. It is probable that palmitic (C_{16}) and stearic (C_{18}) acids were formed in this way then combined, as mentioned above, to form the aglycone. When examined in various tissues, and particularly the uterus, ascarosides were found esterified with volatile fatty acids. After fertilization, the fertilized eggs broke the ester bonds and deposited the free ascarosides in the innermost lipoidal membrane of the egg shell (Tarr and Fairbairn, 1973); this has the effect of hardening it and making it resistant to adverse conditions (see above). At the beginning of their

travel in the oviduct, the oocytes contained large amounts of ascaroside esters and negligible amounts of free ascarosides. Following fertilization, the esters started to break down when oocytes were approaching the uterus and 99·8% of conversion occurred in oocytes localized in the uterus at a distance of 20 mm from the oviduct. Some of them took a longer time and completed the conversion at 100 mm distance from the oviduct. Tarr and Fairbairn (1973) observed that the formation of the ascaroside layer of the shell and the development of an impermeability of the egg to glycerol were always associated with the appearance of free ascarosides. In the uteri which contained only unfertilized eggs having no shells, both free and esterified ascarosides existed throughout their length. All the above results imply that the major function of ascaroside esters synthesized in the ovaries is to provide a source of free ascarosides for the egg shell in the uterus.

V. Lipid Catabolism, Utilization and Excretion

Lipid catabolism seems to be essential in higher animals because it provides the most concentrated source of energy, yielding per gram over twice as many calories as do carbohydrates or proteins (White *et al.*, 1973). Triacylglycerols, which constitute the major bulk of neutral lipids, are hydrolysed by intestinal *lipase* (mainly from pancreatic juice) into fatty acids and glycerol; incomplete hydrolysis yields a mixture of mono- and diacylglycerols. Glycerol derived from triacylglycerols or phosphoglycerides (see below) enters the glycolytic pathway for further degradation. A group of *esterases*, other than the above lipase, is also present in the pancreatic juice of higher animals and catalyse the hydrolysis of short-chain fatty acid esters, e.g. tributyrin, and of other fatty acid esters, notably cholesterol esters. Of the esterases, the phosphatases, which hydrolyse esters of phosphoric acid, are a large and complex group of enzymes, some of which appear to be highly specific and operate on phosphoglycerides and other phospholipids. *Phospholipase A*, also from the pancreas, acts on phospholipids and particularly phosphatidylcholine to produce lysophosphatidylcholine. Fatty acid catabolism is an oxidation reaction requiring oxygen and occurring in one of three ways: (a) β-oxidation, which is the major pathway existing in the mitochondria, (b) α-oxidation, occurring in the microsomes of certain tissues and (c) ω-oxidation, also demonstrated in the microsomes of liver cells and bacteria. In β-oxidation, the fatty acyl-CoA derivatives (formed by the catalysis of *acyl-CoA synthetase*), not the free fatty acids, are involved in these reactions yielding two carbon atoms in the form of acetyl-CoA in the following sequence (1) dehydrogenation catalysed by a flavoprotein, *acyl dehydrogenase*, to yield the α, β-unsaturated derivative, (2) hydration of the

double bond by the help of *enoyl hydrase* to form β-hydroxy compound, (3) dehydrogenation involving NAD^+ and *β-hydroxyacyl dehydrogenase* (or 3-hydroxyacyl dehydrogenase) to yield the β-keto derivative and (4) combination of the β-keto acyl-CoA with a second CoA to yield acetyl-CoA and fatty acid derivative of CoA that is shorter by two carbon atoms. This last reaction is catalysed by a *thiolase* (or *acetyl-CoA acyl transferase*). Successive repetition of this sequence results in the complete degradation of an even-numbered carbon fatty acid into acetyl-CoA. Odd-number acids yield acetyl-CoA and propionyl-CoA. β-oxidation must be tightly coupled with the tricarboxylic acid cycle a mechanism that avoids the accumulation of acetate.

In α-oxidation of fatty acids, the initial step of hydroxylation is catalysed by monooxygenase requiring oxygen and Fe^{2+} and eventually yielding CO_2 instead of acetate. In ω-oxidation, the long-chain fatty acids undergo ω-oxidation to ω-hydroxy fatty acids which are subsequently converted into α, ω-dicarboxylic acids. These are then shortened from either end by the β-oxidation sequence described above. The reaction is also O_2 dependent and requires NADPH.

A. LIPID CATABOLISM

Information on lipid catabolism in parasitic helminths has been fragmentary and scarce. Most of the studies have been confined to the breakdown of fatty acids through β-oxidation, whereas limited work was ascribed to the catabolism of other lipid components. However, not all fatty acids were found free in parasitic tissues; many seemed to originate from hydrolysis of fatty acid esters. In fact, some esterases and more specifically lipases have been demonstrated in parasitic worms. Nonspecific esterases have been detected in various organs of cestodes, trematodes and nematodes (see von Brand, 1973; Jenkins, 1973; Sood and Gupta, 1977; Nwosu and Croll, 1978). Evans (1971) demonstrated the presence of tissue-specific and tissue-nonspecific isozymes of esterase in *Ascaris* sp. The most common esterases studied in helminth parasites have been the cholinesterases and particularly the acetylcholinesterases (specific and nonspecific). Identification was either by biochemical assays in the cestodes, *D. latum* and *H. diminuta*, the trematodes *F. hepatica* and *S. mansoni* and the nematodes, *A. lumbricoides*, *L. carinii*, *N. brasiliensis*, *Oesophagostomum venulosum*, *Dictyocaulus filaria*, *Bunostomum trigonocephalum*, *Leidynema appendiculata*, *S. ratti* and *A. tubaeforme* or by histochemical localization in the various organs of the worms (literature in von Brand, 1973, 1979; Nwosu and Croll, 1978).

Lipases which were specific esterases have been described from numerous helminths. In *H. diminuta*, the lipase was shown to hydrolyse absorbed

monoolein on the body surface or near to it, liberating fatty acids for further metabolism (Bailey and Fairbairn, 1968). Other than this report, no biochemical assay appears to have been carried out on triacylglycerol lipase in parasitic worms, although their existence has been demonstrated in the intestine and tissues of a broad spectrum of parasites (see von Brand, 1973, 1979; Lee and Atkinson, 1976).

Alkaline and acid phosphatases have been found in many different organs of parasitic helminths with higher concentrations in excretory and/or secretory sites such as tegument of cestodes; gastrodermis, excretory canals and tegument of trematodes; intestinal cells of nematodes; and the subtegument of acanthocephalans (Bogitsh, 1975; Terwedow and Huff, 1976).

When considering data on the fatty acid catabolism of helminths, distinctions should be made between volatile and non-volatile acids, mature anaerobic and immature aerobic stages of the same species, and anaerobic and aerobic worms of different species.

Controversial results have been reported on the breakdown of volatile fatty acids and particularly acetate. No utilization was observed by Schwabe (1957) in *Nippostrongylus muris*. Moderate utilization, under aerobic conditions, was reported by Bueding (1949) for *Litomosoides carinii* and substantial amounts of acetate were demonstrated by Frayha (1971) to be oxidized by the protoscoleces of *E. granulosus*, *E. multilocularis* and cysticerci of *T. hydatigena*. Other volatile fatty acids such as 2-methylvalerate were also utilized by *Ascaris* eggs during their aerobic development (Fairbairn, 1955b; Jezyk and Fairbairn, 1969) even at a higher rate than acetate (Saz and Lescure, 1966). These volatile acids were the by-products of muscle metabolism and when esterified (mainly as triacylglycerols) were apparently available for further utilization during egg embryonation (Fairbairn, 1955b). These triacylglycerols became enormously depleted during embryonation. Recently, Ridley *et al.* (1977) found that the larvae of *Cooperia punctata* were capable of partially oxidizing [^{14}C]propionic acid to CO_2 and partially incorporating the label into the lipids and proteins of the parasite.

Regarding the catabolism of non-volatile long-chain fatty acids, almost all of the studies have been centred around β-oxidation. So far, no adult parasitic helminth or its larval parasitic (anaerobic) stage have yet been demonstrated to possess a functional β-oxidation pathway, although in most of them the enzymes of the β-oxidation reactions have been detected in their tissues. In contrast, free-living aerobic immature stages of parasitic helminths show an operational β-oxidation mechanism for fatty acid catabolism. For example, adult *A. lumbricoides*, *H. diminuta* (Ward and Fairbairn, 1970a, b), *S. ratti* (Körting and Fairbairn, 1971), *M. dubius* (Körting and Fairbairn, 1972), *F. hepatica* (Barrett and Körting, 1976), plerocercoids of *Schistocephalus solidus* (Barrett and Körting, 1977) and *L. intestinalis* (Körting and Barrett, 1978) contained either the complete or partial series of the β-oxidation

enzymes namely acyl-CoA synthetase for short chain (acetate, propionate and butyrate) and for long chain (palmitate, stearate, oleate and linoleate), acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase (or β-hydroxyacyl-CoA dehydrogenase) and acyl-CoA acyltransferase. But none of the above mentioned worms were able to utilize the β-oxidation enzymes to catabolize exogenous [U-^{14}C]palmitate. Similarly, but without demonstrating the presence of the enzymes, von Brand (1934) and Greichus and Greichus (1966, 1967) showed that starving *A. lumbricoides* failed to utilize and oxidize [U-^{14}C]palmitate. This non-functional β-oxidation pathway in the above mentioned worms, could be attributed to their anaerobic mode of life, for molecular oxygen is required in β-oxidation to reoxidize the reduced flavoprotein and NADH which is produced. Alternatively, it could be ascribed to the fact that acetyl-CoA, once formed as an end product of β-oxidation, cannot be further metabolized, since the tricarboxylic acid cycle is non-functional in these species (see Barrett, 1976; Körting and Barrett, 1978). In *A. lumbricoides*, the β-oxidation enzymes may catalyse the formation of C_5 and C_6 branched-chain acids (Ward and Fairbairn, 1970b). It has also been shown that β-oxidation enzymes are not involved in volatile fatty acid synthesis in this species (Komuniecki *et al.*, 1981). In addition, it seems that there is no evidence for the co-fermentation of lipids and carbohydrates in helminths. What then is the function of these enzymes? Körting and Barrett (1978) assumed that these enzymes might be involved in the formation of branched C_5 and C_6 acids, as demonstrated in *A. lumbricoides* adults by Ward and Fairbairn (1970b), or in the elongation of long-chain fatty acids by the successive addition of C-2 units via acetyl-CoA, as was shown to occur by Seubert *et al.* (1968). This pathway is independent from malonyl-CoA and operates by direct reversal of the same enzymic steps of β-oxidation except for acyl-CoA dehydrogenase. It is also functional in mammalian mitochondria and uses an NADP-linked dehydrogenase instead of a flavoprotein enzyme (as described above). Another possible function of the β-oxidation enzymes in the worms is their possible catalysing effects in the catabolism of the aliphatic amino acids, valine, leucine and isoleucine, which involved steps similar to those of β-oxidation (see Körting and Barrett, 1978). β-Oxidation enzymes can also catalyse the formation of butyryl-CoA from acetyl-CoA through the reversal of β-oxidation pathway, as shown by Stansly and Beinert (1953). Finally, the enzymes could also be present in these helminths as a 'carry over' from an active β-oxidation sequence functional in free-living aerobic stages, as discussed below.

In contrast to anaerobic adult and larval parasitic helminths, the aerobic immature stages, particularly the free-living stages of parasitic nematodes, have been shown to catabolize readily exogenous long-chain fatty acids through the classical β-oxidation pathway (Ward and Fairbairn, 1970a;

Körting and Fairbairn, 1971). For example, Fairbairn (1957) and Passey and Fairbairn (1957) studied the oxidation of lipid in *A. lumbricoides* eggs and demonstrated the beginning of triacylglycerol disappearance after 5 days of development. This became maximal after 14 days, then proceeded at a slower rate. The authors observed that with lipid utilization there was formation of CO_2 and suggested the occurrence of β-oxidation. Ward and Fairbairn (1970a) later confirmed the presence of a functional β-oxidation pathway. They detected the presence of all the active enzymes in β-oxidation, namely, acyl-CoA synthetase (short and long chain), acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and acetyl-CoA acyl transferase in the developing eggs of *A. lumbricoides*. They also demonstrated the presence of tricarboxylic acid cycle enzymes which must be coupled with those of β-oxidation. Consequently, they showed the breakdown of exogenous [U-^{14}C]palmitate into $^{14}CO_2$ by the eggs. An interesting correlation was reported between the disappearance of triacylglycerol and the specific activities of acyl-CoA dehydrogenase. Furthermore, a short-chain acyl-CoA synthetase was highly active between days 5 and 15 when volatile acids were utilized preferentially, whereas a long-chain acyl-CoA synthetase was maximally active on the 15th day of egg development, at a time when higher fatty acids were primarily utilized. The enzymes enoyl-CoA hydratase and acetyl-CoA acyltransferase kept a pronounced activity during the entire period of egg development. Although the onset of lipid utilization by developing *A. lumbricoides* eggs was accompanied by an increase in the catalytic capacities of the corresponding pathway as described above, there was however, no drop in the catalytic capacities of the pathways when the eggs became dormant, nor was there any change when the dormant egg was activated (Barrett and Beis, 1975).

In *S. ratti*, in contrast to the parasitic adults which were unable to oxidize palmitic acid (C_{16}) because they lacked palmityl-CoA synthetase and NAD-dependent isocitrate dehydrogenase (Körting and Fairbairn, 1971), starving larvae lost great quantities of their triacylglycerols and free fatty acids while their cholesterol and phospholipids remained unchanged, indicating that triacylglycerols and free fatty acids were oxidized for energy production (Barrett, 1969). The used acids were $C_{18:1}$ and $C_{20:1}$, the former being oxidized twice as fast as the latter, in 8 days. Körting and Fairbairn (1971) showed that the free-living adults of *S. ratti* and infective free-living larvae were able to oxidize [U-^{14}C]palmitate into $^{14}CO_2$ via a functional β-oxidation pathway. They demonstrated the presence of all of the β-oxidation enzymes as well as those of tricarboxylic acid cycle. On the other hand, the first-stage larvae, which were considered as intermediate between parasitic and free-living stages, only lacked the NAD^+-dependent isocitrate dehydrogenase (Körting and Fairbairn, 1971).

In *E. granulosus* protoscoleces cholesterol esters are probably hydrolysed into cholesterol (Digenis *et al.*, 1970a). In *H. diminuta* cholesterol, which was absorbed from micellar bile solutions by the worm, was rapidly converted (about 50%) in 1 hour or less to a single unidentified metabolite (Frayha and Fairbairn, 1968). This is probably the same undefined metabolite described by Andrews and Allen (1974) in *T. spiralis* larvae. Homogenates of *S. mansoni* can convert a number of mammalian sterioids into a variety of related metabolites (Briggs, 1972).

B. LIPID UTILIZATION

As shown above, parasitic helminths contained appreciable quantities of lipids. Probably because of their anaerobic mode of life, most of the intestinal helminths studied do not utilize significant amounts of lipids even during starvation and under aerobic conditions. This has been reported, however, in the cestodes, *Moniezia expansa*, *H. diminuta*, *T. taeniaeformis*, *S. mansonoides*, *Schistocephalus solidus*, *Ligula intestinalis*; the trematodes, *F. hepatica*, *F. gigantica*, *P. explanatum* and *G. crumenifer*; the nematodes, *A. lumbricoides*, *S. ratti* and the acanthocephalan *M. dubius* (for references see von Brand, 1973, 1979; Barrett, 1976, 1981; Körting and Barrett, 1978).

Conflicting results have been reported, however, from non-intestinal and, particularly, tissue parasites. For example, *T. spiralis* larvae were shown by von Brand *et al.* (1952) to utilize lipids when they were mobile and only under aerobic conditions. Castro and Fairbairn (1969) failed to confirm these results; but their larvae were incubated under different conditions. Ferguson and Castro (1973) and Ferguson (1973) reported also the inability of *T. spiralis* larvae to oxidize aerobically [U-^{14}C]palmitate, but surprisingly adult *T. spiralis* from the intestine readily oxidized this acid, although endogenous lipid utilization was not proved. In addition, Subrahmanyam (1967) claimed that *L. carinii*, when incubated *in vitro*, did consume triacylglycerols (but no plasmalogen). Popiel and James (1976) stated that there was large endogenous lipid catabolism in the daughter sporocysts of *Cercaria linearis* when they were starved.

Similarly, tissue migratory larvae as well as active free-living larvae of parasitic helminths and particularly nematodes displayed different mechanisms of lipid utilization. Croll (1972) and Croll *et al.* (1975) reported lipid utilization in larvae of *A. tubaeforme* but saw no evidence that lipid could be converted into carbohydrate. Croll and Matthews (1973) related the activity, ageing and penetration of *A. tubaeforme* larvae with lipid consumption rather than lipid level. In contrast, Nwosu (1977) found that there was also no correlation between the drop of the lipid level in the same parasite and

larval penetration of the host; a distinct correlation, however, existed between lipid loss in function of time and larval activity (Nwosu, 1978b; Nwosu and Croll, 1978). Leštan *et al.* (1973) observed a considerable decrease in lipid reserves of *Ascaridia galli* between days 11 and 52 after infection.

Grineva and Chebyshev (1975) observed the depletion of stored lipid and glycogen in 2–4 day-old larvae of *A. suum*. From day 5, larvae fed actively, but only the glycogen content increased. This suggestion of lipid conversion into glycogen was also reported by Rubin and Trelease (1975) who observed, in the posterior region of *Ascaris* larvae, dense granules among lipid bodies during the resynthesis period, suggesting triacylglycerol–glycogen interconversion. Wilson (1976) also suggested that *H. contortus* larvae utilized lipids and converted them into carbohydrates.

In trematode larvae some conflicting results have been reported. Hoskin (1973) suggested the utilization of lipids in the rediae of *Himasthla quissetensis*, and Popiel and James (1978) reported endogenous lipid and carbohydrate utilization together with autolysis in the daughter sporocysts of *Cercaria stunkardi* and *C. linearis*. In contrast, DiConza and Basch (1976) found no specific esterase or lipase activity in mother and daughter sporocysts of *S. mansoni*.

C. LIPID EXCRETION

Only a limited number of parasitic helminths are able to degrade lipids into CO_2 and hence gain energy, and not all lipids have been utilized for metabolic processes. In addition, it seems most unlikely and uneconomical in terms of energy preservation to consider lipids and, in particular, higher fatty acids, as end products of carbohydrate metabolism as suggested by von Brand (1966, 1973), for as Barrett (1976) pointed out, it takes more energy to rebuild a fatty acid from acetate than that obtained in breaking down glycogen to acetate. As a matter of fact, Reid (1942) after starvation of the host, could find no increase (or decrease) in lipid concentration in the fowl cestode, *Raillietina cesticillus*, although there was a drastic reduction in glycogen. Similarly, Fairbairn *et al.* (1961) found that, in fasted hosts, *H. diminuta* lived mainly at the expense of its carbohydrate but did not use any of its lipids. Triacylglycerols apparently had no value in the energy metabolism of cestodes nor do they appear to accumulate due to synthesis from end products of carbohydrate metabolism (Buteau and Fairbairn, 1969). What is therefore the biological significance of these lipids in worm tissues? Could some of them serve as structural compounds or growth factors, others as transport agents in transport mechanisms, and the rest only as secretory and excretory waste products? In fact, Bolla *et al.* (1974)

found that the free-living stages of *N. brasiliensis* required sterol along with porphyrin or heme compound for growth and survival. Farnesol, as well as its aldehyde farnesal, which were found to exist naturally in the protoscoleces of *E. granulosus* hydatid cysts (Thorson *et al.*, 1968; Frayha, 1974) and the tissues of *H. diminuta* (Frayha and Fairbairn, 1969; Fioravanti and MacInnis, 1977) has been found to display distinct biological activities, similar to juvenile hormone, in the development and growth of certain helminth parasites such as *T. spiralis*, *H. diminuta* and *Mesocestoides corti* (Meerovitch, 1965; Thorson *et al.*, 1968; Tofts and Meerovitch, 1974; Kowalski and Thorson, 1976). Vercelli-Retta *et al.* (1975) showed that there were highly differentiated metabolic areas in the germinal membrane of hydatid cysts of *E. granulosus*. These areas accumulated lipids and enzymes and were topographically related to the origin and insertion of the brood capsules in which the metabolism of lipids and their resultant products served in the development and growth of the protoscoleces. In support of a possible transport function for lipids, Gupta *et al.* (1974) studied the function of sudanophilic lipids, mainly triacylglycerols, phospholipids and lipoproteins which were associated with walls of the excretory canal and vesicle of the protonephridia of 12 species of digenetic trematodes. The lipids seemed to be involved in the transport of lipid-soluble metabolic products through the wall of the protonephridial system. Numerous studies, both early and recent, have described lipids as secretory or excretory products. Most of this work, however, has been confined to trematodes and particularly *F. hepatica*. von Brand and Weinland (1924) demonstrated histochemically that fat droplets were excreted via the excretory system of *F. hepatica*. Weinland and von Brand (1926) reported the production and excretion of higher fatty acids by this parasite during anaerobic carbohydrate metabolism *in vitro*. Stephenson (1947) reported similar results and suggested that this excretion could be due to a need of the parasite to eliminate certain lipids and particularly cholesterol which was absorbed from the cholesterol-rich environment. Mansour (1959) observed that the volatile fatty acids, propionic and acetic, accounted for most of the eliminated products of anaerobic carbohydrate metabolism of *F. hepatica*. The excretion of several classes of lipids including cholesterol has been shown to occur in this trematode under aerobic conditions (Burren *et al.*, 1967). Agreeing with the results of Weinland and von Brand (1926), Burren *et al.* (1967) demonstrated that lipid excretion was as much as 2% of the parasite's wet weight per day. Bennett and Threadgold (1973) saw lipid droplets in the excretory system of newly excysted juvenile *F. hepatica* and considered them to be excretory products. Hrzenjak and Ehrlich (1976) studied the polar lipid secretions in *F. hepatica*, *Paramphistomum microbothrium*, *Moniezia benedeni*, *A. lumbricoides* and *E. granulosus* and found that cerebroside ester was only secreted by *F. hepatica*, phosphatidylserine

only by the protoscoleces of *E. granulosus*, and phosphatidylinositol by all worms parasitic in the liver. Lipid excretion was also noticed in other trematodes besides *Fasciola* sp. For example, Fried and Shapiro (1975) found that lipid droplets in the intestine of the metacercariae of *Leucochloridiomorpha constantiae* consisted mainly of triacylglycerols and cholesterol esters. They also observed the excretion of sterols. Halton and Stranock (1976) demonstrated the release of lipid droplets by exocytosis in the apical vacuoles of the caecal epithelial cells of *Calicotyle kröyeri*. In *E. revolutum*, neutral fat, consisting of free fatty acids, free sterols, sterol esters, triacylglycerols and diacylglycerols were seen to be excreted by cultured metacercariae (Butler and Fried, 1977) and metacercariae and adults of *Cotylurus* sp. (Fried and Butler, 1977). Senft *et al.* (1978) made the unusual observation that the gynaecophoral canal tegument of *S. mansoni* seemed to be a site of active lipid secretion.

For cestodes, the work of Reissenweber *et al.* (1975) suggested lipid elimination through the excretory pore of protoscoleces and brood capsules of *E. granulosus*, because high quantities of lipids and intense activities of simple esterase and lipase along with other enzymes were demonstrated in this region.

In nematodes, a great variety of fatty acids, mainly volatile, varying in number between C_2 and C_6 were reported to be excreted by some. For example, *A. lumbricoides*, *Parascaris equorum*, *T. spiralis*, *Trichuris vulpis*, *Ancylostoma caninum*, *Angiostrongylus cantonensis* displayed various degrees of fermentation by excreting a complex mixture of volatile fatty acids, the most important of which were acetic, proprionic, n-valeric, n-methylbutyric, tiglic, 2-methylvaleric, n-caproic (for references see Greichus and Greichus, 1966; Castro and Fairbairn, 1969; Warren and Pool, 1970; von Brand, 1973, 1979). The spirurid, *Tetrameres fissispina* is reported to extrude from its digestive organelles (phagolysosomes) lipid droplets accumulated during digestion (Riley, 1973).

VI. Comparison Between Host and Parasite Lipids

Studies on the comparison of lipids in parasites and their hosts, have produced conflicting results and no distinct pattern has been discernible. For example, Buteau *et al.* (1969) found that a high quantity of unsaturated fatty acids existed in cestodes parasitizing sharks, of which the polyunsaturated C_{20} and C_{22} acids were identical to those of the hosts. However, the C_{16} and C_{18} acids which were also commonly found in the worms were different from those of sharks. Cain and French (1975) reported that the fatty acid profile of the lung fluke, *Haematoloechus medioplexus* exhibited a

relatively high degree of unsaturation and more closely resembled the lung tissue than the blood of the bullfrog host. McManus *et al.* (1975) observed that the lipid fractions of the daughter sporocysts of *Microphallus similis* and those of the digestive gland cells of their snail host were qualitatively identical although quantitative variations were apparent. A slight decrease in triacylglycerols and fatty acids were shown in infected digestive glands; most neutral lipid levels were lower in the parasite than in the host tissue but sterols, sterol esters and phospholipids were higher. Pronounced quantitative differences between the polyunsaturated acids of linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) series of *S. mansoni* and the host serum have been found by Fripp *et al.* (1976). Similarly, Hutchison *et al.* (1976) observed that the level of unsaturated fatty acids was twice that of saturated fatty acids, with C_{16} and C_{18} acids representing 70% of total fatty acids in *D. immitis*, *D. viteae* and *L. carinii*. The fatty acid pattern of the parasites differed considerably from those of the serum of the dog host. The recent work of Vykhrestyuk *et al.* (1977) added more complexity to the picture. They compared the lipids of the platyhelminths *Eurytrema pancreaticum*, *Paramphistomum* sp., *Bothriocephalus scorpii*, *Nybelina* sp. larvae and *Raillietina tetragona* with those in the tissues of their normal hosts. In general, the neutral fats and phospholipids were similar in the parasite and its host tissue but *B. scorpii* and *R. tetragona* had no sphingomyelin; *E. pancreaticum* contained almost no phosphatidylserine, but more cardiolipin than the host. The fatty acid composition was similar in helminth and host but not identical. The authors suggested that helminths were capable of manufacturing specific lipids from the host fatty acids.

VII. General Comments

This review has attempted to provide a reasonably comprehensive review of the literature on lipid metabolism. Because of the difficulties of assessing the reliability of the various methods used, especially by earlier workers, the data presented is more encyclopaedic than analytical and each result must therefore be examined critically in the light of the techniques used and the protocols followed.

Compared with the carbohydrate metabolism of helminths, lipid metabolism has been relatively rarely reviewed, although there are a number of useful accounts in some monographs and text books (Barrett, 1981; Fairbairn, 1969; von Brand, 1952, 1966, 1973, 1979). The reasons for this are not hard to seek and are probably related to the fact that: (a) some of the literature has been published in less well-known journals; (b) the techniques of lipid

identification and analysis have improved enormously within recent years and, as mentioned above, much of the value of earlier work is open to question; (c) some areas of metabolism have been rather neglected, so that it is difficult to obtain an integrated view of the lipid metabolism; (d) workers have tended to concentrate on those species which are readily available in quantity (e.g. *Ascaris*, *Fasciola*), with the result that the metabolism of only a limited number of species has been investigated in any depth.

Nevertheless, in spite of these limitations and of individual doubtful or conflicting results, one general metabolic pattern is emerging—namely that parasitic helminths, with a few exceptions, have lost the ability to synthesize complex lipids *de novo*. Exceptions to this are, for example, the nematode *Dirofilaria immitis* and (to a lesser extent) the cestode *Spirometra mansonoides*. This raises the intriguing question of how this situation arose during evolution. When initial parasitization took place were a few individuals 'pre-adapted' to this situation or did it arise as the result of mutation and selection? Comparative studies on the metabolisms of closely related free-living and parasitic species may throw some light on this question.

The dependence of parasitic helminths on host lipids may have other implications which may not have been altogether appreciated by parasitologists. For example, to what extent could the nature of a host lipid—in relation to the parasite's requirements—determine host specificity? Is it not possible to suppose that a particular species may have become narrowly adapted to the availability that it can only survive in a host providing such a molecule? Could this be the explanation of the failure of certain 'strains' of helminth to survive in a certain host? This is a problem which is less likely to arise in relation to carbohydrate or protein metabolisms where (in most cases) complex carbohydrate or proteins can be built up from relatively simple molecules of a non-specific nature.

The failure of parasitic helminths to synthesize lipids *de novo* could also have important implications in the field of *in vitro* culture. Most media used for *in vitro* culture are based on that used for culture of mammalian tissues and pay scant attention to any possible specific lipid requirements. This could explain the failure to grow many helminth species *in vitro*, although, of course, numerous other factors are also likely to be involved.

From the data reviewed above, it is evident that the most neglected area in lipid research is that of lipid catabolism. This is, perhaps, understandable, because earlier workers had not at their disposal the sophisticated techniques now readily available in most laboratories. Hopefully, the next decade should see the appearance of data on all stages of many more species than those investigated in the past and so build up a more comprehensive picture of helminth metabolism than is at present available.

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Subject Index

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