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Ahari, Esmaeil Ebrahimzadeh

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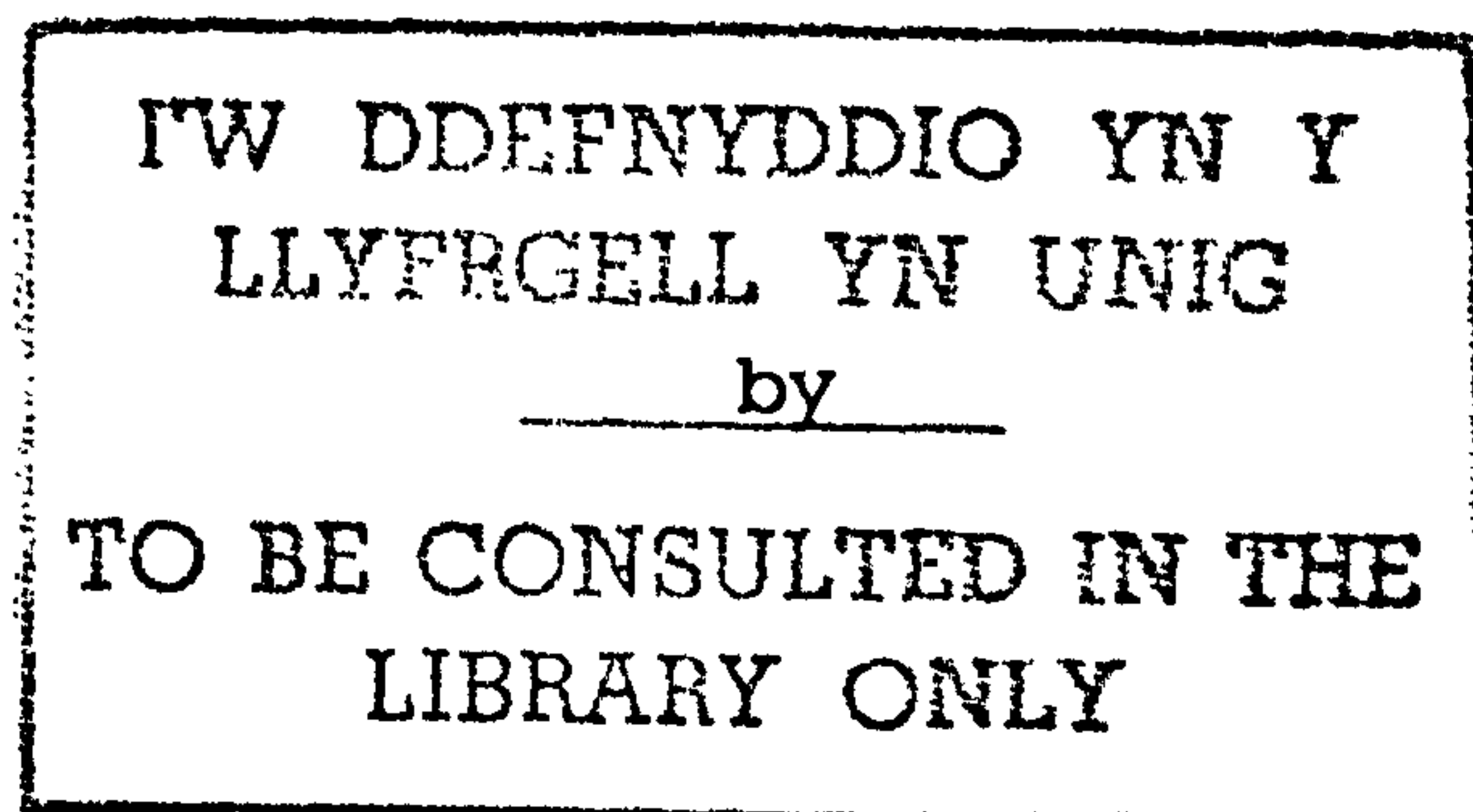
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STUDIES ON THE BIOLOGY OF SCHISTOSOMA MARGREBOWIEI

A thesis submitted to the University of Wales

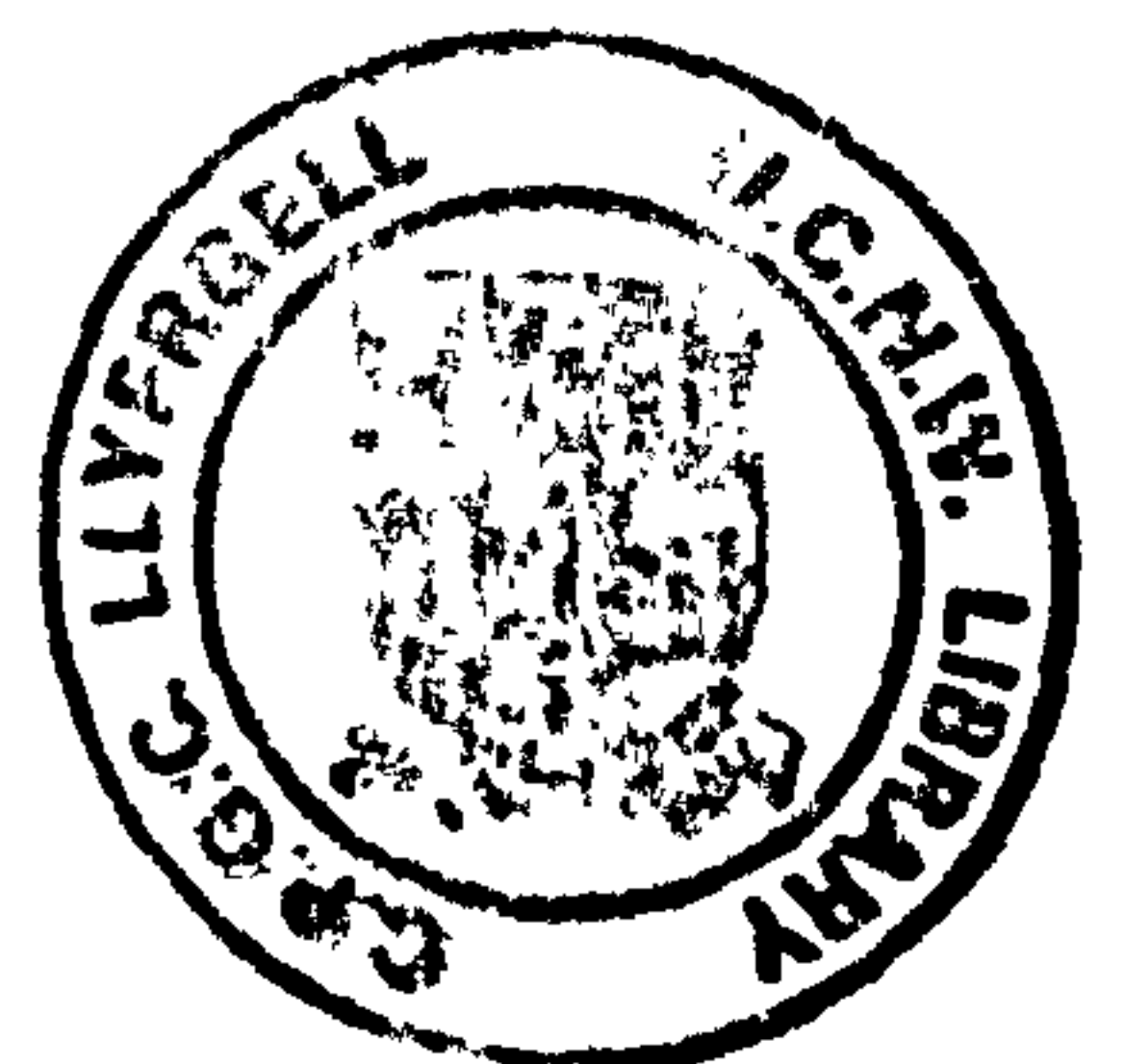


Esmaeil Ebrahimzadeh Ahari, BSc, MSc

In candidature for the degree of Doctor of Philosophy.

School of Biological Sciences,  
University of Wales, Bangor,  
Bangor,  
Gwynedd.

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## Summary

Studies on the biology of Schistosoma margrebowiei include, a simple means of culturing and infecting Bulinus natalensis snails; the morphology and ultrastructure of various stages in the life-cycle; pathology; cercarial longevity and infectivity; cross-reactivity with S.mansoni rabbit anti-sera and the possible use of S.margrebowiei egg homogenate in the serodiagnosis of S.haematobium patients.

A simple method of maintenance and infection of B.natalensis snails en masse, was found to yield a rapid and continuous supply of material. The results indicate that the size of snails at the time of exposure is an important factor in successful infection.

A wide range of morphological and ultrastructural similarities were found between S.margrebowiei larval stages and those of other species of the genus. Whereas the adult worms are among the largest, the eggs, miracidia and cercariae of S.margrebowiei, are among the smallest in the genus.

The pathology associated with S.margrebowiei, is due to deposition of large numbers of eggs in various organs of the infected animal. Eggs were not only recovered from the liver and intestine but following 50 days post-infection, from the spleen. A large number (10-15%) of the total eggs recovered from mice 45 to 65 days post-infection were deposited in the spleen.

The cercariae of S.margrebowiei by utilizing their glycogen reserves, can live for up to 70 hours in fresh water at temperatures of 26-28°C. This observed life-span can be prolonged when water temperatures were decreased to 8-12°C. Cercariae kept in cold water although physically active and still infective, were found to be attenuated as measured by a reduced percentage of recovered worms compared with controls.

The potential for immunizing mice with the hepatopancreas from infected and uninfected snails against schistosomiasis has been evaluated using S.mansoni. Although a reduction in the number of worms and eggs was observed in mice immunized with infected hepatopancreas when compared to the controls, this decrease was not significant.

Sera from 53 patients infected with schistosomiasis were studied by ELISA using S.margrebowiei crude soluble egg antigen (SEA), S.mansoni SEA and cationic S.mansoni egg antigen (CEF6). It was found that S.margrebowiei SEA was more specific for the identification of S.haematobium infections.

## **CHAPTER ONE**

**A general introduction to schistosomiasis.**



## CHAPTER ONE

### Introduction:

Schistosomes are digenetic trematodes belonging to the family Schistosomatidae, super-family Schistomatoidae, sub-order Strigeatida, order Prosostomata, sub-class Digenea, class Trematoda and phylum Platyhelminthes. They are blood-feeding helminths which parasitize mammals and other vertebrates (Jordan & Webbe, 1982).

Schistosome literally means "Split body" and refers to the presence of a deep longitudinal groove, " Schist " or the gynaecophoric canal, on the ventral surface of the male, in which the female lives in copulo. Here the function of the female is to produce eggs. Her position in the host vein is therefore maintained using hardly any energy. It is believed that the female receives nutrients from the male by passive transfer across the tegument (Smyth & Halton, 1983). So unlike other trematodes (which are, with the exception of the family Didymozoidae, hermaphroditic) schistosomes have developed separate sexes.

The disease schistosomiasis is also known as bilharzia or bilharziasis named after the German pathologist Theodor Bilharz who first discovered the parasite in Egypt in 1851. The infection is endemic in 76 countries from East to West across the tropics and sub tropics. The disease affects 200 million people with another 500-600 million considered at risk (WHO, 1990). The incidence of the disease is increasing due to the introduction of large scale agricultural projects involving irrigation in endemic areas (WHO, 1973). Poor

personal hygiene, indiscriminate urination and defecation into water together with water contact due to occupational, domestic and religious practices are among the main factors which maintain transmission of the disease. At the same time due to movement of populations connected with commerce, tourism and displacement of refugees, there are increasing opportunities for the spread of the disease provided the appropriate snail is present in the new area.

Species of the genus Schistosoma have a complex life cycle involving a number of stages typical of digenetic trematodes. The stages involved are the egg, miracidium, sporocyst, cercaria, schistosomulum and adult. Unlike many other digeneans there is no redial stage and no metacercaria. The cercaria of Schistosoma species enters the definitive host by direct penetration. The alternation of generations, that is, an asexual phase followed by a sexual phase, which occurs in schistosomes is a typical digenean characteristic.

There are about eighteen species of Schistosoma currently recognized, three of which are of paramount medical importance: Schistosoma mansoni (Sambon, 1907), S.japonicum (Katsurada, 1904) and S.haematobium (Bilharz, 1852). The former two species occupy the superior and inferior mesenteric veins of the intestine respectively; but the third one lives within the plexus of veins around the bladder, hence the terms intestinal and urinary schistosomiasis. A fourth species, S.mekongi (Voge et al., 1978) also occurs in man and dogs, it is endemic on Khong Island, Vietnam, Southern Laos and some areas of Northern and Central



Kampuchea (Jordan & Webbe, 1982, Soulsby, 1982). A fifth species, S.intercalatum (Fisher, 1934), a form of intestinal schistosomiasis has also been reported from man in Equatorial Guinea, Cameroon, Gabon, Central African Republic, Chad and Congo (WHO, 1989). Furthermore Greer et al., (1988) reported the presence of a S.japonicum-like organism in Malaysia which has been named S.malayensis. Man can be infected also by other species. Zoonotic schistosomiasis can be divided into two categories: 1) schistosome dermatitis due to penetration of the skin by cercariae of the schistosome species which naturally occur in birds and mammals<sup>and</sup> which often cause dermatitis in man in many temperate countries of the world (Trichobilharzia ocellata for example is a duck schistosome which often causes dermatitis in man) and 2) patent zoonotic schistosomiasis, this group of mammalian schistosomes may or may not produce dermatitis in man but do develop to maturity in man. These species include, S.bovis, S.mattheei, S.curassoni, S.rodhani and S.margrebowiei.

Schistosomes are usually considered as belonging to one of three groups; those with a lateral egg spine as typified by S.mansoni, those with a terminal egg spine as seen in S.haematobium and those with round, minute spined eggs as seen in S.japonicum.

Host-parasite relationships vary considerably according to the combination of host and schistosome. Mice and primates, for example allow all three principal human schistosome species (S.mansoni, S.japonicum and S.haematobium) to develop into sexually mature adult



parasites that deposit eggs in the tissue. They also cause the development of immunopathological changes as a consequence of egg deposition. ~~Some~~ rats on the other hand, allow S.mansoni to develop for only four weeks, after which the majority of worms are expelled. The few parasites that remain are stunted and produce few non-viable eggs. The guinea pig is intermediate between these two rodents in that it permits mature egg-laying worms of S.mansoni to develop, but the eggs are non-viable and not excreted in the faeces. Other examples of host-parasite combinations which allow full development of the parasite are S.japonicum in rabbits, S.haematobium in hamsters or baboons, S.bovis in mice (Smithers & Doenhoff, 1982), and S.margrebowiei in mice and hamsters (Ogbe, 1985).

#### **Pathological Consequences Of Schistosome Infection:**

There is no increase in the number of adult worms in the definitive host by reproduction and most infected individuals in fact carry low worm burdens. Schistosomiasis patients frequently suffer intestinal disorders such as vomiting and diarrhoea. Later in the course of the disease, those infected with S.mansoni or S.japonicum develop hepatosplenomegaly, portal hypertension, eosinophilia, intestinal polyposis, portal fibrosis etc. Generally, liver function is intact despite egg-induced granulomatous and fibrotic lesions. Those infected with S.haematobium suffer bladder damage and haematuria. The major cause of pathology is the infected person's immune reaction to eggs in the form of granulomata.

### Concomitant Immunity:

A state of resistance to re-infection has been observed in many experimental hosts, including the rhesus monkey (Macacca mulatta) (Smithers & Terry, 1965a) the grivet monkey (Cercopithecus aethiops) (Cheever & Duvall, 1974) the baboon (Papio anubis) (Damian et al., 1974) the rat (Smithers & Terry, 1965b) and the mouse (Olivier & Schneidemann, 1953). These hosts, when infected with S.mansoni conform to the concept of "concomitant immunity" a term used by Smithers and Terry (1969a and 1969b) to describe the situation where the host is resistant to re-infection but unable to rid itself of the primary infection. However, the induction mechanisms in these hosts are not the same. For example, in the rhesus monkey the adult worms alone are thought to be responsible for the induction of resistance (Smithers & Terry, 1967, Smithers, 1968), whereas in the mouse the development of resistance is dependent upon egg production by adult worms (Long et al., 1978), since neither single sex (non-patent) infections nor pre-patent bisexual infections were capable of rendering the host insusceptible to a challenge infection.

The presence of host material on the surface of the worm might explain the mechanism of concomitant immunity. According to Smithers (1972), the adult worm liberates antigens that induce a state of immunity against the migrating schistosomula of a challenge infection; but this immunity is ineffective against the adult worms because host material on their surface serves in some way to block or mask the sites that are vulnerable to the host's immune response.



As a result, the parasite would not only avoid destruction by the host but, through the agency of the host's immunity, would create a barrier against continual reinfection that might otherwise lead to overcrowding and death of the host (Smithers, 1972).

#### Heterologous Immunity:

A number of workers have shown that acquired immunity to schistosomes is both intra and inter-specific. Hsu and Hsu (1961 and 1963) found that previous inoculations of rhesus monkeys with cercariae of the zoophilic strain of S.japonicum induced immunity against a challenge with the human strain. Sadun et al., (1961) reported similar results in mice. In rhesus monkeys S.bovis, S.mattheei and S.japonicum, induced protection against S.mansoni (Eveland et al., 1969, Amin et al., 1968). In baboons, S.rodhaini and S.bovis immunized against S.mansoni (Taylor et al., 1973). Immunity also developed after exposure to a schistosome of a different genus to that of the challenge infection. It has been shown in mice that S.mansoni protects against Schistosomatium douthitti (Hunter et al., 1961) and Ornithobilharzia turkestanium protects against S.bovis, S.haematobium and S.mansoni (Massoud & Nelson 1972).

Eveland et al., (1969) concluded that the degree of cross-protection between different strains within a species and between different species within a genus is related to the phylogenetic<sup>et</sup> relationships of the schistosomes concerned.

## Diagnosis:

For diagnosis of schistosomiasis one usually relies on direct parasitological methods, including microscopy and immunodiagnostic assays.

Microscopical techniques are based on the microscopic examination of excreta for the demonstration of the parasite eggs. To increase the sensitivity of microscopical diagnosis of schistosome infection, the faecal specimen has to be concentrated. Techniques include Bell filtration method (Bell, 1963), the glycerin concentration method (Faust, 1946), and the zinc-flotation method (Zaman & Cheong, 1967). For large scale field application, the Kato thick smear technique (Katz et al., 1972) is often used for the diagnosis of S.mansoni and S.japonicum infections. The urine filtration method (Peters et al., 1976) is commonly applied for the diagnosis of infections with S.haematobium.

A vast number of serological tests have been developed for the diagnosis of schistosomiasis. These assays include tests for the detection of antibodies, antigens and immune complexes. Many antigen preparations have been used as target antigens in tests demonstrating schistosome antibodies. These include crude, semi-purified and highly purified egg and worm antigens. Among these the isolation of a fraction CEF6 from crude egg homogenate by chromatography has enhanced the degree of sensitivity in serodiagnosis of S.mansoni infections (Dunne et al., 1984).

## Intermediate host:

Schistosomes are transmitted from infected vertebrates



to susceptible vertebrates via intermediate stages developing in aquatic snails. The regions of the world in which the snails are distributed reflect the endemic areas of the disease. These hosts are snails of the sub-class Pulmonata the genus Biomphalaria for S.mansoni, Bulinus for S.haematobium, S.margrebowiei and S.intercalatum and the sub-class Prosobranchia, genus Oncomelania for S.japonicum and S.suifilum. (Jordan & Webbe, 1982, Loker, 1983). The snails are aquatic, although Oncomelania species maybe amphibious and are associated with large bodies of open water, including man-made lakes.

#### Control:

The control of schistosomiasis depends upon an understanding of the disease complex, the biology, ecology and distribution of the parasites and their intermediate hosts. At present control continues to be largely based on drugs (chemotherapy), use of molluscicides and improvements in public health/education, the latter two are difficult and costly to implement. A number of drugs damage the worm's tegument and cause their destruction, these include Praziquantel (Becker et al., 1980, Mehlhorn et al., 1981, Shaw and Erasmus, 1983a, 1983b), Oxamniquine (Kohn et al., 1982) Amoscanate (Voge & Bueding, 1980, Leitch & Probert, 1984), 153C51 (Watts, 1978), Antimony (Standen, 1955), Hycanthone (Hillman et al., 1977) Niridazole (Huang, 1981) and BW484C (Watts, 1986). The most commonly used drugs are praziquantel and oxamniquine. Low activity in the search for and development of other antischistosomal drugs is explained

by the absence of any signs of resistance towards the present drugs. This is probably a complacent attitude since resistance has invariably resulted against most antiparasitic compounds. Molluscicides such as niclosamide are commonly used with chemotherapy. Although no vaccine against schistosomes has yet been developed to a stage that would be suitable for use in man, several candidate vaccine antigens have been identified and investigations of their effectiveness are in progress in laboratory models. The majority of these molecules with immunoprophylactic potential are present in the schistosomula surface and many fall within a restricted molecular weight range, 15-38kDa (reviewed in Simpson and Smithers, 1985). Among these p28 or GST (glutathione S-transferase) has been proven the most promising (Capron et al., 1992).

## Ruminant Schistosomiasis:

Domestic livestock and wild game in Africa have long been known to harbour schistosomes. Much controversy and confusion has existed regarding the number of valid African bovine schistosome species. S.magna, S.faradji, S.hippopotami and S.edwardiensi are not considered valid species, and various reports of S.spindali and S.japonicum are all believed to refer to S.leiperi and S.margrebowiei, respectively. The following four species, all belonging to the terminal-spined <sup>43</sup>schistosomes species complex, are accepted as valid schistosome species: S.bovis, S.mattheei, S.leiperi and S.margrebowiei (Christensen et al., 1983).

### Schistosoma margrebowiei:

S.margrebowiei was first described and recorded by Le Roux (1933). It was named in honour of his wife Dr.Margaret Gregor Le Roux (née Bowie). The specimens, on which the diagnosis was made, were collected from cattle, a zebra, lechwe kobs (Kobus leche), reedbuck (Redunca arundium), pukus (Kobus vardoni) a blue wildebeest (Connochaetes taurinus), a sitatunga (Tragelaphus spekei), and a roan antelope (Hippotragus equinus) Le Roux (1933). It is endemic in two focal areas. The first is in South East Africa, through Zambia; it spreads from Katanga to Botswana (Pitchford, 1976). The second is in the Chad Republic, where schistosomes in small numbers were collected in 1965, 1967 and 1968 (Graber, 1969).

The intermediate hosts of S.margrebowiei are bulinid snails which also serve as hosts for S.haematobium and



S.bovis. The only known naturally infected intermediate hosts for this schistosome are Bulinus forskalii, B.scalaricus and B.tropicus (Wright et al., 1979, Southgate and Knowles, 1977).

Pitchford and Du Toit (1976) successfully infected snails of the diploid B.tropicus complex : B.depressus and B.natalensis in the laboratory and Southgate and Knowles (1977) have obtained infections in a wide range of diploid populations as well as in tetraploid B.truncatus snails.

Although not a parasite of man, there are reports of human patients suffering from S.margrebowiei infections. Eggs were recovered in stools of 4 out of 39 persons in Kwenda in June 1976, but only 2 from 471 persons examined in the whole of East Caprivi strip in September 1975 were positive which led to the conclusion that man is a poor host with low grade short term infection acquired during early Summer after the floods have receded (Pitchford and Wolstenholm, 1977). Other reports include findings in Botswana (Pitchford, 1976), Zaire (Walkiers, 1928), Mali (Lapierre and Hein, 1973) and a Zambian student in Czechoslovakia (Giboda et al., 1988).

Pitchford (1976) suggested that there is an "inverse" relationship between lechwe schistosomes (S.margrebowiei and S.leiperi) and the human schistosomes (S.mansoni and S.haematobium). So in areas where lechwe were scarce high levels of S.mansoni and S.haematobium were found and in areas with high lechwe or puku populations, very low S.mansoni and S.haematobium infections were observed. This cross protection is also evident in Sicily, where S.bovis is prevalent in cattle but no S.haematobium occurs in man, although the local

strain of B.truncatus is highly susceptible to both parasites. So in places where man is constantly exposed to cercariae of schistosomes which do not normally mature in man, some degree of immunity to infection with human schistosomes is evident (Le Roux, 1961). Furthermore, Pitchford (1977b), pointed out that S.mattheei did not occur in cattle in areas where lechwe schistosomes were prevalent. This heterologous immunity between these four species becomes more complex when one considers the studies of Wright et al., (1979) in Lochnivar National Park. These workers showed that S.margrebowiei infection was only present in very young lechwes, while older animals were only infected with S.leiperi, and concluded that, S.margrebowiei infection in lechwes was suppressed by a later exposure to S.leiperi. However, this pattern of infection could be explained by the movement of the herds throughout the flood season and their contact with suitable intermediate hosts.

Although S.margrebowiei has a large number of intermediate hosts, its distribution is limited due to a combination of factors including, limited primary definitive host distribution, "introggressive" ~~infection~~ i.e. the suppressive nature of mixed infections (as explained above), heterologous immunity and the physical behaviour of the cercariae. Southgate (1978) had already postulated that the restricted distribution of S.intercalatum could be due to the aggregations formed by the cercariae in response to small increases in temperature. The same phenomenon was observed in S.margrebowiei. The implication being that aggregation is due



to the secretions from the post-acetabular glands hence rendering the parasite less infective. The shedding pattern of S.margrebowiei at dawn and dusk (Raymond and Probert, 1991) could also explain the restricted distribution since optimum infection would only occur in those animals which come to water to drink at these times.

Little was known about the life-cycle of S.margrebowiei until Pitchford (1975) managed to culture the eggs and infect B.tropicus. In infected snails, two peaks in cercarial output are observed, one at dawn and the other at dusk, the rhythm being termed "ultradian" by Pitchford & Du Toit (1976). Other rhythms such as the diurnal one of S.mansoni and the nocturnal one of S.rodhaini are thought to be genetically based (Théron, 1989) and in mixed infections have little interference with one another (Théron & Moné, 1986). These rhythms are thought to be closely related to the behavioral patterns of the vertebrate host, so as to maximize the chance of host-parasite contact (Combes & Théron, 1977).

## Aims and organization of this study:

The intermediate hosts of schistosomes are the vital link in the life-cycle. In order to carry out long term experiments and to obtain a large amount of parasite material, an efficient and successful method of culturing the snail population is required.

Since obtaining S.margrebowiei from the British Museum (N.H.) in 1985, it has proven to be a difficult parasite to culture and indeed on four occasions the culture collapsed and the parasite was lost. The main causes of this collapse can be attributed to a high rate of mortality among young snails, low fecundity of mature snails, a small turn over in the snail population and a build up of resistance to infection. This problem is also exacerbated by the continual use of infected snails leaving uninfected snails as the breeding stock i.e susceptible genes were being removed from the population. With the availability of the new snail room, with a better controlled environment and larger space, it has been possible to explore other methods of culturing and indeed expanding the snail population.

Chapter two compares the different methods of maintenance and infection in order to find a suitable (time saving, less labour intensive and efficient) way to maximize the production of parasite material. The importance of the size of the snail intermediate host at the time of infection has been examined to determine the optimal size for infection. A comparison of infection of mice by the paddling and ring method has also been carried out.

Chapter three is concerned with a detailed investigation of the larval stages of S.margrebowiei using light, scanning and transmission microscopy to study the morphology and ultrastructure of the cercaria and miracidium of this parasite.

Chapter four deals with the deposition of eggs in different tissues of BKTO mice and compares results with previous observations.

Chapter five is a study of the longevity of the non-feeding cercariae and their infectivity as they age.

Chapter six establishes cross reactivities between different stages in the life-cycle of schistosomes (in particular S.mansoni and S.margrebowiei) and evaluates the use of snail digestive gland as a potential vaccine.

Chapter seven evaluates the potential for using S.margrebowiei egg antigens in the serodiagnosis of S.haematobium.

Chapter eight discusses the various observations made in this study with respect to the biology of S.margrebowiei and emphasizes the usefulness of non-human animal schistosomes as models for the human species.

## **CHAPTER TWO**

**Procedures for the culture and maintenance of schistosomes  
and their intermediate hosts.**



## CHAPTER TWO

### Introduction:

#### The Maintenance Of S.margrebowiei And It's Intermediate Host:

The successful breeding of Bulinus species of snails in the laboratory is an important limiting factor for the experimentation with S.haematobium complex of schistosomes. Reduced fertility and fecundity together with increased mortality, are often observed in the laboratory environment (Sodeman & Dowder, 1973).

Webb and James (1971) have reviewed many different methods for the laboratory maintenance of schistosomes in their molluscan intermediate hosts. These methods vary with the individual investigators concerned, the amount of available space and time and the number of cercariae required. A successful method of maintenance should yield a rapid and continuous growth in the snail population, and at the same time require as little time and labour as possible. With this aim in mind, two methods of maintenance were tried.

#### Materials and Methods:

S.margrebowiei, originally obtained from the Lochinvar National Park, Zambia, was maintained in the laboratory using Bulinus natalensis (<sup>Lake</sup>Sibaya, Natal) as the intermediate host. The original stock of infected snails was obtained from the Experimental Taxonomy Unit of the British Museum (Natural History) London.

#### Method One : Non-Aerated "Lunch Packs"

This is a modification of the method used at the Experimental Taxonomy Unit of the British Museum (Natural



History). Snails were maintained in 500 ml (17x11x5 cm) clear polystyrene "lunch packs" with lids (Stewart Plastic PLC), no more than 20 snails per container, and the cultures kept in a 12 hour light/dark cycle at 26°C.

The water for the snail culture was obtained from an artificial pond on site. This water was double filtered, through a wire wool filter and Whatman No. 4 filter paper. The filtrate was diluted by adding an equal volume of copper-free water. 1ml of Nolan-Carriker salt mixture (Malek and Cheng, 1974) was added to every litre of the filtered water to increase it's inorganic calcium content. The Nolan-Carriker stock solution was made up in three litres of distilled water containing, 50g calcium carbonate, 5g magnesium carbonate, 5g sodium chloride and 1g potassium chloride. The snails were fed on dried sycamore leaves (Acerpseudo plantanus) and "Aquarian Tropical Fish Flakes", two flakes every day. Cultures were cleaned out weekly by removing leaf skeletons, dead snails , uneaten food and replacing with fresh water and food. Although quite successful, this method was very time consuming and laborious.

Method Two : Aerated 15 Litre Plastic Aquarium Tanks.

Snails were kept in copper-free water filled 15 litre capacity plastic aquarium tanks (25x35x20 cm) in the same condition as above. The tank water was constantly aerated using air-stones connected to air pumps by plastic tubing. A small population of water fleas was also introduced to keep the culture clean. No more than 50 snails were kept in each

tank. The snails were fed every day on fish flakes (5 flakes per tank) alone. The tanks were cleaned once a week of some faecal material and dead snails by suction using a long plastic tube. Lost water was replaced by fresh warm copper-free water. Once a week, 5ml of Nolan-Carricker salt mixture (from the stock solution) was added.

#### **A Comparison Of The Efficiency Of These Two Culture Methods:**

One hundred B.natalensis less than three days old and measuring less than 0.5mm in length were selected and divided into two groups of 50.

The first group were placed in the 15 litre capacity tank and the second group were sub-divided into three groups of 17, 17 and 16 and placed in 3 separate "lunch packs".

Both cultures were maintained as described previously. Each culture was examined weekly for the presence of dead snails and mortality rates recorded. The effects on growth rates of the two methods of culture were not carried out since handling of the snails itself could contribute to mortality. The experiment was repeated under identical conditions.

**Results:**

Table 2.1 is the pooled results of two sets of observations regarding the number of B.natalensis snails kept in the two sets of conditions.

Time Week	No. of snails alive Large tank	No. of snails alive Small tanks	X <sup>2</sup> value	P value
0	100	100		
1	93	91	0.07	0.7944
2	92	80	5.02	0.0250
3	92	70	14.33	0.0002
4	86	61	4.79	0.0001
5	80	55	13.13	0.0003
6	73	52	8.53	0.0035
7	72	50	9.27	0.0023
8	71	48	10.04	0.0015
9	67	47	7.36	0.0067
10	67	47	7.36	0.0067
11	66	46	7.33	0.0068
12	65	46	6.55	0.0104
13	65	46	6.55	0.0104

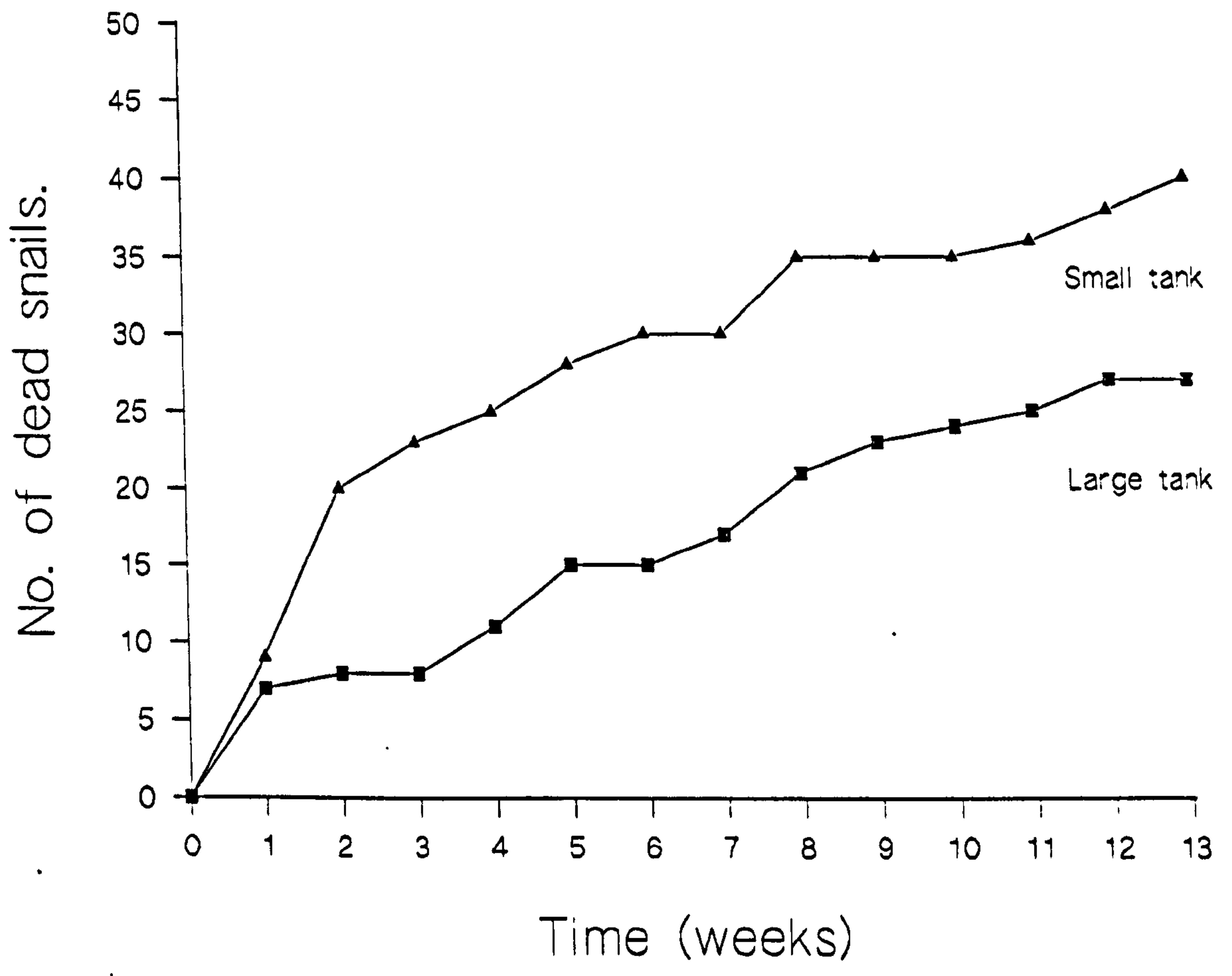


Figure 2.1 Mortality with time in two culture systems over a thirteen week period.



## Discussion:

With the exception of the first week ( $X^2 = 0.07$ ,  $p = 0.7944$ ) significantly more snails died in the small containers compared with the large ones throughout the duration of the experiment (the  $X^2$  value was always greater than 5.00 and the  $p$  value less than 0.05). Individuals died throughout the duration of the experiment but to varying degrees at various times. For example while 8% and 20% of snails died within the first two weeks in large and small tanks respectively, from thence to the termination of the experiments, on average, 2.7% and 3.9% mortality per week was observed in the large and small tanks respectively. At the termination of the experiment in week 13, a total of 35% of the snails in the larger tanks had died as opposed to 54% in the smaller tanks. The graph (fig. 2.1) of mortality against time clearly demonstrates that not only is the initial death rate lower in the large tanks, but that the survival rate is higher in the larger aerated tanks.

It has been well established that snails bred in small volumes of water grow less rapidly than those bred in larger volumes. Chernin and Michelson (1957a), who studied the effects of population density on the growth and fecundity of Australorbis glabratus and Simoes et al., (1974) working with Bulinus species of Continental Portugal, reached the same conclusion. It is believed that overcrowding leads to the secretion of growth inhibiting factors which cause a decrease in growth and fecundity (Chernin and Michelson, 1957b).

From the results of the present study, it is clear that

the use of larger tanks is a more efficient method of culturing this species of snail. The  $X^2$  analysis demonstrates that after the first week, the number of snails dying in the large tanks is significantly lower than that in the small containers at any given time. However, since in both containers, <sup>higher</sup> death rates were seen during the first few days it was therefore decided that for infection purposes snails older than three weeks of age should be used.

Since there are so many variables in the two methods for example space, food, air and water volume it is difficult to determine which factor(s) is the most important one affecting survival. However, it is clear that the use of large aerated tanks, containing copper-free water together with the use of fish flakes gives optimal survival. Since we no longer needed pond water and sycamore leaves considerable saving in time and labour was achieved. Cleaning the large tanks of dead snails and faecal material, was also simpler and less time consuming. A long plastic tube was used to clean the debris from the bottom of each tank by suction. This procedure did not take long and the small amount of water loss was replaced by fresh copper-free water. The only draw back to this system was the rapid build up of unwanted organisms such as ostracods. Ostracods irritate snails causing them to move up above the water line where they often die from desiccation. It is essential therefore to keep their numbers in check. This can be achieved during routine cleaning of the tanks and if numbers become too large by changing the water completely. Oligochaetes also develop in large tanks but are harmless to

snails. Oligochaetes are known to feed on miracidia and can reduce snail infection rates. It is important therefore to make sure that whenever large tanks are used for infecting a vast number of snails, the tank is free from these organisms.

From these results it is concluded that because of the larger space, continuous oxygen supply and constant temperature provided by the large volume of water, large tanks were more suitable for culturing B.natalensis snails than small "lunch packs".



### Maintenance of the parasite:

S.margrebowiei eggs were isolated from the liver of mice with heavy adult 50-day infections by maceration of the liver through a 500 $\mu$ m sieve into a urine flask. The sieve was subsequently flushed with isotonic saline (0.9%) and the sieved material poured into 30 ml plastic tubes and centrifuged at 2000 rpm for 2 mins. The supernatant was discarded and the settled eggs re-suspended in copper-free water. After a second centrifugation, the supernatant was once again discarded and the eggs poured into a plastic petri dish with 10 ml of copper-free water at room temperature and illuminated with a desk lamp. After 10-20 minutes the eggs hatch and the miracidia are ready to infect the snails.

### Snail infection:

Once again in order to reduce the time and effort involved in infecting snails, two methods of infection were compared.

Previous work by Raymond (1991) had concluded that sexually mature, egg laying B.natalensis snails were less susceptible to infection with miracidia of S.margrebowiei. For this reason, all snails used here were "immature" and non-egg laying. Although non-linear, size is closely related to age and age to the sexual state of the snail (Anderson et al., 1982). For these reasons and for simplifying the parameters, size was chosen to be the discriminating factor in infection studies. Furthermore in order to measure susceptibility, five parameters were recorded, death of snails during the prepatent period (i.e before cercariae

appear), the percentage of surviving snails infected, the percentage of the original population infected, the number of cercariae shed and mortality of infected snails during the period 90 days post-infection.

#### **Materials and methods:**

The length of each snail was measured in millimetres using a ruler. Snails were blot dried and positioned on the ruler and measured under a microscope. The experiment was carried out in three runs under identical conditions, with 100 snails in each size class. Three size classes were used, small (1-2mm), medium (2-3mm) and large (3-4mm). Snails were infected either individually or en masse.

#### **Individual Infection:**

Clear plastic vented culture petri-plates (Flow labs. Irvine, Scotland) were used for the exposure of snails to miracidia. Each lidded petri-plate consists of 25 compartments of 2cm<sup>2</sup>. Five miracidia per snail were transferred to each compartment in approximately 2.5ml of water using a pasteur pipette (i.e 5 miracidia/2.5ml/snail). All miracidia used were collected within one hour of hatching. Individual snails (B.natalensis) were placed in each compartment containing miracidia. The lid was placed on the petri-plate and left for 8 hours. Exposed snails were subsequently kept in 15 litre capacity tanks and maintained as before.

#### **En masse Infection:**

One hundred snails, of the same size, were placed in a "lunch pack" container. 250ml of copper-free water was added.



Hatched eggs from the liver of a previously infected mouse were added to the tank, approximately 20 miracidia per snail (i.e 20 miracidia/2.5ml/snail). The snails were left for 8 hours, after which the water was removed and the snails transferred to 15 litre capacity tanks with 10-13 litres of fresh copper-free water.

During the infection period snails often crawled above the water line and it was necessary to check the infection tanks hourly and gently push the snails into the water. On day 15 post-infection, all the "infected" snails were transferred into a dark chamber and kept there until required.

The "infected" snails were screened for the presence of cercariae 22 days post infection as follows:- At 07.00 hours each snail was placed in a compartment of a petri plate together with 2.5ml of copper-free water, the lids replaced and subjected to bright illumination. After 5 hours, each compartment was screened for the presence of cercariae using a light microscope. Positive snails were removed and the negative snails re-screened 3 days later. The snails which did not shed after 25 days were considered to be refractory to infection and removed from the main breeding culture.

**Results:**

Table 2.2 A comparison of the infection of three size classes of snails: A) Individually exposed and B) Exposed en masse.

A)

Size at exposure(mm)	1-2	2-3	3-4
No. exposed	300	300	300
Death during pre-patency	141	80	78
Survivors by day 25	159	220	222
% of survivors infected	78	65	60
% of exposed infected	41	47	45
% death during pre-patency	47	28	26

B)

Size at exposure(mm)	1-2	2-3	3-4
No. exposed	300	300	300
Death during pre-patency	111	95	81
Survivors by day 25	189	205	219
% of survivors infected	90	93	89
% of exposed infected	57	64	65
% death during pre-patency	37	32	27



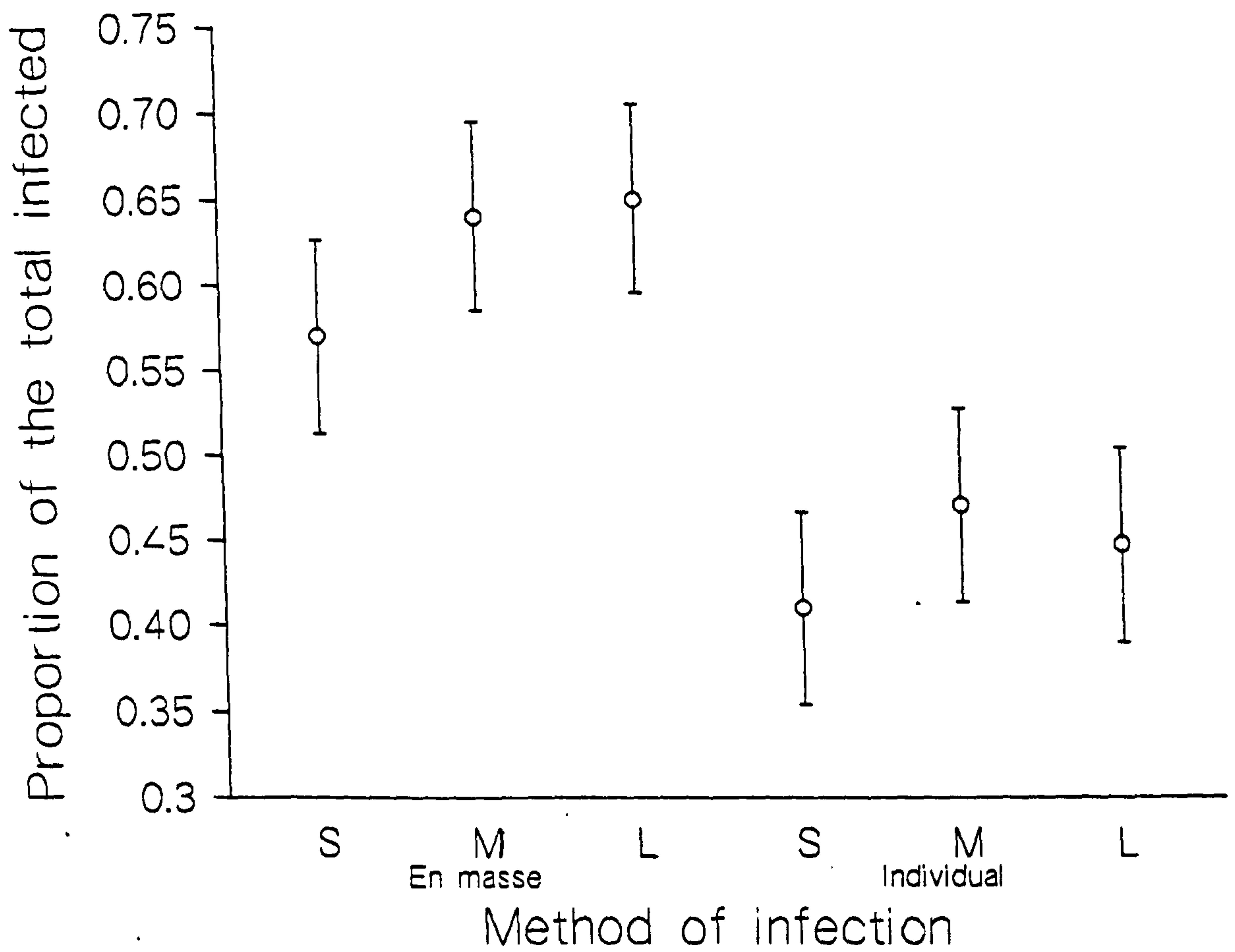


Figure 2.2 A comparison of the infection rates in different size classes of snails using two methods of infection with 95% confidence interval.

Table 2.3 A comparison of the percentage of infected snails surviving during the 90 days post-infection when A) Individually exposed and B) Exposed en masse.

A)

Size/mm	No. Infected	% surviving (days post-infection)						
		30	40	50	60	70	80	90
1-2	124	63.7	35.5	11.3	3.2	0	0	0
2-3	140	79.2	55.7	43.6	30.7	14.3	10.0	5.7
3-4	134	80.6	70.9	62.0	47.8	30.0	20.9	16.4

B)

Size/mm	No. Infected	% surviving (days post-infection)						
		30	40	50	60	70	80	90
1-2	171	63.1	24.0	6.4	0	0	0	0
2-3	192	61.5	33.3	18.2	12.5	4.7	2.6	1.0
3-4	195	87.7	66.1	50.2	36.4	32.8	29.8	22.6

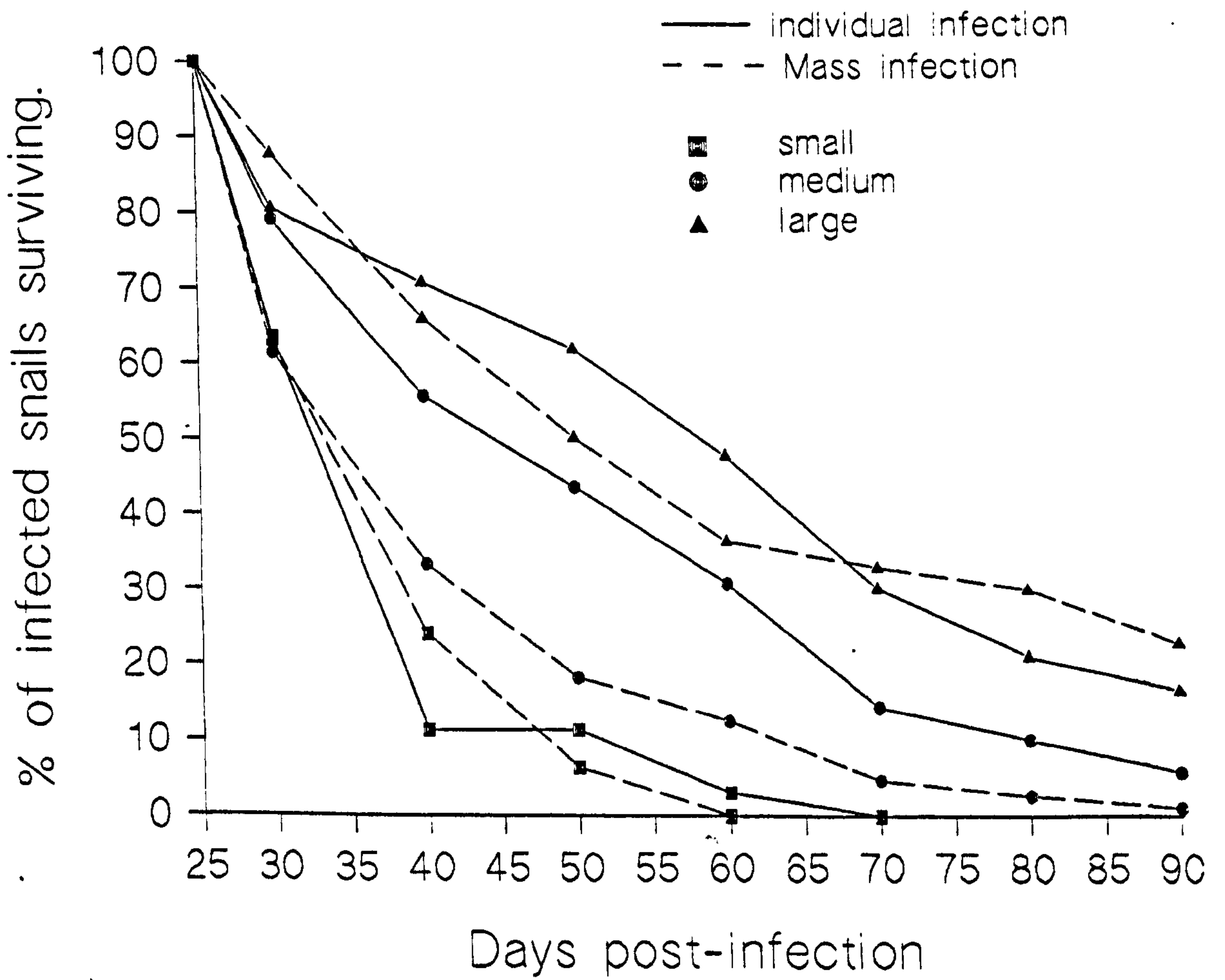


Figure 2.3 A comparison of the percentage of infected snails surviving during the 90 days post-infection when infected individually and en masse.

### Cercarial output:

In order to determine the cercarial productivity of infected snails it was necessary to count the number of cercariae emerging from these snails. This was carried out as follows: on day 32 post-infection, 10 infected snails were removed from each size class; these snails were placed in 50ml beakers containing 20ml of copper-free water and allowed to shed cercariae under artificial light. Snails were left to shed from 07 00 to 12 00. At 12 00 the water containing cercariae was poured through a sieve into another 50ml beaker and all snails returned to their tanks. The beaker containing the cercariae was stirred as five 100 $\mu$ l aliquots were taken using an automatic pipette. The number of cercariae in each aliquot was counted under X40 magnification after staining with Lugol's Iodine. The mean value for the five samples was obtained, and the average number of cercariae shed by one snail was calculated from this. If the counts of any individual aliquot was more than 10% from the mean, further aliquots were counted. This entire procedure was repeated three times and the results given in table 2.4.



Table 2.4 The mean number of cercariae shed per snail (n = 30) when infected A) Individually and B) En masse.

A)

Size/mm	No. of cercariae shed per snail 32 days post-infection from 07.00 to 12.00 noon ( $\pm$ s.d.)
1-2	73.3 $\pm$ 18.4
2-3	104.6 $\pm$ 17.1
3-4	122.6 $\pm$ 21.5

B)

Size/mm	No. of cercariae shed per snail 32 days post-infection from 07.00 to 12.00 noon ( $\pm$ s.d.)
1-2	87.3 $\pm$ 16.5
2-3	160.0 $\pm$ 13.2
3-4	216.3 $\pm$ 19.6

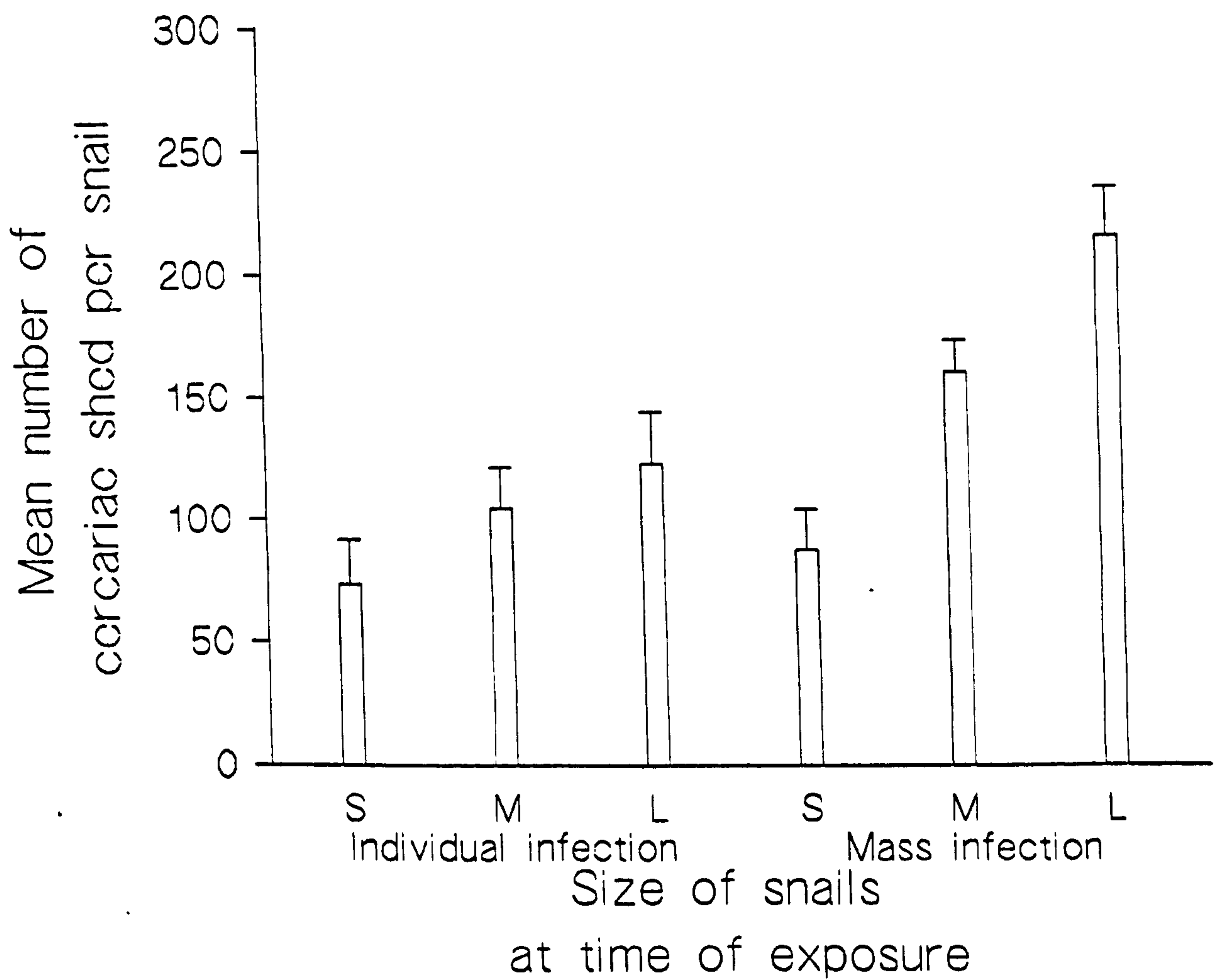


Figure 2.4 The mean cercarial output of one snail in each size class using two different methods of infection (n = 30). Error bars S.D.

The following parameters were chosen as indicators of the susceptibility of B.natalensis to infection with S.margrebowiei.

1) Mortality during the pre-patent period: A  $X^2$  analysis indicates that death during pre-patency among small, medium and large snails infected individually is not significantly different when compared with their counterparts in the en masse infection group (small:  $p = 0.197$ , medium:  $p = 0.643$  and large:  $p = 1$ ). However, among the individually infected group, significantly more small size snails died when compared to both medium ( $p = 0.008$ ) and large ( $p = 0.003$ ) snails. No such differences were observed among the en masse infected snails.

2) The percentage of surviving snails infected: Apart from a significantly greater percentage of surviving small snails becoming infected compared with the large size snails in the individually infected group ( $p = 0.009$ ), no significant differences were observed between size groups in both infection methods. However, when small, medium and large snails from the individually infected group are compared with their counterparts in the en masse infected group, significantly more snails were infected in the en masse infected group in all three size classes (small:  $p = 0.033$ , medium:  $p = 0.000$  and large:  $p = 0.000$ ), Table 2.2.

3) The percentage infection of the total snails exposed: There were no significant differences within the two methods of infection in the percentage of exposed snails infected (Table 2.2 and Fig.2.2). However, when comparing small,



medium and large snails with their counterparts in the en masse infected group, significantly more snails were infected in the en masse infected group (small:  $p = 0.039$ , medium:  $p = 0.228$  and large:  $p = 0.006$ ).

4) The number of infected snails surviving by day 90 post-infection: The percentage of infected snails remaining alive during the 90 days post-infection in both groups was lower in the smaller size group. Thus by day 40, only 10-25% of the small snails remained alive while 60-70% of the larger snails were still alive. By day 60-70 all the small infected snails had died. This trend was similar in both infection groups (Table 2.3 and Fig. 2.3).

5) The mean number of cercariae shed per snail: An analysis of variance shows that there is no significant difference in the number of cercariae shed by the three size classes within the individually infected group,  $F = 5.09$ , which is smaller than the critical  $F$  value of 5.14. However, a significant difference was observed in the en masse infection group ( $F = 45.26$ ). Furthermore Students'  $t$  Test indicates that although there was no significant difference in the number of cercariae shed by small size snails in both methods of infection ( $p = 0.3827$ ), significantly more cercariae were shed by medium ( $p = 0.0115$ ) and large snails ( $p = 0.005$ ) in the en masse group than their individually infected counterparts (Table 2.4 and Figure 2.4).

## Discussion:

The probability of a miracidium coming into contact with a snail depends principally on the number of snails available given of course that the volume of water is constant. This is in some way increased by the natural behaviour of the two organisms since both the miracidium and snail prefer to congregate near the outer-margins of the container at the water-air interface. Both methods of infection should enable the parasite to enter its host, however, not all snails are able to support a patent infection. Infectivity can be considered as the ability of the infective stage to penetrate a host, develop and produce the next stage in the life/cycle. So a truly susceptible snail should provide these requirements. In some cases, a miracidium may enter the snail but fail to develop to the stage of cercarial production. In resistant snails, the penetrating larval stage is actively destroyed. Since snails lack lymphocytes, immunoglobulins and anamnestic responses to specific antigens, the system used by resistant snails to combat invading organisms is called an "internal defence system", (Van Der Knapp and Loker, 1990). The mobile haemocytes associated with this defence system are responsible for the killing of non-self and in schistosome infections, the recognition of non-self is thought to be at the miracidial level (Kechemir and Combes, 1982, Bayne, 1983 and Daniel et al., 1992). Furthermore experimental cross-breeding strains of resistant and susceptible snails have shown that susceptibility is a heritable character involving several genetic factors (Newton, 1953). Susceptibility of a

snail is the ability to attract an infective miracidium, allow it to enter and develop into cercaria-producing daughter sporocysts. According to this definition it is clear that small snails are the most susceptible with 78-90% of the survivals being infected in both methods of infection (Table 2.2).

Among susceptible snails many factors affect the rate of infectivity and parasite development. These include environmental factors such as food and water quality, temperature (snails are poikilothermic) and physiological factors including previous experience of infection. In laboratory conditions the environment can be kept relatively constant. So that once inside the snail the development of the invading miracidium would mostly depend on the physiological status of the snail host. This includes size (Loker et al., 1987, Niemann & Lewis 1990, Raymond and Probert, 1992) age (Sturrock 1968, Lo 1972) and the sexual state (Wright 1971, Raymond and Probert, 1992) of the snail. Regarding which of these factors are the most important, there are conflicting reports.

The laboratory studies of Pan (1965) and Chu et al., (1966) have shown that infection with schistosomes adversely influences the survival of the molluscan host, and the results of this present study confirm this finding. Anderson and May (1979) also concluded that infection tends to reduce life-expectancy of the snail often by a factor of three. Pan (1965) also reported that mortality increases once cercarial shedding begins and that the survival during the pre-patent



period of an infection is virtually identical to that of uninfected control snails. The results of the present study, however, indicate that infection of B.natalensis with miracidia of S.margrebowiei, increases the rate of mortality among snails of different size groups throughout the pre-patency and patent period. From the control tanks it was calculated that 2.7% of snails died per week. In snails exposed to infection, during pre-patent period this increased to 8-12% while, a week following cercarial emergence, the death rate rose to 15-40% (Table 2.3 and Figure 2.3).

After a successful penetration, miracidia transform to mother sporocyst. These mother sporocysts developing in the foot region provoke little reaction (Pan 1963, 1965), but a few days later, as the daughter sporocysts migrate to the digestive diverticula they provoke a slightly higher tissue reaction. It is only when some cercariae, during the process of migration and emergence within the snail die, that a strong tissue response occurs leading to higher rate of mortality among the infected snails. This was confirmed by Lengy's (1962) results working with B.truncatus infected with S.bovis. He observed a higher rate of mortality in snails 12-17 days post-exposure and again 2-3 weeks after shedding. The results of the present study indicate the occurrence of the same pattern of ever increasing death rate as the parasite develops and emerges from its snail host.

According to Ward et al., (1988), the total cercarial production is a direct reflection of the number of primary sporocysts which become established. Niemann and Lewis

(1990), clearly showed that cercarial production is higher in large snails since there was more space for the development of secondary sporocysts. This effect of size is also evident in our study where the largest snails produced the largest number of cercariae (Fig. 2.4). Barbosa (1975) had also reported a positive correlation between successful invasion of susceptible snails and cercarial production. However, it was pointed out by Sluiter et al., (1980) that even among susceptible snails, cercarial output varies and that according to Théron (1981), the daily fluctuations in cercarial output should be taken into consideration before any conclusion regarding size and cercarial output could be reached. These variables were minimized in our study by calculating the cercarial output of all snails on a single day.

In conclusion, the size of the snail at exposure is very important since this affects its subsequent longevity and also the total cercarial output. Mass infection produced the highest rate of infection and cercarial output in all three size groups, emphasizing that miracidia dose given at the time of exposure governs cercarial output and rate of infection. The high mortality and the high infection percentage in surviving snails of the smaller size group, indicates that smaller snails are more susceptible to infection. This agrees with the findings of Evans (1982). Finally infection en masse of snails 2-4mm in length is the best method of achieving maximal infection of B.natalensis with S.margrebowiei.

## Establishment of adult worms in laboratory mice:

S.margrebowiei adults were passaged through random bred BKTO and random bred coloured mice bred on site. At the time of experimentation they were 6-8 weeks old. Cercariae, shed from infected snails (at least 20), were concentrated using a Millipore concentration apparatus (Millipore Corp., USA), incorporating a vacuum pump to gently remove excess water through a 8 $\mu$ m pore size Millipore filter, the positively phototactic cercariae being attracted away from the filter by overhead illumination.

Infection of mice with S.margrebowiei was performed using the paddling and the ring method of infection.

### Paddling Method:

The paddling method followed the technique described by Moore et al., (1949). Mice were placed in a 5 litre glass beaker containing enough warm water (25-28°C), to reach halfway up the abdomen of the mice. After 20 minutes, the dirty water was discarded and each mouse was transferred into a separate 5 litre beaker. Sufficient warm, copper-free water, with a known number of cercariae (usually 100) was added. One hour was allowed for infection, after which the animals were returned to labelled cages until required. The contents of the beaker were filtered through Whatman No. 1 filter paper using a Buchner flask. Any remaining cercariae were thus trapped and counted after spraying the filter paper with a ninhydrin aerosol in a fume cupboard. It was found that approximately 55-75 % of the cercariae entered the host.



### Ring Method:

The ring method followed the technique described by Doenhoff et al., (1978) which was adapted from the method previously described by Swithers and Terry (1965a). Mice were anaesthetized with sodium pentobarbitone (0.06 mg per gram of body weight of sagatal) which was injected intraperitoneally. The abdominal skin of the mice was shaved and they were placed on their backs in wooden holding racks and gently secured with sellotape. A plastic ring with an internal capacity of 1 ml was placed over the shaved abdominal skin and secured with sellotape. Water containing the desired number of cercariae (usually 100) was then applied to the ring and the cercariae allowed to penetrate the skin for 30 minutes. Following infection, the water inside the ring was collected and examined for the presence of cercariae. Very few cercariae were found and about 90-95% penetrated the host.

The mice were left to recover in their cages in a warm place. Six BKTO mice were infected by each method. Each mouse was given 100 cercariae of S.margrebowiei and on day 50 post infection sacrificed, perfused, and the number of adult worms counted.

## Results:

Table 2.5 The mean recovery of adult worms in each infection group.

Infection method	No. of mice	Mean worm burden
Ring	6	43.8 ± 13.75
Paddle	6	26.4 ± 3.13

One way analysis of variance indicates that there is a significant difference in the mean worm burdens using the two methods ( $F = 7.61$  and  $p < 0.05$ ).

## Strain Differences:

For the collection of egg and worm material a very large number of mice are required. Coloured mice bred on site were considered as possible hosts (the coloured mice not only bred faster but there was a large number of them available in the animal house). An experiment was therefore carried out to find out their degree of susceptibility in comparison with the BKTO mice.

## Materials and Methods:

100 cercariae of S.margrebowiei were administered to each mouse using the ring method and on day 50 post-infection mice were sacrificed, perfused, and adult worms counted.

Table 2.6 The mean worm recovery from two strains of mice.

No. of mice	Strain	Mean worm burden
5	BKTO	39.8 ± 6.6
5	coloured	38.6 ± 8.2

There was no significant difference in susceptibility between the two strains ( $F= 0.06$  and  $p> 0.05$ ).

#### Discussion:

There are a number of different methods of infecting animals with schistosomes that are at present practised in laboratories throughout the world. These include, paddling, ring, tube, cover-slip, tail immersion, oral, gastric injection (ruminants only), subcutaneous and intraperitoneal injection. Of these the most commonly used are the ring method (Smithers & Terry, 1965a), the paddling method (Moore et al., 1949), and the subcutaneous injection technique (Peters & Warren, 1969). The ring and paddling methods of infection are by far the most popular techniques used in laboratories today. Preston and James (1972) working with S.mattheei and S.haematobium concluded that the ring method was the most efficient means of infecting hamsters. They noted that for experimental purposes the paddling method was unsuitable because marked variation in worm load occurred. Watson and Azim (1949) however, found that the paddling method of infection was more reliable and constant. The present results agree with those of Preston and James (1972).

Students' t Test Analysis of the two methods clearly demonstrates a significantly higher rate of worm return using the ring method. ( $T = 2.75$  and  $P < 0.05$ ).

The fact that there was no significant difference between the BKTO and coloured mice in terms of susceptibility



(p = 0.8) was a bonus. As mentioned previously coloured mice bred faster and they proved to be easier to handle and shave.

## CHAPTER THREE

The developmental stages of S.margrebowiei.

## CHAPTER THREE

### Introduction:

Due to the ease of culturing S.mansoni and its medical importance, the majority of microscopic studies of the genus Schistosoma have been carried out on this species.

This chapter is an attempt to redress an aspect of this imbalance by focusing attention on S.margrebowiei and in particular its larval stages. The two non-feeding larval stages of schistosomes are the miracidium and cercaria.

Scanning electron microscopy (SEM) studies have been carried out on the morphology of a wide range of schistosome species. These include, S.mansoni (Silk et al., 1969; Race et al., 1971; Miller et al., 1972; Voge et al., 1978), S.haematobium (Kuntz et al., 1976; Hicks and Newman, 1977; Leitch et al., 1984), S.japonicum (Sakamoto and Ishii, 1977; Sobhon and Upatham, 1990), S.mattheei (Tulloch et al., 1977), S.intercalatum (Kuntz et al., 1977); S.bovis (Kuntz et al., 1979; Southgate et al., 1986), S.curassoni (Southgate et al., 1986), S.leiperi (Southgate et al., 1981), S.mekongi (Sobhon and Upatham, 1990) and S.margrebowiei (Ogbe, 1982; Probert and Awad, 1987, Awad and Probert, 1989, 1990 and 1991).

Hockley (1968) was the first to use SEM to study the cercariae of S.mansoni, S.haematobium and S.japonicum. His results confirmed most of the light microscopy observations. LoVerde (1976) and Sakamoto and Ishii (1978), carried out similar studies on these species. The majority of work, however, has been carried out on S.mansoni. Race et al., (1971), studied the cercaria of S.mansoni which confirmed



Hockley's findings. Robson and Erasmus (1970) studied the oral sucker region of S.mansoni. In their studies the delicate sensory papillae were reported. Short and Cartrett (1973), used both light and scanning microscope to study the "argentophilic papillae". Stirewalt and Dorsey (1974) made a SEM study of S.mansoni cercariae during penetration of the epidermis of mouse ear skin. The most detailed studies on the cercarial tegument of schistosomes have been carried out by Smith et al., (1969), Race et al., (1971), Morris (1971) and Ebrahimzadeh (1974).

Light, scanning and transmission microscopy studies by Wajdi (1966), Wilson (1969), Kinoti (1971), Schutte (1974), Wikel and Bogitsh (1974), LoVerde (1975), Koie and Frandsen (1976), Sakamoto and Ishii (1978), Pan (1980), Eklun-Natey et al., (1981), and Sobhon and Upatham (1990) have, in the main concentrated on the morphology of the miracidium of S.mansoni, S.haematobium, and S.japonicum respectively.

### Schistosoma margrebowiei

Adult worm: The adult worms inhabit the anterior mesenteric venules of the ruminant host. The male worm is shorter (23.3mm) and thicker than the female worm (33.8mm) (Southgate and Knowles, 1977) and the mature male worm has both spined and unspined tubercles on its tegument while the female lacks tubercles (Probert and Awad, 1887). Thus contrary to previous belief (Ogbe, 1982), S.margrebowiei does not resemble S.mattheei or S.bovis.

Eggs: The eggs are yellow-brown in colour, are non-operculate and are round to oval with one small terminal spiny appendage. They measure  $87.2\mu\text{m} \pm 9.6$  by  $62.3\mu\text{m} \pm 7.6$  and the egg shell stains positively with the Ziehl-Neelsen technique in a similar way to those of S.intercalatum, S.mansoni, S.japonicum and S.rodhaini (Southgate and Knowles, 1977). Each fertilized female lays eggs in groups, and the mean number of eggs produced per day is 837 which is four times greater than some strains of S.haematobium and 14 times greater than an Iranian strain of S.bovis (Southgate and Knowles, 1975).

Miracidia: The newly-hatched miracidium is pyriform in shape and has a maximum survival time of 13 hours, at 26°C (50% die within 4-9 hours) (Evans, 1985). It measures 130µm long and 40µm wide and has four tiers of ciliated epithelial cells. The number of cells in each tier varies, the first has 6, the second 9, the third 4 and the fourth 3, giving a total of 22 cells (Southgate and Knowles, 1977).

Cercariae: The free-swimming cercaria, has a pear-shaped

body, on average measuring 192 by 61 $\mu$ m, a long narrow tail stem measuring 202 by 44 $\mu$ m and furcae each 69 $\mu$ m long. This gives a total length of 463 $\mu$ m, however, the fixed specimen only measures 345 $\mu$ m (Southgate and Knowles, 1977) which indicates a 25% shrinking.



## Materials and Methods:

S.margrebowiei originally obtained from the Lochivar National Park, Zambia was maintained in the laboratory using Bulinus natalensis (Sibaya, Natal) as the intermediate host and BKTO mice as the final host.

### SEM preparation techniques:

Adult worms, eggs, miracidia, the digestive glands of snail and cercariae, were immediately fixed in 2.5% glutaraldehyde made in 0.1M sodium cacodylate buffer (pH 7.2) for 2 hours at 4°C and then they were pipetted into metal tubes closed at both ends by grids, rinsed in a series of buffer washes (0.1M sodium cacodylate buffer, pH 7.2) and left over night in a final buffer wash. The specimens were then dehydrated through a series of graded ethanols to 70% and stored or treated as follows:

For the critical point dry<sup>ing</sup> procedure, further dehydration was carried out using graded ethanol to pure ethanol. The specimens were then treated via a series of graded acetone\ethanol to pure acetone. The tubes were then placed in a Polaron Critical Point Drying apparatus for five hours using carbon dioxide as the critical point liquid. The dried specimens were then removed from the tubes and mounted onto aluminium stubs using double sided sellotape, coated with a thin layer of gold 20nm thick, in a Polaron E500 gold coating unit. Each stub was then examined in a Hitachi S520 Scanning Electron Microscope. For closer study of the surface of the miracidia, after fixation, and dehydration with 70% ethanol, some miracidia were placed in an ultrasonic cleaner (Dawe

Sonicleaner ) for 15 mins. The vibration results in breaking off the cilia and exposing the surface. These were also examined using SEM as described above.

#### **TEM preparation techniques:**

For TEM studies, specimens were fixed <sup>as before</sup> and post-fixed in 1% osmium tetroxide for 1 hour at 4°C and then washed in buffer to remove excess osmium. The dehydration procedure was repeated three times using pure ethanol and the specimens were embedded in Spurr resin (Taab Laboratories Reading U.K.). This was a mixture of E R L 4206 (5.0g), D E R 735 (3.0g), N S A (13.0g) and S-1 (0.25). The blocks were polymerised for 48 hours at 60°C. Thin sections (70nm) were cut with a glass knife and mounted on copper 100 mesh grids coated in pioloform. These sections were stained with 10% alcoholic uranylacetate for 45 minutes and lead citrate for 15 minutes. Stained sections were viewed in GEC Corinth 275 Transmission Electron Microscope.

#### **Silver nitrate staining:**

Eggs, miracidia and cercariae were pipetted into a hot 0.5% silver nitrate solution for 10 minutes in the dark, washed 3 times with distilled water and placed under light for 10 minutes. After this they were mounted in a 10% glycerine solution for examination. Photographs of stained eggs, miracidia and cercariae were taken. The number of papillae on the surface of the cercariae was recorded using a microscope.

#### **Measurements:**

**Adults:** The perfused worms recovered from day 25 to 65

post-infection were relaxed in cold Hank's balanced <sup>Salt</sup> solution for 4 hours and measured using a binocular microscope fitted with a calibrated eyepiece. At least 15 pairs were counted for each time interval

Eggs: Silver nitrate impregnated eggs were measured.

Miracidia and cercariae: A single miracidium or cercaria was pipetted onto a clean slide and examined with a microscope set at X25. A drop of Lugol's iodine was added. This immediately killed and immobilised the miracidium and cercaria. Excess fluid was removed by using filter paper and the length and width of each specimen recorded. Over 50 eggs, miracidia and cercariae, respectively, were measured.

#### Sex determination:

Fifty B.natalensis snails were individually exposed to a single miracidium of S.margrebowiei. The cercariae shed from each infected snail were used to infect a mouse. Cercariae shed by each snail were also sexed using the C-band technique (Liberatos and Short, 1983). Cercariae from singly infected snails were fixed in cold, fresh methanol-acetic acid [3:1 mixture]. They were then centrifuged at 1,300 r.p.m. for 5 mins. The supernatant was discarded, the pellet resuspended, and the procedure repeated once more with cold fixative. This time all but a small volume of the supernatant was discarded and the pellet was resuspended and the suspension placed on clean wet slides. The slides were air dried and treated with 0.2 N HCL for 15 mins., saturated barium hydroxide for 15 mins., and 2X SSC (saline sodium-citrate, pH 7.0) at 60°C for 2 hrs. Finally the preparation



were stained with 4% Giemsa (buffer pH 7.0) for 5 mins. Sex was determined by the presence or absence of the W heterochromatin in the W-chromosome of females. This early sexing, was confirmed by the recovery of adult worms from mice 50-55 days post-infection.

## Results:

Adults: Data showing the growth of adult worms given in figure 3.1, show that the cross over point (the point at which the male and females are the same length) for S.margrebowiei, is 28 days post-infection in BK-TO mice, when both sexes measure 10.8 mm. By day 40 post-infection, however, the female worms have doubled in size and continue to grow to  $29.7\text{mm} \pm 3.36$  by day 65 post-infection. The males grow at a slower rate, reaching a maximum size of  $19\text{mm} \pm 3.65$  by day 65 post-infection.

The SEM studies on the mature paired adult worms (Fig. 3.2) recovered from mice showed that the slender female worms are kept within the gynaecophoric canal of the shorter male. The surface of the female appears smooth when compared to the male (Fig. 3.2a). The anterior end of the female worm with its well developed oral sucker, has a smooth appearance pitted with minute holes. The spines are short and sparse on the rims of oral sucker (Fig. 3.2b). The surface of the male, immediately posterior to the ventral sucker, is supplied with tubercles (Fig. 3.2c). Some of these tubercles are heavily spined; uniciliate sensory organs are present in the spine-free regions between the tubercles (Fig. 3.2d).

Eggs: The eggs of S.margrebowiei are laid in groups (Fig. 3.3a). Eggs measure  $80.2\mu\text{m} \pm 18.6$  by  $64.8\mu\text{m} \pm 9.8$ . They are oval shaped with a small terminal spine measuring  $1.6\mu\text{m} \pm 0.6$  (Fig. 3.3b), which is not always visible. Microspines, cover the surface of the egg (Fig. 3.3c).

Miracidia: The miracidium measures  $120\mu\text{m} \pm 12.5$  by

49.52 $\mu\text{m}$   $\pm$  7.7. It is positively phototropic and negatively geotactic. Apart from the anterior region, it is covered with cilia (Fig. 3.4a). The anterior apical papilla (a dome-shaped structure), consists of anastomosing microfolds giving it a "rosette" pattern. It is surrounded by tactile apical cilia (Fig. 3.4b). The surface of the miracidium is covered by microvilli, and apart from the prominent apical papilla, there are two more lateral papillae on the body, an excretory pore is also evident in the "bald" miracidium (Fig. 3.4c). The apical papilla with its associated uniciliated, multiciliated pits and secretory ducts is the main organ involved in penetration (Fig. 3.4c). There are four rows (tiers) of ciliated plates immediately after the apical papilla, clearly divided by three rings (Fig. 3.5a). The entire body surface is covered with 22 plates. Starting from the anterior end, the first tier has 6, the second 9, the third 4 and the fourth tier 3. These tiers are separated by non-ciliated intercellular ridges. Numerous sensory receptors are scattered in between the ciliated plates on the anterior (13) and median (20) rings, but no receptors were seen on the posterior ring (Figs. 3.5a,b,c and d).

TEM studies confirm that the outer surface of the ciliated plates have numerous long cilia. These extend at a right angle to the surface and between the cilia are shorter microvilli. There are two types of granules found within the tegument, one has an ovoid shape and a mottled appearance and the second whilst also ovoid is more lightly stained. Both measure 0.08-0.1 $\mu\text{m}$ . Desmosomes, join the adjacent cells



(Fig. 3.6a). From the epidermal plates, cytoplasmic projections extend into the submuscular region. Longitudinal and circular muscles are surrounded by a large number of mitochondria situated in rows in the *connective tissue cells*. Beneath the muscle layer, the excretory ducts are evident (Fig. 3.6b). Multiciliated pits in the anterior region of the miracidium *are evident* (Fig. 3.6c). The flame cells with their associated cilia are also seen near the anterior end (Fig. 3.6d). A pair of penetration glands, the contents of which are secreted through secretory ducts, are a prominent feature of the anterior region (Fig. 3.6e). *Below the glands, there is a large nerve ganglion, with an outer layer of nuclei* (Fig. 3.6f). There is a lateral sensory papilla on either side of the body between the first and second tier (Fig. 3.7a). There are two types of plate, one which is heavily ciliated, and another which is not (Fig. 3.7b). The sensory organs include uniciliated (Fig. 3.7c) and multiciliated pits (Fig. 3.7d) with their associated cilia and vacuoles (Fig. 3.7e).

Upon entering a suitable snail host, the miracidium undergoes a series of developmental stages leading to the formation of a sporocyst. The sporocyst is a long, thin sac-like structure, which is covered with microvilli (Fig. 3.8a). Inside the sporocyst, immature cercariae develop (Fig. 3.8b). The sporocyst wall of *S. margrebowiei* consists of a syncytial layer with a microvillous surface, underneath which are the irregular shaped tegumental cells (Fig. 3.8c). After four weeks post-infection, both mature and immature cercariae are

seen inside and outside the sporocyst (Figs. 3.8d,e and f). Bulinus natalensis, the intermediate snail host of S.margrebowiei, grows to 6mm in the laboratory (Fig. 3.9a). When infected, numerous cercariae occupy its hepatopancreas (Fig.3.9b).

Cercariae: Upon emerging from an infected snail, the cercariae measure  $351\mu\text{m}$  in total length. They have a long pear-shaped body measuring  $142\mu\text{m} \pm 35$  and a longer cylindrical tail stem measuring  $154\mu\text{m} \pm 25$  which is divided into two furcae measuring  $55\mu\text{m} \pm 11$  at the posterior end (Fig. 3.9c). The excretory pores are located at the end of each furca, and sensory hairs are located on the tail and furcae (Fig 3.10a). The muscular ventral sucker is situated in the centre of the body and is covered with numerous, large sharp, backwardly directed spines Figure 3.10b. The anterior tip of the body is spine-free, and possesses a circular depression with a number of papillae and ducts which are the openings of penetration glands (Fig. 3.10c). The remainder of the body tail stem and furcae are covered with spines and the body is separated from the tail stem by a collar which is also spined (Fig. 3.10d).

TEM studies on the ultrastructure of the cercariae reveal that the entire body, collar, and tail are covered by an electron-dense tegument. This tegument is a  $0.1\text{-}0.2\mu\text{m}$  thick syncytium that overlies the extracellular matrix, muscle and subtegumental cells. The muscle layer consists of an outer layer of circular and an inner layer of longitudinal muscles, and large numbers of mitochondria can also be seen in this

region (Fig. 3.11a). Irregular subtegumental cells, with their associated projections into the tegument are found just below the matrix. Flame cells are also evident in this region (Figs. 3.11a and b). The excretory system consists of flame cells which have long, branching microvilli which extend into the excretory cell lumen. Glycogen is distributed throughout this region (Figs. 3.11c and d). The uneven appearance of the tegument is due to folds, microvilli and unciliated processes, (with their associated sheathed cilium projecting from a sensory bulbous process) (Figs. 3.11e and f). The spines covering the cercariae are recurved and dagger shaped, pointed at the apical tip but rounded at the base, and are covered with a glycocalyx which extends over the entire tegument (Fig. 3.12a). This glycocalyx is 0.5-1.5 $\mu$ m thick. The body and tailstem are connected by a delicate collar, which is easily severed (Figs. 3.12b and c). The excretory duct seen in this region, extends to the tail. Within the tailstem large amounts of glycogen are found together with an ~~outer~~ muscular layer and numerous mitochondria (Fig. 3.12d). The excretory pores are situated at the apex of each furca (Fig. 3.12 e).

Apart from the head gland (Fig. 3.12f), there are three pairs of post-acetabular and two pairs of pre-acetabular glands, the contents of which are secreted from a number of ducts (Figs. 3.13a,b and c). The secretory granules are surrounded by an array of microtubules (Fig. 3.13c) while the anterior tip of the cercaria, with its tegumental folds (Fig. 3.13d) and papillae (Fig. 3.13e) is the site where secretion occurs



(Figs. 3.13 e and f).

Silver nitrate staining shows (Fig. 3.14) that there are a large number of papillae, ducts and pores which stain on the body and tail of S.margrebowiei. The papillae are arranged in a bilaterally symmetrical pattern. On the anterior (head) and posterior ends (furcae), the concentration of papillae and opening ducts or excretory pores are too close to differentiate. However, the papillae on the tail stem are fewer and easier to count. Counts from 30 cercariae indicate that there are 32-37 such papillae on the tail (Figs. 3.14a,b,c and d).

Sex determination: From a total of 50 snails, each infected with a single miracidium, Only 12 produced cercariae (24% infection rate). From these, only 9 shed sufficient cercariae to infect 9 mice.

Figures 3.15a, b, c and d are four groups of cercariae used to infect four individual mice. Sex was successfully predicted in 3.15a (Female) and 3.15b (Male). However, cercariae in 3.15c were identified as males but the worm recovery indicates that they were in fact females. The opposite was true in 3.15d where male cercariae were wrongly identified as females.

For the other 5 groups of cercariae, 3 were identified as males and 2 as females. The worm recovery indicated that 4 groups were males and only 1 female. Overall, 6 groups of cercariae were identified correctly, which is a 66.6% rate of accuracy.



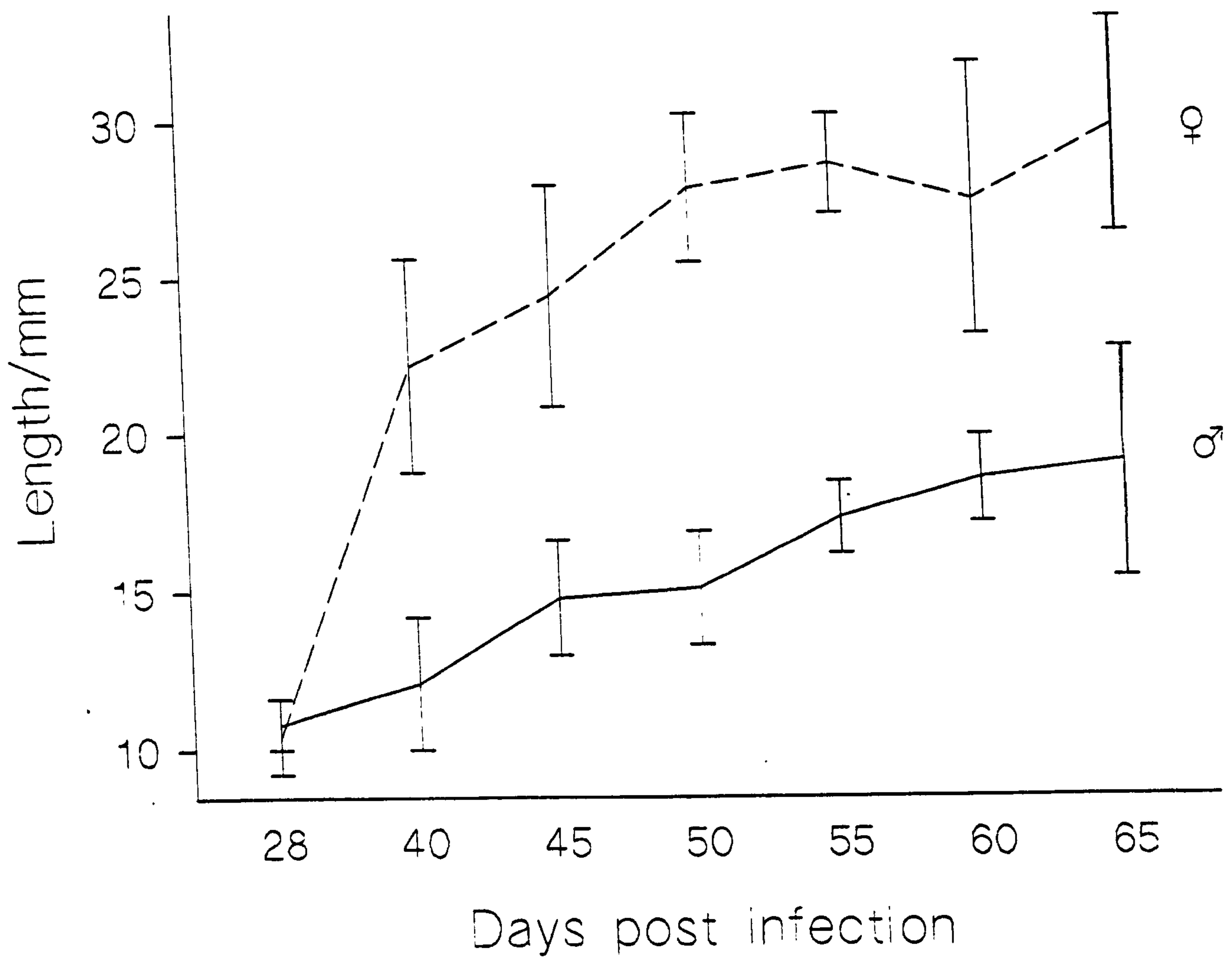


Figure 3.1 The rate of growth of adult male and female S.margrebowiei worms in BKTO mice.

Figure 3.2 SEM micrographs of adult male and female S.margrebowiei.

a) The body of the female (Fe) emerges from the male (Ma) gynaecophoric canal (arrow) at both ends. Note the smooth appearance of the female worm and the oral (Os) and the ventral (Vs) sucker of the male. Bar = 215 $\mu$ m

b) The anterior region of the female showing the oral sucker (Os) which has a rim with numerous spines. The spines (Sp) are also scattered in the anterior region. Note the pitted (Pi) appearance of the body. Bar = 10 $\mu$ m

c) Dorso-lateral region posterior to the ventral sucker of the male worm showing tubercles (Te). Bar = 50 $\mu$ m

d) Tubercles often have spines (Sp). There are a number of sensory organs (So) scattered in the region between the tubercles. Bar = 6 $\mu$ m



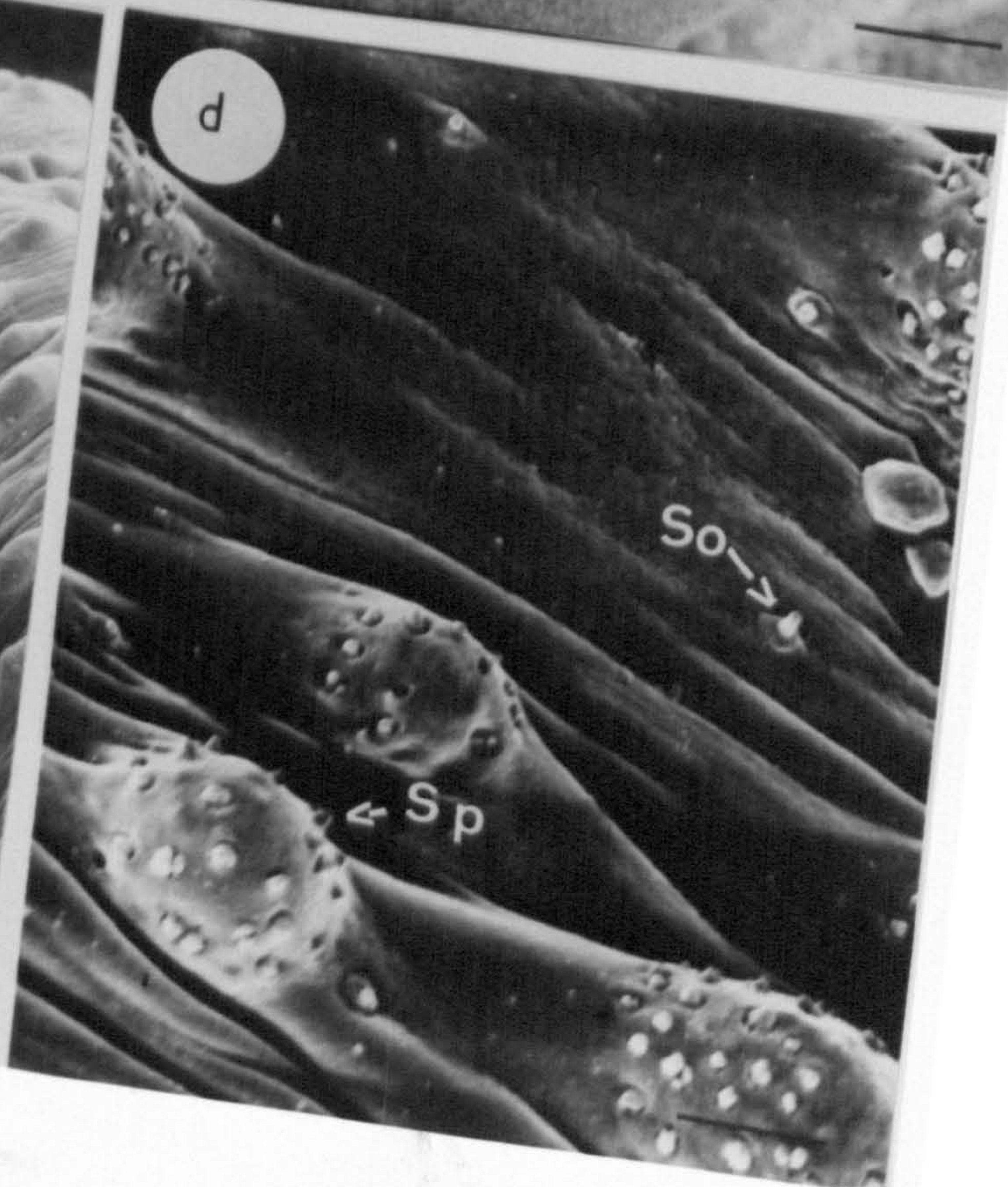
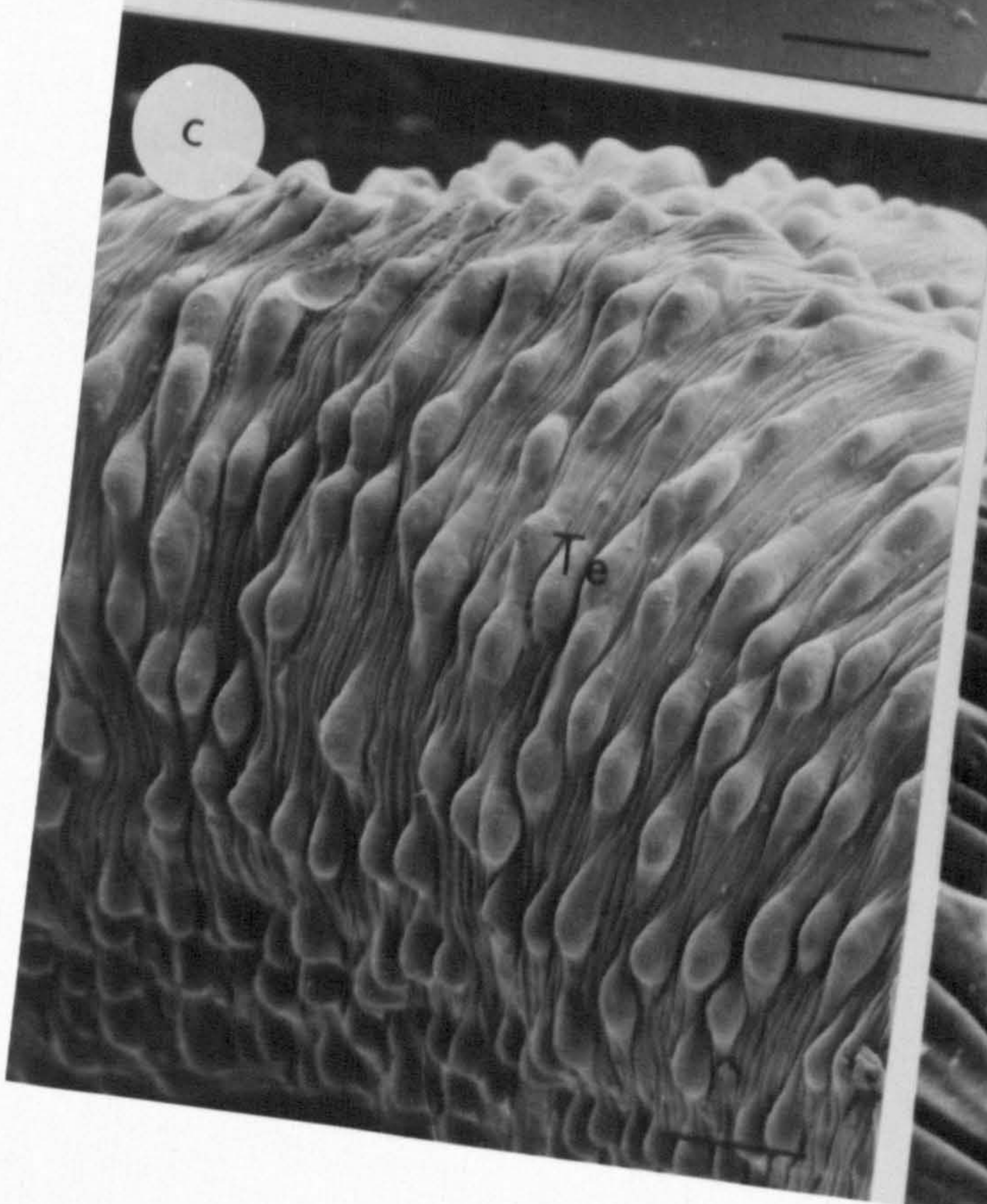
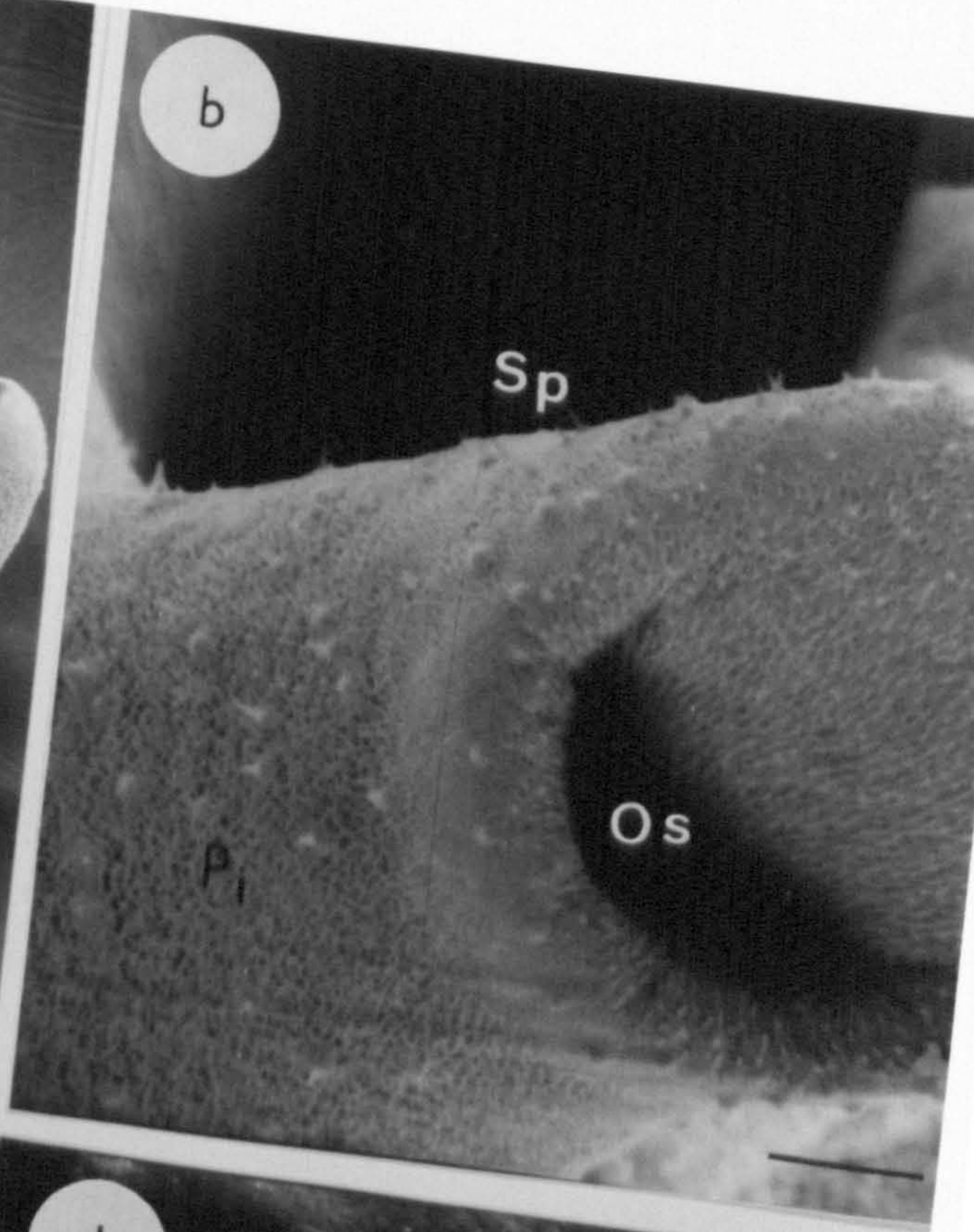
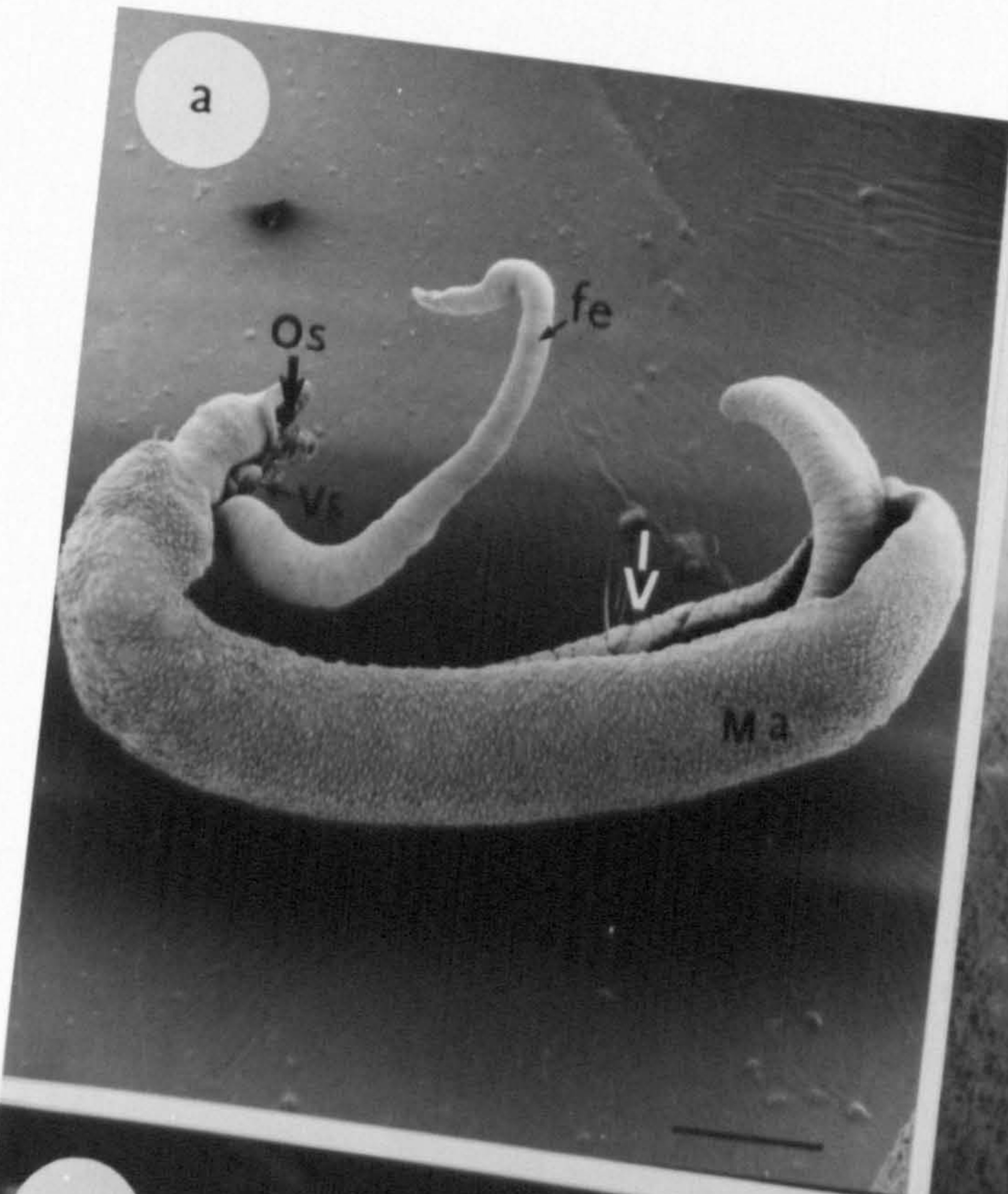




Figure 3.3 Light and SEM micrographs of the egg of S.margrebowiei.

a) Light micrograph of eggs (e) inside the female (Fe) worm.

Bar = 80 $\mu$ m

b) Light micrograph of an egg with a minute, terminal spine

(Sp). Bar = 18 $\mu$ m

c) SEM of eggs (e). Note the surface of eggs is covered with

microspines. Bar = 15 $\mu$ m



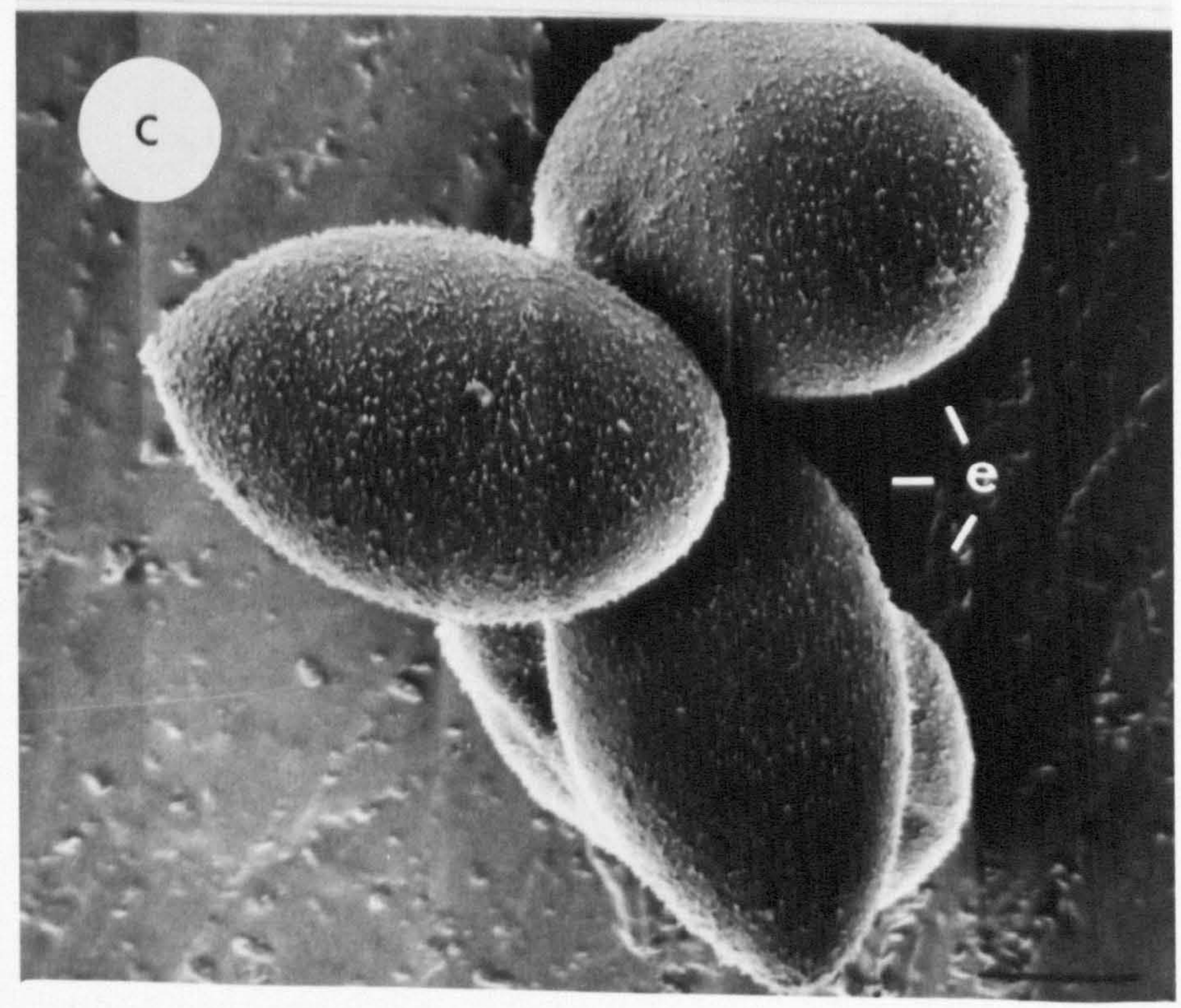
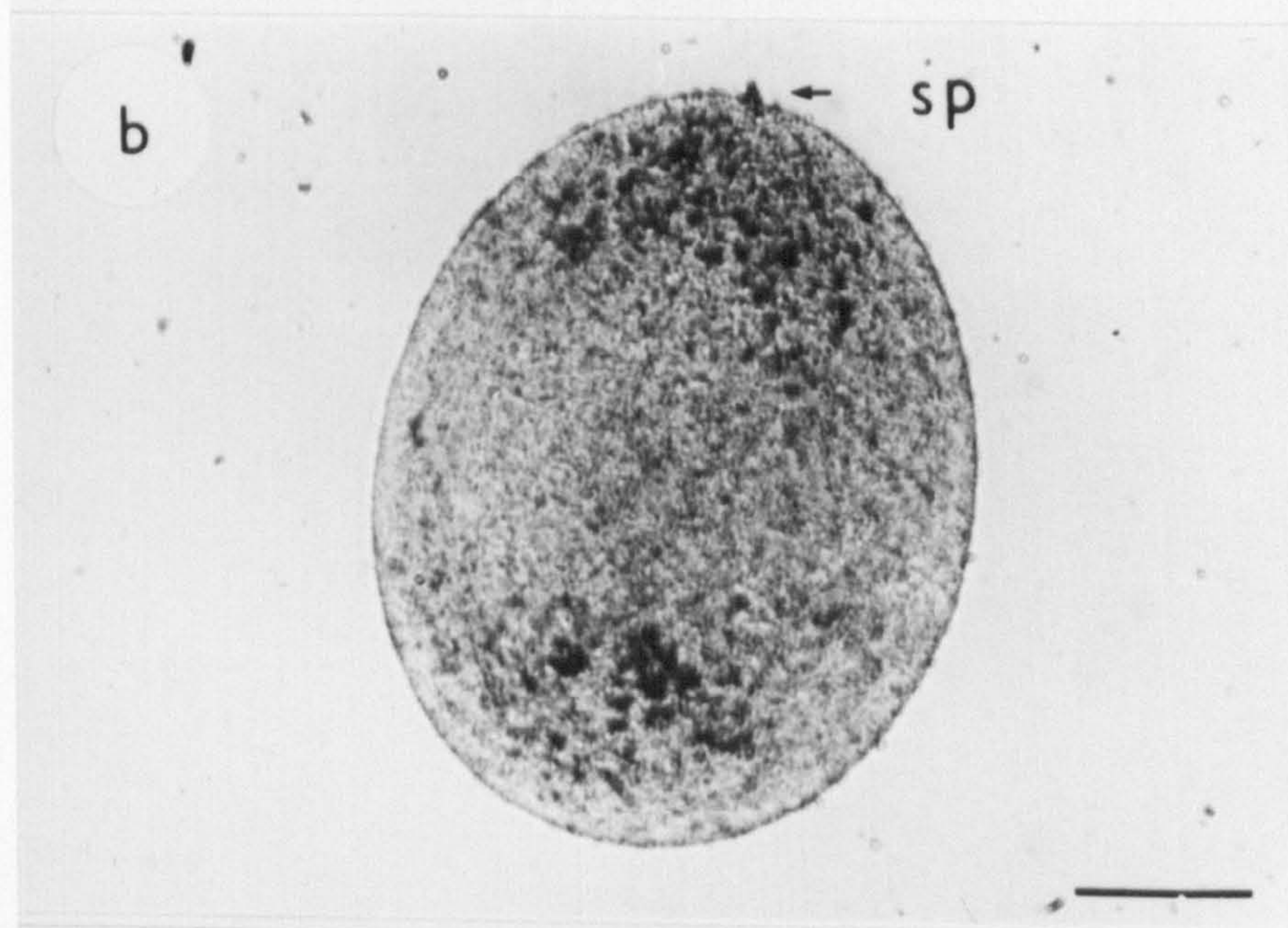
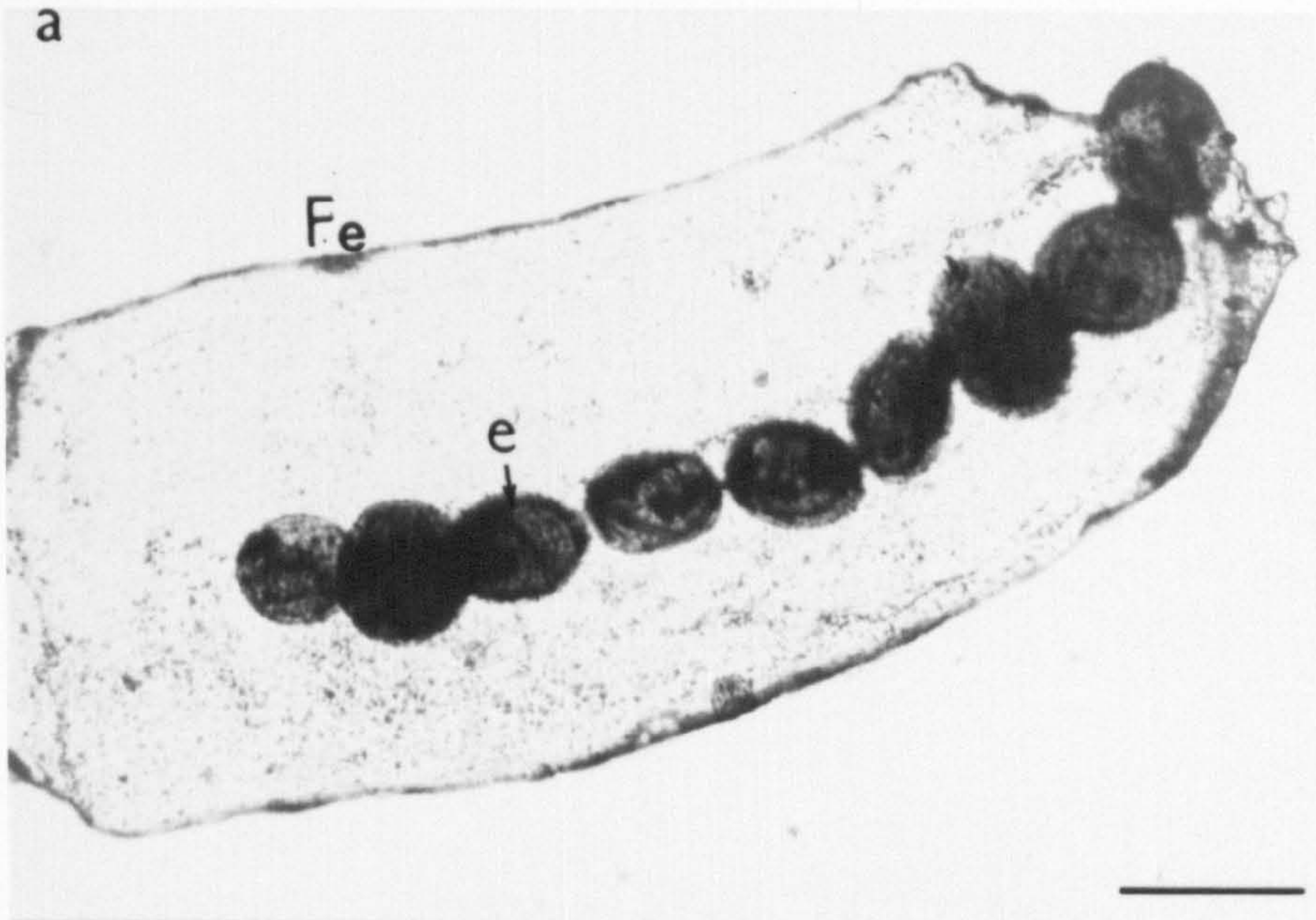




Figure 3.4 SEM micrographs of the miracidium of S.margrebowiei,

a) General body shape. Note the richly ciliated (Ci) appearance of the miracidium. Bar = 12 $\mu$ m

b) En face view showing the "rosette" pattern of the apical papilla (AP) surrounded by tactile apical cilia (Ta).

Bar = 2.5 $\mu$ m

c) The surface of a miracidium treated with ultrasound to break off the cilia showing the apical papilla (AP), Lateral papilla (LP) and an excretory pore (EP). Note that the body is covered with microvilli (Mv). Bar = 15 $\mu$ m

d) Side view of the apical papilla showing the presence of multiciliated pits (Mc), ducts (d) and unciliated pits (arrow). Bar = 1.5 $\mu$ m



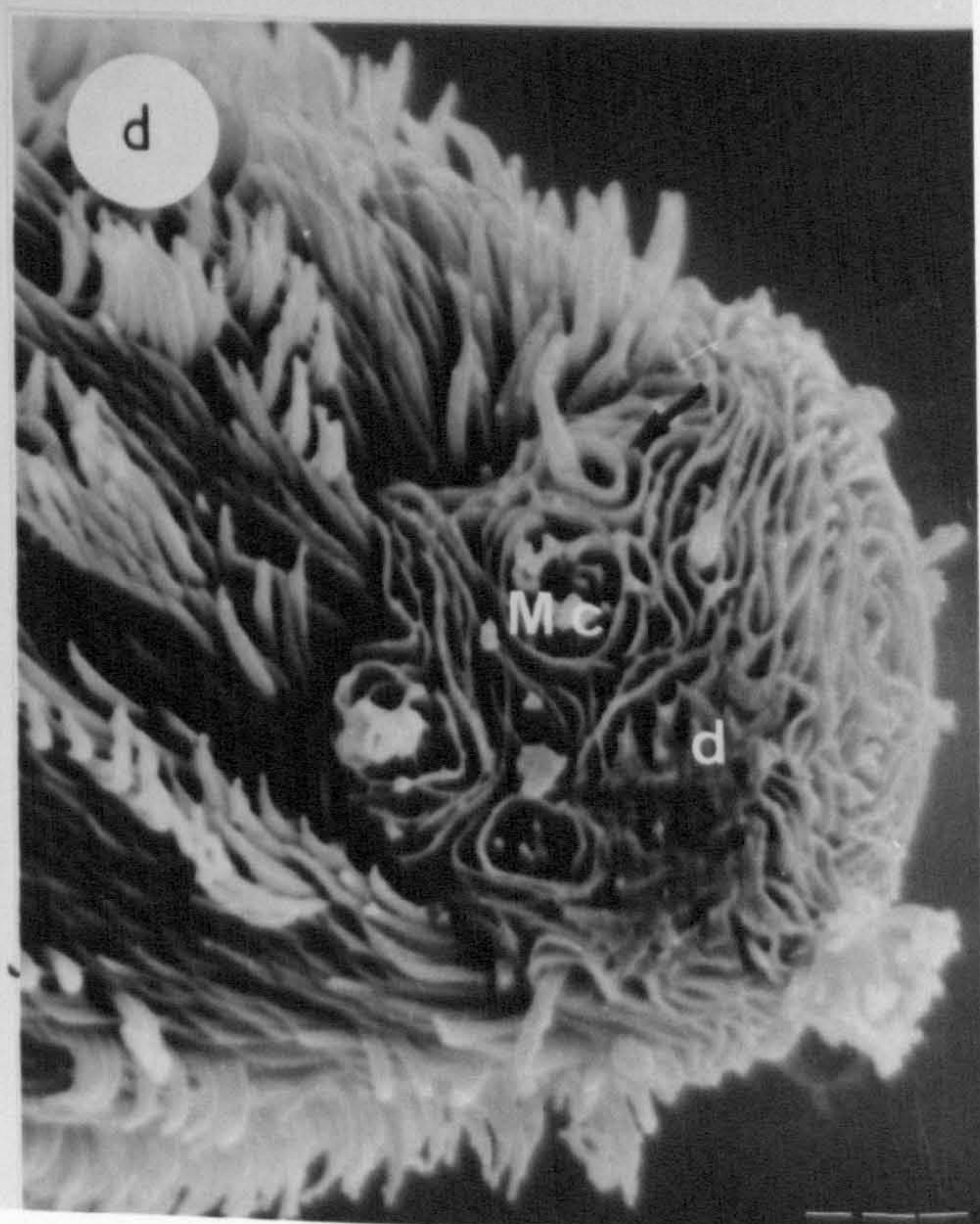
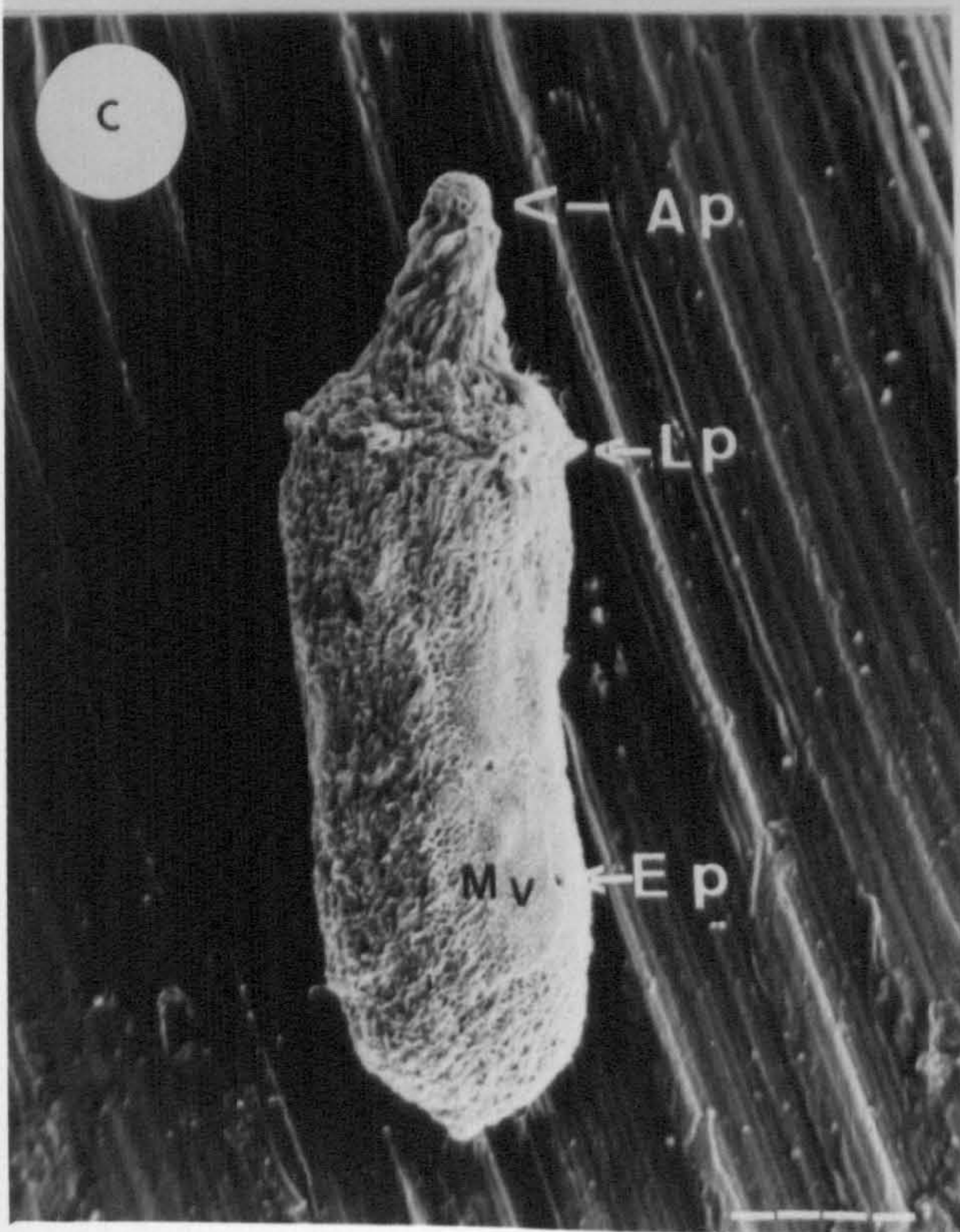
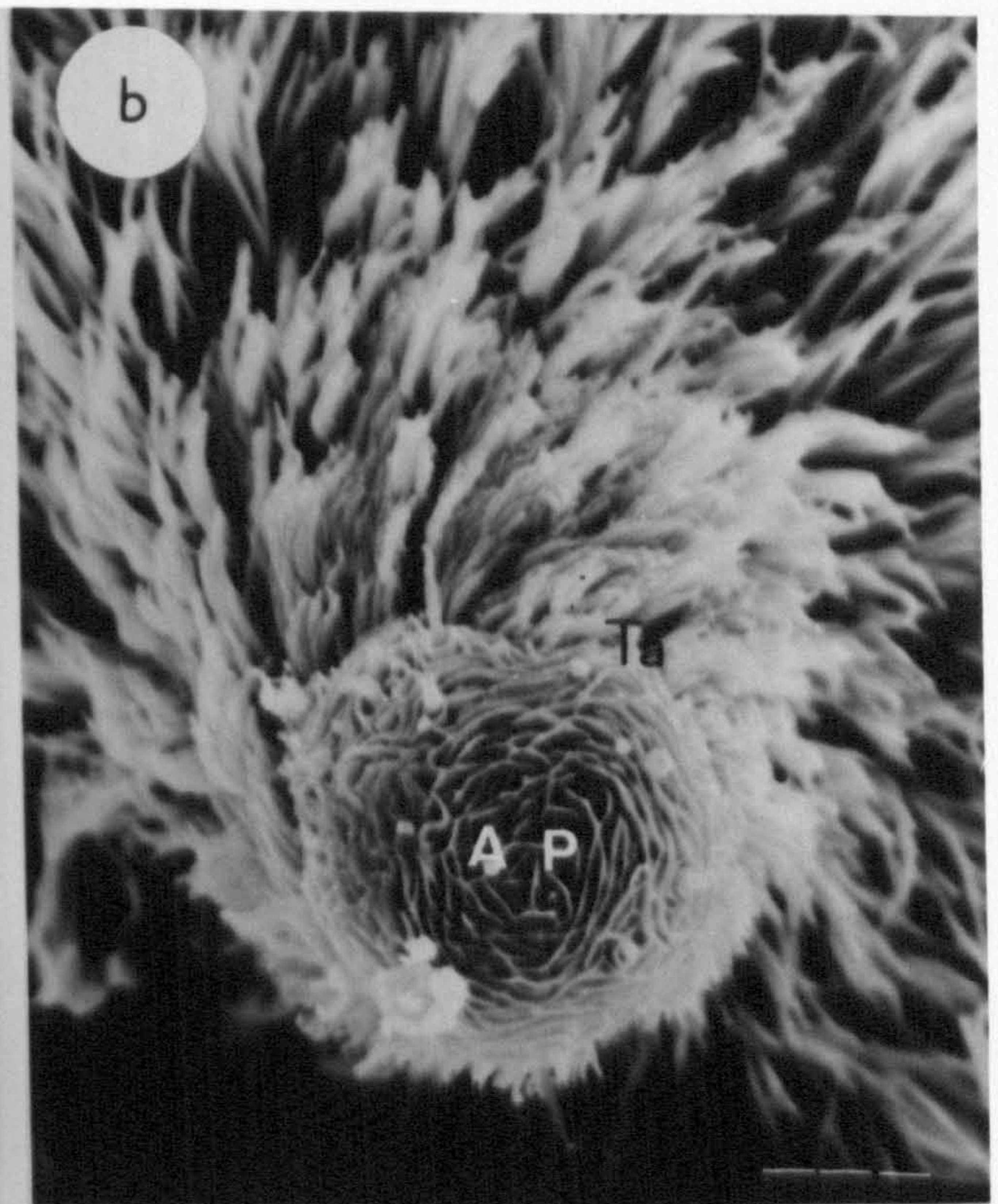
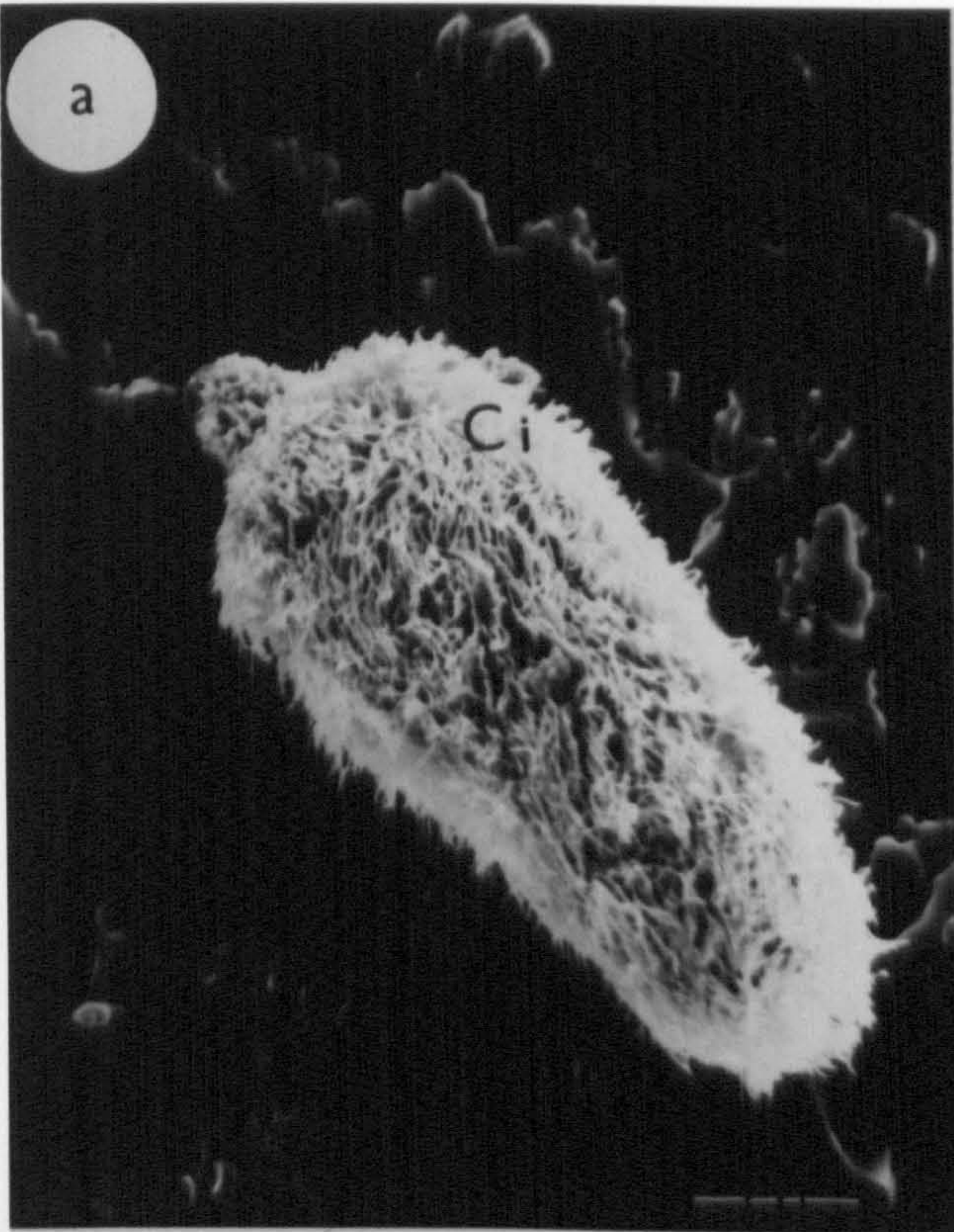




Figure 3.5 Light micrographs of the silver nitrate stained miracidium of S.margrebowiei.

a and b) The same specimen. The entire body is divided into four tiers. The number of epidermal plates on the second (2), third (3) and the fourth (4) tier are as follows: 9, 4, and 3 respectively. Bar = 22 $\mu$ m

c) The four tiers of epidermal plates are immediately after the apical papilla (AP). These plates are separated by intercellular ridges (ir). Note the presence of sensory receptors (sr) only on the anterior and the median rings.

Bar = 22.5 $\mu$ m

d) The first tier surrounding the apical papilla has 6 epidermal plates. Bar = 18 $\mu$ m



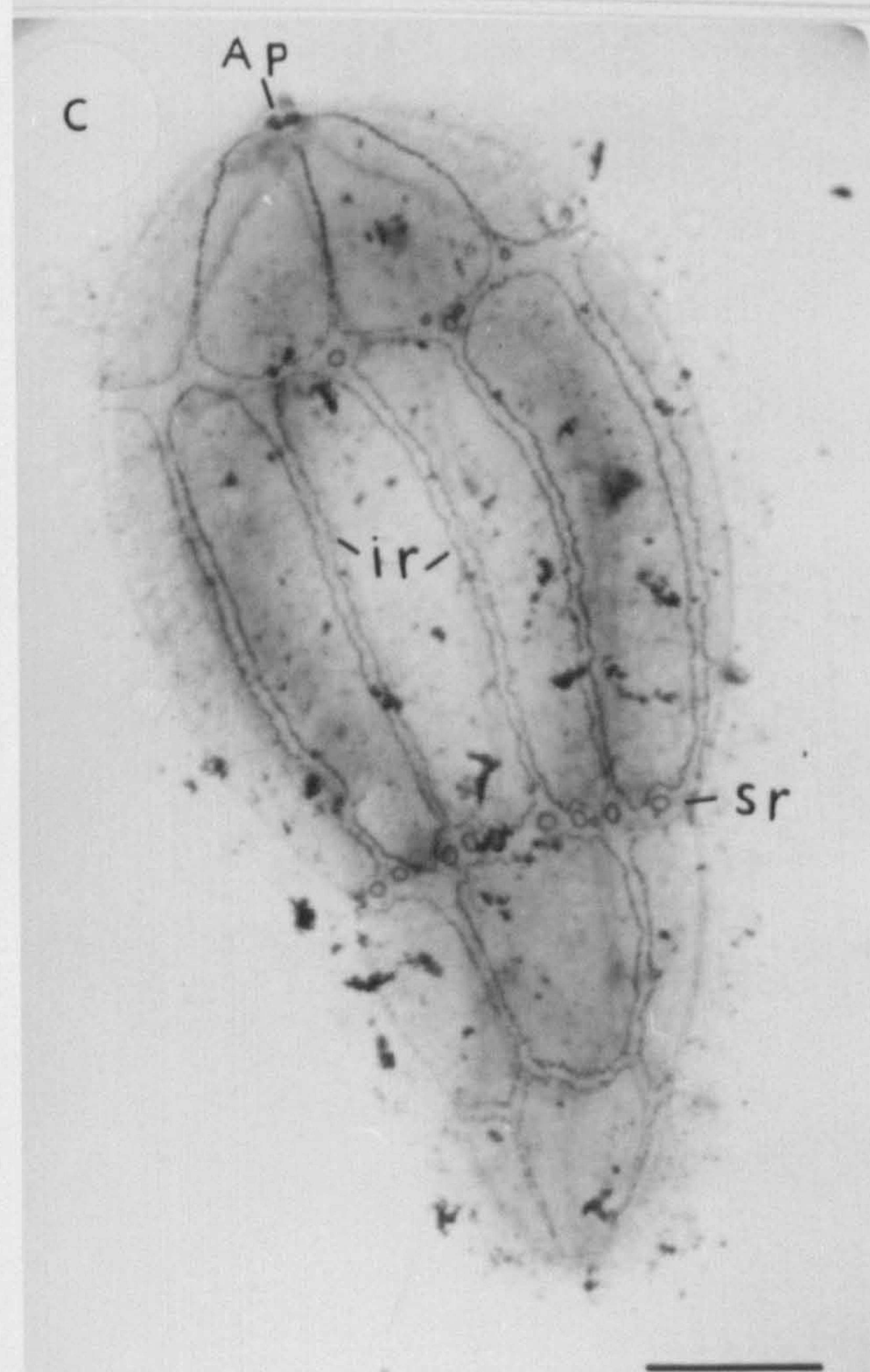
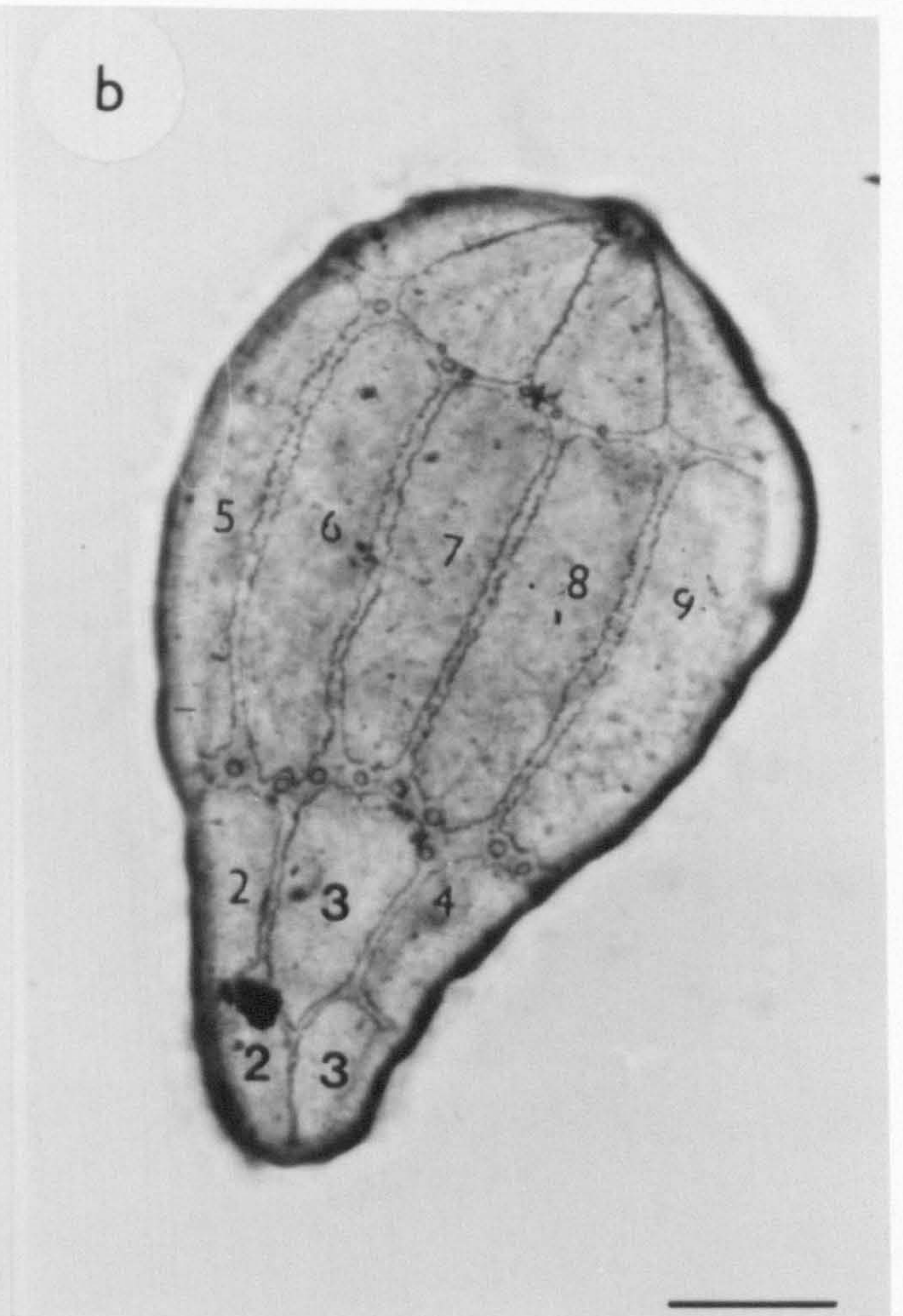
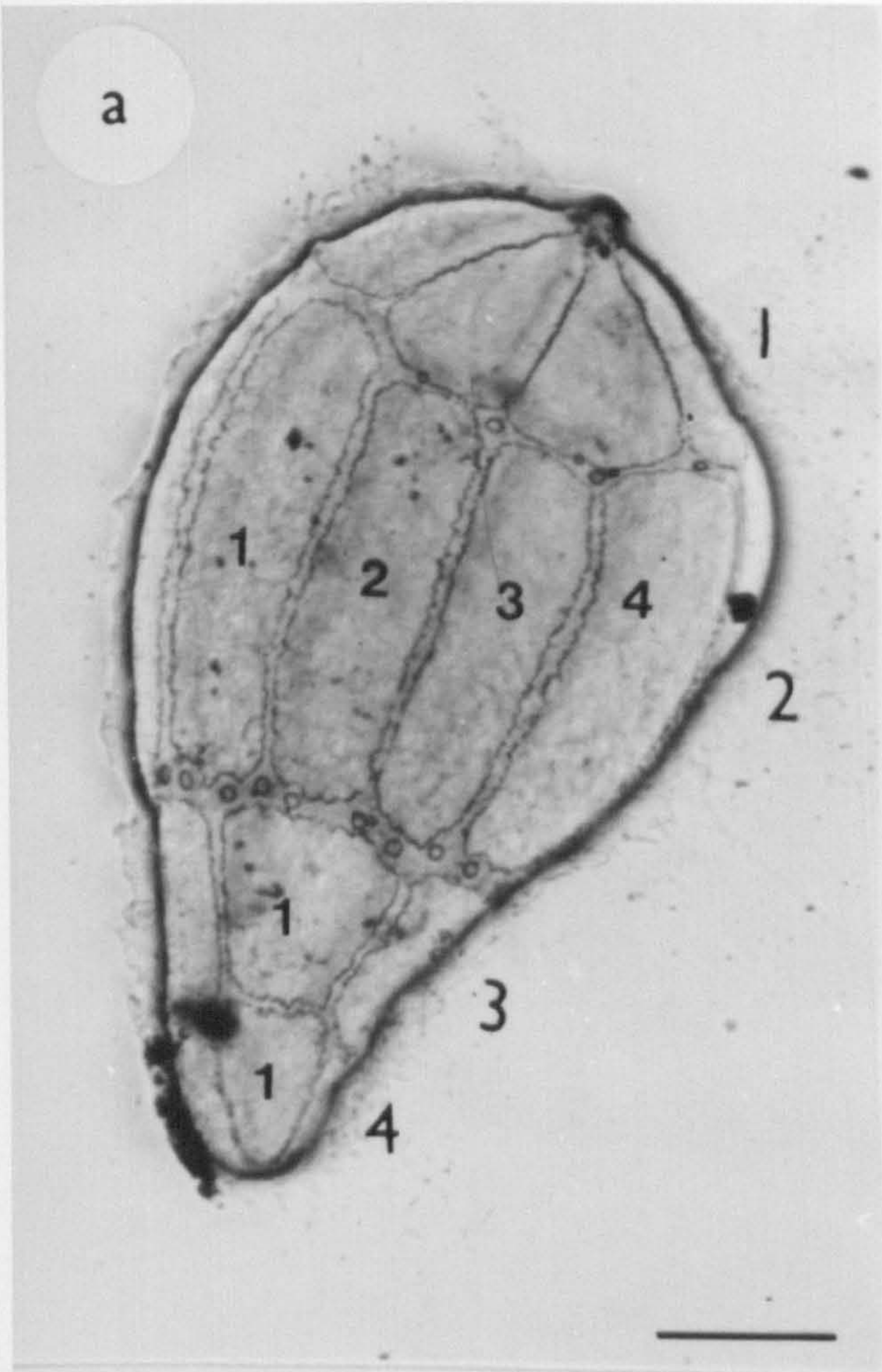




Figure 3.6 TEM micrographs of the miracidium of S. margrebowiei.

a) A low magnification of a ciliated epidermal plate with numerous cilia (Ci) and microvilli (Mv) on the outer surface. Note the presence of mottled dense ovoid granules (Dog), light ovoid granules (Log) and desmosomes (De) immediately under the surface. Bar =  $0.6\mu\text{m}$

b) A low magnification of the submuscular region with longitudinal (Lm) and circular (Cm) muscles. Note the presence of large number of mitochondria (Mi). Excretory ducts (ed) with their associated desmosomes (arrow) and cytoplasmic projections (Cp) are also evident. Bar =  $0.88\mu\text{m}$

c) The anterior region of the miracidium showing a multiciliated pit (Mc) and numerous mitochondria (Mi).

Bar =  $2.2\mu\text{m}$

d) Transverse section of a flame cell (Fc) with its associated cilia (Ci). Bar =  $0.64\mu\text{m}$

e) Longitudinal section of the anterior region of the apical paila (AP) showing two penetration glands (P) with their associated secretory pores (arrow). Bar =  $0.375\mu\text{m}$

f) Longitudinal section of the region immediately below the penetration glands showing the neural mass (Ne) with the outer nuclei (N). Bar =  $0.375\mu\text{m}$



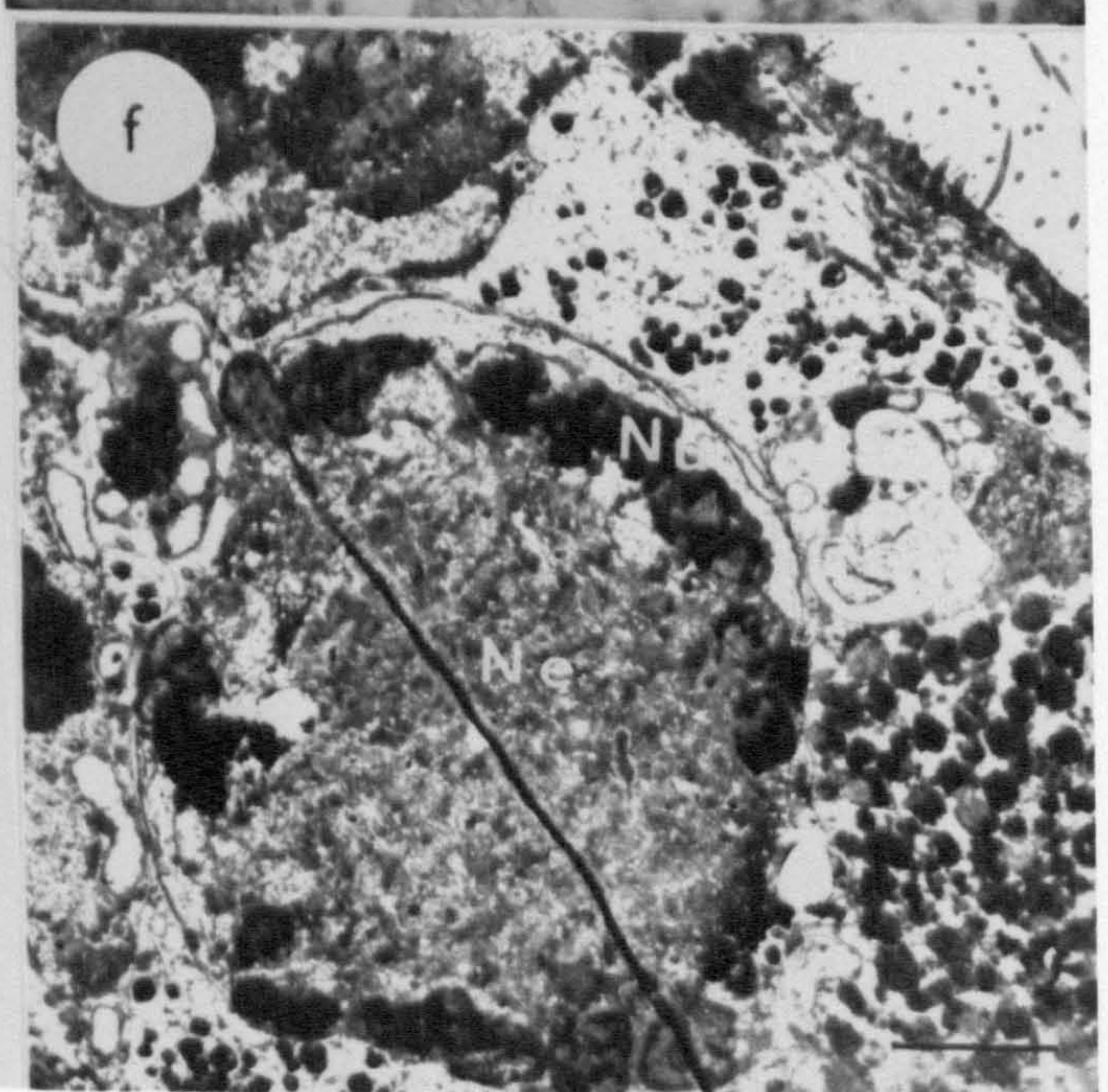
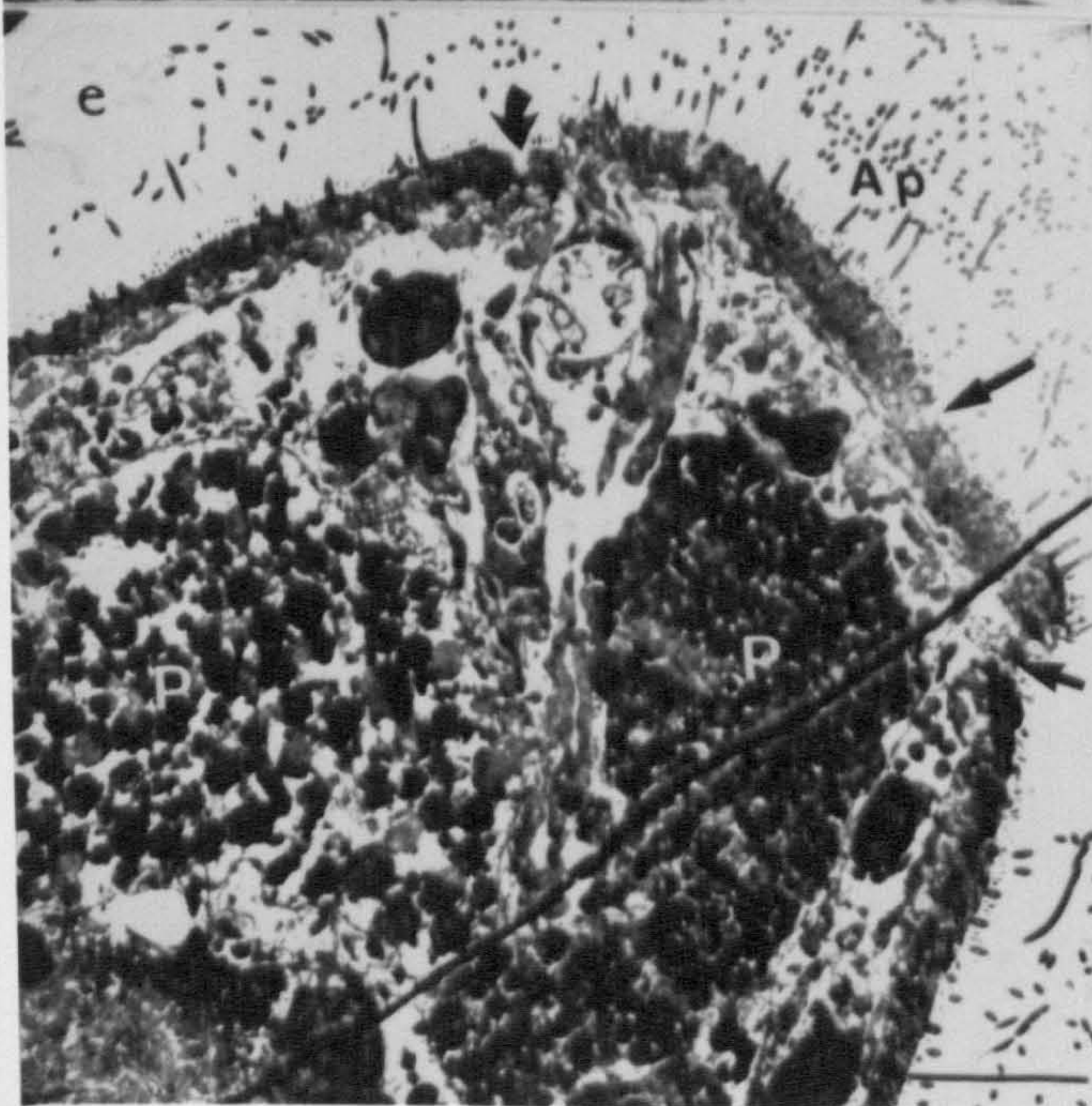
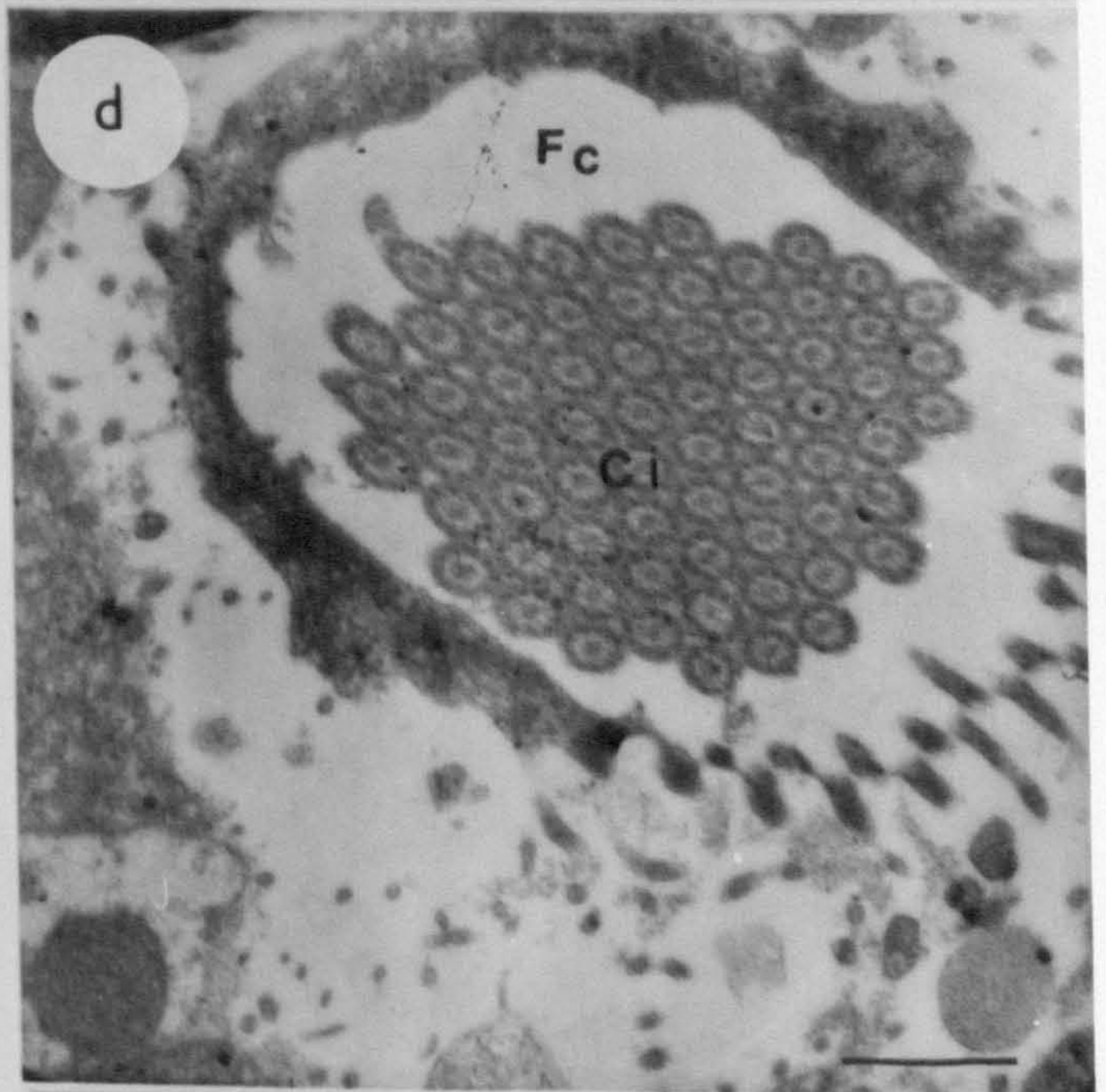
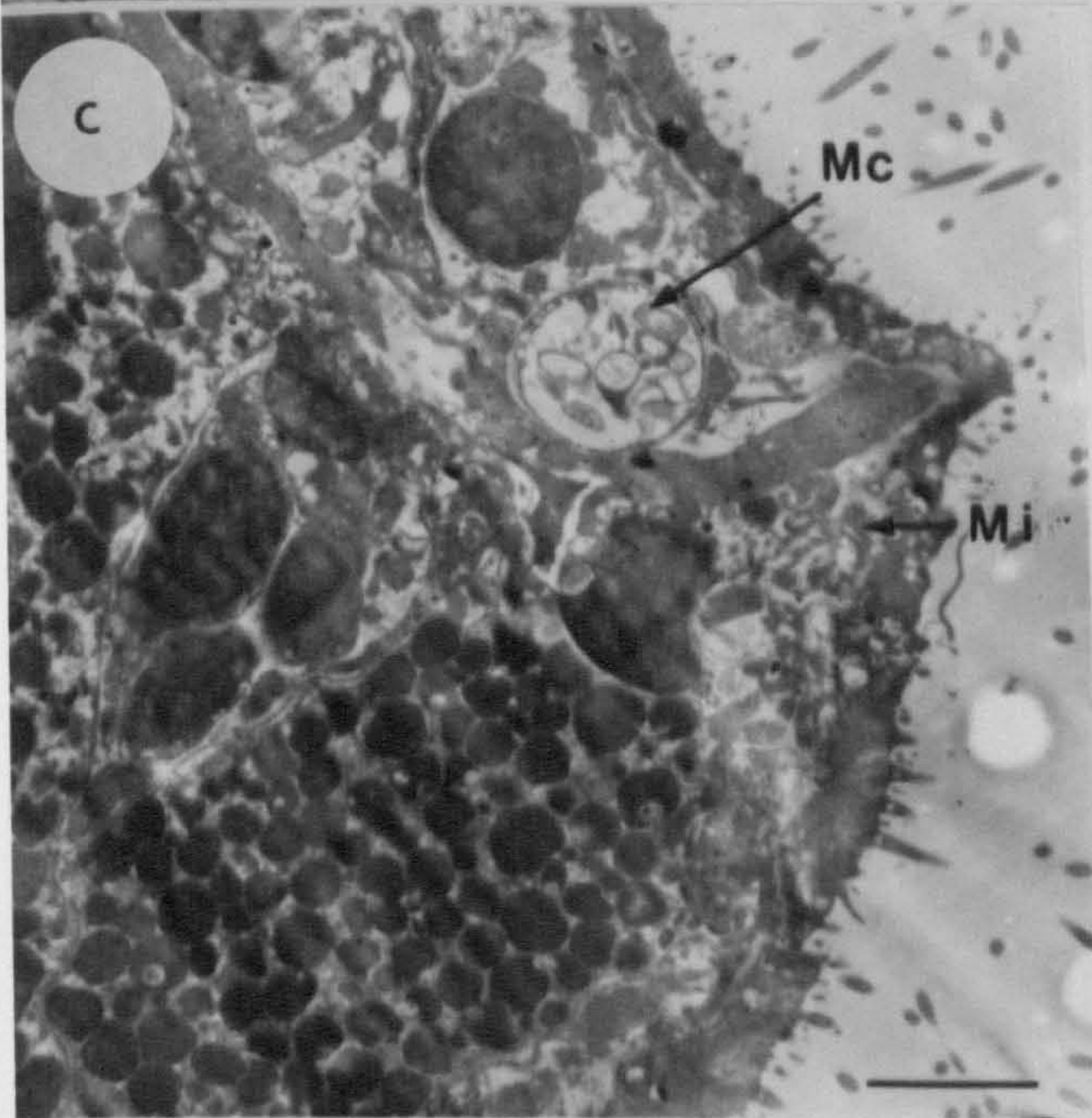
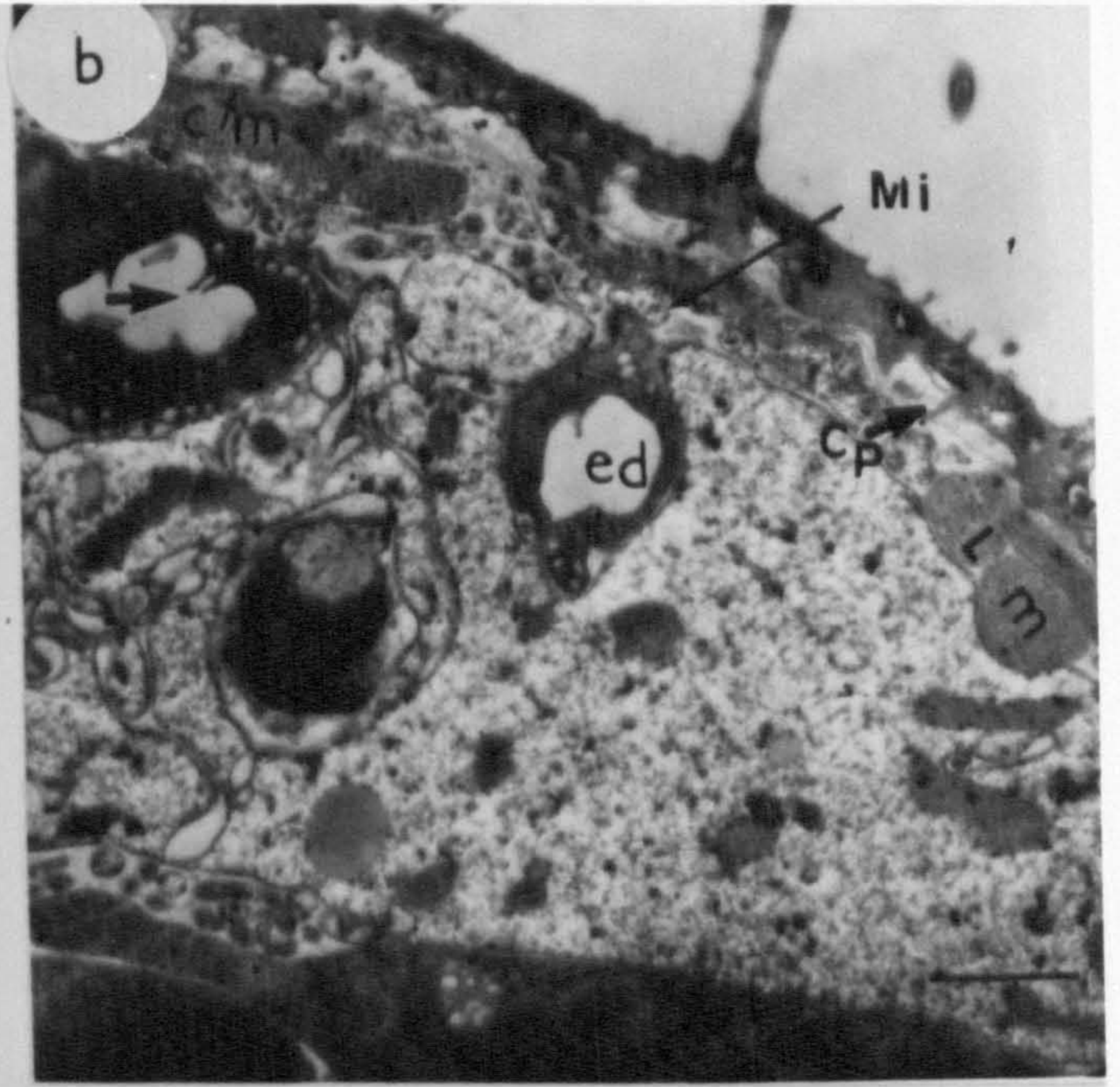
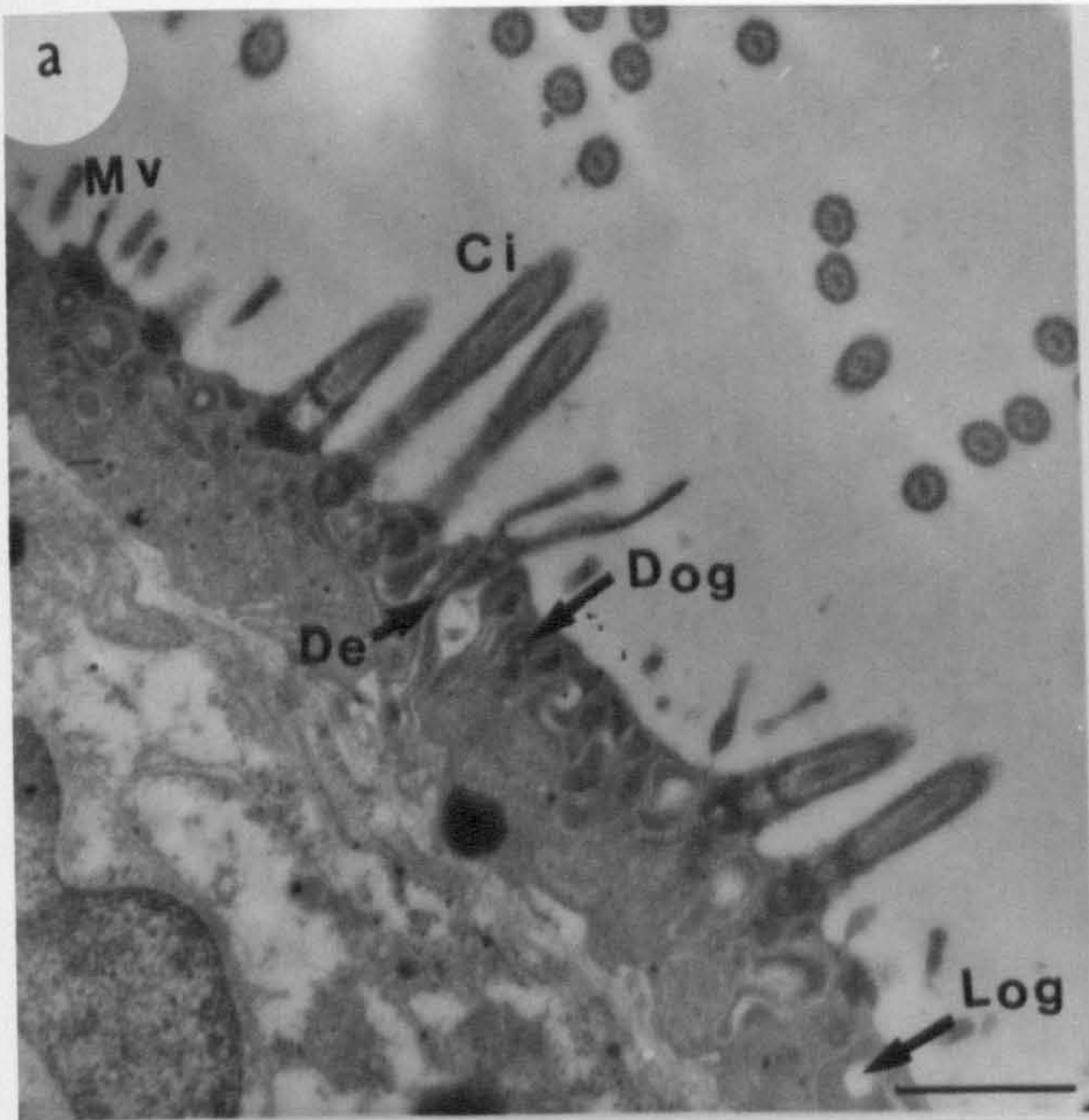




Figure 3.7 TEM micrographs of the miracidium of S. margrebowiei.

a) Transverse section of a lateral papilla (LP) with its associated nerve  $\leftrightarrow$ . Bar =  $3.6\mu\text{m}$

b) A low magnification of epidermal plates (arrow). One with numerous cilia (Ci) and one without. Bar =  $0.35\mu\text{m}$

c) Anterior region of the miracidium showing a uniciliated sensory organ (Uc). Bar =  $0.588\mu\text{m}$

d) Anterior region of a miracidium showing a multiciliated sensory pit (Mc) with a number of cilia (Ci). Bar =  $0.38\mu\text{m}$

e) A multiciliated pit with its associated cilia (Ci) and vacuoles (V). Bar =  $0.36\mu\text{m}$ .



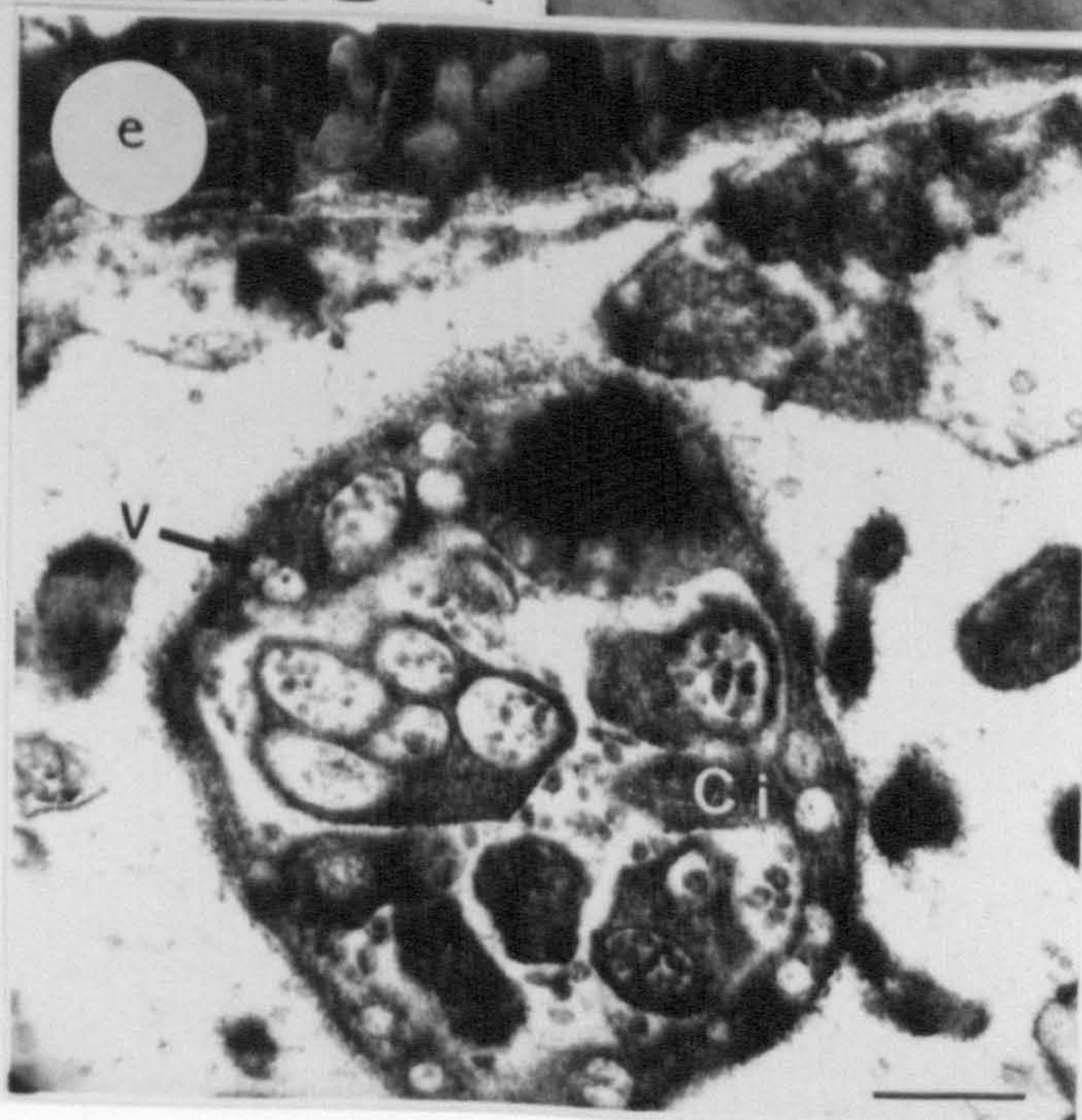
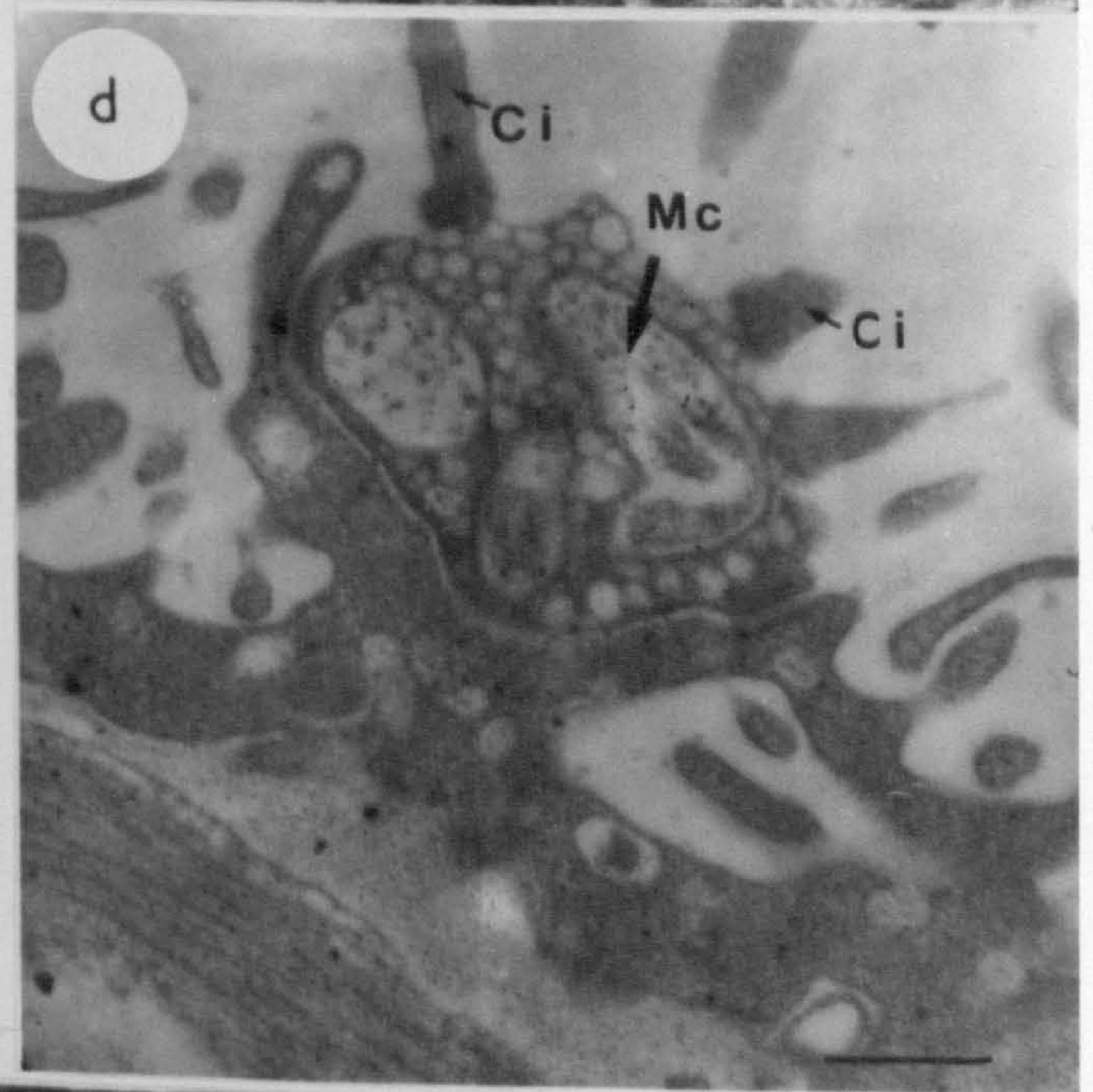
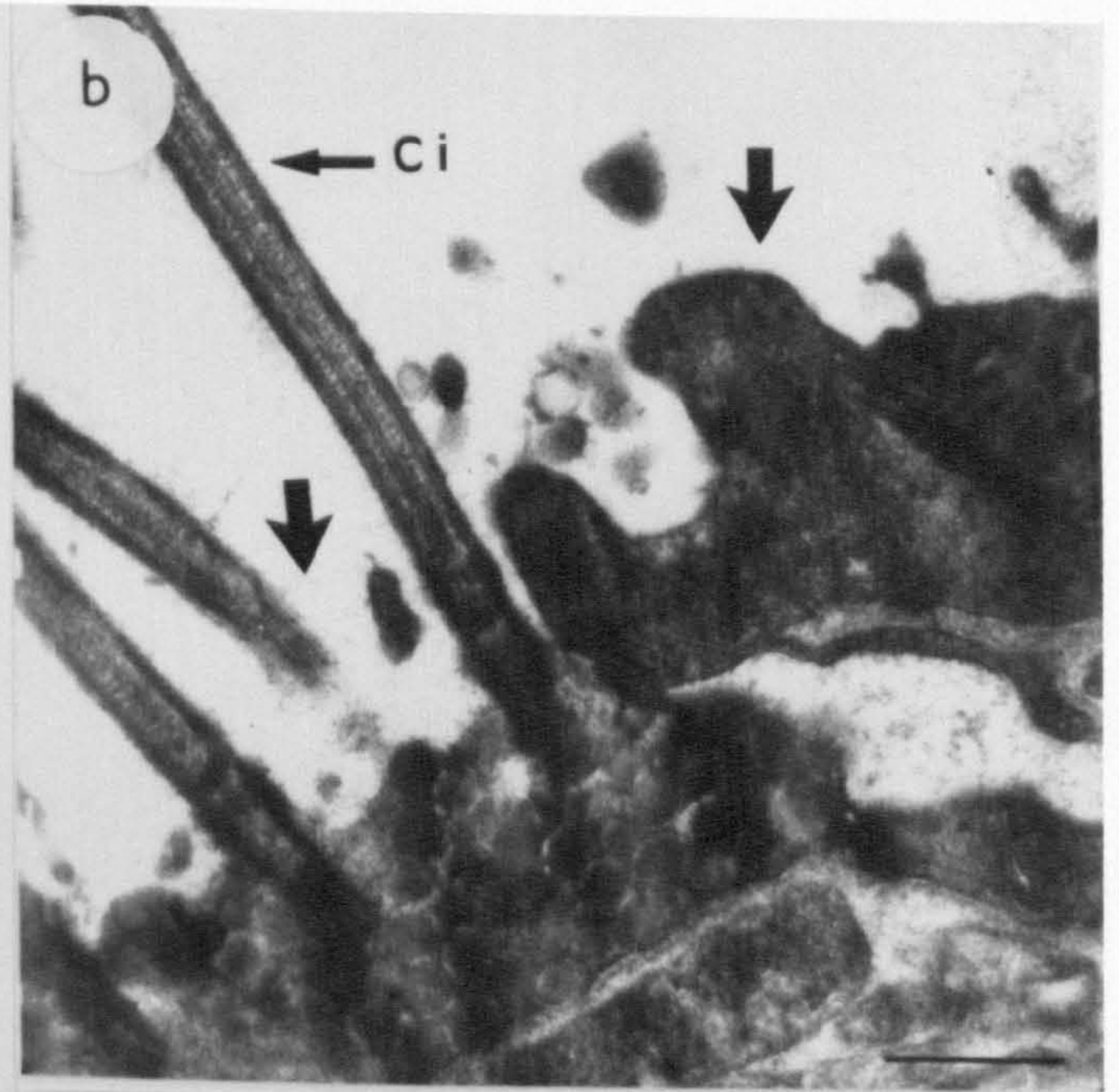




Figure 3.8 TEM micrographs of the sporocyst of S.margrebowiei.

a) Transverse section of a sporocyst (Sp) which is covered with long microvilli (Mv). Bar = 4.64 $\mu$ m

b) A developing cercaria (Ce) of S.margrebowiei found in the hepatopancreas of the snail host. Note the undifferentiated and developing glands (arrows) of the cercaria. Bar = 7.2 $\mu$ m

c) The surface of the sporocyst is covered with microvilli (Mv) underneath which, irregular shaped tegumental cells (Tc) are found. Bar = 3 $\mu$ m

d) A cercaria (Ce) found in the hepatopancreas of the snail host. Bar = 6.12 $\mu$ m

e) A mature cercaria with its associated spines (arrow) and a flame cell (Fc). Bar = 1 $\mu$ m

f) A developing cercaria of S.margrebowiei. Note the immature state of its secretory glands (arrow). Bar = 2.2 $\mu$ m



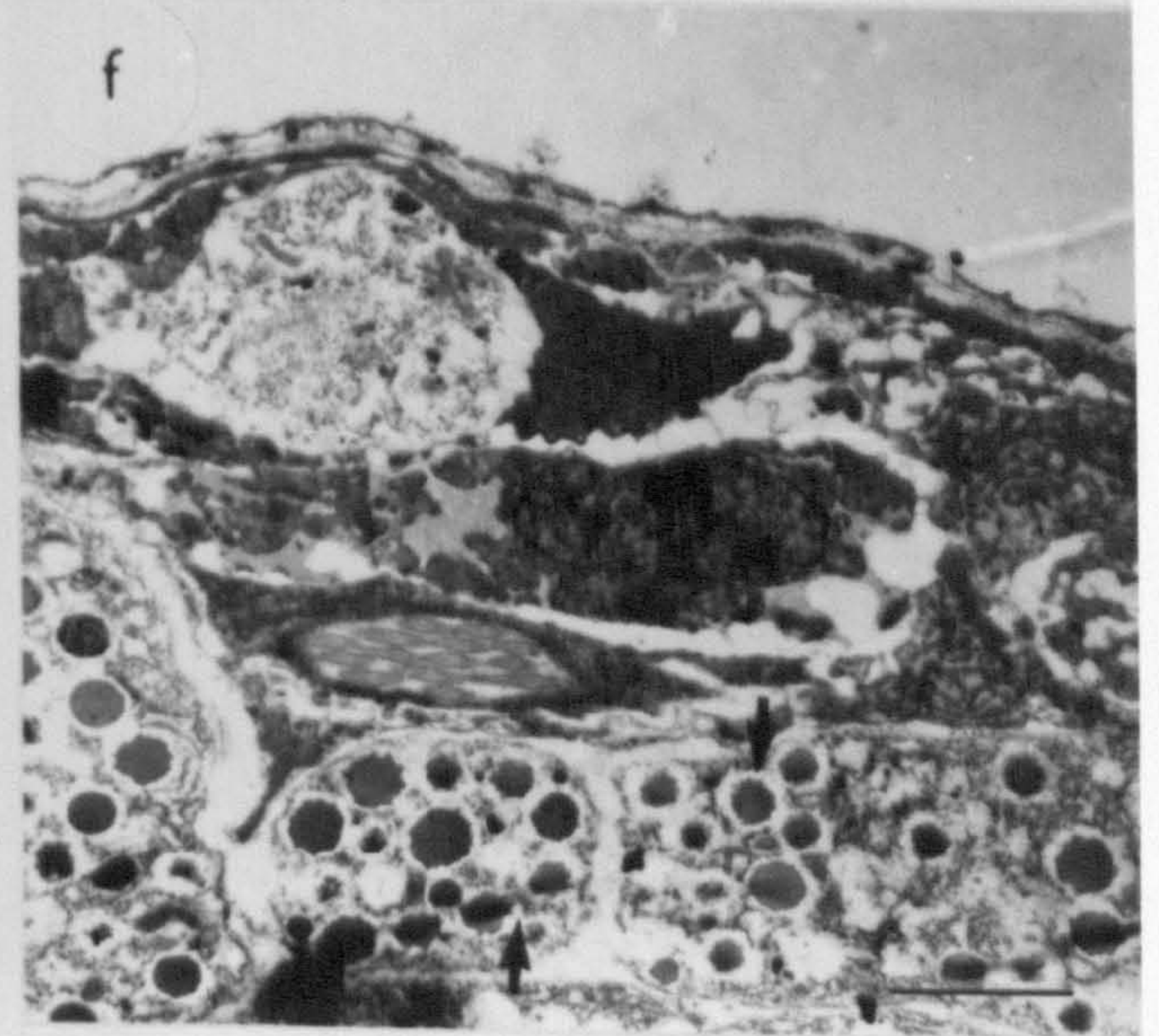
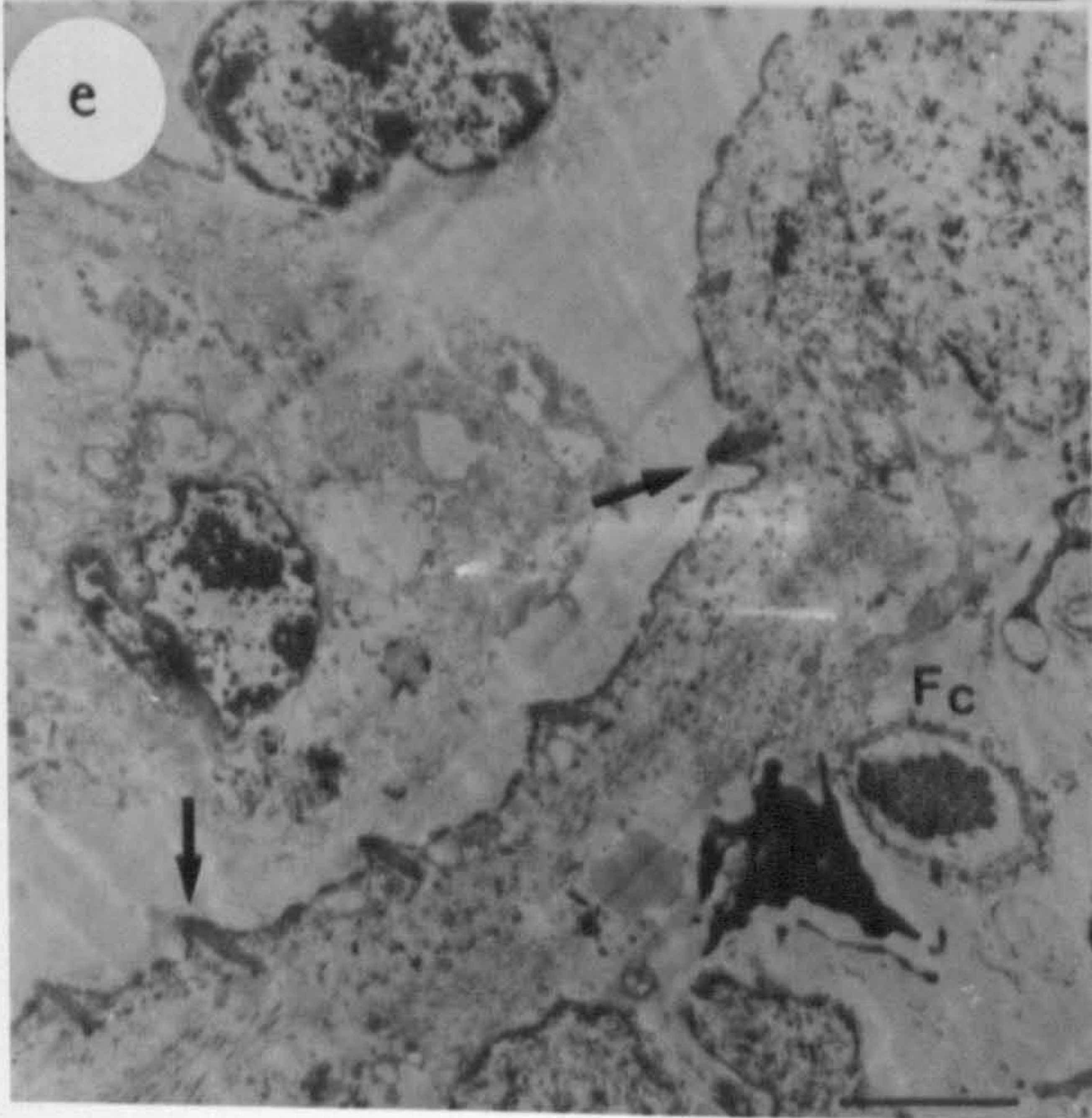
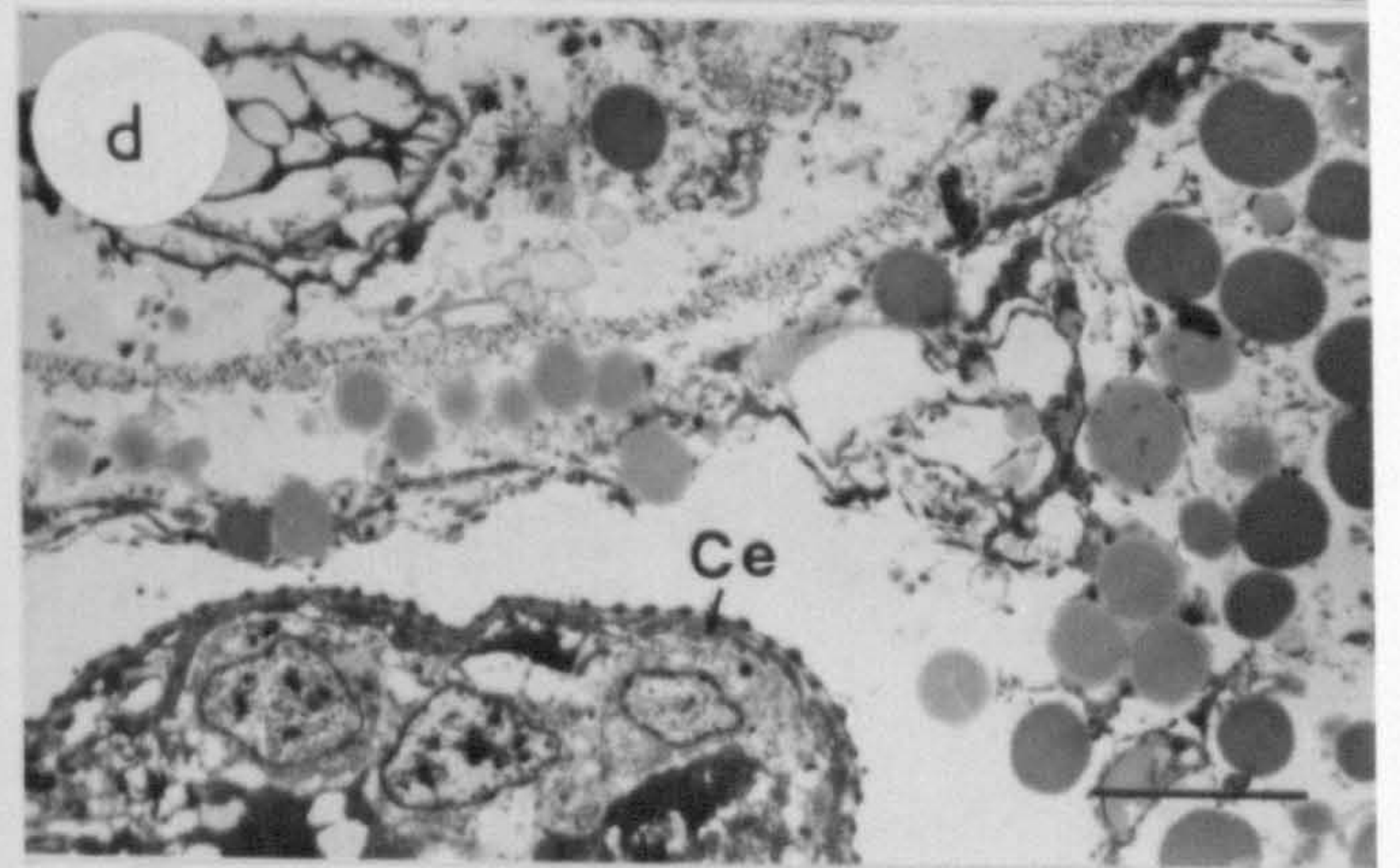
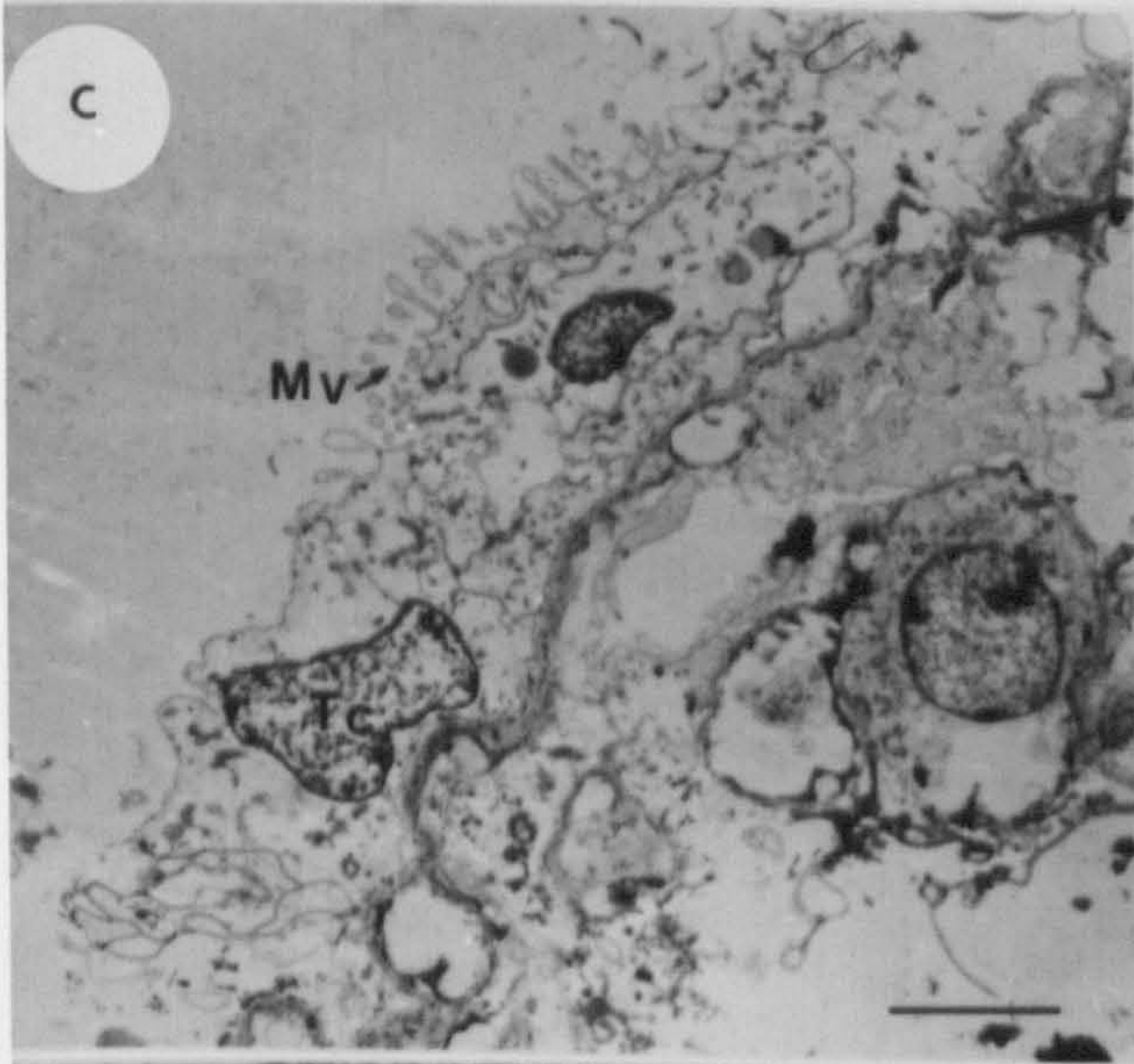
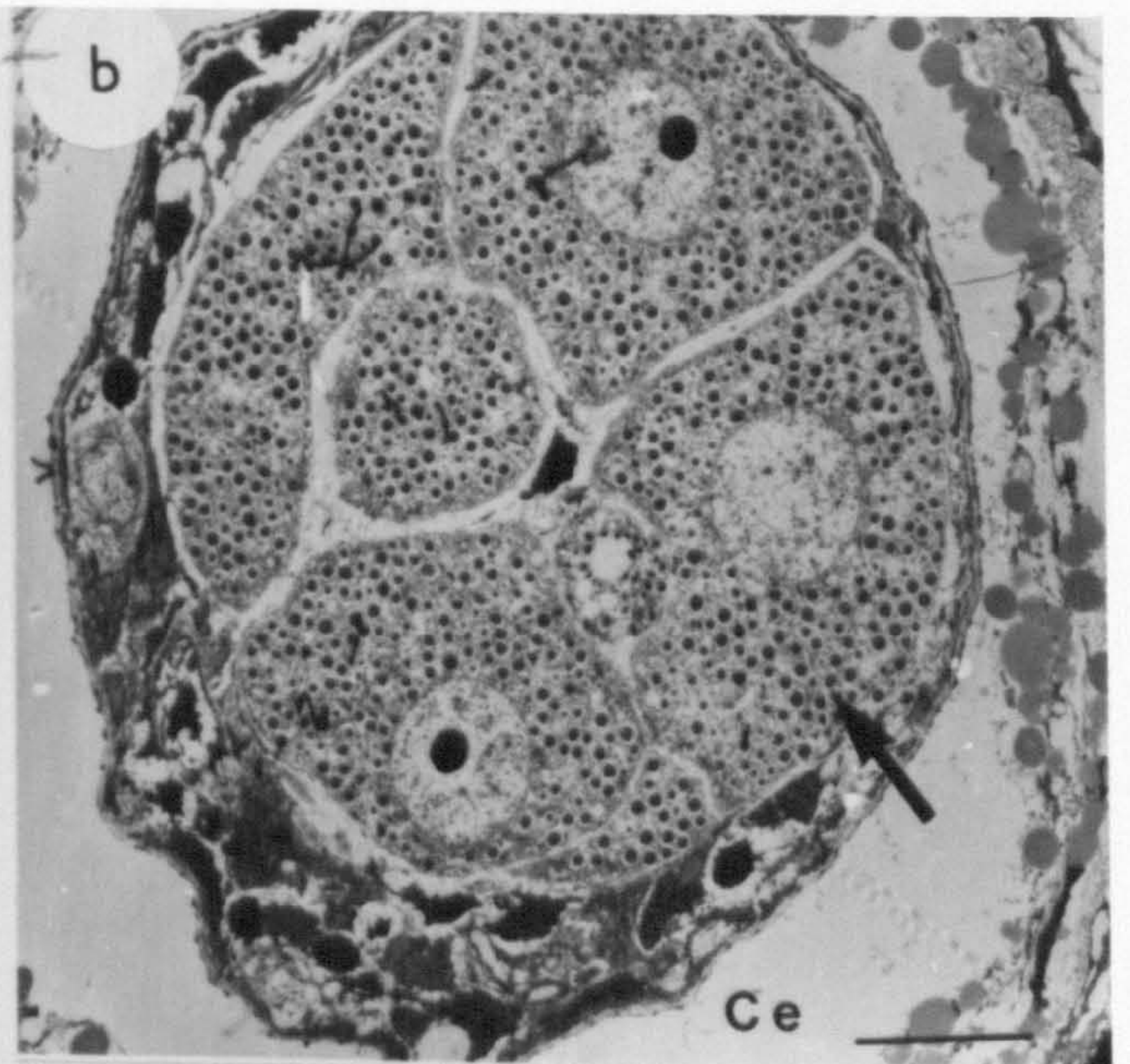
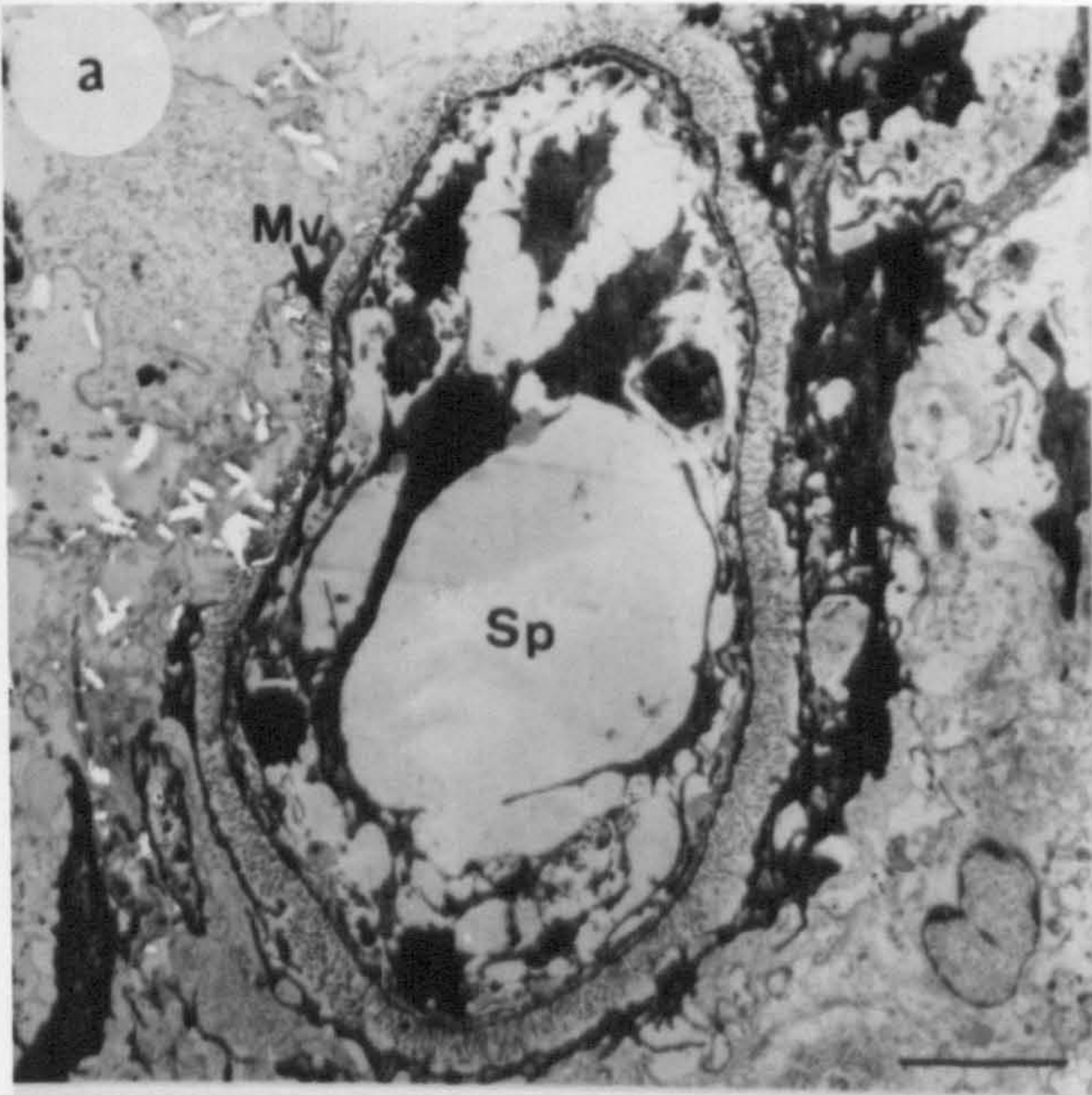




Figure 3.9 Light micrographs of the snail host Bulinus natalensis and SEM of the cercaria of S.margrebowiei.

a) A micrograph of different sized shells of B.natalensis.

b) SEM of the hepatopancreas (HP) of an infected snail. Note the presence of numerous cercariae (Ce). Bar = 37 $\mu$ m

c) SEM micrograph of a fully mature cercaria showing the head (H), the body (B), the collar (C), tail stem (T) and furcae (Fu). Note the position of the ventral sucker (Vs) on the body. Bar = 26 $\mu$ m



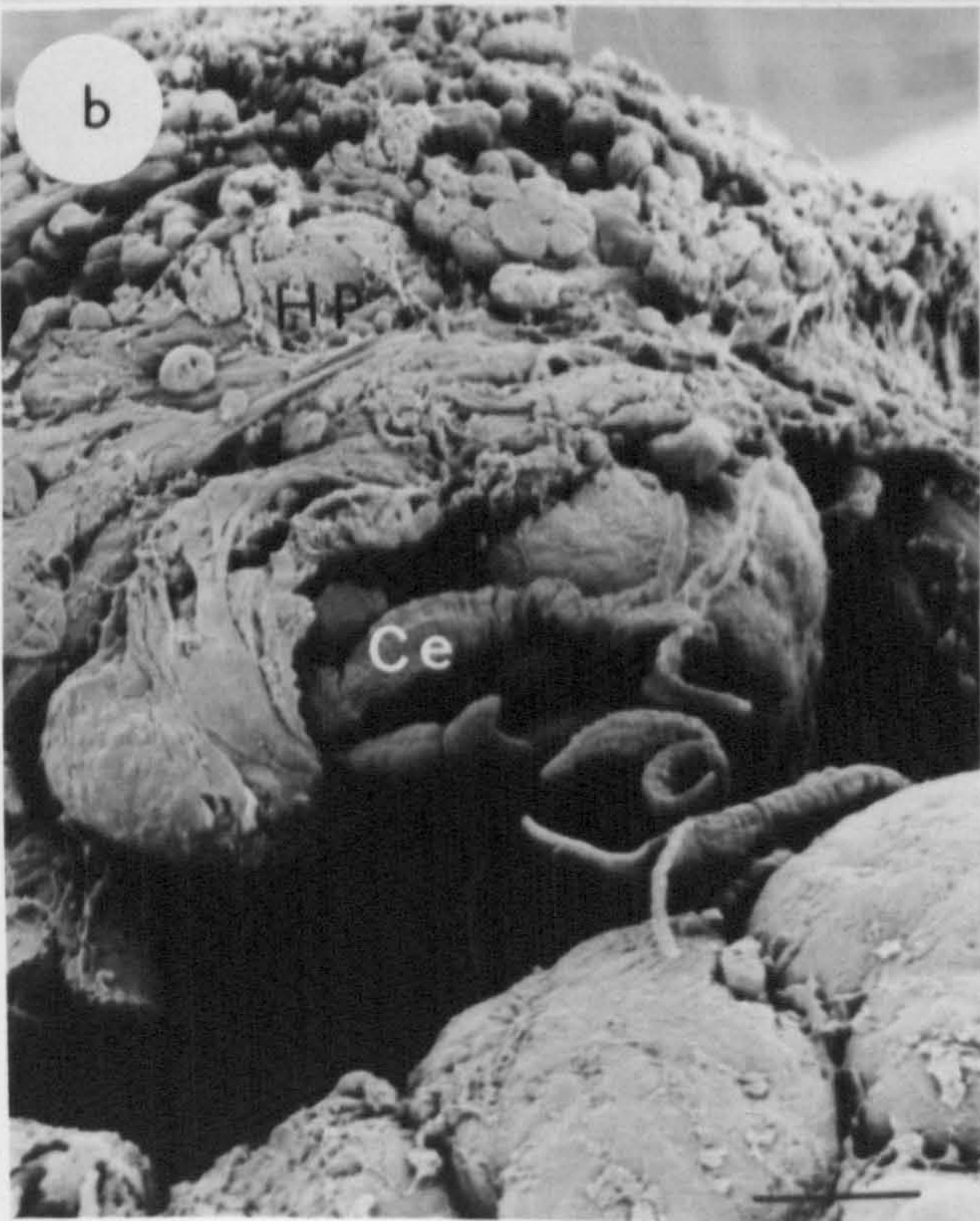
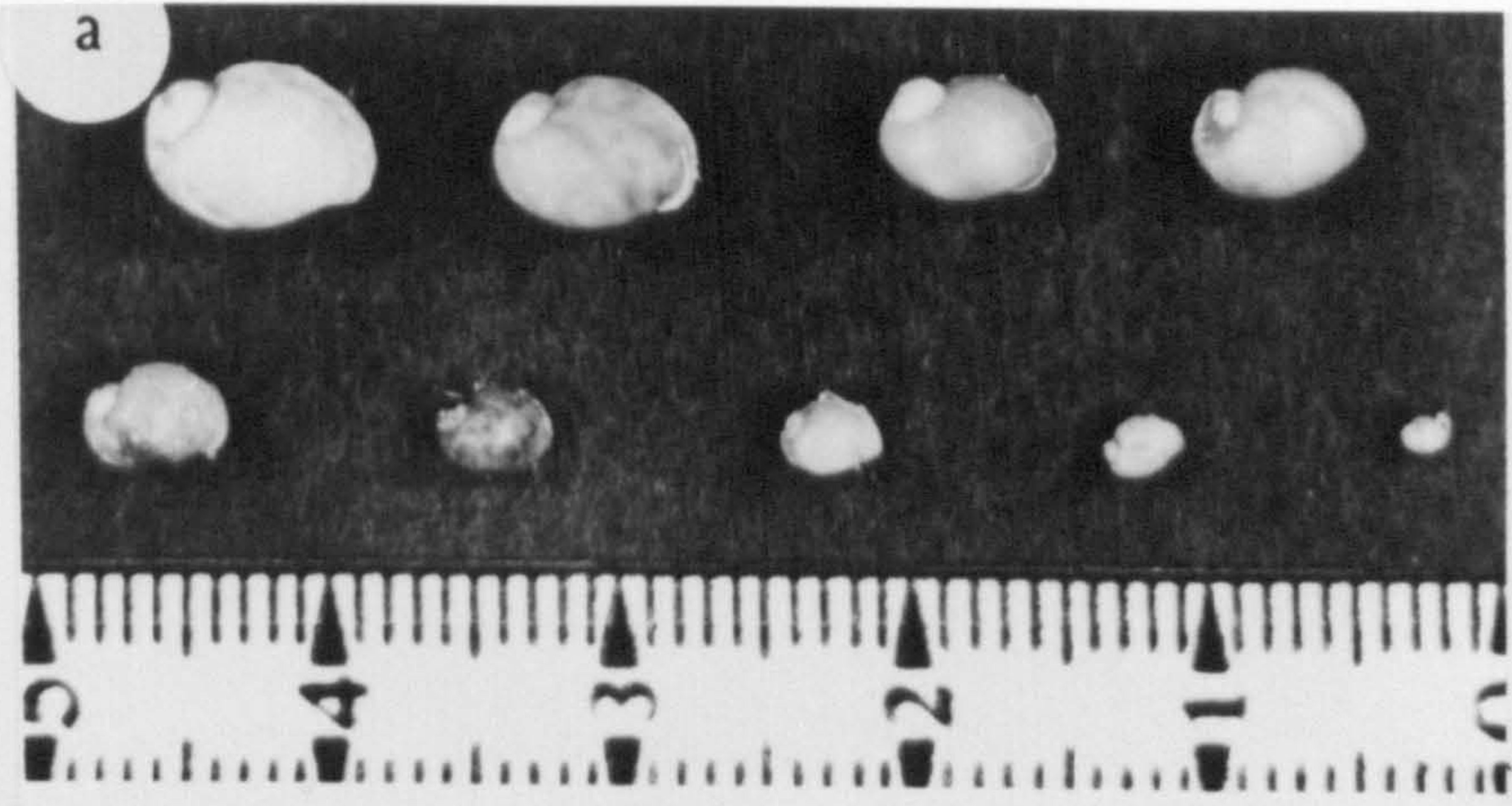




Figure 3.10 SEM micrographs of the cercaria of S.margrebowiei.

a) Posterior region of the cercaria showing the presence of sensory hairs (SH) on the tail stem and furcae. Note the excretory pore (Ep) positioned on the end of each furca.

Bar = 10.5 $\mu$ m

b) Side view of the ventral sucker (Vs), with its inwardly directed spines. Note the posteriorly pointed spines (Sp) on the body. Bar = 2.5 $\mu$ m

c) The anterior region showing a number of papillae (P) and ducts (arrow). Bar = 1.15 $\mu$ m

d) The collar region (C) separating the body (B) from the tail stem (T). Bar = 2 $\mu$ m



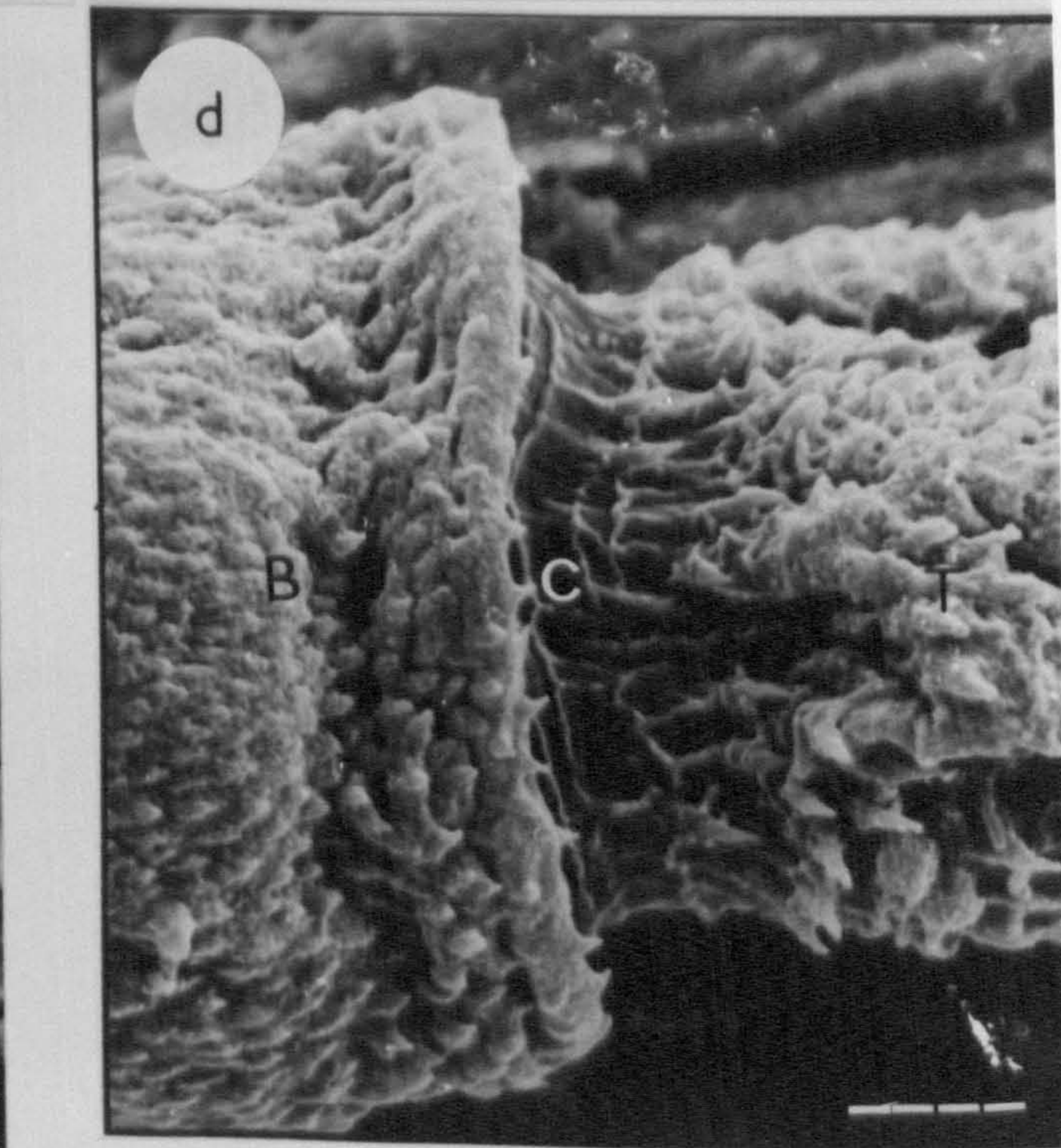
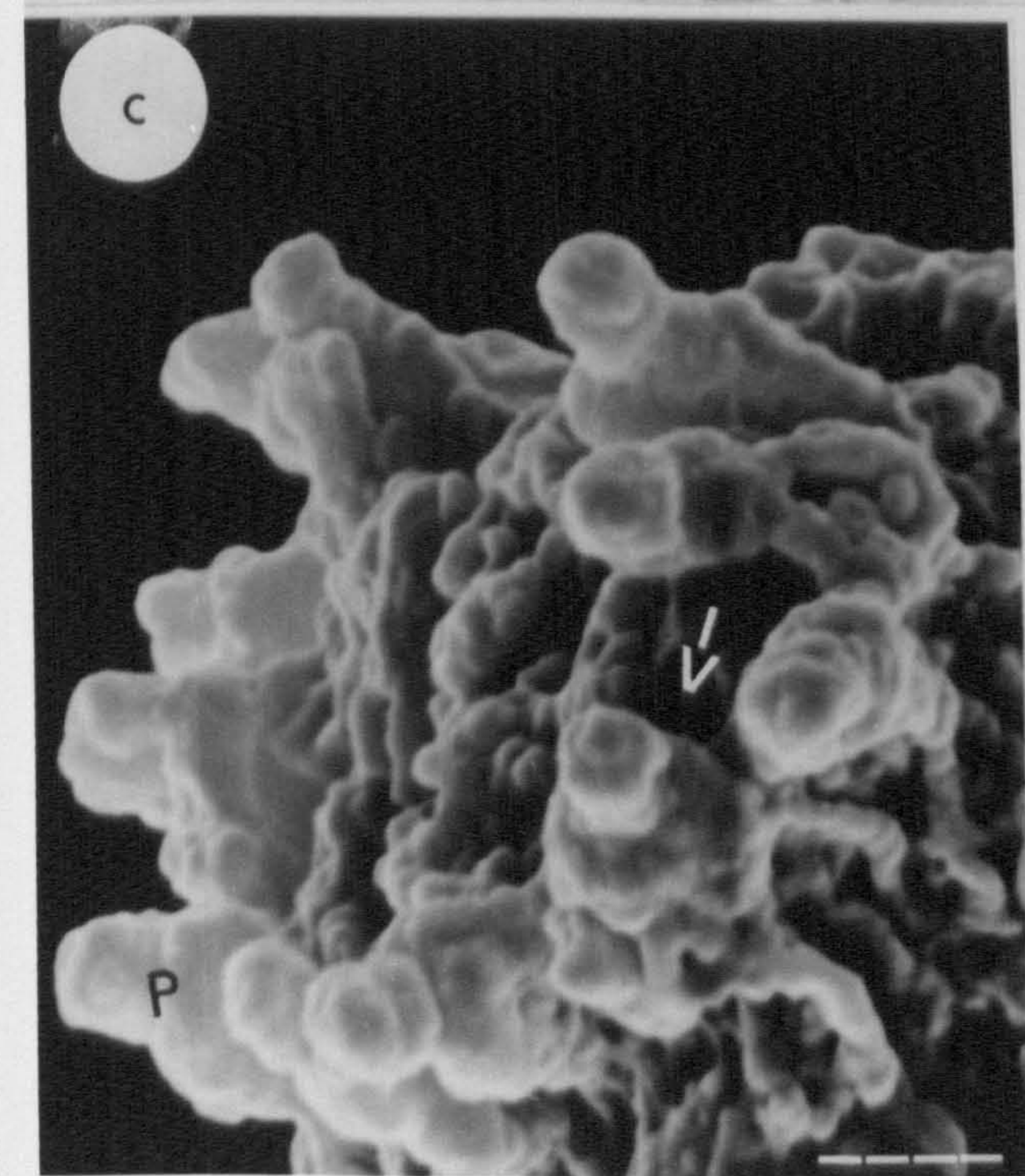
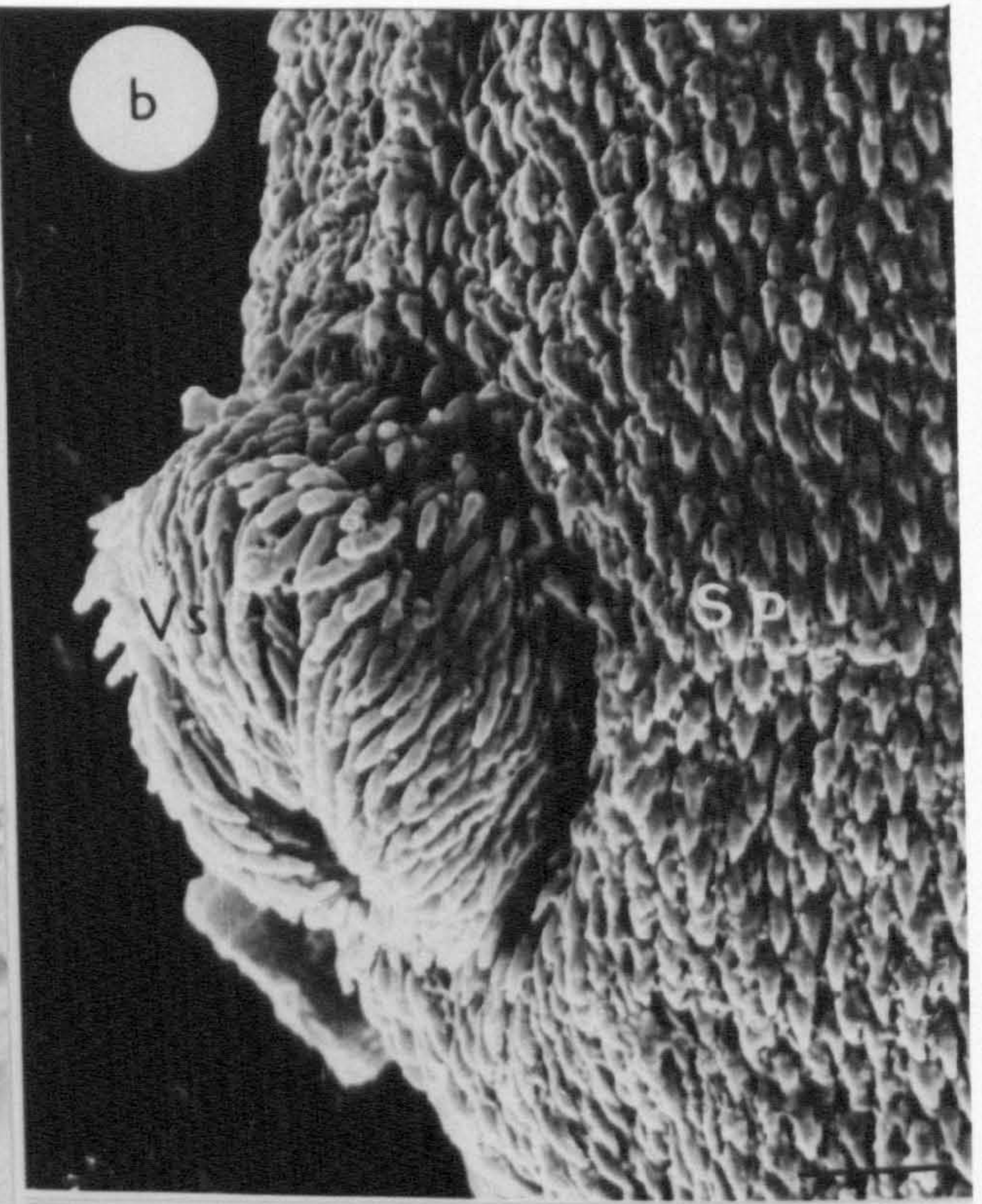
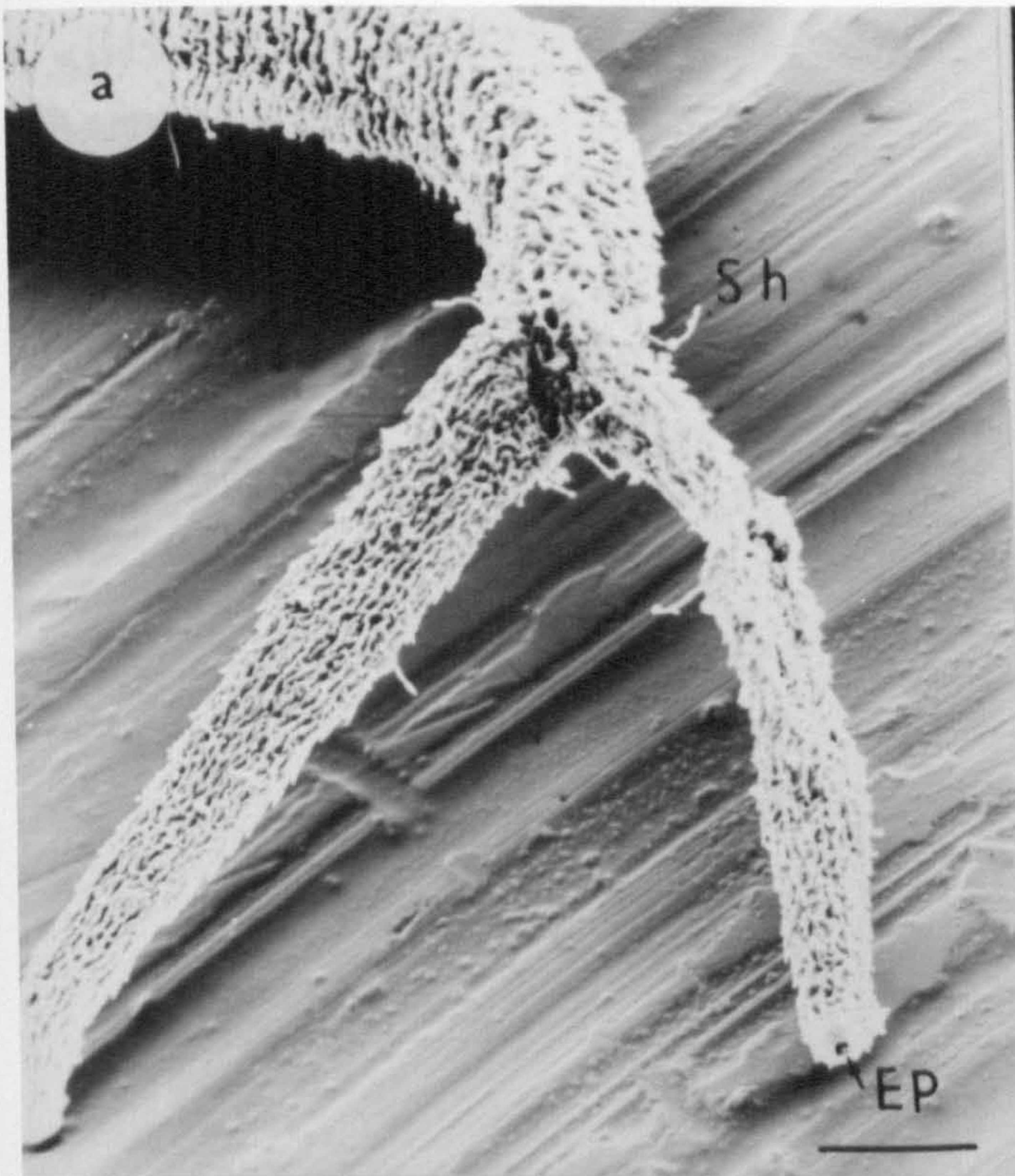




Figure 3.11 TEM micrographs of the cercaria of S.margrebowiei.

- a) TEM of the cercarial tegument (Te). The tegument overlies the extracellular matrix, muscular region consisting of longitudinal (Lm) and circular (Cm) muscles. Note the irregular shaped tegumental cells (Tc), flame cells (Fc) and nucleus (Nu) present in this region. Bar =  $0.93\mu\text{m}$
- b) TEM showing the tegumental cell (Tc) which often have long irregular cytoplasmic process (arrow) and are packed with granules. Bar =  $0.65\mu\text{m}$
- c) Transverse section showing an excretory lumen (L) with its associated long and branching microvilli. Note that glycogen (Gl) is distributed throughout this region. Bar =  $1.56\mu\text{m}$
- d) Transverse section showing a flame cell (Fc). Note the presence of glycogen (Gl). Bar =  $9.9\mu\text{m}$
- e) Anterior region of the cercaria showing a uniciliated pit (Uc) with its associated cilium. Note that microvilli (Mv) are numerous in this region. Bar =  $0.9\mu\text{m}$
- f) High power micrograph of a uniciliated pit. Note The sheathed (Sh) cilium (Ci) emerging from the sensory bulb (S). Bar =  $1.45\mu\text{m}$



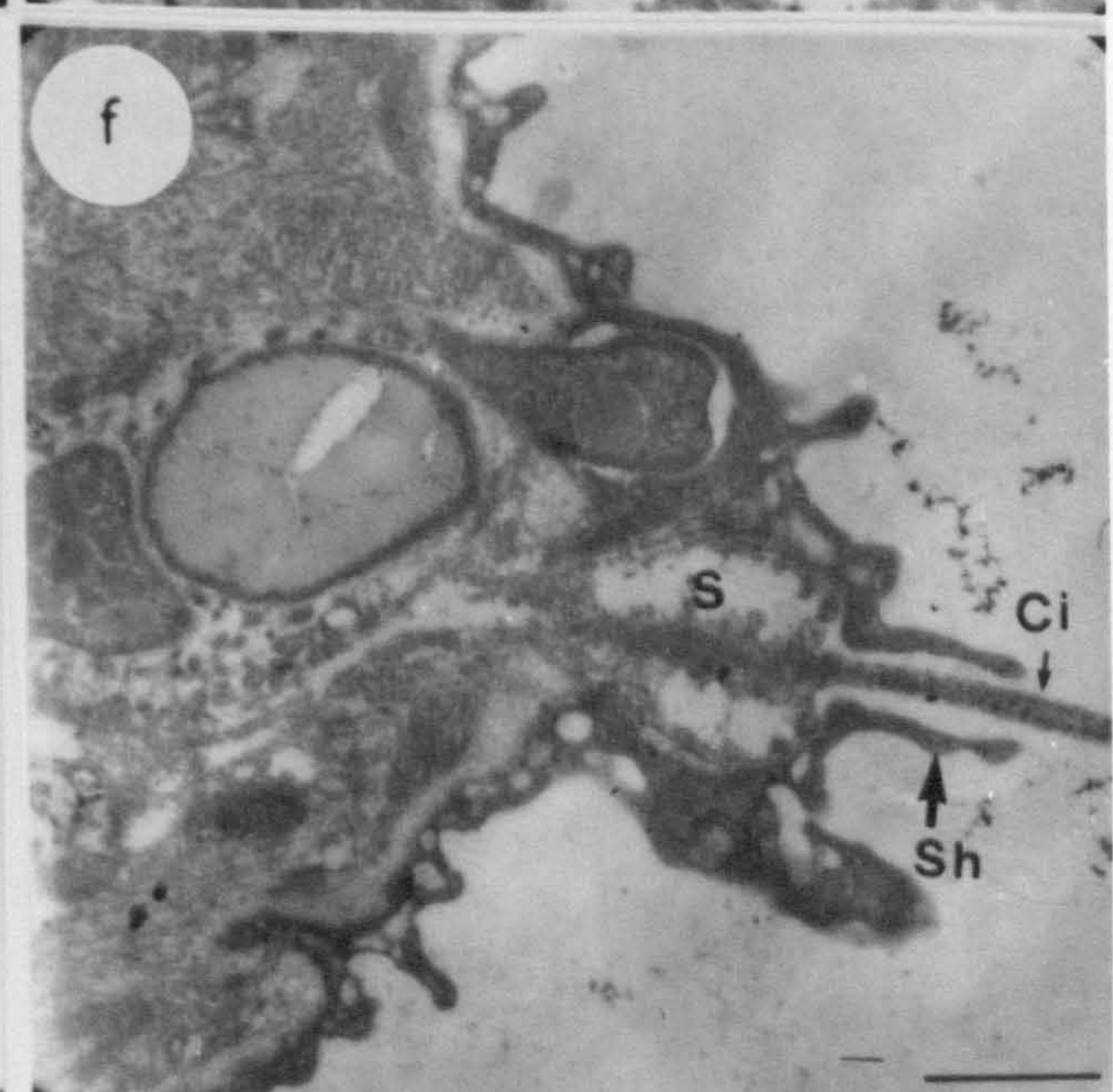
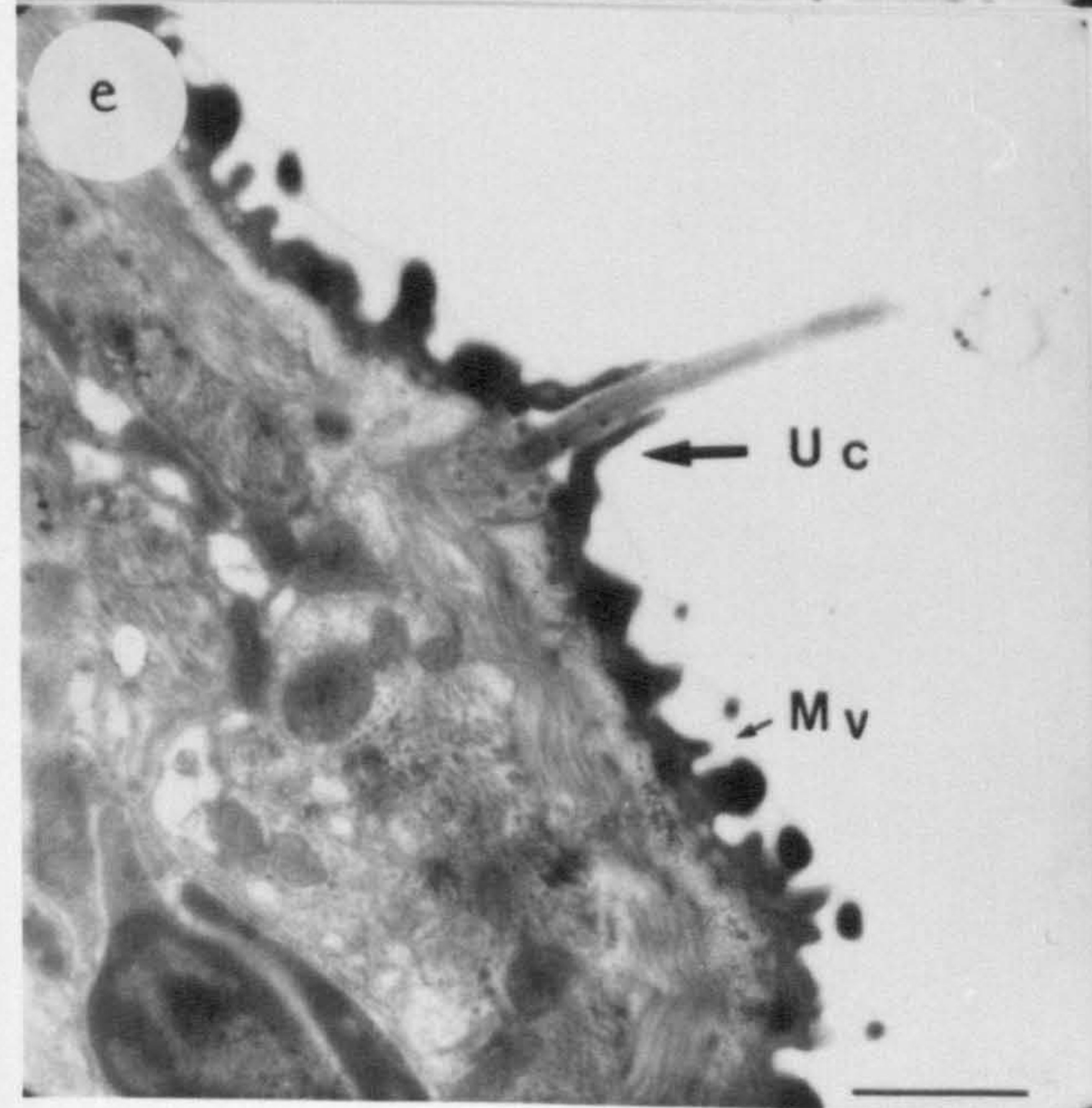
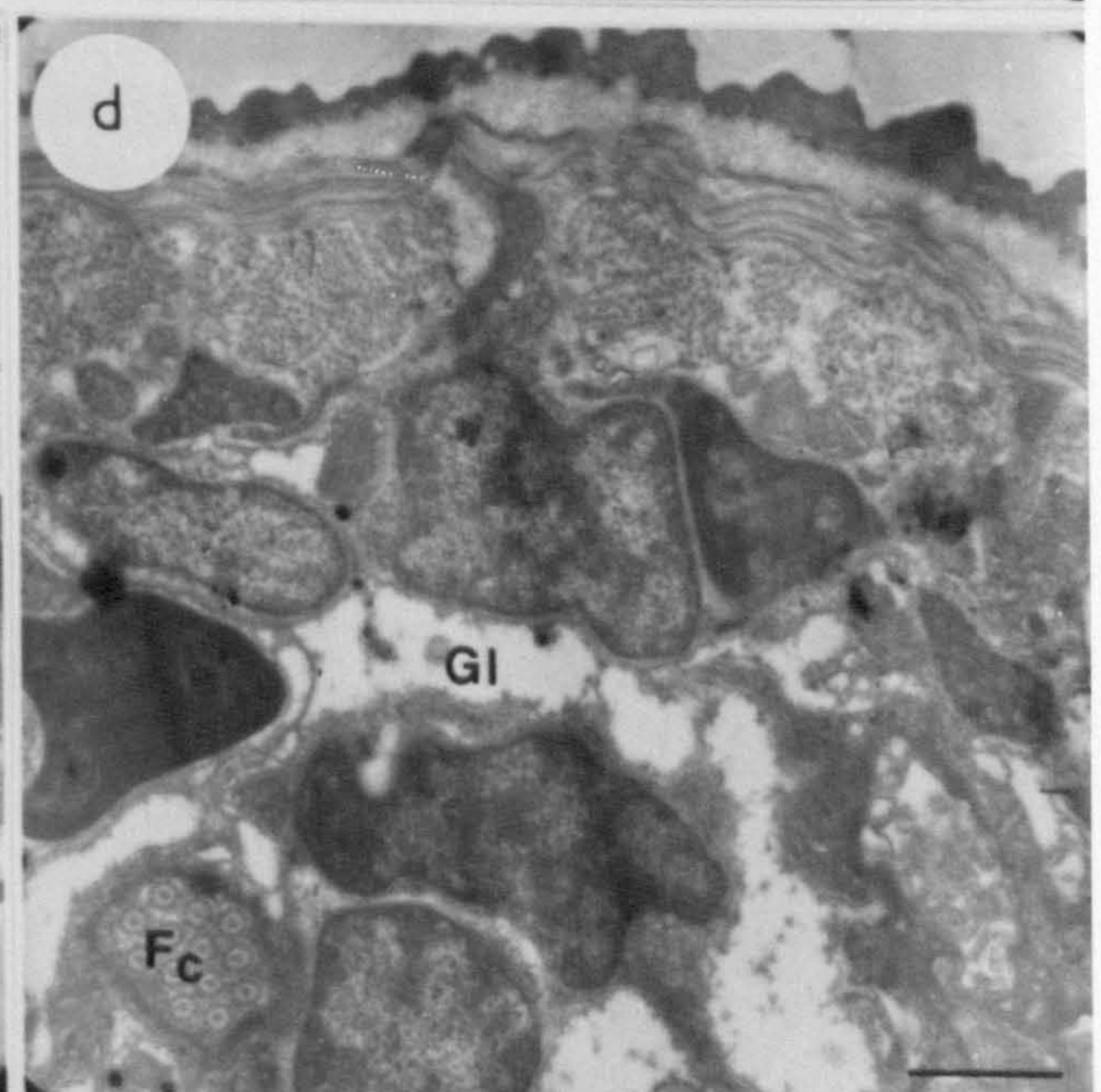
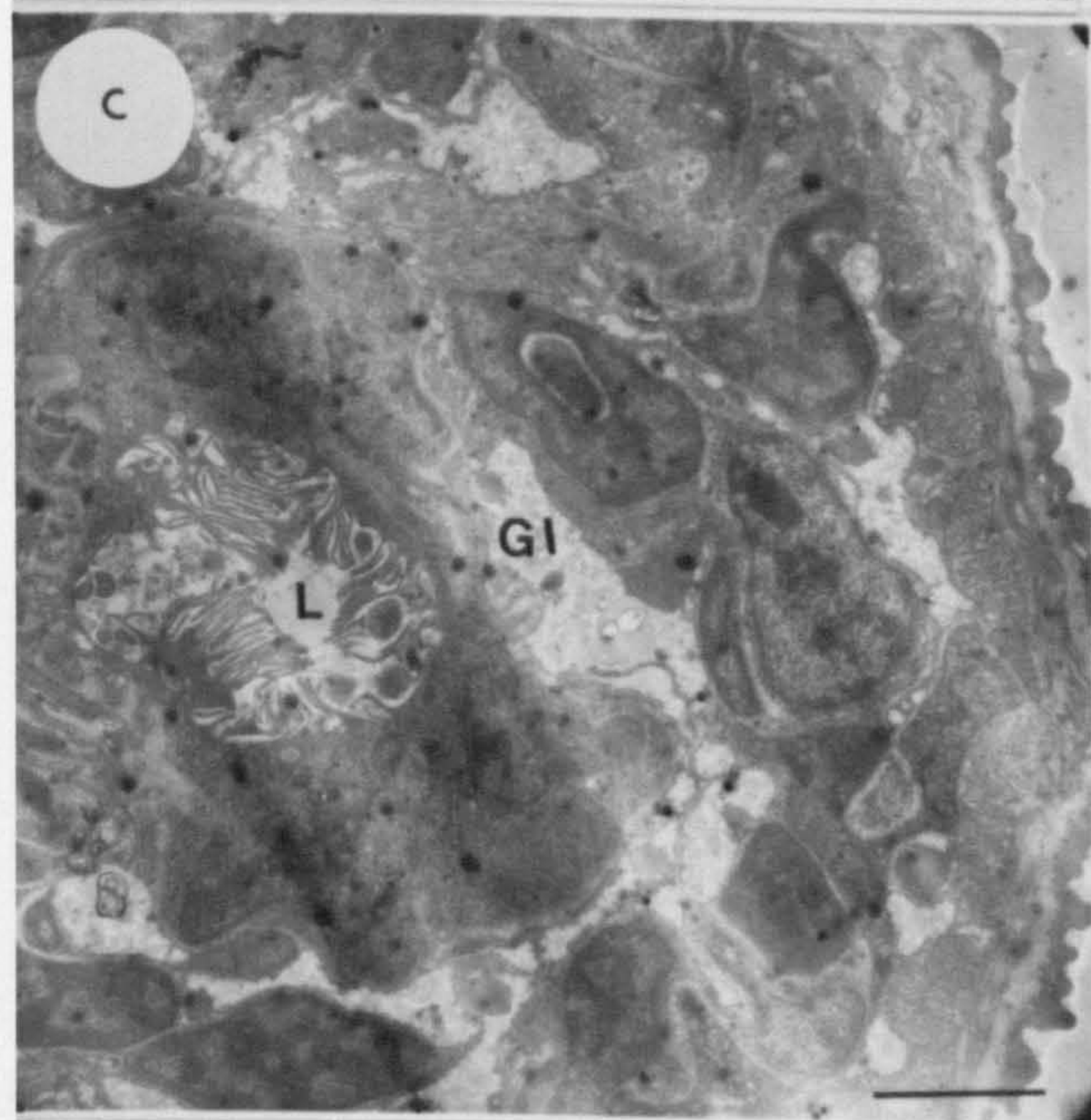
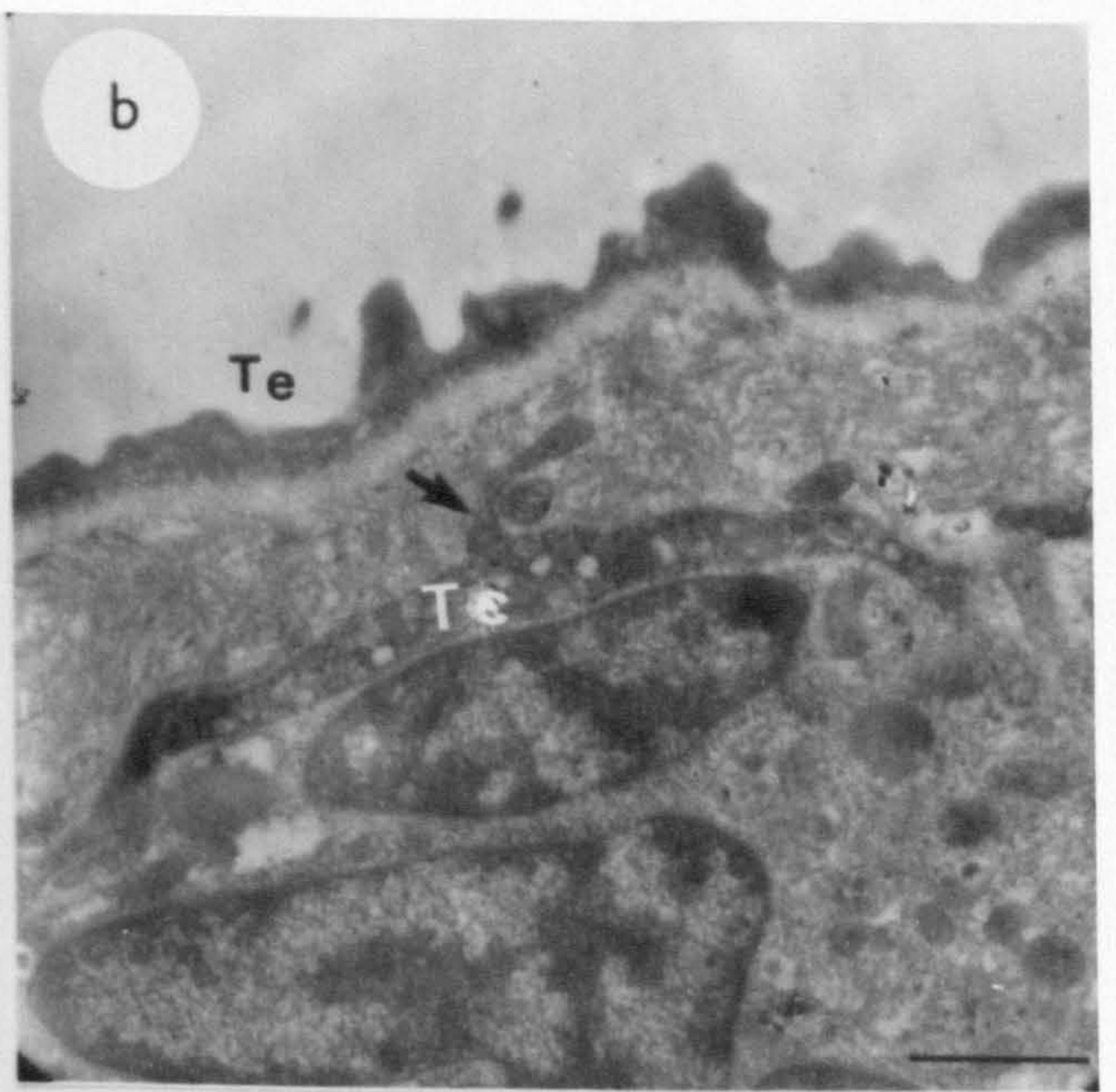
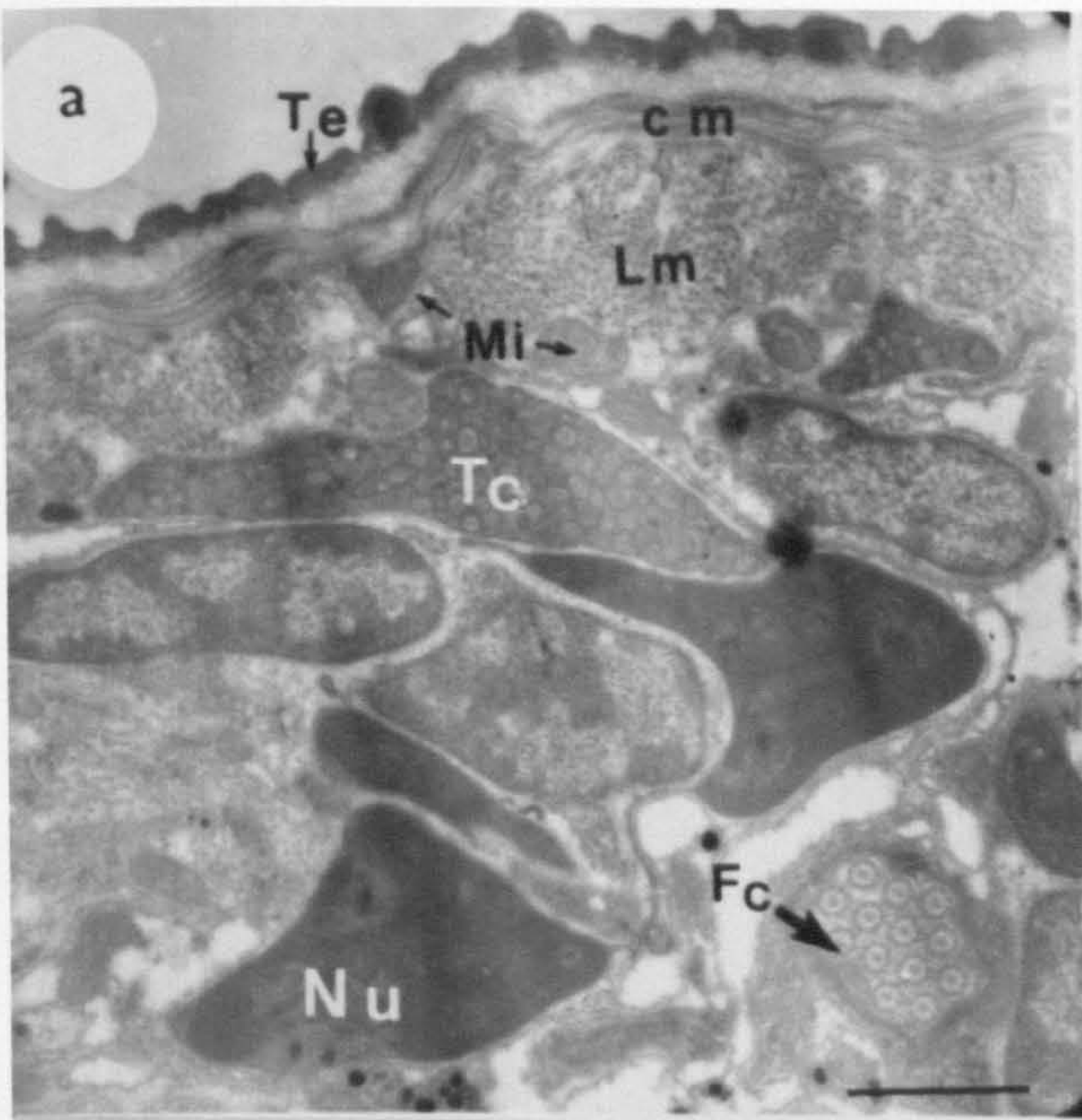




Figure 3.12 TEM micrographs of the cercaria of S.margrebowiei.

- a) TEM micrograph showing the posteriorly pointing spines (Sp), and a thick layer of glycocalyx (G). Glycocalyx is composed of finely branched and anastomosing filaments covering both the syncytium and the spines. Bar =  $0.57\mu\text{m}$
- b) TEM micrograph of the collar region separating the body (B) from the tail stem (T). Note the position of an excretory duct (Ed) and muscle (Mu) layers at this region. Bar =  $3.68\mu\text{m}$
- c) TEM micrograph showing the separation of the body (B) from the tail stem (T). Note that the glycocalyx ~~varies in thickness~~ ~~is~~ ~~of~~ ~~variable~~ ~~thickness~~. Bar =  $3.68\mu\text{m}$
- d) TEM of the tail stem showing that the underlying striated muscle (Mu) is thin and incomplete. Rows of mitochondria (Mi), nucleus (Nu) are also present. Note that vast areas of the tail stem are filled with glycogen (Gl). Bar =  $1.5\mu\text{m}$
- e) TEM micrograph of two furcae showing the excretory duct (Ed). Bar =  $2\mu\text{m}$
- f) Anterior region of the cercaria showing the head gland (Hg) and post-acetabular glands (Po). Note the position of ducts (arrow) through which secretory products are emptied. Bar =  $3.75\mu\text{m}$



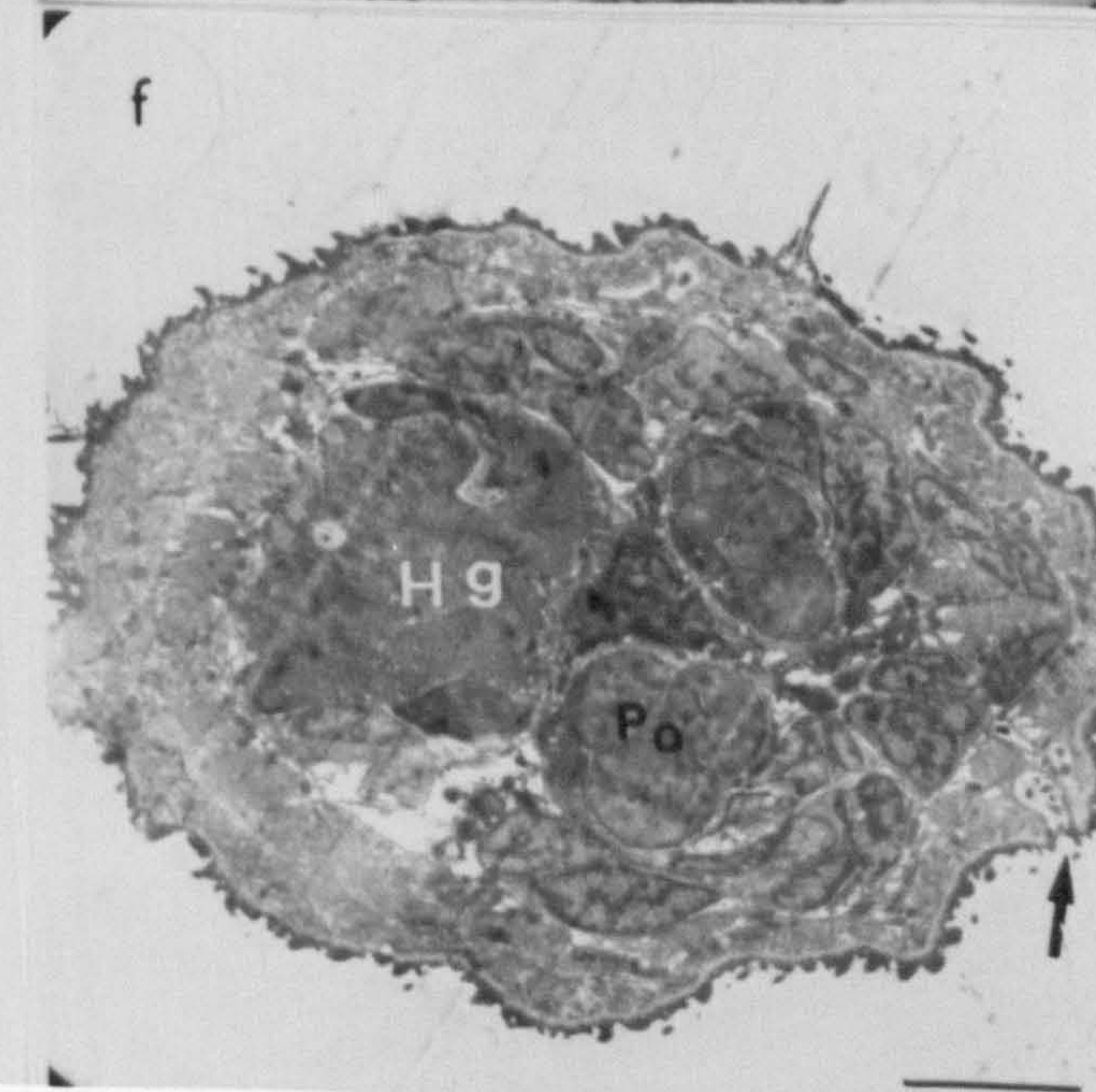
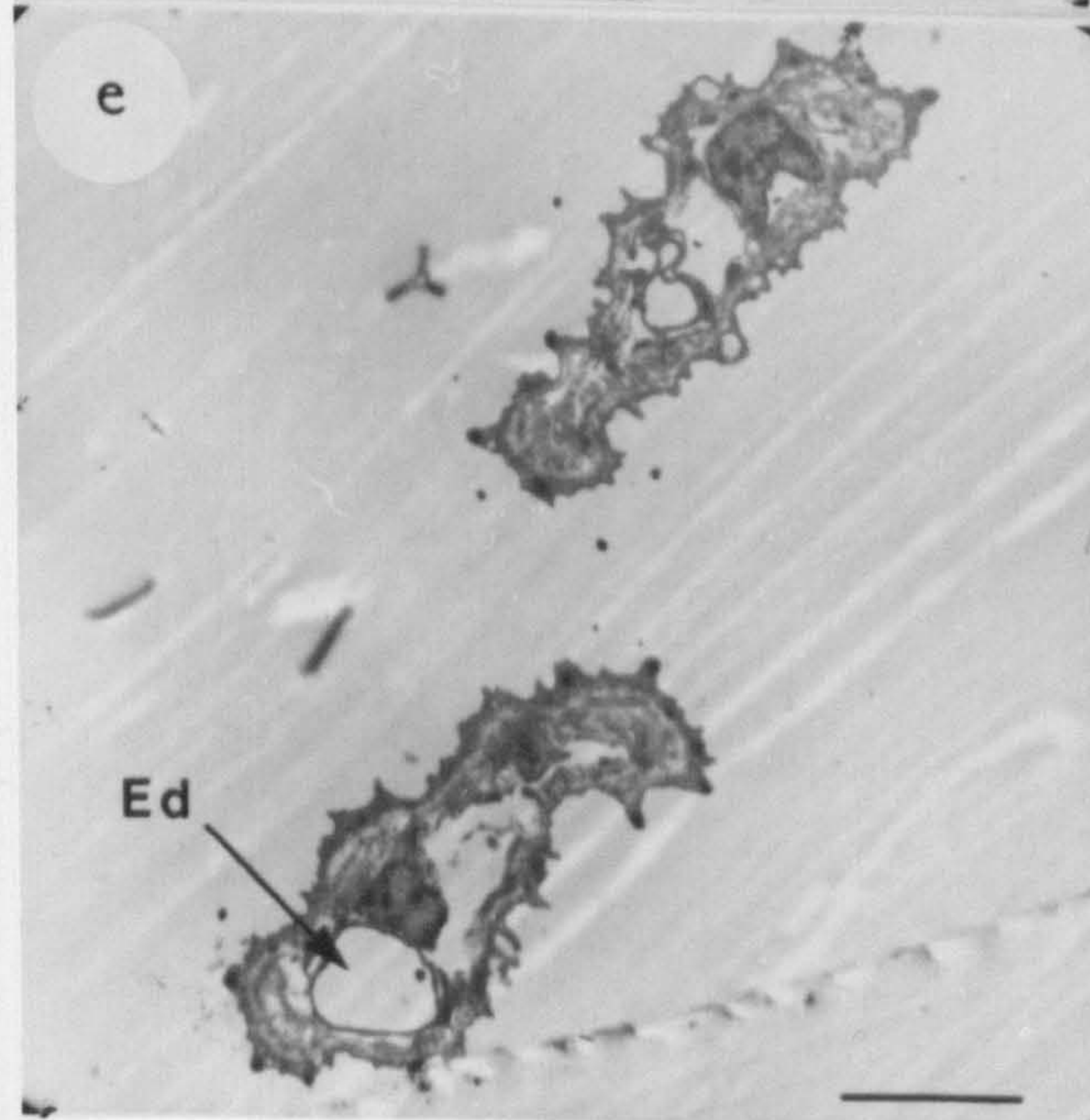
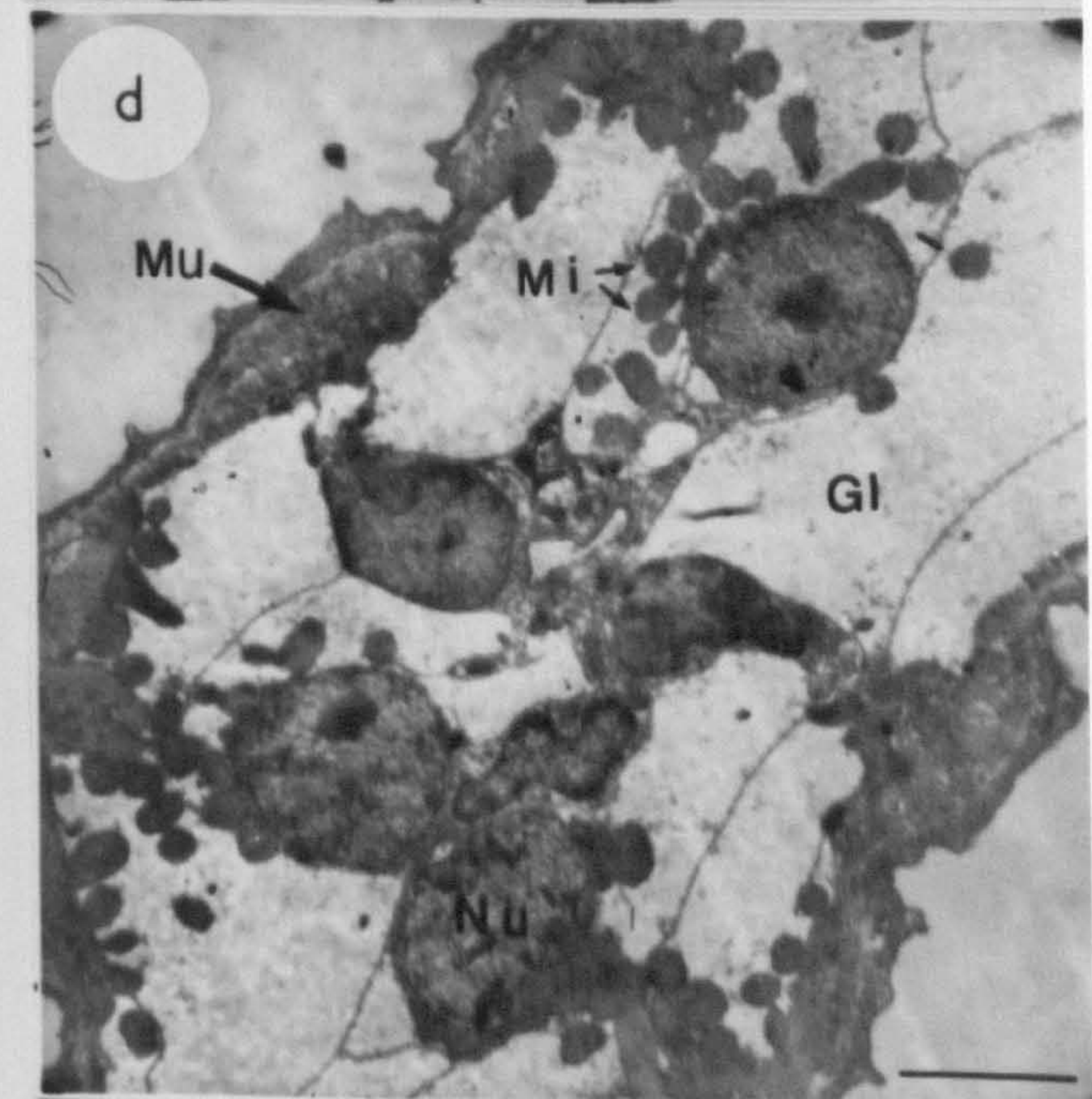
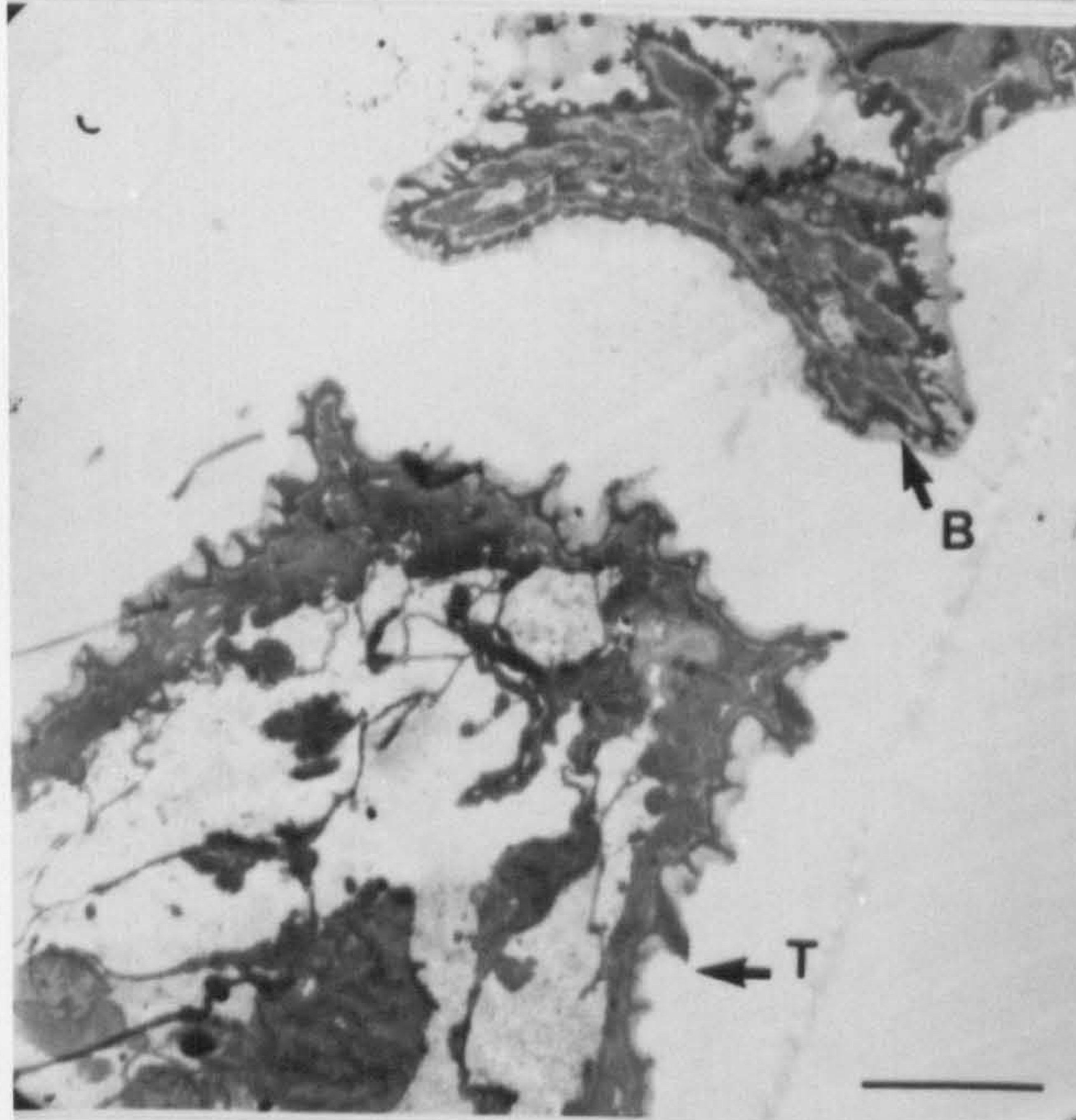
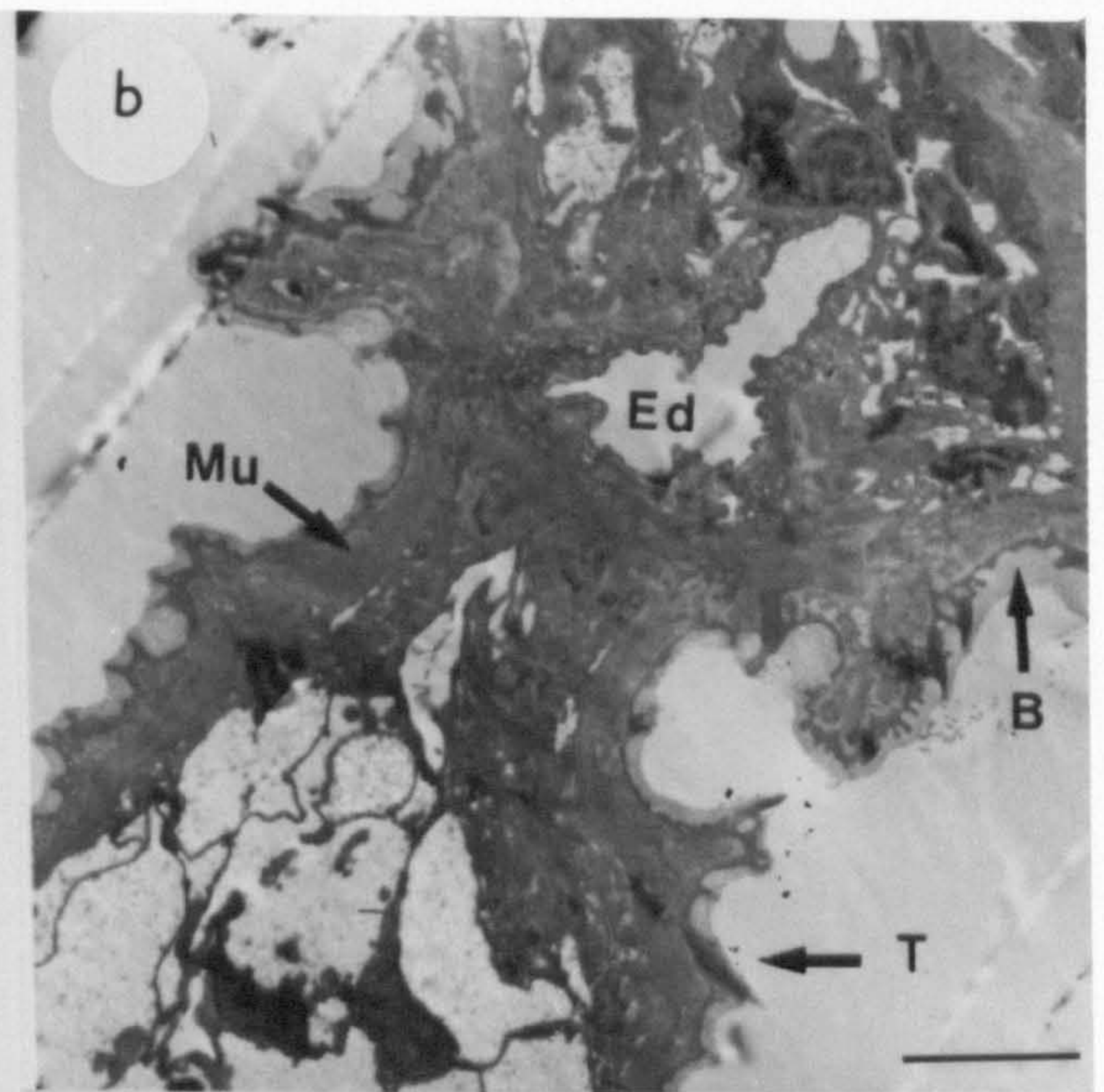
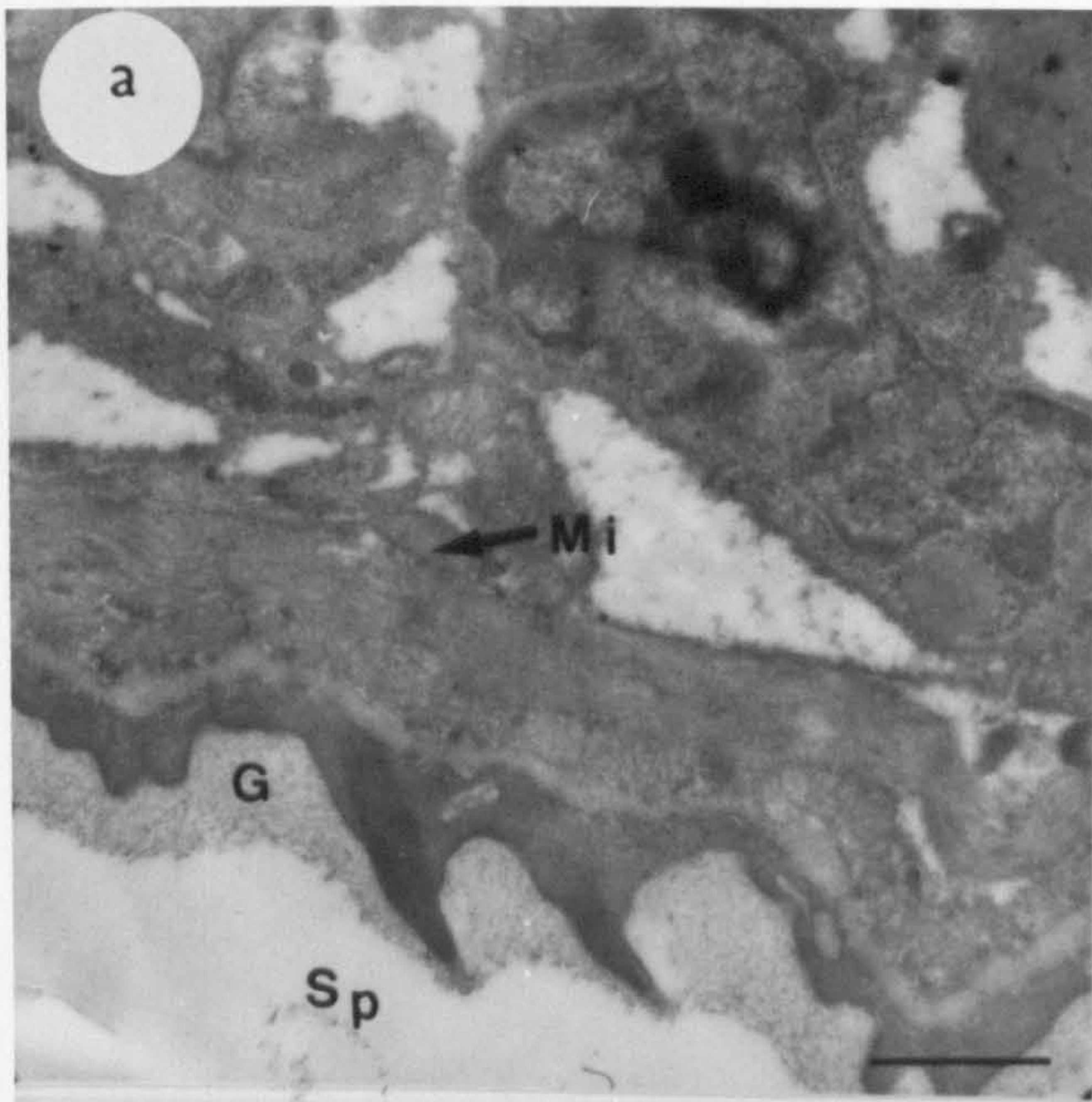




Figure 3.13 TEM micrographs of the cercaria of S.margrebowiei.

a) TEM micrograph of the anterior region of the cercaria showing pre-acetabular gland (Pr) and secretory ducts (d). Nucleus (Nu) is also shown. Bar =  $2.4\mu\text{m}$

b) Transverse section showing the three pairs of post-acetabular (Po) glands. Bar =  $2.3\mu\text{m}$

c) Transverse section showing both the pre-acetabular (Pr) and post-acetabular (Po) glands. The glands are surrounded by microtubules (Mt). Bar =  $0.62\mu\text{m}$

d) Anterior region of the cercaria showing the tegumental folds (Tf). Bar =  $1.5\mu\text{m}$

e) Anterior region showing a papilla (P) and secretory granules (arrow). Bar =  $0.6\mu\text{m}$

f) Secretory granules (arrow) are secreted through ducts which are surrounded by tegumental folds. Bar =  $0.9\mu\text{m}$



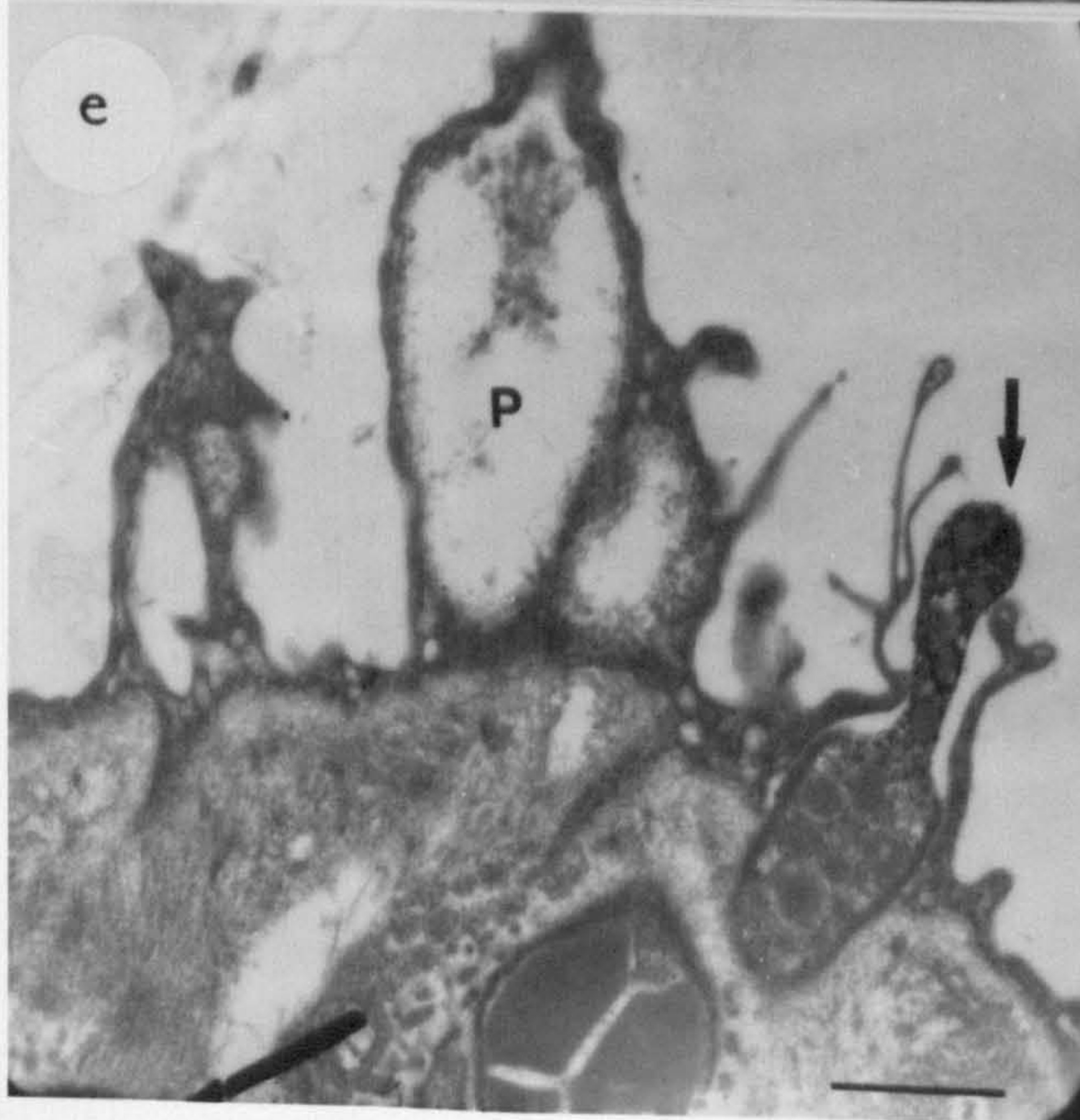
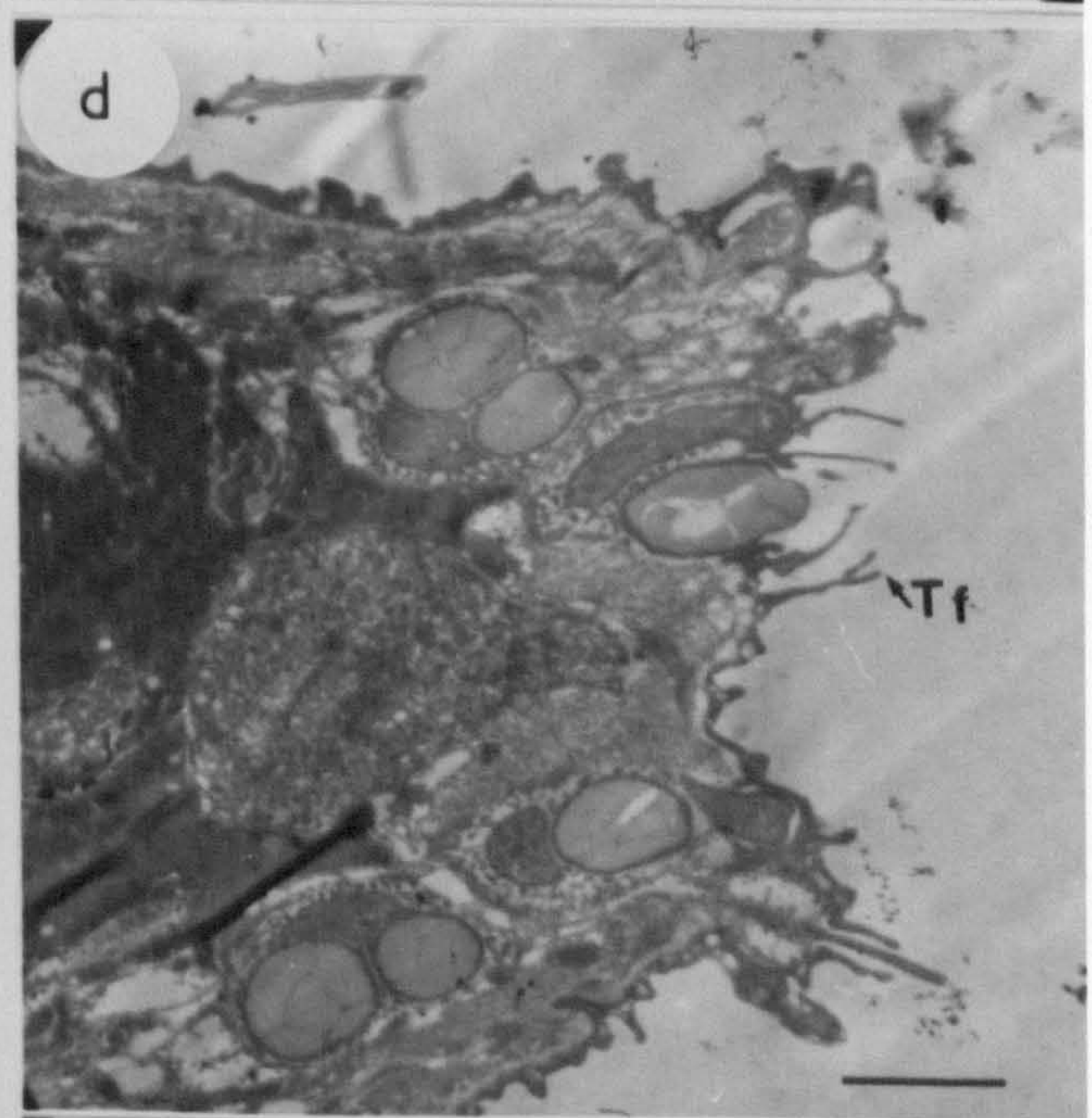
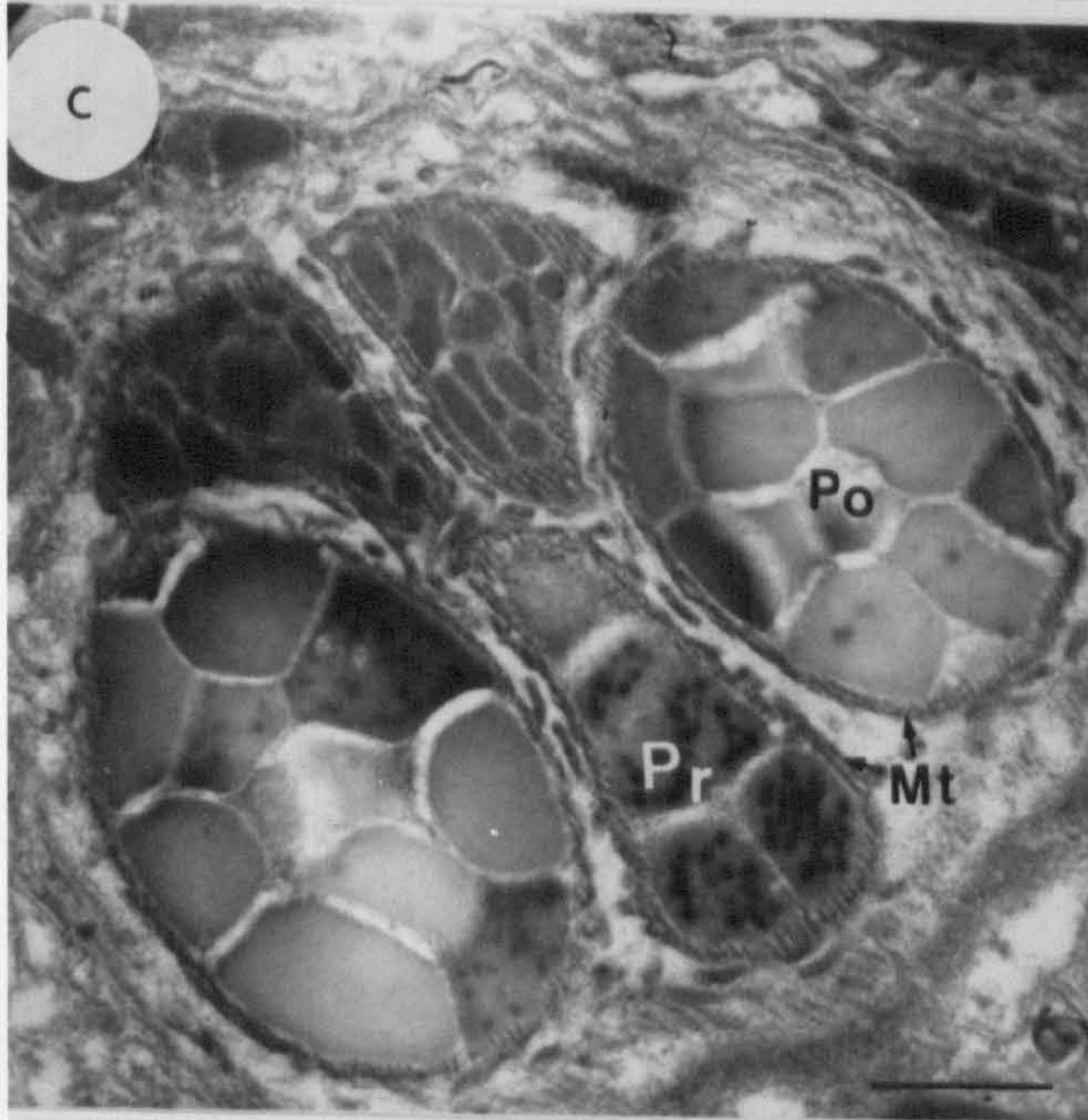
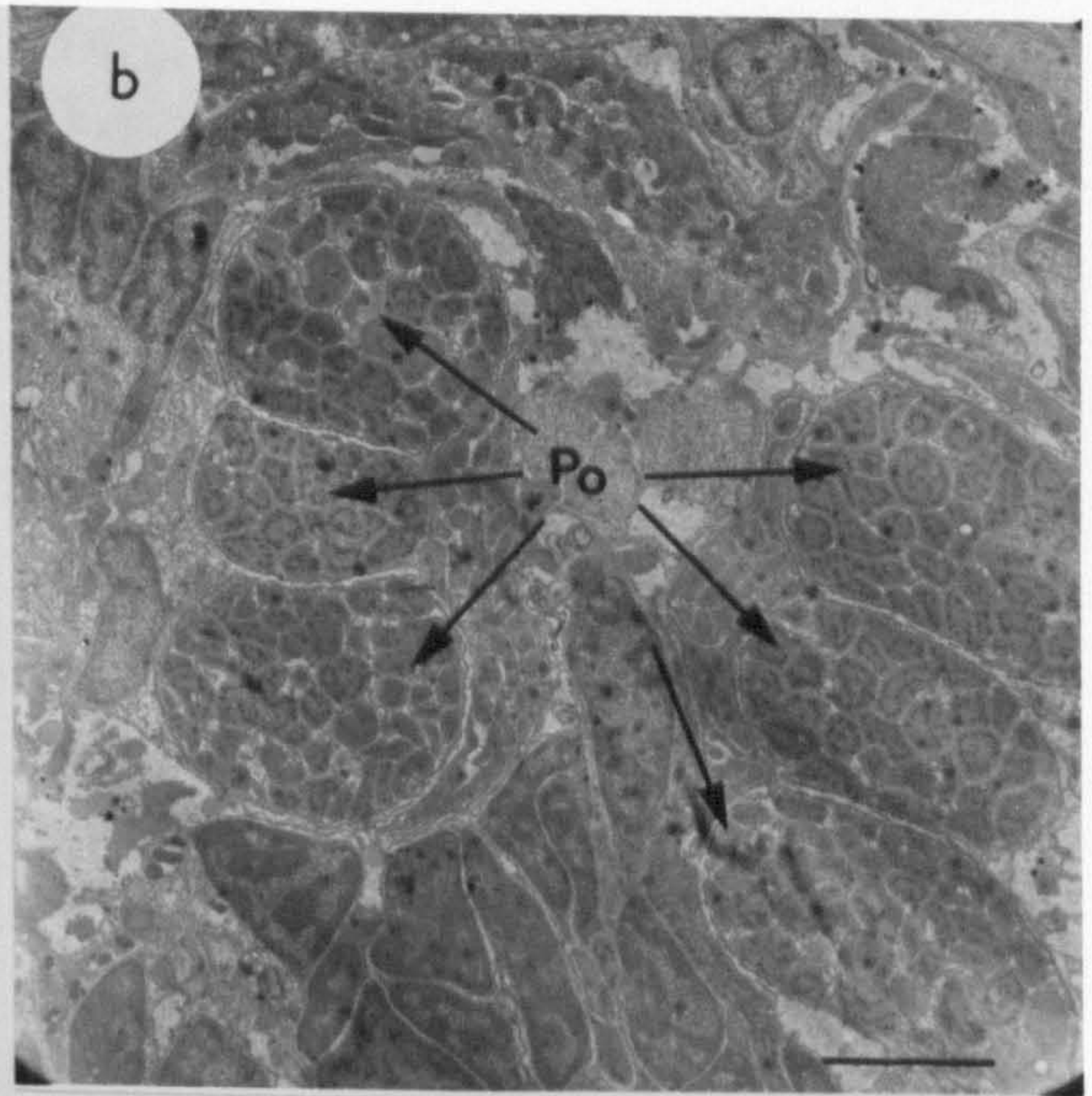
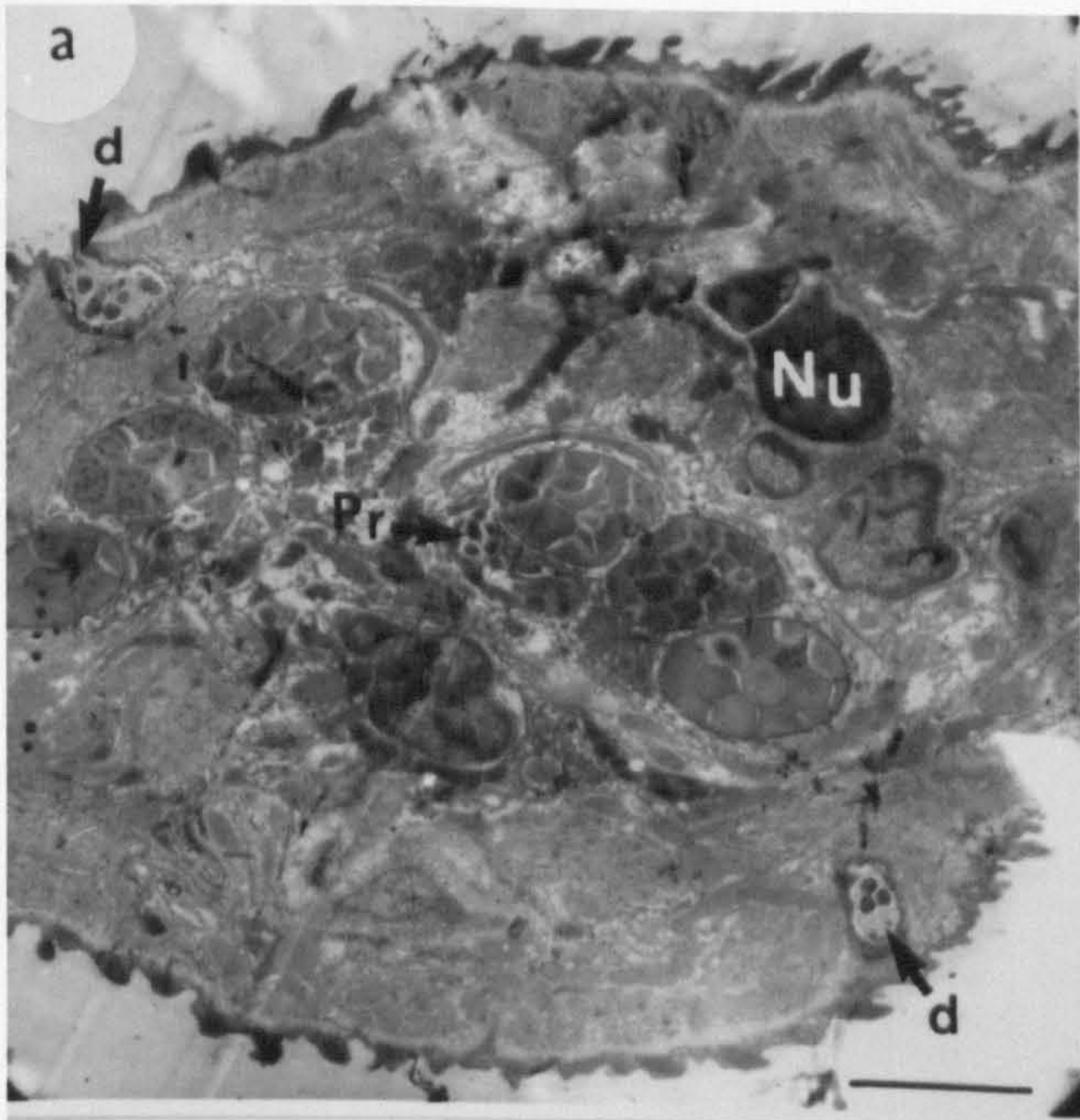




Figure 3.14 Light micrographs of silver nitrate stained cercaria of S.margrebowiei showing the distribution of the papillae on the entire surface of a single cercaria.

a) Ventro-lateral view of the body of the cercaria showing the distribution of papillae in this region. Arrow indicate the position of one such papilla which is seen clearly from the dorso-lateral view (b). Bar = 25.5 $\mu$ m

b) Dorso-lateral view of the same cercaria. Bar = 25.5 $\mu$ m

c) Ventro-lateral view of the tail stem and furcae.

Bar = 27.5 $\mu$ m

d) Dorso-lateral view of the tail stem and furcae. Arrow indicates the position of one papilla on this region.

Bar = 27.5 $\mu$ m



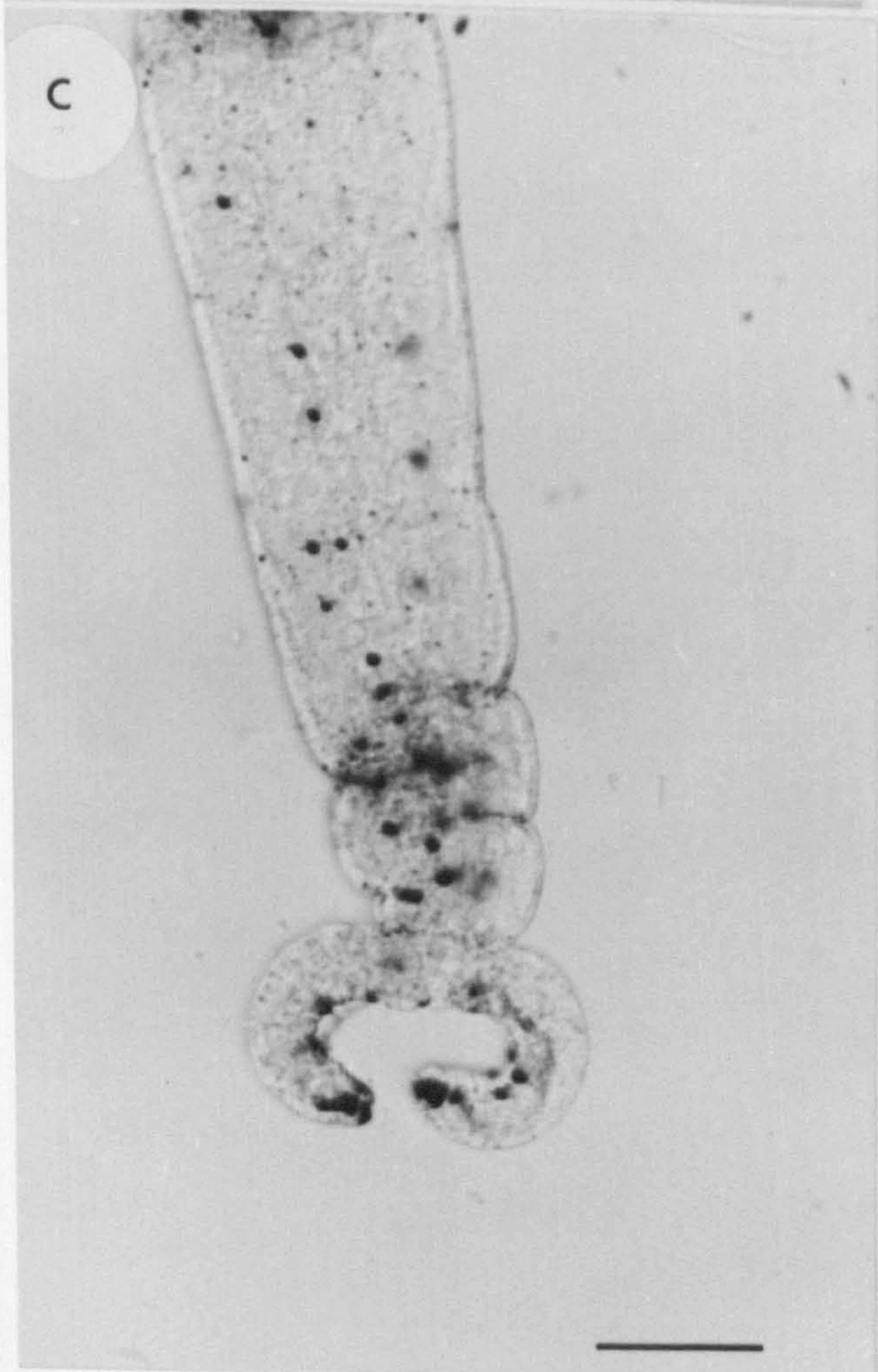
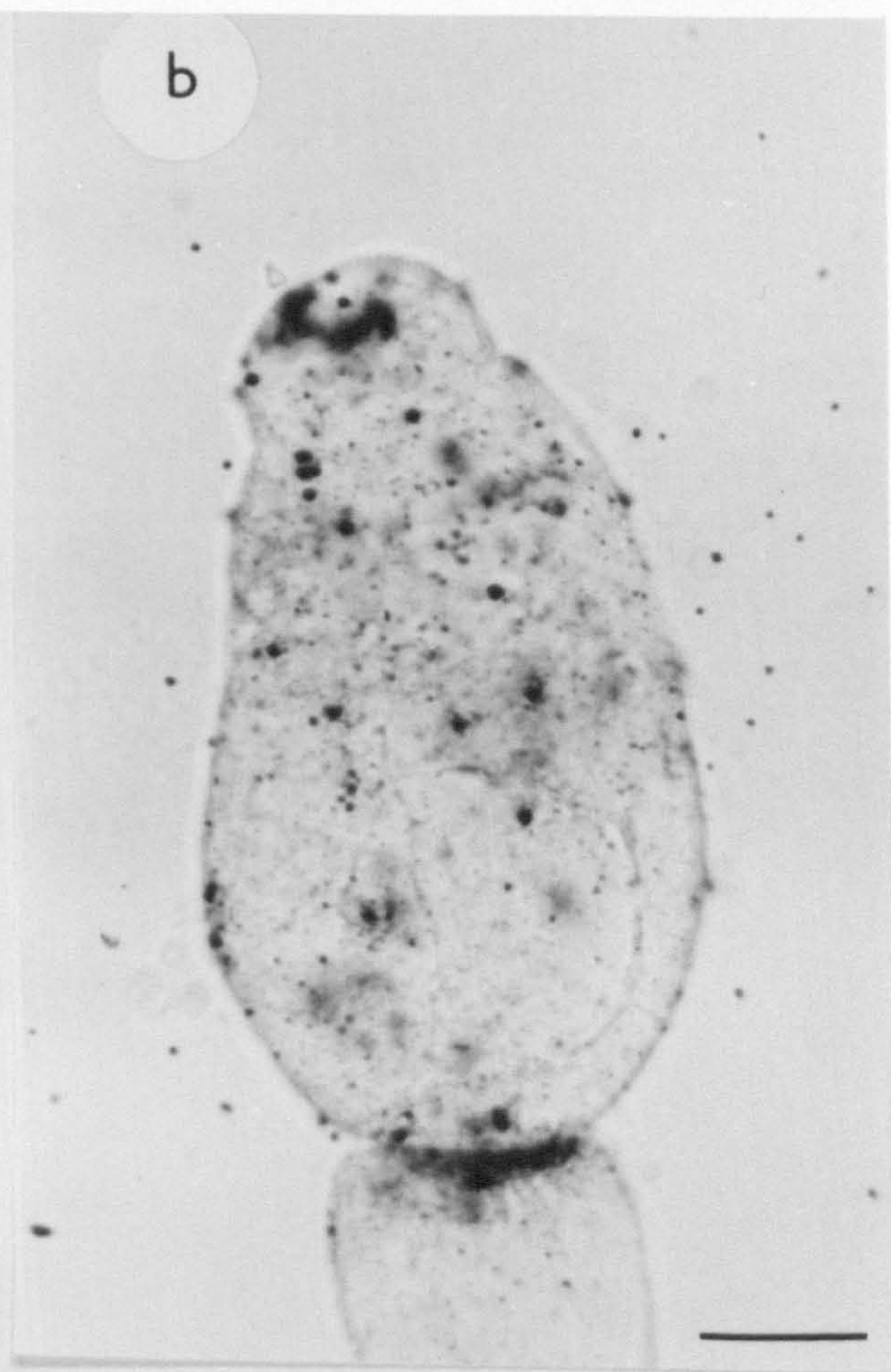




Figure 3.15 Light micrographs of the nuclei in distal end of tail stem of cercariae of S.margrebowiei.

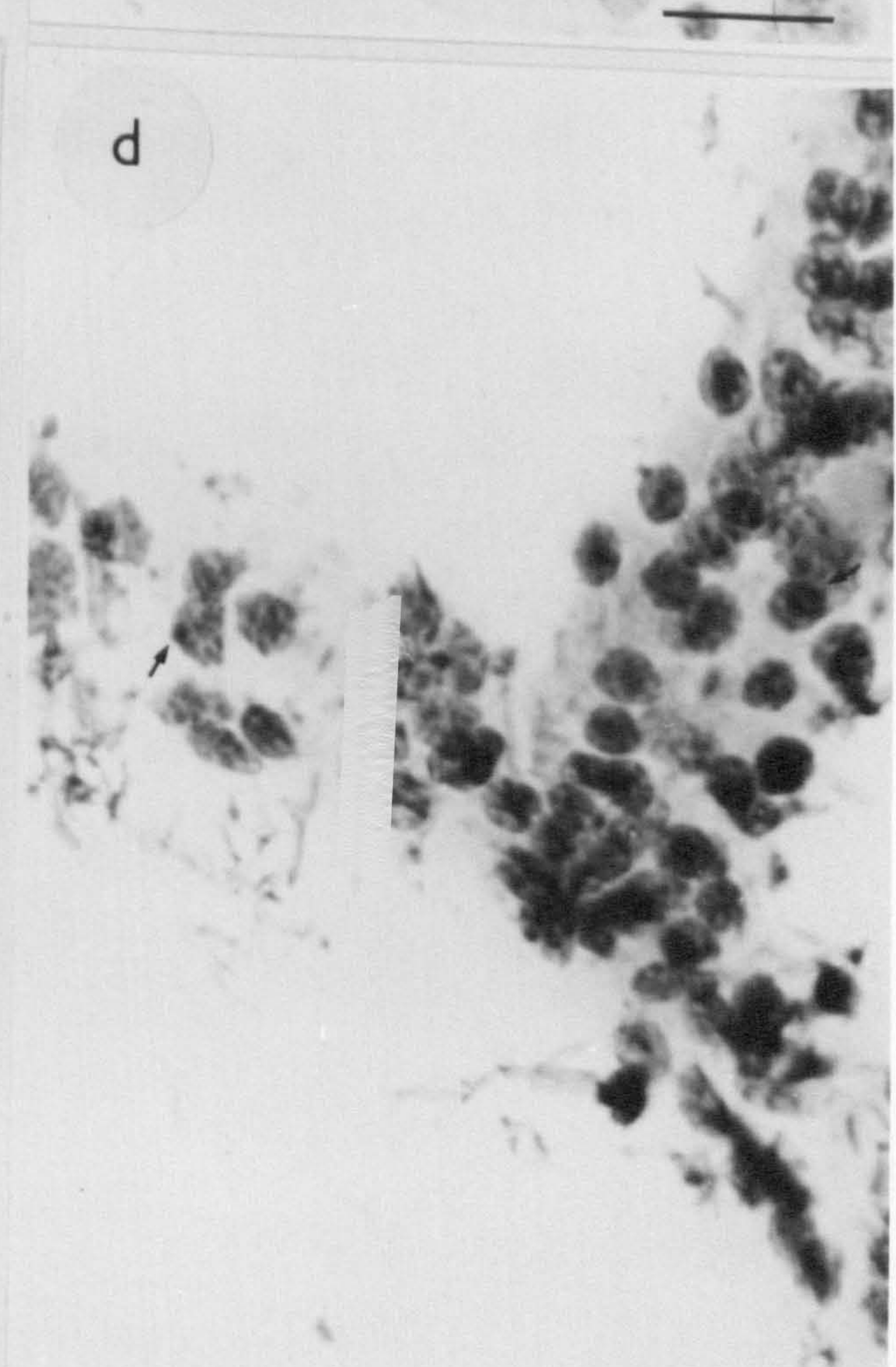
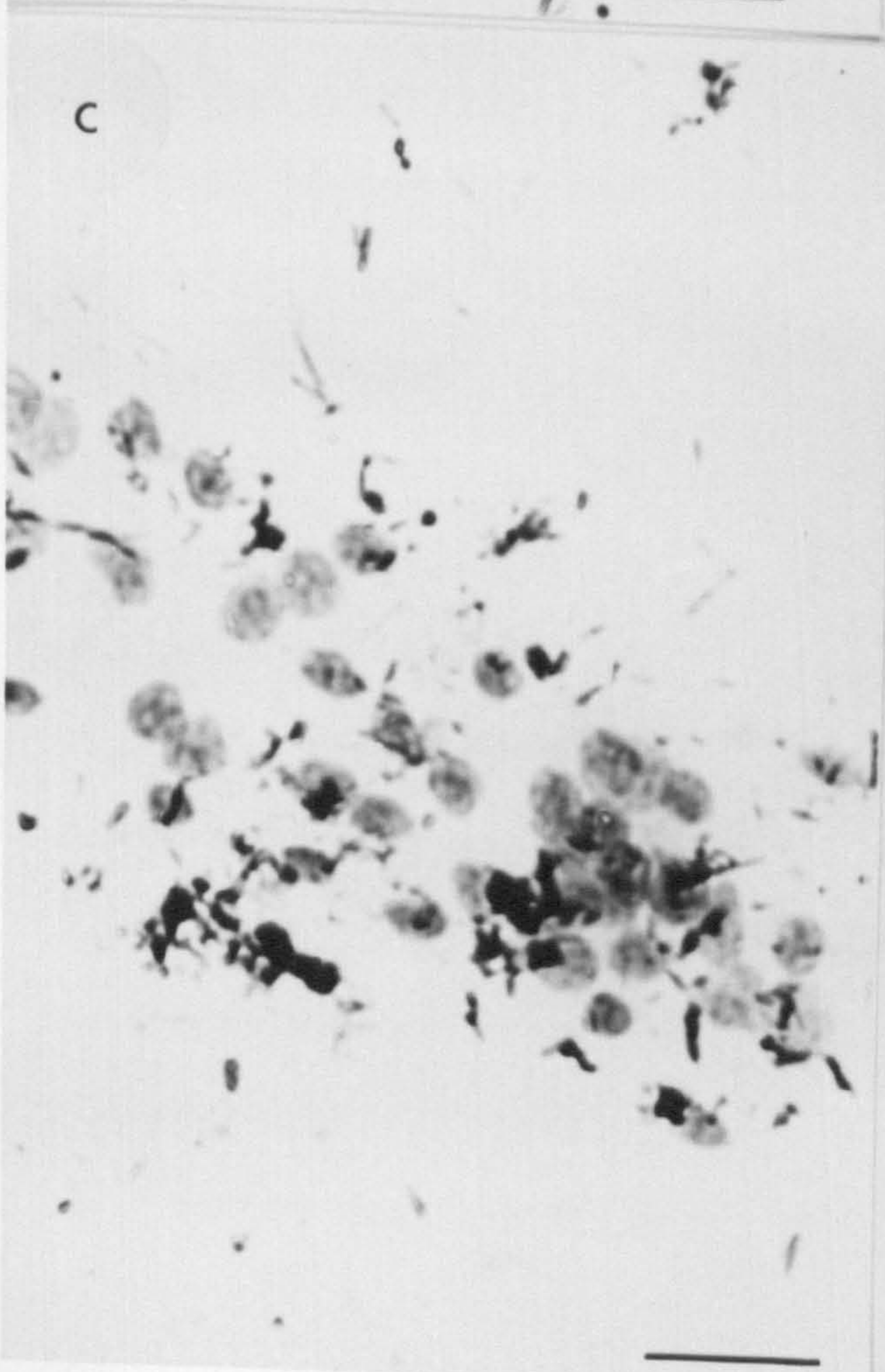
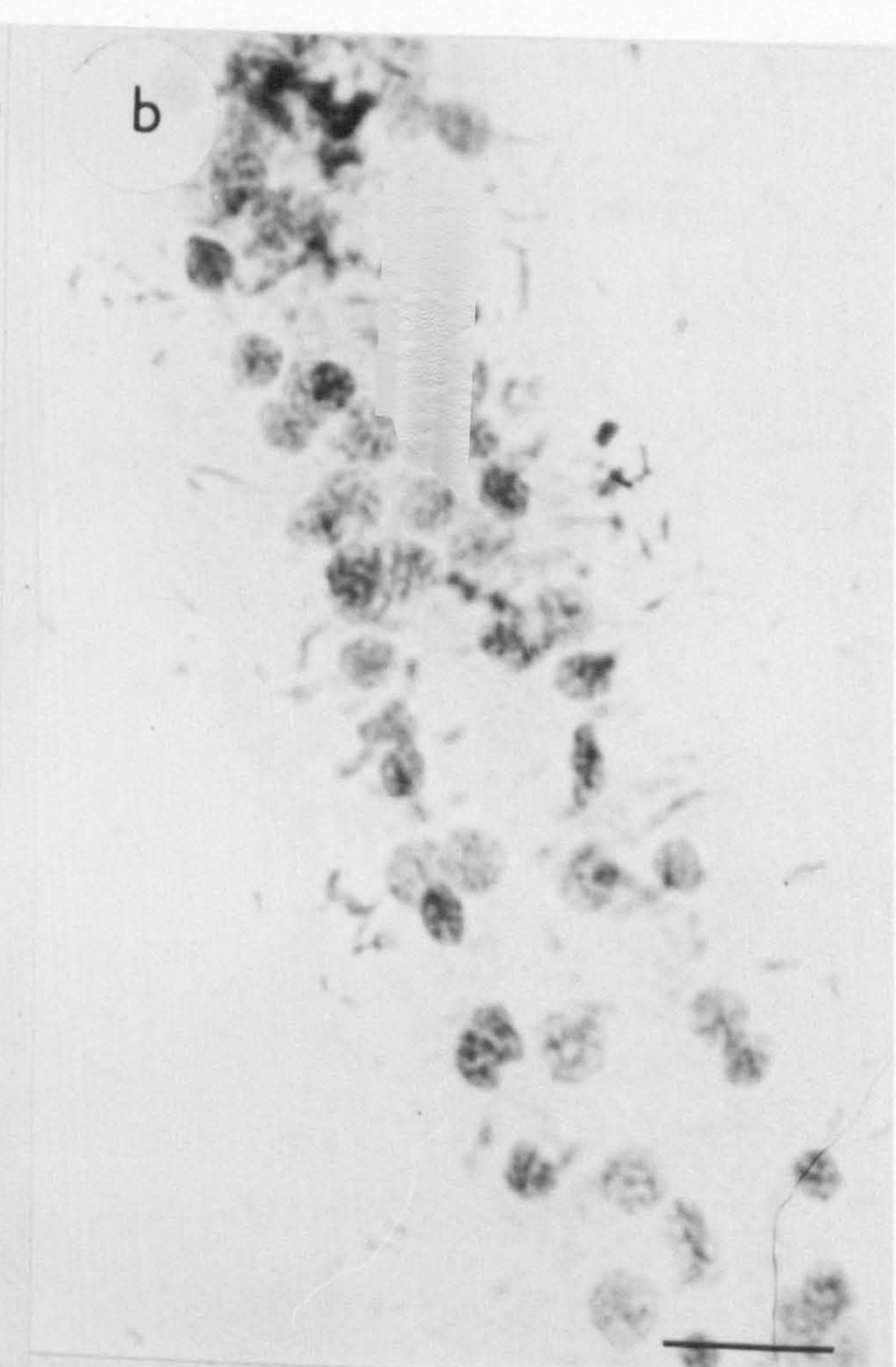
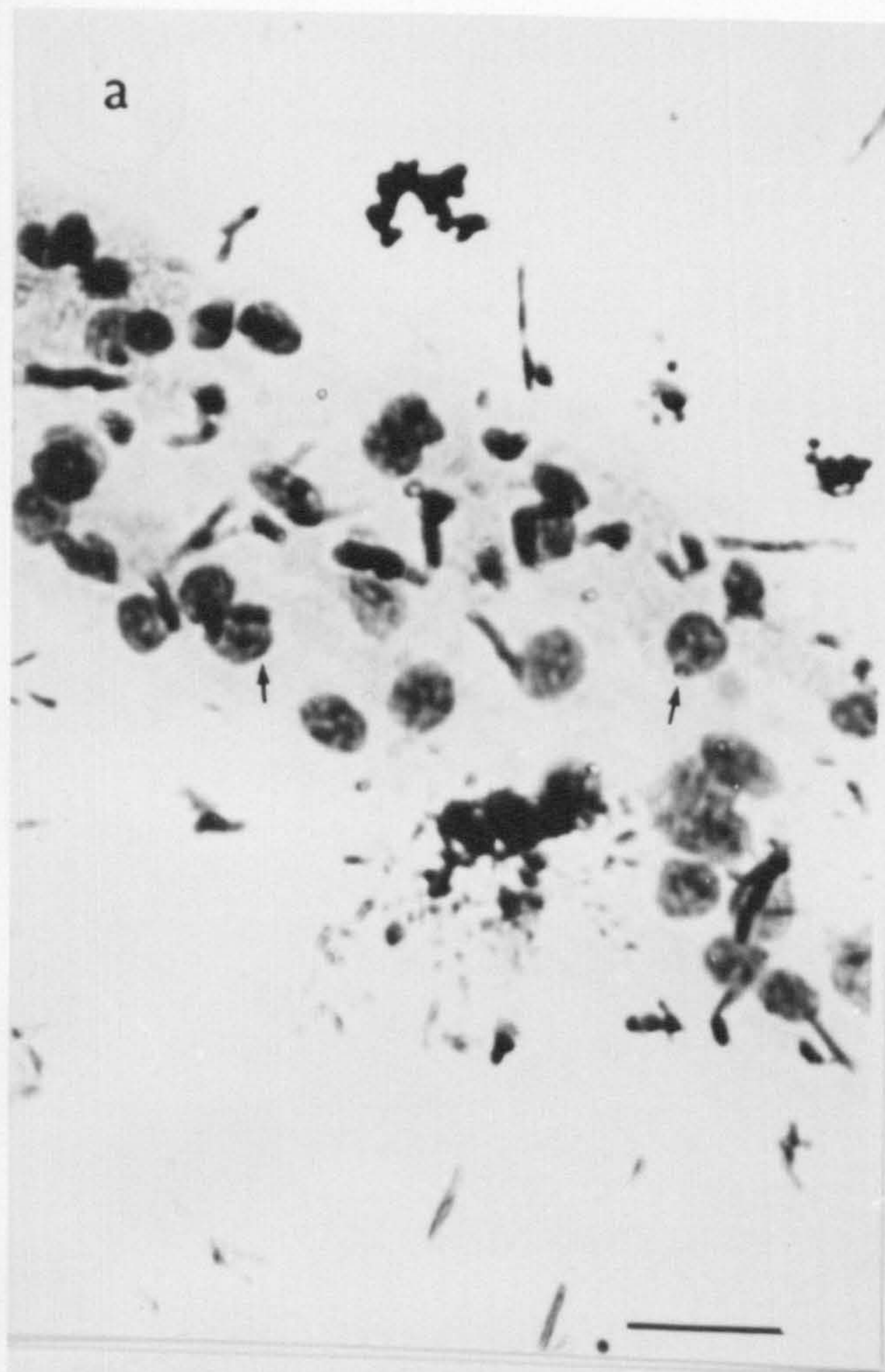
a) Arrows indicate W-chromatin in the W chromosome of the female cercaria. Bar = 9.5 $\mu$ m

b) Nuclei of a male cercaria. Note the apparent absence of W-chromatin and thus W chromosomes. Bar = 9.5 $\mu$

c) Nuclei of a male cercaria. Bar = 9.5 $\mu$ m

d) Arrows indicate W-chromatin of a female cercaria.  
Bar = 9.5 $\mu$ m







## Discussion:

The rapid rate of growth of S.margrebowiei in BKTO mice, confirms earlier findings by other workers that S.margrebowiei develops more rapidly than most other species of schistosomes. The cross over point of 28 days post-infection reported in the present study (Fig. 3.1), compares favourably with that reported by Southgate and Knowles (1977) and Ogbe (1985) of 28, 28 and 31 days in hamsters, gerbils and mice respectively. The cross over point for S.haematobium is reported to be 62 days post-infection, Southgate et al., (1985).

The mature adult worms are also among the largest in the genus. By 65 days post-infection the slender female measures 29.7mm  $\pm$  3.36 and the male 19mm  $\pm$  3.65 in length. Although these figures are in agreement with those of Southgate and Knowles (1977) and Ogbe (1985), (in the case of Ogbe 1985, the text is correct but Fig.3 is incorrectly labelled), they are larger than Le Roux's (1933) original description. This can be explained by Le Roux's own comment:"It cannot be over-emphasized that the various measurements of all trematodes, but more especially of the genus Bilharzia, are subject to enormous variations brought about by methods of collection, mode of preservation and the state of putrefaction of the viscera of the host at the time of collection". In addition to these factors, the sex and species of the definitive host, duration and intensity of infection should also be included. The present SEM observations (Fig. 3.2), confirm the findings of Probert and Awad (1987) regarding the presence of spines

on some tubercles of the mature male worm recovered from hamsters. Male worms recovered from infected mice also had spiny tubercles. This confirms the view of these workers that both hamsters and mice are permissive hosts for S.margrebowiei. The spined tubercles are thought to enable male worms to attach themselves to the endothelial lining of the blood vessels, hence sustaining their position (Probert and Awad, 1987). In a similar fashion to S.haematobium and S.japonicum, mature, fertilized female S.margrebowiei worms, lay eggs in aggregates (unlike S.mansonii). These small, (80 X 64 $\mu$ m) terminally spined, eggs are similar to those described by Southgate and Knowles, (1977). However, whereas Southgate and Knowles (1977) recorded spines as 0.25-0.6 $\mu$ m in length, the present observations found them to be longer, measuring 1.6 $\mu$ m  $\pm$  0.6 (Fig. 3.3). This should enable one to clearly distinguish them from S.japonicum eggs which, although similar in size (81 x 63 $\mu$ m), have a small lateral spine.

Previous electron microscopy studies of the fine structure of S.mansonii eggs has revealed the presence of two features on the eggshell 1) micropores, (Stenger, et al., 1967); Race et al., (1968, 1969) and 2) microspines. Stjernholm and Warren (1974) demonstrated the possibility of the uptake of host metabolites via these micropores while Hockley (1968), considered the role of the microspines was for the abrasion of the host tissue, increase in surface area and possible secretion of enzymes. Present observations, demonstrate the presence of microspines on the eggs of S.margrebowiei.



When S.margrebowiei eggs are placed in copper-free water, the mature miracidium is seen to swell and become hyper-active. The egg shell ruptures at variable sites allowing the ciliated miracidium to escape. Miracidia (measuring 120 by 49 $\mu$ m) swim actively using their cilia. The present SEM and TEM studies indicate that the basic surface feature and ultrastructure of the miracidium of S.margrebowiei, is similar to those of other species of the genus (as described by many authors, see Introduction). These features include, 1) apical papilla: a dome-shaped structure with a "rosette" pattern consisting of anastomosing microfolds which are thought to act as small suckers holding the miracidia in position when entering a snail (Wright, 1971, and LoVerde, 1975). 2) A lateral papilla on each side of the body, which contains a large number of nerve endings thus, suggesting a possible sensory role. However, Rees (1940) studying the miracidia of Parorchis acanthus, reported that the lateral papillae were capable of considerable extension and suggested that they could play a major part in the emergence of the miracidium from the egg. 3) Uniciliated and multiciliated sensory pits.

The tegument of the miracidium consists of ciliated plates covered by long cilia and shorter microvilli while the sub-tegumental region is packed with circular and longitudinal muscle layers. These muscles are particularly important in the penetration process. A row of mitochondria provide the necessary energy for the ciliary and muscular movements of the miracidium. The excretory system consists of

two anterior and two posterior flame cells. The excretory ducts from these flame cells, fuse and pass to two excretory pores situated on the third tier. The secretory glands, which consist of two lateral penetration glands, are situated above the neuron mass (Figs. 3.4, 3.5 and 3.6). Wajdi (1966) postulated that the content of these glands not only help the miracidia to adhere to the snail, but also serve as a lubricant in assisting their entry.

According to Eklun-Natey et al., (1985), who studied miracidia of S.haematobium, S.japonicum, S.intercalatum and S.mansonii, the four species could be differentiated by the following characteristics: 1) the dimensions of the miracidia, 2) the shape of the epidermal plates, 3) the configuration of the apex of the apical papillae, 4) the position of the excretory pores and 5) the number and distribution of the sensory receptors. However, we believe that the dimensions should only be used if a standard preparation procedure is followed, since considerable variation in size occurs otherwise. Unfortunately, direct comparisons of the size recorded by other workers are somewhat limited due to the variation in techniques used. These include measurements of live and fixed (in different fixatives) specimens. The dimensions reported in the present study are based on stained specimens. The use of fixatives, coupled with the preparation processes used for SEM studies, leads to 25-35% shrinkage, as seen in this study and reported elsewhere (Southgate and Knowles, 1977).

Results from the present study show that the miracidia of S.margrebowiei, have four tiers of epidermal cells with



6,9,4, and 3 plates giving a total of 22 ciliated epidermal plates. This is in agreement with the findings of Southgate and Knowles (1977) and are similar to those for S.haematobium (Capron et al., 1965) and S.mansoni (Schutte, 1974). The number of tiers is thought to be similar among trematode species within a particular family (Eklun-Natey et al., 1985). However, the number of plates inside a particular tier, shows interspecific variation. With the exception of S.rodhaini, which has 5 plates in the first tier (Fripp, 1967), all species of Schistosoma have 6 plates in the first tier. The number of plates in the third and fourth tier in all species is constant, 4 and 3 respectively. So interspecific variations are mainly restricted to the number of plates in the second tier (being either 8 or 9). S.intercalatum (Wright et al., 1972) and S.bovis (Lengy, 1962) which have 8 plates while S.margrebowiei, S.mansoni, and S.haematobium have 9. The actual shape of the plates in S.margrebowiei resembles that of S.mansoni, S.haematobium and S.intercalatum in that the shape of the plates of the first and fourth tier are triangular, whereas those of the second and third tier are oblong (Fig. 3.7). The "rosette" pattern observed in the en face view of the miracidium of S.margrebowiei (Fig. 3.4b), is also similar to that of S.haematobium and S.intercalatum, but differs from the "honeycomb" pattern seen in S.mansoni and S.japonicum (Eklun-Natey et al., 1985). The excretory pores, in common with most species of the genus, are located on the third tier. Sensory receptors were clearly visible on the anterior and median rings. Thirteen receptors were recorded

on the anterior, and 20 on the median rings (Fig. 3.7), which is in agreement with the descriptions of Southgate and Knowles (1977). The number of these receptors (13, 20), resemble those of most other species of schistosomes apart from S.japonicum which lacks these receptors on its median ring. Although it is possible to confuse the eggs of S.japonicum and S.margrebowiei, the miracidium of S.japonicum can easily be distinguished from S.margrebowiei by the absence of sensory receptors on its median rings and the "honey-comb" pattern of its apical papillae.

The miracidium of S.margrebowiei has only a few hours to find and penetrate a suitable, susceptible snail. In the case of susceptible snails they fail to recognise the miracidia as non-self and infection is readily achieved. Although no in vivo studies of the transformation of the miracidia of S.margrebowiei into sporocysts within the snail have yet been carried out, Daniel et al., (1992) described the in vitro transformation of this species. It is initiated by the cessation of ciliary beating, followed by expansion of the intercellular ridges, the shedding of the cilia and the transformation of the tegument into that of the mother sporocyst. Within 3-4 weeks post-penetration, sporocysts are seen in the interstitial areas of the hepatopancreas adjoining the testicular tissue of B.natalensis (Fig. 3.8). The surface of sporocyst is covered with microvillus-like structures which increase the surface area and are likely to be involved in absorption of nutrients. The sporocyst has no gut, unlike the rediae of other trematodes, and must provide



sufficient nutrients to generate large numbers of cercariae. Within ~~the~~ sporocysts, cercariae gradually mature and eventually emerge from the snail (Fig. 3.9) approximately 3-4 weeks post infection.

The fully mature, and free-swimming cercariae of S.margrebowiei, are together with S.japonicum amongst the smallest of the species of the genus. The cercaria of S.margrebowiei measures 351 $\mu$ m in total length whereas those of S.mansoni, S.haematobium, S.intercalatum and S.bovis measure greater than 450 $\mu$ m (Loker, 1983). In general, the cercariae of schistosomes have a similar basic structure. The most anterior region is the head. It is a conical structure and is demarcated from the rest of the body by a distinct constriction. There are a number of sensory papillae and ducts in this region which are thought to be openings of the secretory penetration glands. The remainder of body is thicker and oval in shape. It is clearly marked by the presence of the ventral sucker and is separated from the tail stem by the collar. The tail stem is long and thin and divided into two furcae at the posterior extremity. The body, collar, tail stem and furcae are covered with spines, all of which point posteriorly thereby probably facilitating the penetration process (Figs. 3.9 and 3.10). The heavily spined collar of the cercariae of S.margrebowiei is similar to that of S.haematobium (LoVerde, 1976) and S.japonicum (Sakamoto and Ishii, 1978).

TEM studies have shown that the surface membrane of the head and body is covered by a glycocalyx, which extends over the

spines. This glycocalyx is a labile structure which is difficult to demonstrate by SEM. The thickness of the glycocalyx varies considerably, but it is much greater over the body than tail surface. The intact glycocalyx is thought to be involved in the control of surface permeability, while the cercariae lives in fresh water (Stirewalt, 1974). The subtegumental region is packed with muscles, mitochondria, subtegumental cells and glycogen. Glycogen, which is an important energy store of cercariae, is abundant in both the body and tail. However, it is dissolved during the preparation procedure. The sensory structures, include sensory hairs on the tail, ensheathed uniciliated structures, which are thought to be mechanoreceptors (Nuttman, 1971) and sensory papillae which are only found at the anterior end of the body. The excretory system is composed of flame cells and their associated ducts. The single median excretory canal branches posteriorly at the base of tail furcae. Each branch then enters a furca and terminates in the excretory pore (Figs. 3.11 and 3.12).

Three morphologically distinct types of unicellular glands were observed during the TEM studies. These consist of a large head gland, the contents of which are almost homogenous, two pairs of post and three pairs of pre-acetabular glands, the contents of which are secreted via ducts on the anterior end of the head (Fig. 3.13). A number of workers have studied the structure and role of these glands in penetration. Dorsey and Stirewalt, (1971) concluded that morphologically, these glands, resemble vertebrate



exocrine glands, and functionally they are holocrinic. It is thought that the contents of the post-acetabular glands are secreted during search for and exploration of the skin, whereas secretions from preacetabular glands occur only after the duct openings are within the keratogenous zone (Stirewalt and Kruidenier, 1961). The enzymes involved in cercarial penetration, which originate from these glands, have received considerable attention. Initial work by Milleman and Thornard (1959), Lewert et al., (1959), Dresden and Asch (1972) and Stirewalt (1973 and 1974), indicated that proteases secreted from the acetabular glands were important mediators of cercarial invasion. One such enzyme, elastase, a protease with a molecular weight of 24-30kDa, was found to degrade a variety of substrates in the connective tissue matrix, including keratin (Tzeng et al., 1983), fibronectin, laminin, Type IV collagen (McKerrow et al., 1985), and elastin (McKerrow et al., 1985). The crucial role of cercarial elastase in penetration was demonstrated when synthetic peptide inhibitors of cercarial elastase were shown to prevent cercariae from invading human skin (McKerrow and Doenhoff, 1988). This cercarial elastase, is synthesized as a proenzyme in the post-acetabular glands during cercarial development within the sporocyst.

Silver nitrate staining shows the position of various papillae on the body and tail of the cercariae (Fig. 3.14). The position of these structures are bilaterally symmetrical. However, due to their close proximity with the openings of ducts and pores, they are difficult to count accurately on

the body and the furcae. The papillae on the tail stem are in contrast fewer and more widely spread and more easily counted. There are 32-37 of these structures on the tail stem. Previous workers have shown that the number of papillae on the tail stem show a greater variation than those on the body (Short and Cartrett, 1973, and Sakamoto and Ishii, 1978) and are thus an unreliable feature for any direct comparison with other species of schistosomes.

#### Sex determination:

Schistosomes are dioecious digeneans with clear differences between the adult male and female (Fig. 3.2). However, this marked difference is not apparent during other stages of development although sexual dimorphism is nonetheless maintained. The DNA of all Schistosoma species is contained within eight pairs of chromosomes (N=16) Short (1963) and Grossman and Short (1981). Unlike mammals, it is the female that is the heterogametic with nonhomologous sex chromosomes ZW pair. The male worm is homogametic and contains the homologous ZZ pair. Attempts to sex the larval stages of schistosomes include morphological studies (Short and Cartrett, 1973), cytological studies (Liberatos and Short, 1983), the use of DNA probes (Walker et al., 1989) and polymerase chain reaction (Gasser, 1992). The simplest method for determining the sex of larval stages involves the cytological demonstration of the heterochromatin in the W-chromosome of the females by the C-banding technique. Liberatos and Short (1983) using this method, successfully sexed cercariae of S.mansoni with 100% accuracy, but failed



to sex the cercariae of S.haematobium, S.japonicum and S.douthitti. Their inability to sex the cercariae of these three species was explained as follows: in the case of S.haematobium, the separation of the very large block of heterochromatin of the W chromosome into several bands at metaphase makes it impossible to distinguish from the Z and indeed other autosome chromosomes. S.japonicum, lacks a very large block of interstitial heterochromatin on the W chromosome and is thus difficult to distinguish from the Z chromosome and in the case of S.douthitti, there is no noticeable difference in the heterochromatin content of the W and Z chromosomes. The results of the present study using the C-band technique gave a 66% rate of accuracy. Since the W chromosome of S.margrebowiei like S.haematobium has a very large block of heterochromatin (Short, 1983), we think that as in the case of S.haematobium (Liberatos and Short, 1983), the separation of this heterochromatin into many bands, makes it impossible to distinguish. In conclusion, the low rate of uni-miracidial snail infection coupled with the low number of cercariae emerging from the infected snails and the 66% rate of success, makes the C-band technique unsuitable in this species.

## CHAPTER FOUR

Deposition of S.margrebowiei eggs in different tissues of BKTO mice.



## CHAPTER FOUR

### Introduction:

The degree of parasitic disease often depends on the ability of the parasite to reproduce itself within the host. Schistosomes however, do not replicate within their definitive hosts, instead the paired males and females copulate and lay eggs. These eggs, produced by the adults, actually cause the disease. In contrast the adult worms although they feed on the host's red blood cells cause very little damage. Eggs laid by female worms of S.margrebowiei must pass from the venules into the lumen of the intestine so that they can reach the outside environment. However, most eggs fail to do so and instead lodge in different tissues. Thus leads to the initiation of tissue injury and other events in a complex pathogenesis which is typical of all species <sup>causing</sup> mammalian schistosomiasis. It is the antigenic material secreted by the egg which elicits the granulomatous and other related responses. Oliver-Gonzalez (1954) demonstrated that when live eggs were incubated at 37°C in sera from individuals (human and monkeys) infected with S.mansoni a precipitate was formed around the egg shell which increased with continual incubation. This was the result of interaction between substances diffusing through the shell and antibodies present in the sera. This reaction was found to be both stage and species specific. Furthermore Von Lichtenberg and Raslavicius (1967) showed that eggshell alone did not elicit granuloma formation and concluded that to induce maximum granulomata, the eggs needed to be both intact

and viable. So it is the antigenic material secreted from the egg of S.mansoni which elicits the granulomatous and other immune responses and not any other possible structural components.

In schistosomiasis, the severity of the disease and the probability of complications is related to the intensity and duration of infection. The intensity of the disease can be assessed directly by counting the number of worms at autopsy or perfusion and indirectly by tissue egg counts. Egg accumulation in different tissues is a product of the duration of oviposition and the magnitude of infection (Smith and Christie, 1986).

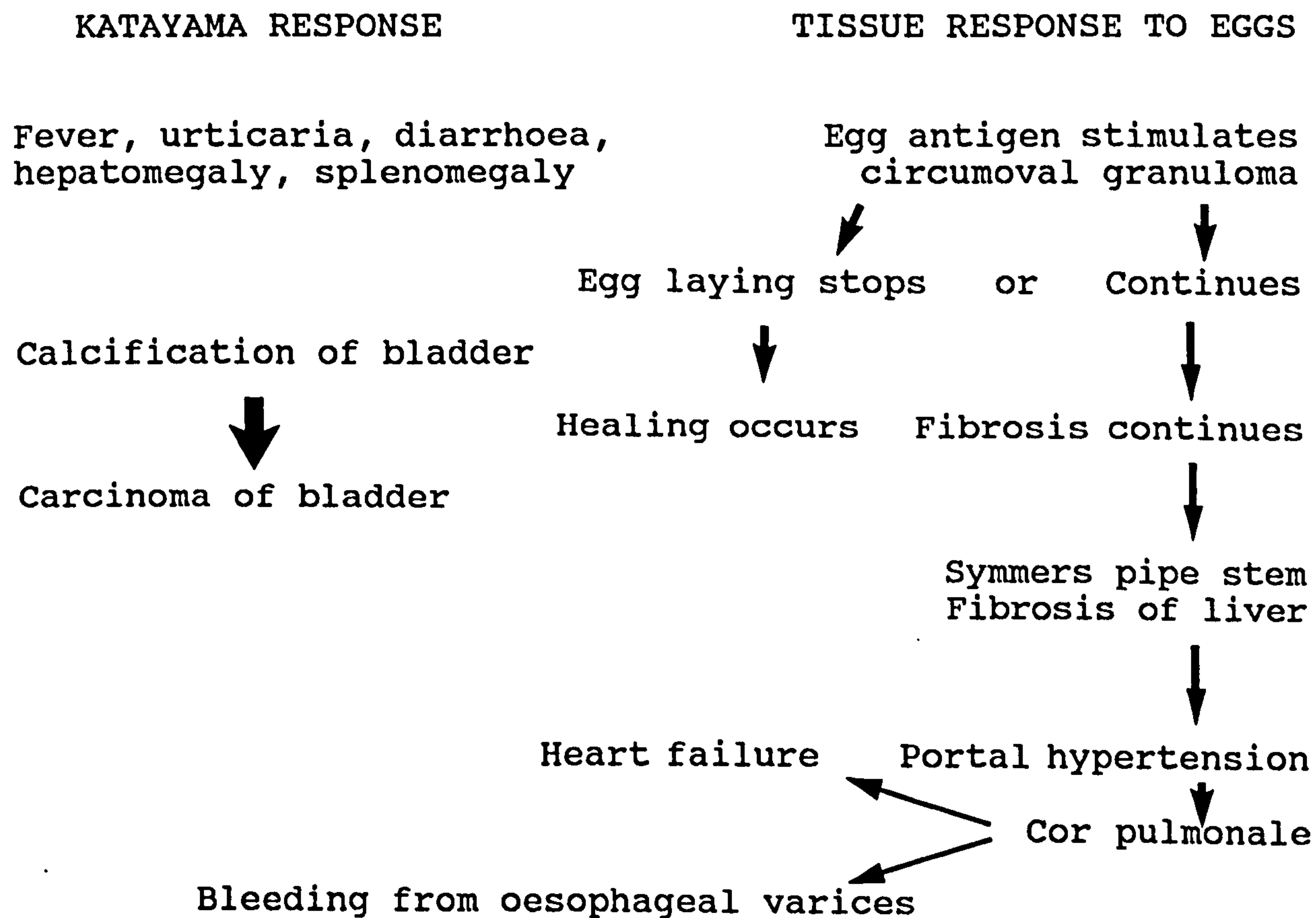
Among the three main species of schistosomes infecting man (S.mansoni, S.japonicum and S.haematobium), obvious differences exist with respect to location of adult worms within the host, fecundity and pattern of egg deposition.

Hepatic schistosomiasis caused by S.mansoni, is probably the world's most prevalent chronic liver disease. The disease begins with the formation of schistosome egg granulomata in portal tracks. As chronic disease evolves, infected individuals show the presence of humoral antibodies and also cell-mediated responses to schistosome-derived antigens. Each granuloma blocks the portal blood flow, but the reduced flow rate is compensated for by an increased blood supply via the hepatic artery leading to portal hypertension. As the disease progresses and as fibrous scar tissue forms, new blood vessels develop. By this stage other clinical symptoms include hepatomegaly, splenomegaly and oesophageal varices.



A summary of the pathogenesis of schistosomiasis is as follows:

### The pathology of schistosomiasis



Splenomegaly can be substantial caused by passive congestion in the portal circulation and hyperplasia of cellular elements, thought to be due to continual antigenic stimulation.

Warren (1975) described schistosomiasis as an "immunologic disease" because in the case of S.mansoni in a mouse model, most of the symptoms mentioned above are directly the result of egg granuloma formation. Domingo et. al., (1967), suggested that by suppressing the granuloma formation, most of the clinical symptoms associated with schistosomiasis could be averted. Experimental results of Byram & Von Lichtenberg, (1977) and Lucas et al., (1980) do not support this hypothesis. Indeed Von Lichtenberg's (1964) earlier assumption that the immune granulomata benefit the host in sequestering toxic schistosome egg products was proved to be correct. In immune-suppressed mice granuloma formation is much reduced, yet mortality is higher. In such mice, around the eggs trapped in the liver there are areas of necrosis due to toxins released by the eggs. So the encapsulation of eggs seemed to be advantageous to the host in that the spread of such toxins is minimized. However, in a series of experiments Doenhoff et. al., (1986) showed that when lymphoid cells were transferred from intact mice with a patent S.mansoni infection to infected deprived (no thymus) mice, although the recipients mounted a granulomatous response, they failed to protect against the hepatotoxic reaction. Also working with S.bovis, these workers found no evidence of egg-induced hepatotoxicity in T-cell deprived



mice, despite their inability to mount a granulomatous response. So the immunopathological nature was not evident in S.bovis. In fact it was noted that T cell deprived, infected mice survived longer than infected yet intact mice. However, the immune-dependency of egg excretion (the immune response help the eggs to escape from the tissue and to pass out with the faeces) was demonstrated in both S.mansoni and S.bovis in mice, confirming earlier findings of Dunne et. al., (1983) and Doenhoff et. al., (1978 and 1981). It was therefore concluded that granuloma formation facilitates the passage of eggs through the tissue and out with the faeces. Hence granuloma formation is advantageous to the parasite. This was further confirmed by the fact that immune-suppressed mice were unable to void eggs in their faeces.

At the cellular level, Pearce and Sher (1991) concluded that granulomata are Delayed Type Hypersensitivity reactions mediated by T helper cell 1 (Th 1), but as egg production begins the Th2 response is elevated and the Th1 depressed. Th2 products, interleukin-4 and 5 are normally associated in providing help for B cells and inducing immediate hypersensitivity. However, Amiri et al., (1992) working on infected Severe Combined Immunodeficient (SCID) mice (which have normal macrophages but lack functional B or T lymphocytes) reported that these mice failed to produce granulomata. However, when spleen cells from infected BALB/c mice were injected into SCID mice, a granulomatous reaction was observed. Crude supernatant from an antigen-stimulated Th-2 T-cell clone also gave the same result. Further studies

demonstrated that tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) alone was sufficient to restore granuloma formation in SCID mice. Furthermore it was found that the adult worms use host TNF $\alpha$  to increase their fecundity.

Urinary schistosomiasis, caused by S.haematobium which dwells principally in the perivesical venous plexus (of the bladder) in humans is the worlds leading cause of haematuria (Smith and Christie, 1986). Urinary schistosomiasis has also been linked to urothelial (bladder) cancer. Observations from animal studies have shown that infection with S.haematobium, supplies the proliferative stimulus necessary to accelerate the development of tumour foci from cells initiated or altered by exposure to low doses of bladder carcinogens. The predisposition to secondary bacterial infection of the urinary tract, caused by pathological changes of S.haematobium infection resulting in the production of nitrosamines, could also be a contributing etiological factor for bladder cancer (Koroltchouk et al., 1987). Work in our own laboratory in collaboration with the Paterson Research Institute has shown that promutagenic methylation damage occurs in the liver-DNA of mice experimentally infected with S.mansoni (Badawi et al., In Press). Such changes are early indicators of tumour formation. The immunological response induced in the tissues of experimental animals by S.haematobium eggs appears to be similar to that provoked by those of S.mansoni. The only physical difference is that in the case of S.haematobium, eggs are more often found within granulomata as aggregates, which is the same for S.japonicum



(Erickson et al., 1974) and S.margrebowiei (Present study).

Domingo and Warren, (1968) coined the term "endogenous desensitization" and Boros et al., (1975) "spontaneous modulation" which refer to the earlier findings of Andrade and Warren (1964) who reported that in chronic S.mansoni infected mice, the granulomata formed around mature eggs decrease in size as the infection progresses. This decrease is paralleled by a decline in reactivity of other cell-mediated parameters. Thus lymphocyte blastogenic responses induced by egg antigens, in vitro, reach a peak at 7-9 weeks post infection in mice and thereafter decline (Colley, 1972). Delayed foot-pad swelling in infected mice, elicited by injection of egg antigen, also peaked at 8 to 10 weeks post infection but declined by 12 weeks (Boros et al., 1975).

Studies on the pathology of S.margrebowiei in experimental animals include, Southgate and Knowles (1977) and Ogbe (1985) who reported the deposition of eggs in different tissues of hamsters, and hamsters, mice and gerbils, respectively. In both cases the presence of eggs in the spleen was not reported. However, during the course of the present study it became clear that large numbers of eggs were deposited in the spleens of BKTO mice. Consequently, an experiment was designed to investigate this unusual observation, and also to examine the effects of prolonged infection on the egg density in tissues.

## Materials and Methods:

S.margrebowiei cercariae from infected snails were used to infect 30 BKTO female mice (100 cercariae per mouse) by the ring method. All mice were infected using the same batch of cercariae thus reducing the chance of differences in terms of cercarial infectivity occurring.

### Faecal egg examination:

In order to check that the adult schistosomes were patent, the faeces of the mice were examined for the presence or absence of parasite eggs. The technique used was an adaptation of a method originally reported by Bell (1963) which was described by Doenhoff et al., (1978). Mice were placed individually in 500ml plastic beakers and allowed to defecate. Single faecal pellets (25-50mg) were each placed in 10ml of normal saline and then disrupted by repeated aspiration in a 10 ml syringe. The larger particulate material was removed by filtration through a 300 mesh metal sieve. The filtrate was passed through a Whatman No.4 filter paper (Whatman Ltd. Kent England) which was stained with saturated ninhydrin solution, dried at 37°C for 5hrs and examined under X40 microscope.

### Perfusion of mice and determination of adult worm burden:

Adult schistosomes were recovered from the mice by portal perfusion using the method of Smithers and Terry (1965a), as modified by Doenhoff et al., (1978). Infected mice were sacrificed using 30mg/mouse sagatal containing 25 units heparin/ml. The thoracic and abdominal cavities dissected, and the rib cage removed. The mice were then suspended from



a vertical perspex board by means of clips on their front and hind legs. The liver lobes were suspended over a funnel leading into a 25ml universal tube. The intestines were held vertically to reveal the hepatic portal vein which was severed at the point of entry into the liver. Perfusion commenced by injecting 25ml of perfusion fluid with a 50ml syringe and 18g needle into the right ventricle. With the injection of the perfusion fluid, the liver and the intestines were gently massaged to facilitate worm expulsion. Perfusion was considered to be successful only if the liver and mesenteric veins were fully cleared of blood. On completion, the organs of the mouse and the funnel were checked for any adhering worms.

The worms were left to settle at the bottom of the universal tube, during which time the supernatant was aspirated and the remaining 2mls of perfusate cleared from blood cells by lysis with a few drops of 5% saponin (BDH Chemicals Ltd). The perfusate was poured into a plastic petri dish with a grid and the worms counted under a low power microscope.

#### **Tissue egg counts:**

The tissue egg count technique used was according to Doenhoff et al., (1978). Following perfusion, the liver, small intestine (duodenum, jejunum and ileum), colon and spleen were removed from the animal. Each organ was washed in saline and cleaned of faecal material. They were then blot dried and weighed. These organs were then stored in numbered polythene bags at -20°C. When required each organ was digested separately in 20ml 5% potassium hydroxide at 37° C

for 18 hours (Cheever ,, 1968). The digests were individually emptied into 100ml beakers, agitated and three 100 $\mu$ l aliquots were pipetted onto microscope slides, covered with a coverslip and eggs counted under X40 magnification. The mean result of three counts were was multiplied by 200 and expressed as total number of eggs per tissue.

#### **Light microscopy:**

Fresh infected mouse spleen was fixed in SUSA solution for 24 hours, washed in industrial methylated spirit (I.M.S.) 3 times, and using a LKB 2218-500 Histo-resin Embedding Kit, embedded as follows: the spleen was placed in I.M.S. and histo-resin infiltration solution (1:1) for 8 hours at 4°C. It was transferred to histo-resin infiltration solution for 7 days with several changes after 3 days. The spleen was embedded in histo-resin infiltration solution : hardner (15:1) and cut at 4 $\mu$ m with a disposable metal knife using a microtome (Historange). Sections were stained with Ehrlich's haematoxylin and eosin.



## Results:

### Total worm recovery:

The mean overall worm recovery (expressed as a percentage of the cercariae administered) for S.margrebowiei from 20 female BKTO mice exposed individually to 100 cercariae by the ring method was, 38.8%. The average worm return for different times post infection from four mice in each group was,  $39.5 \pm 3.1$ ,  $48.2 \pm 16.3$ ,  $28.7 \pm 4.7$ ,  $41.2 \pm 8.3$  and  $36.2\% \pm 9.00$  for 45, 50, 55, 60, and 65 day old infections respectively. The highest worm recovery from an individual mouse was 69% from a 50 day old infection, and the lowest was 22% from a 55 day old infection.

The percentage of paired worms was 87.8%. The ratio of male to female worms was 1:0.87.

Eggs recovered from liver, intestine and spleen: Eggs of S.margrebowiei were first detected in mouse faeces 33-35 days post infection. The deposition of eggs in tissues was examined from day 45 to 65 at 5 day intervals.

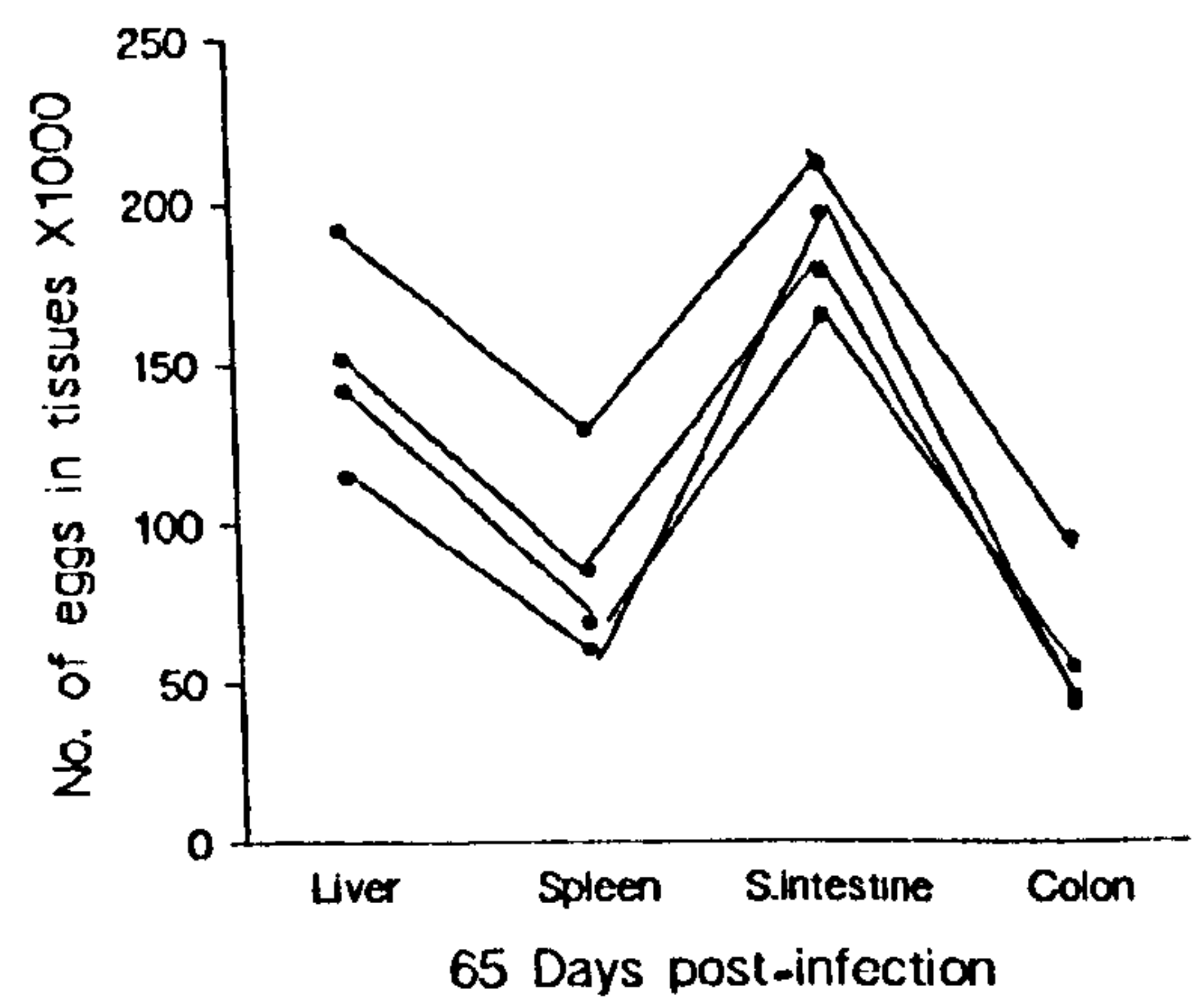
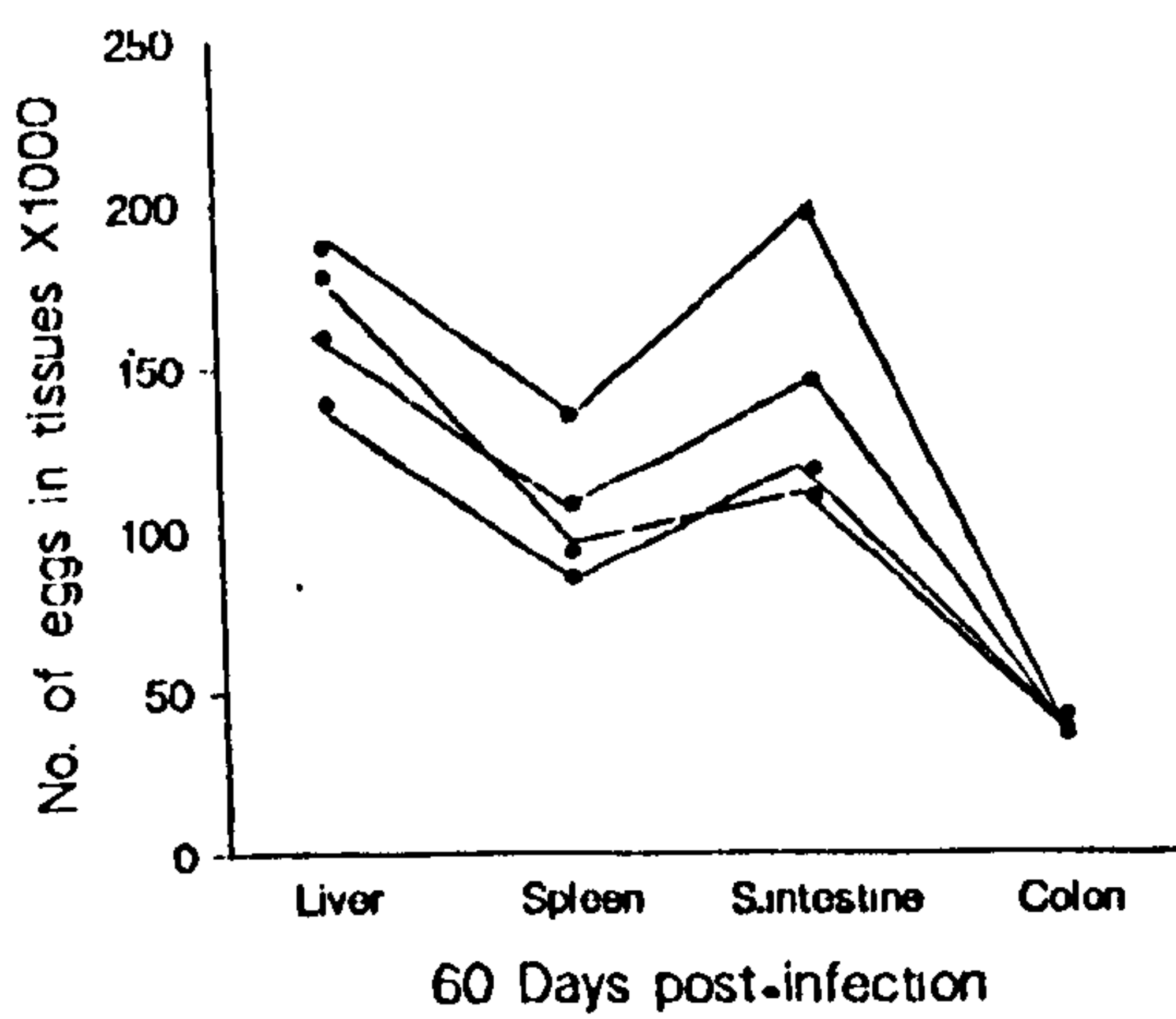
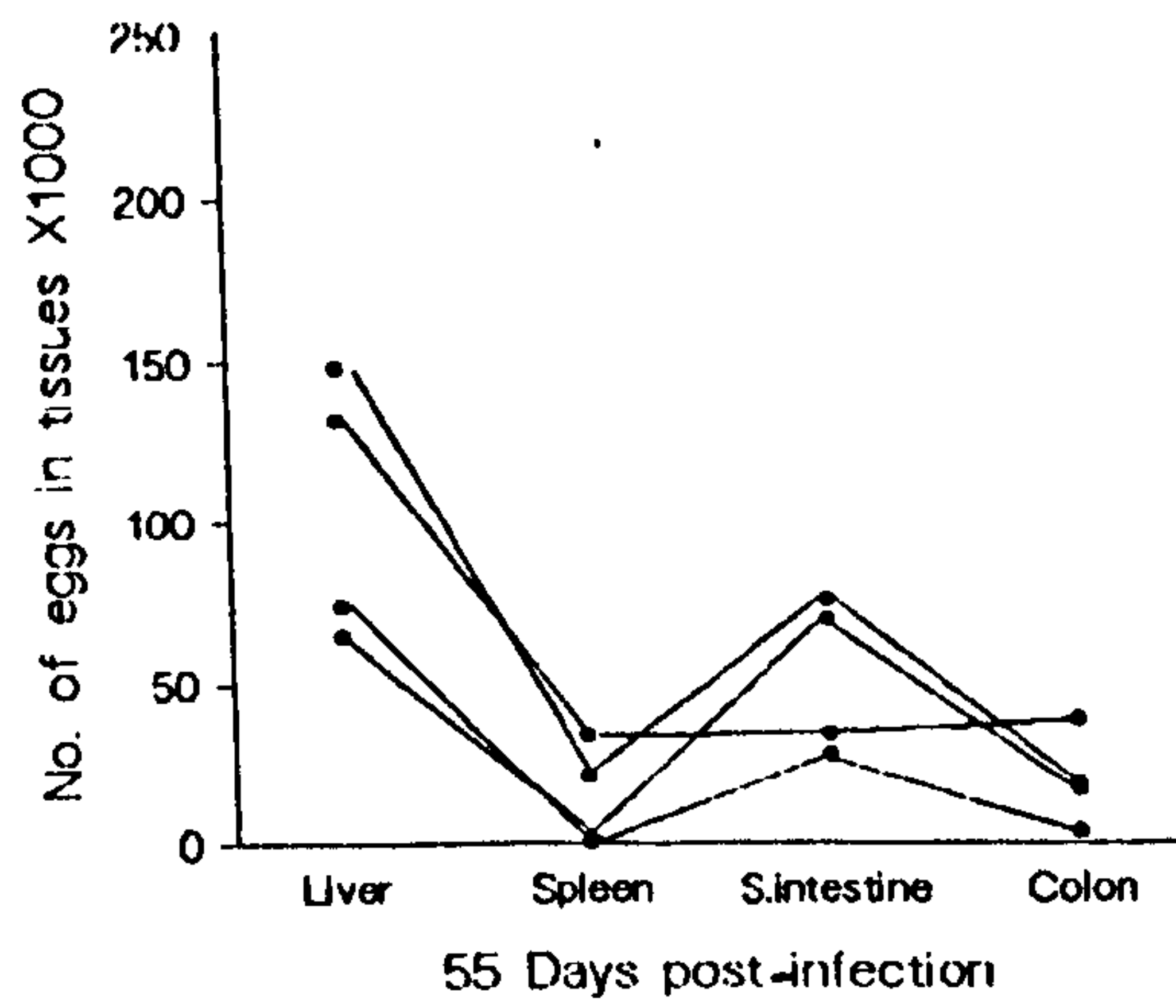
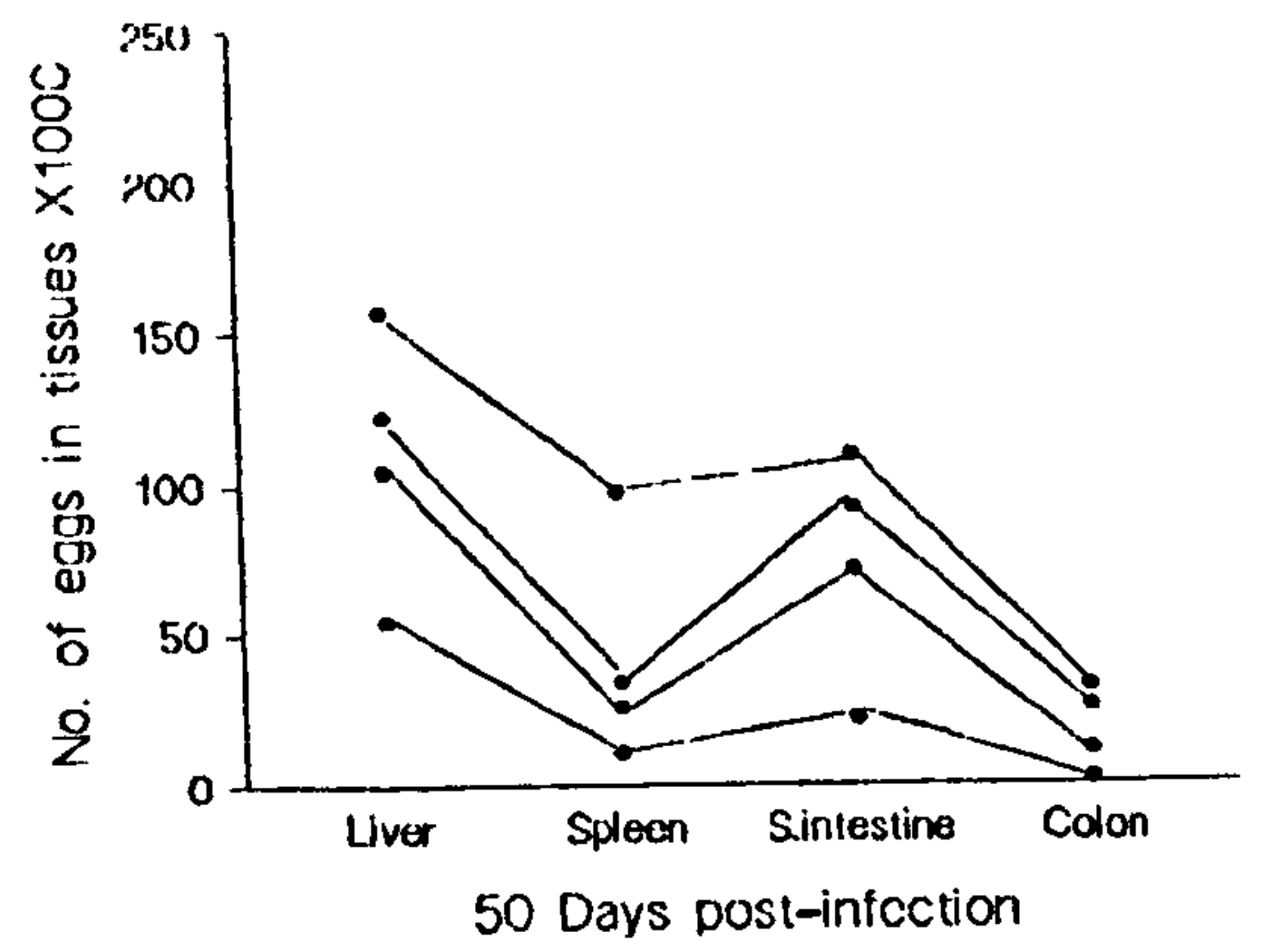
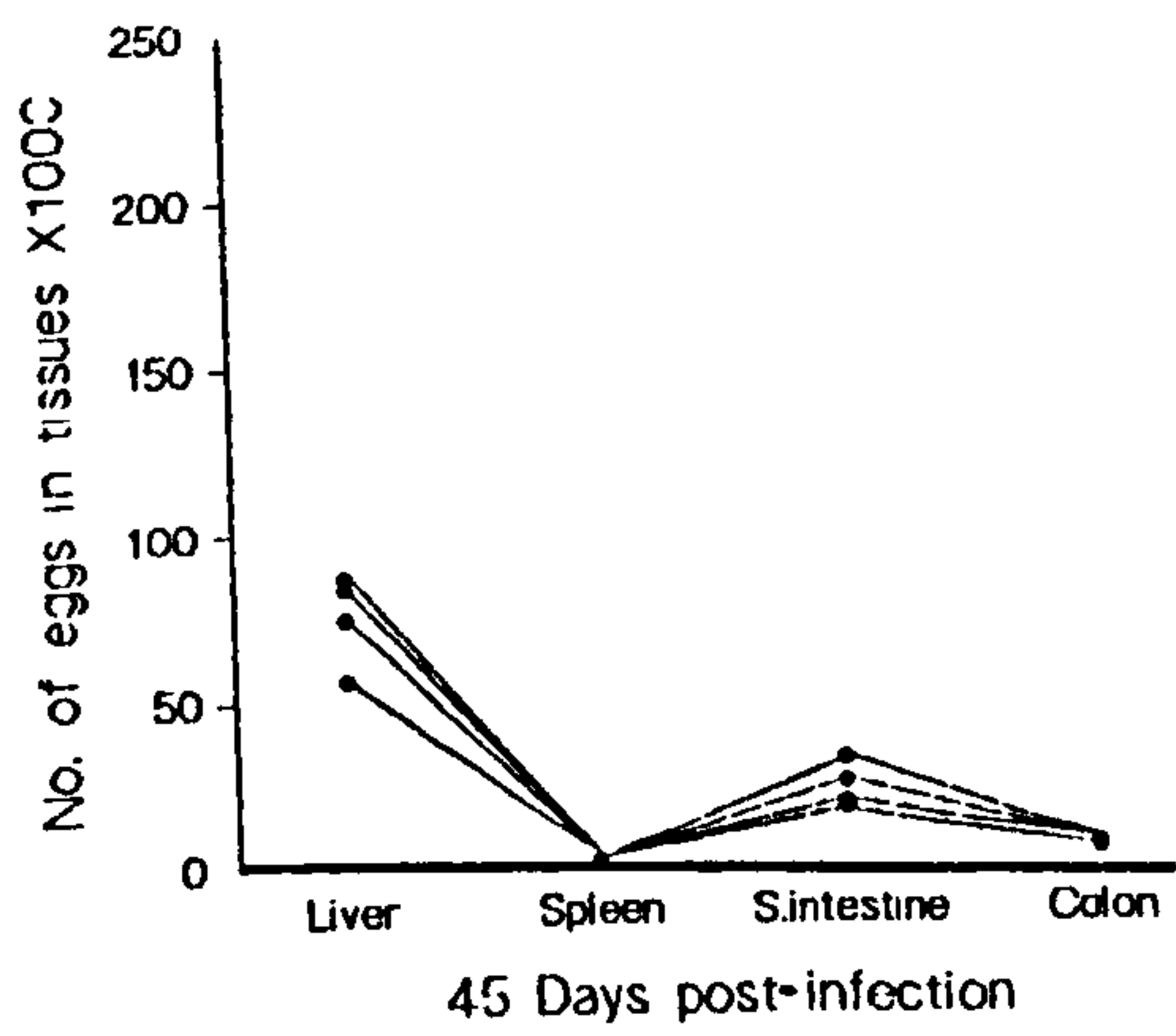


Figure 4.1 The number of eggs recovered from the tissues of four individual mice at different times post-infection. The result for each individual is connected.



Table 4.1 The mean number of eggs recovered from each tissue (X 10<sup>3</sup>) of four mice in each group

Days p.i	Liver	Spleen	S.intes.	Colon
45	74.7±13.8	0.5± 0.3	22.3±06.5	3.8± 0.7
50	109.3± 4.0	41.8±38.2	73.4±37.9	14.3±14.0
55	104.4±41.3	14.0±16.3	51.4±23.9	19.3±14.3
60	166.0±21.3	84.3±50.4	142.8±39.0	39.3± 2.9
65	150.0±31.9	86.3±30.6	188.1±20.5	60.4±23.9

± represents standard deviation of the mean.

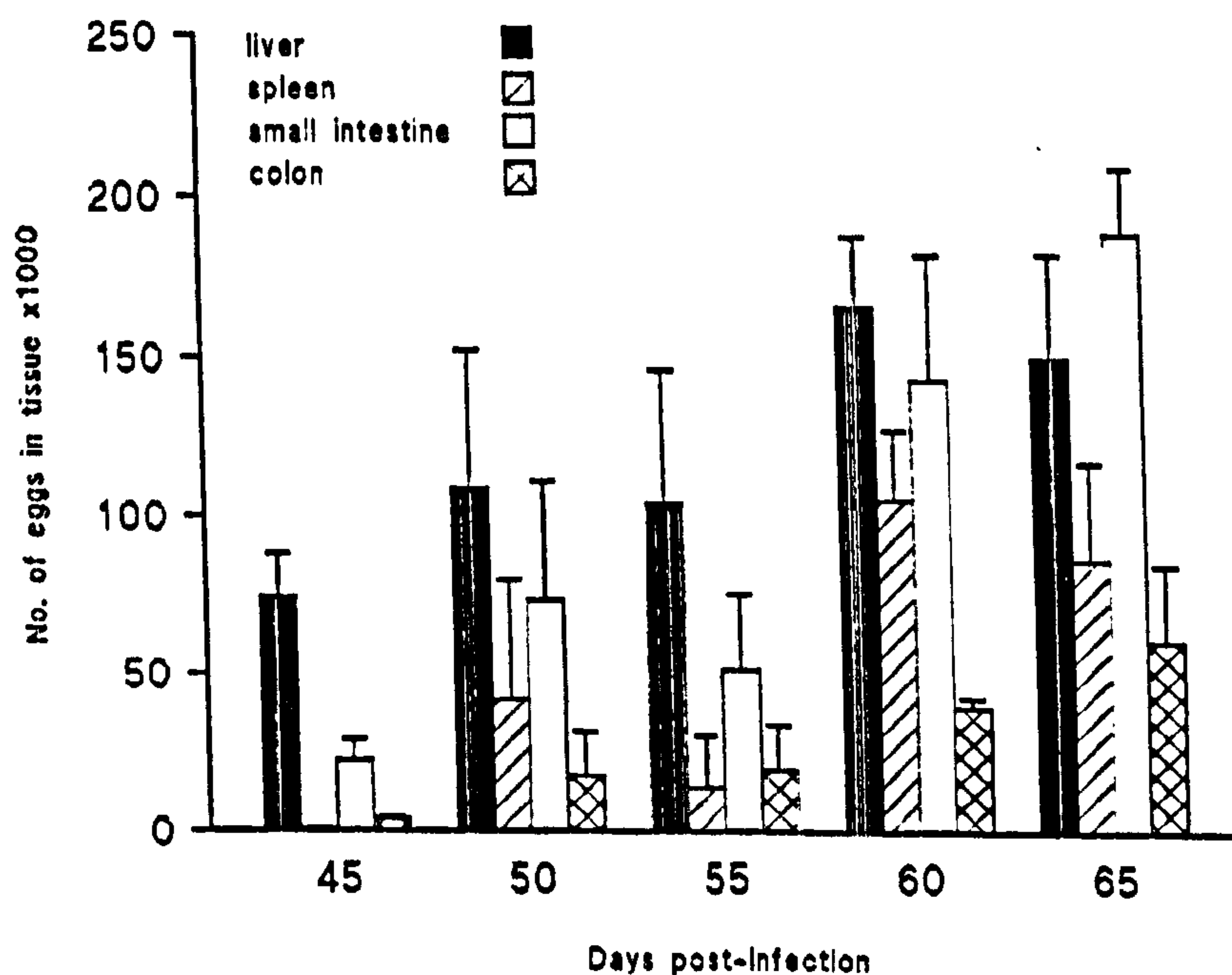


Figure 4.2 The mean number of eggs recovered from different tissues of four mice in each group.

Bars ± S.D.

Table 4.2 The mean number of eggs/g recovered from each tissue ( $\times 10^3$ ) of four mice in each group.

Days p.i.	Liver	Spleen	S.intes.	Colon
45	37.1 $\pm$ 5.4	1.98 $\pm$ 1.2	17.48 $\pm$ 5.0	10.47 $\pm$ 1.9
50	48.3 $\pm$ 19.2	73.00 $\pm$ 64.2	54.95 $\pm$ 24.7	44.00 $\pm$ 35.1
55	38.9 $\pm$ 10.6	16.38 $\pm$ 18.3	25.2 $\pm$ 10.6	37.28 $\pm$ 28.0
60	71.2 $\pm$ 11.6	134.71 $\pm$ 33	69.53 $\pm$ 13.9	75.1 $\pm$ 14.8
65	55.4 $\pm$ 6.4	94.36 $\pm$ 28.4	78.7 $\pm$ 5.7	73.9 $\pm$ 18.3

$\pm$  represents standard deviation of the mean

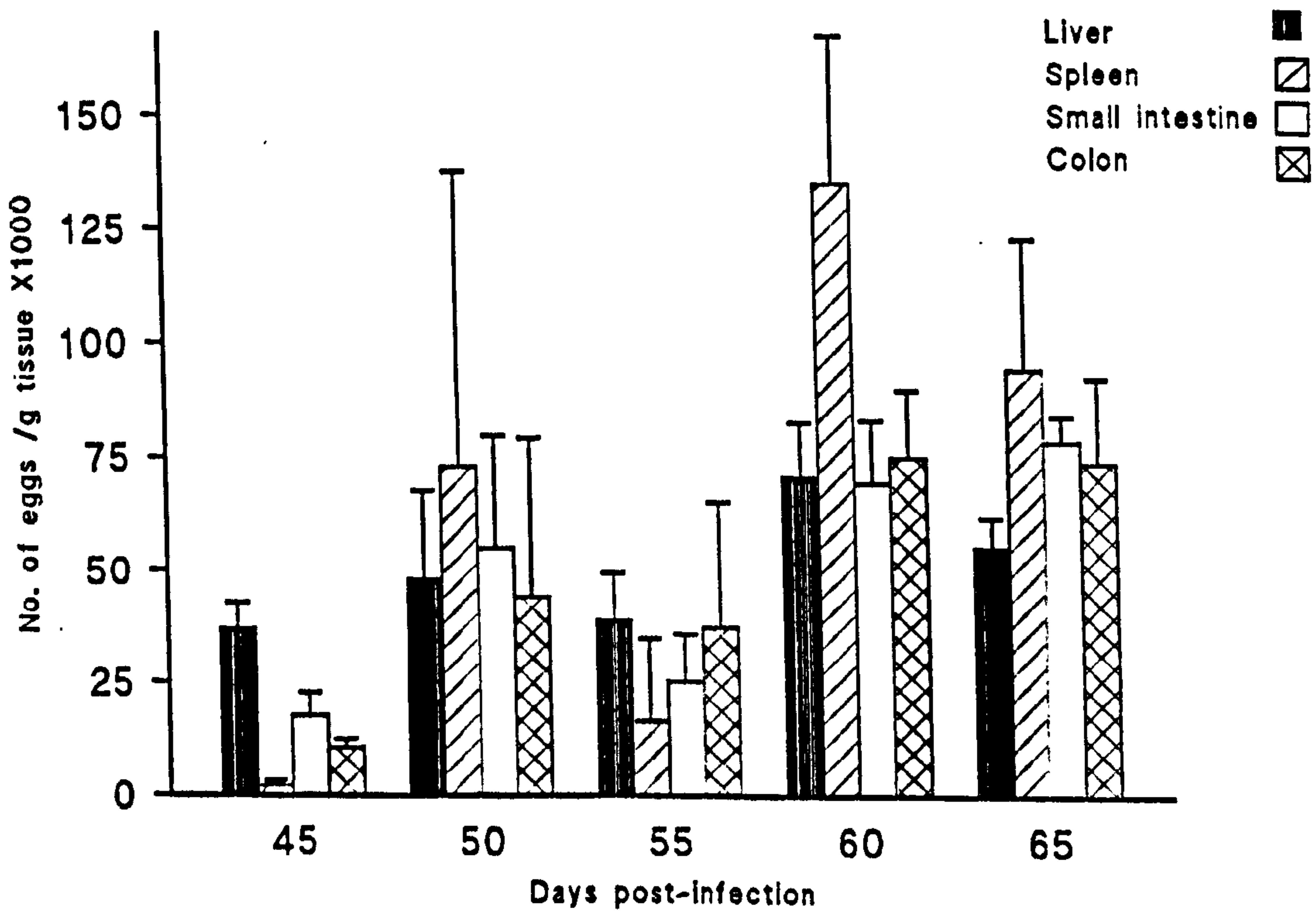


Figure 4.3 The mean number of eggs/g recovered from each tissue of four mice in each group.

Bar = S.D.



Table 4.3 The mean total percentage of eggs recovered from different tissues of four mice in each group.

Days p.i	Liver	Spleen	Intestine
45	73.7±3.0	0.4±0.3	25.7±3.3
50	48.6±9.2	15.1±6.2	36.0±6.7
55	56.0±8.5	5.8±6.6	45.3±3.5
60	36.8±3.9	22.9±0.92	40.3±3.5
65	30.8±2.6	17.4±2.6	51.6±4.4

± presents standard deviation of the mean.

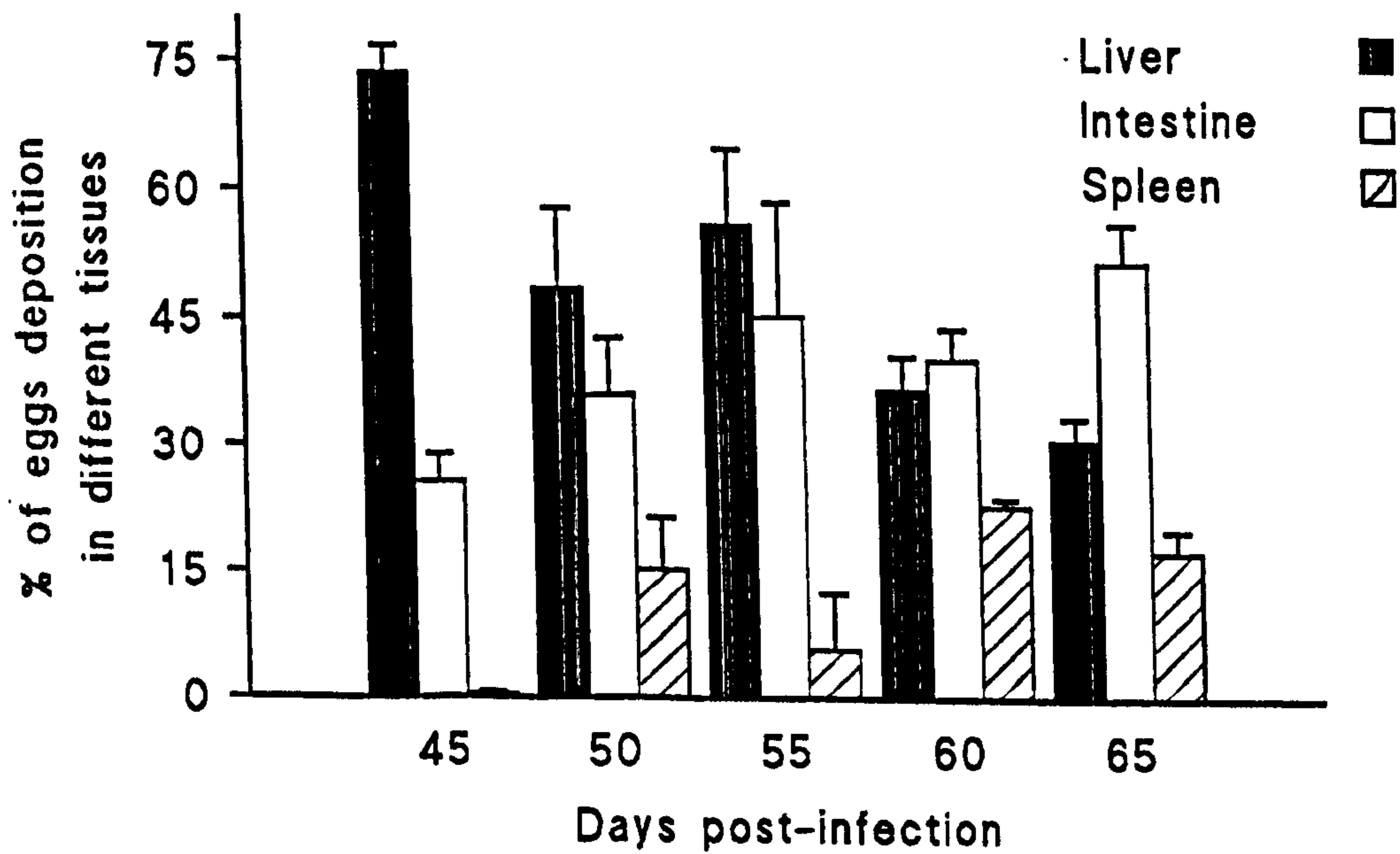


Figure 4.4 The mean total percentage of eggs in different tissues of four mice in each group.

Bar = ± S.D.

Figures 4.5 Light micrographs of the liver and spleen of BKTO mice infected with S.margrebowiei.

a) The gross appearance of liver and spleen from an infected (In) BKTO mouse 50 days post-infection compared with the spleen and liver of an uninfected (Un) control mouse. Note the marked hepatomegaly and splenomegaly such that these organs have increased by a factor of 2-3. Arrow indicates areas of granuloma. Bar = 1cm

b) Transverse section ( $4\mu\text{m}$  thick) of the spleen of an infected mouse stained with Ehrlich's haematoxylin and eosin (H & E). Note an area of white pulp (WP) and germinal centres (g). Bar =  $265\mu\text{m}$ .

c) Transverse section ( $4\mu\text{m}$  thick) of the spleen of an infected mouse stained with Ehrlich's H & E. Note eggs of S.margrebowiei (E) deposited in the spleen and the presence of trabeculae (Tr) which project from the connective tissue capsule (Co) into the substance of spleen. Bar =  $250\mu\text{m}$ .



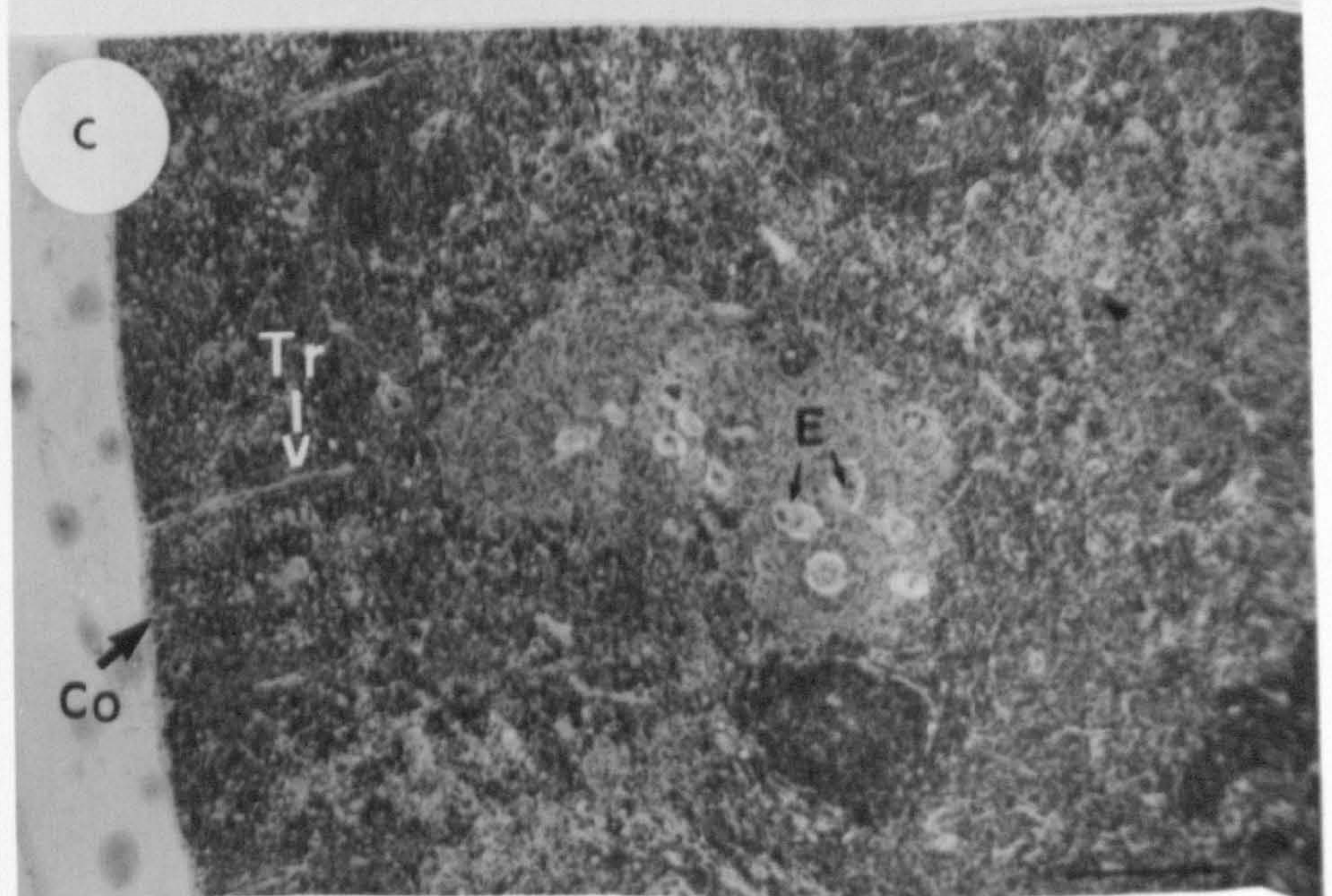
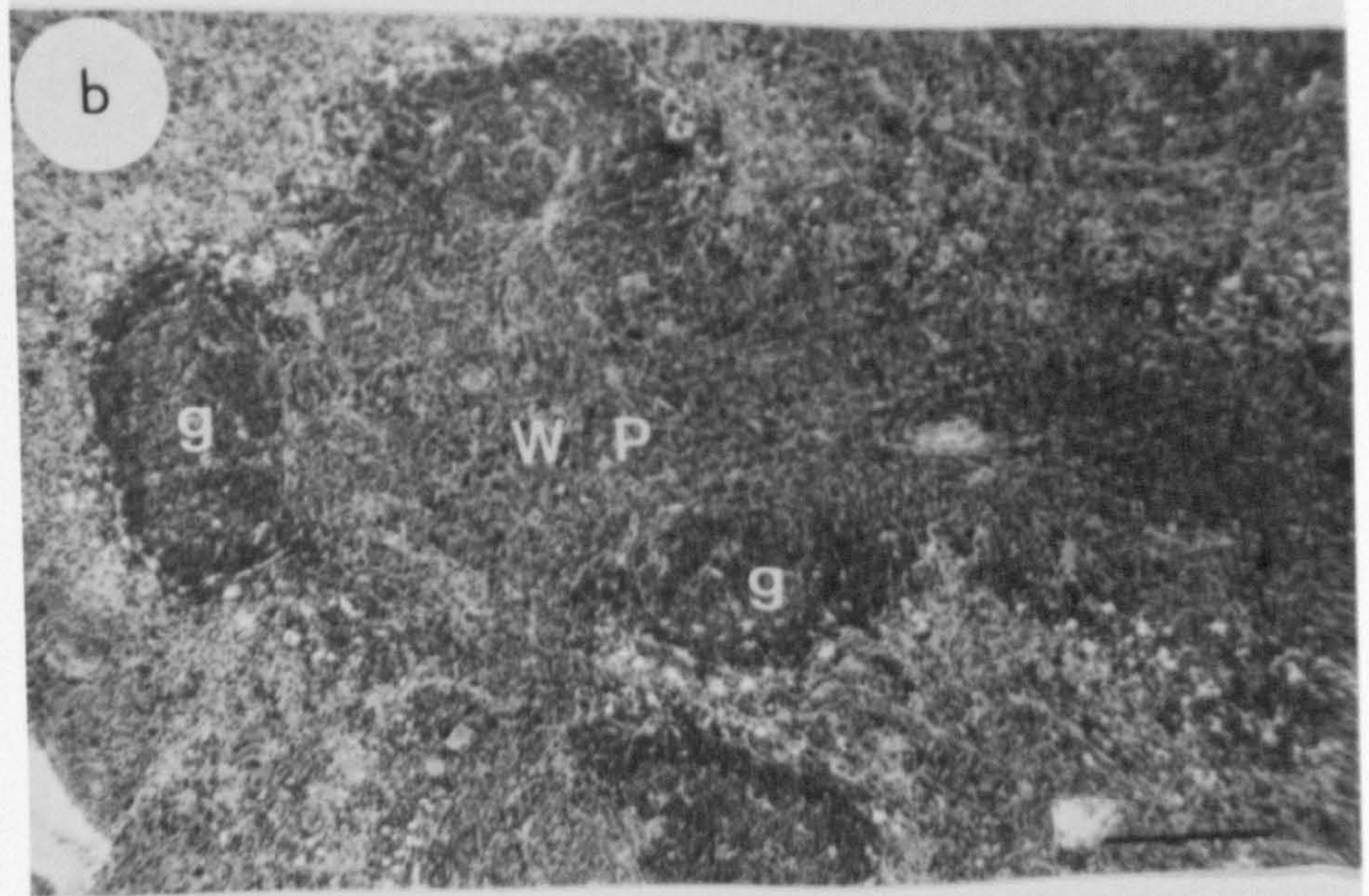
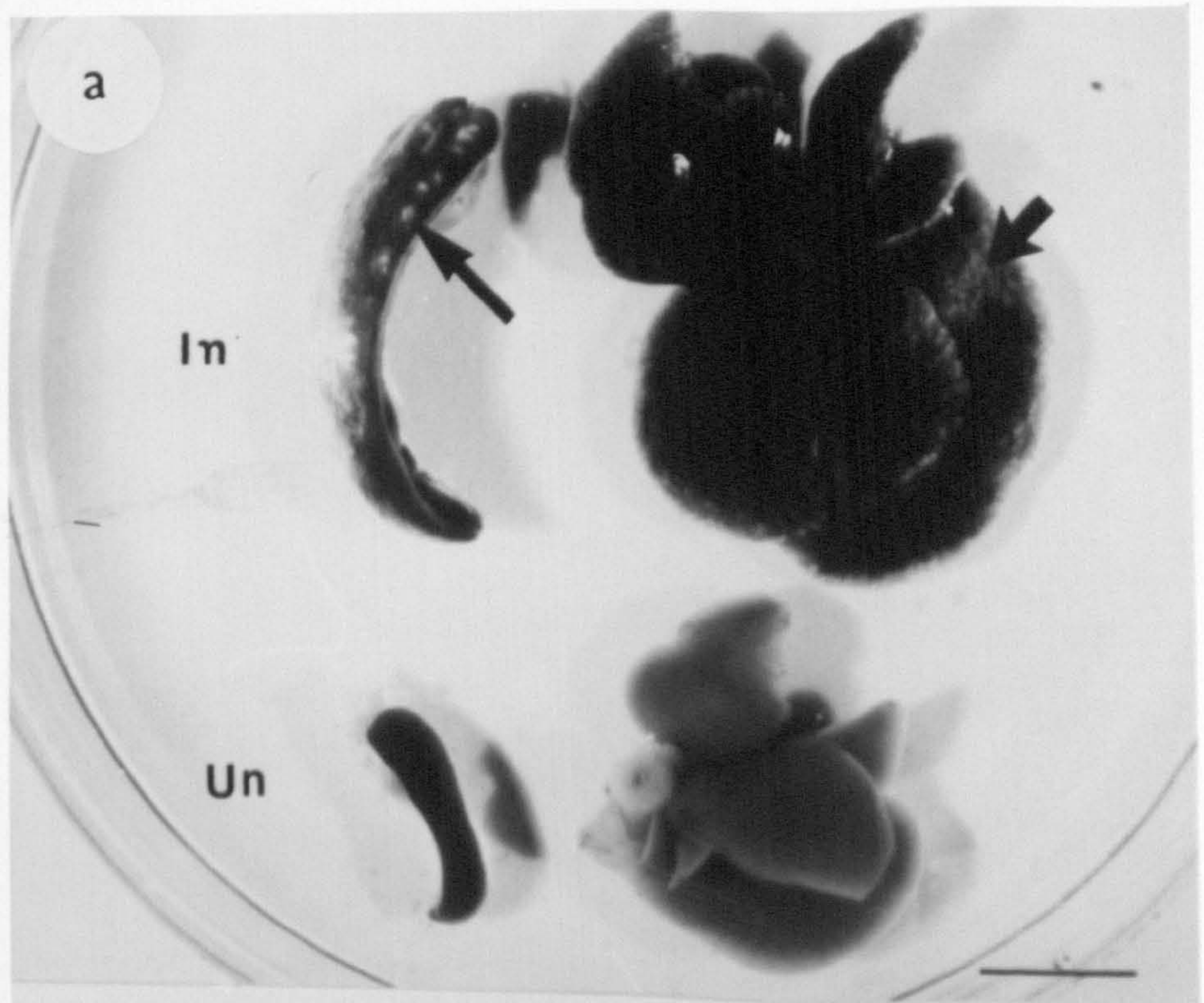




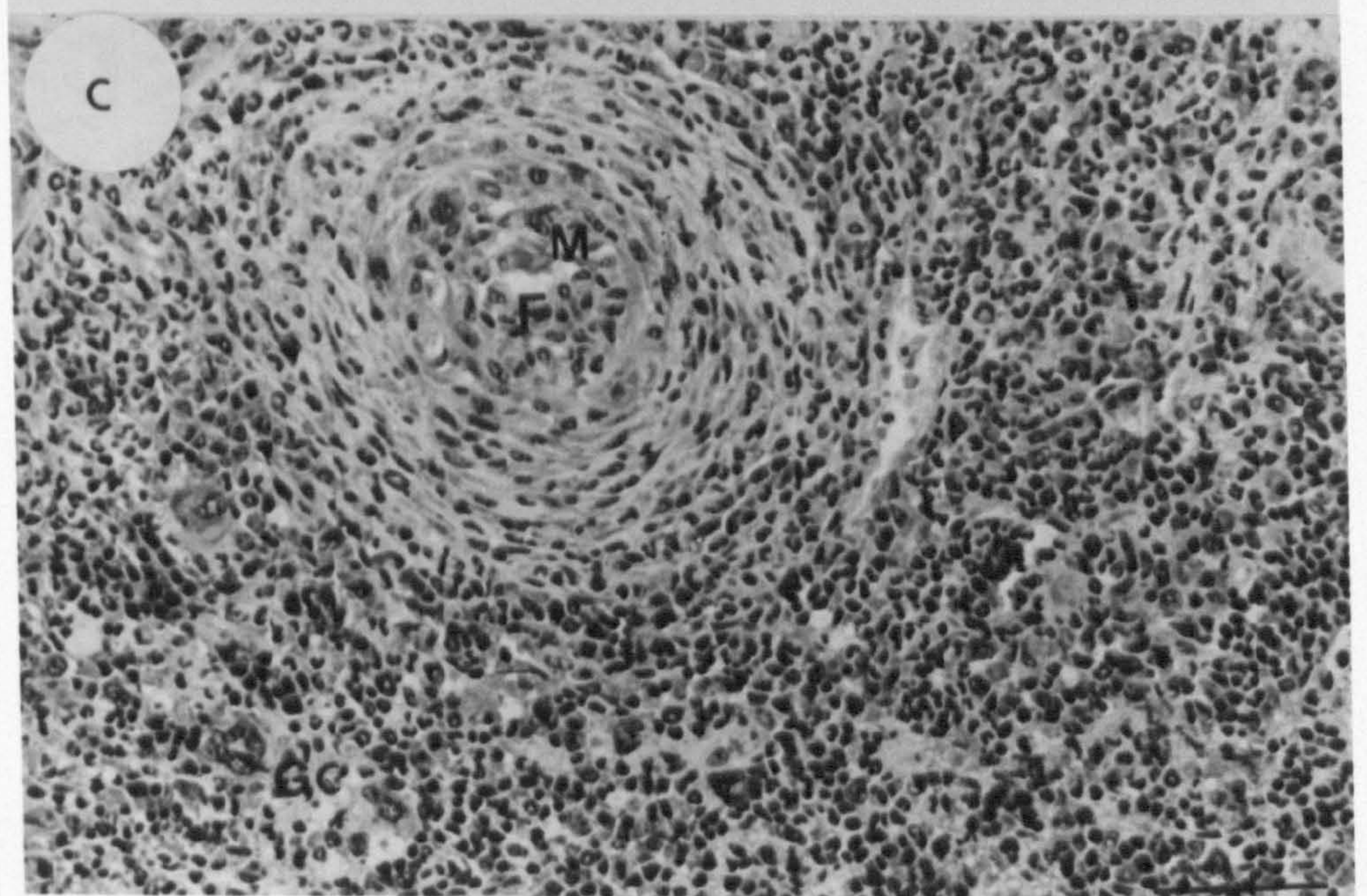
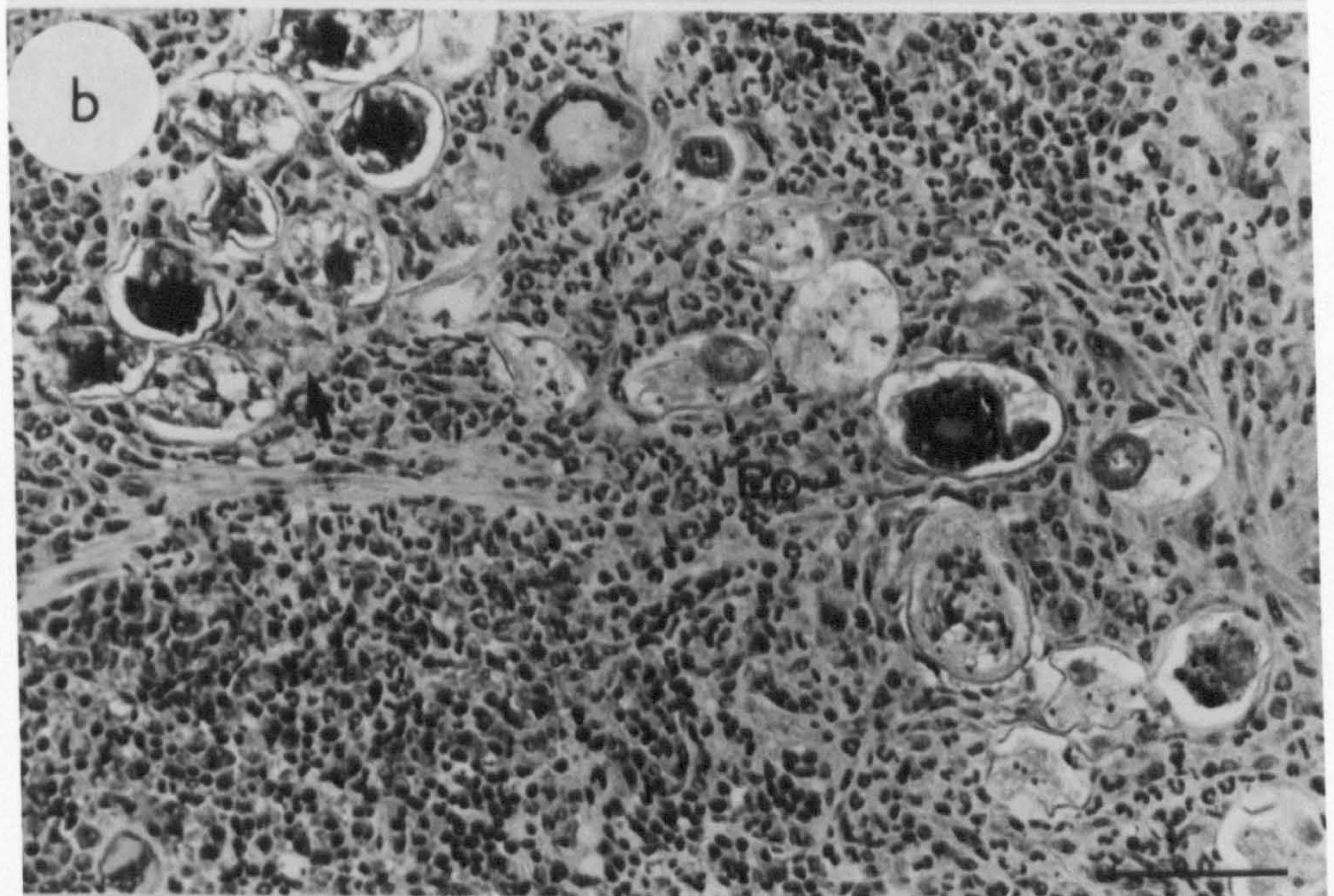
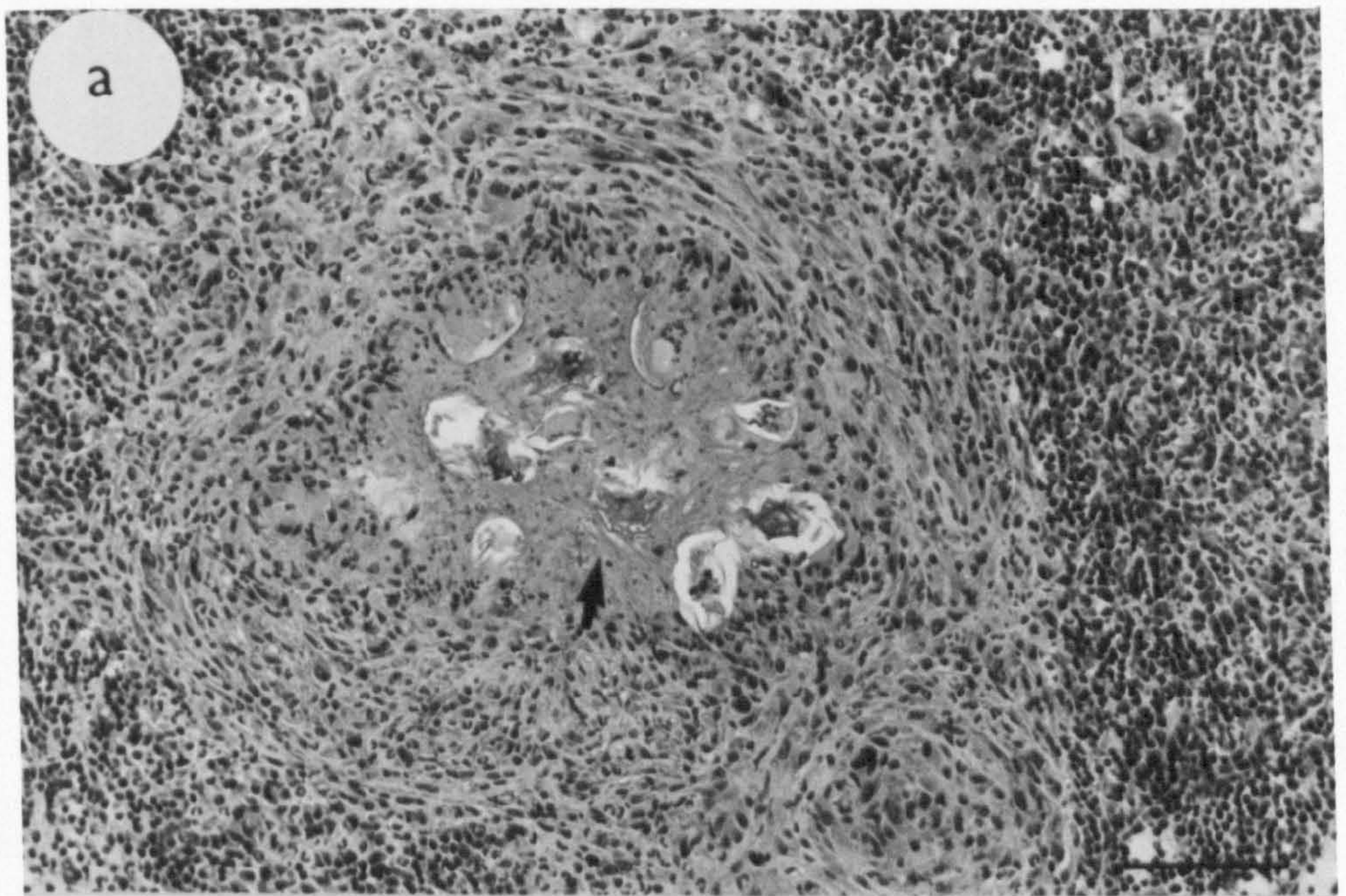
Figure 4.6 Light micrographs of the spleen of S.margrebowiei infected BKTO mice.

a) Transverse section ( $4\mu\text{m}$  thick) of the spleen of an infected mouse stained with Ehreich's H & E. Note the gradual destruction of eggs in the spleen by the host's immune response which forms an area of necrosis (arrow). Bar =  $110\mu\text{m}$

b) Transverse section ( $4\mu\text{m}$ ) of the spleen of an infected mouse stained with Ehreich's H and E. Note the aggregation of S.margrebowiei eggs (arrows) surrounded by eosinophils (Eo).  
Bar =  $60\mu\text{m}$

c) Transverse section ( $4\mu\text{m}$  thick) of the spleen of an infected mouse stained with Ehreich's H & E. Note fibroblast formation (F) around remains of an egg and the presence of macrophages (M) and giant cells (Gc). Bar =  $60\mu\text{m}$







**Mortality rates:**

S.margrebowiei is not very pathogenic in BKTO mice, of the 30 female mice infected only 7 died before they were due to be sacrificed (23% mortality rate). However, the timing of death was not uniform. Table 4.4, indicates the number of mice that died at different time intervals.

Table 4.4 Number of mice which died at different times post infection

Days p.i	0-44	45-49	50-54	55-59	60-64
No. dead	0	0	4	2	1



## Discussion:

The measurement of the number of eggs present in host tissue, is notoriously difficult. Furthermore, individual variability is seen in the numbers of eggs shed by the same size population of worms in different host individuals. Before the biological basis of this variability can be ascertained, attempts must be made to ensure that the methods used for counting eggs yield reliable and reproducible estimates. These methods most commonly involve flotation (using NaCl, Mg SO<sub>4</sub> or Zn SO<sub>4</sub>) and of these, the McMaster technique is perhaps the most well known. However, due to the density of the eggs of S.margrebowiei they sink in 4% potassium hydroxide, which makes counting difficult and inaccurate. It was therefore decided to use glass slides and coverslips as recommended by Doenhoff et. al., (1978). At least three 100 $\mu$ l aliquots were taken and if the number of eggs in any individual aliquot was found to vary by more than 10% from the mean, further aliquots were taken. This ensures consistency and decreases the variability in the estimated number of eggs present.

Southgate and Knowles (1977) were the first workers to study the deposition of S.margrebowiei eggs in an experimental definitive host. In their study hamsters (Mesocricetus auratus) were used. Hamsters were infected with 100 cercariae by partial immersion for one hour. Twenty hamsters were killed in batches of five at 10-day intervals commencing at 30 days i.e 30, 40, 50 and 60 days post-infection. Their results demonstrated that 80.9% of eggs were

deposited in the intestine (small intestine, caecum and colon) and only 18.2% were found in the liver. An insignificant number of eggs, 0.05% were recovered from the spleen. The data were presented as percentage of eggs recovered from the particular tissue.

Southgate and Knowles (1977) also concluded that S.margrebowiei was very pathogenic in hamsters with a premature death rate of 71.8%. This relatively high rate of mortality is in agreement with the findings of Hsu and Hsu (1960) that there is a direct relationship between the length of the prepatent period and the survival time of the host. A parasite with a shorter prepatent period is more virulent and results in the death of the host sooner. Among schistosome species, S.margrebowiei has one of the shortest prepatent periods and development. The cross-over point (the point at which both the male and female worms are of the same size) is 28-31 days. The cross-over points of 61 days for S.mattheei (Wright et al., 1972), 46 days for S.leiperi (Southgate et al., 1981), 42, 62 and 40 days for S.curassoni, S.haematobium and S.bovis (Southgate et al., 1986) are all longer than S.margrebowiei.

Ogbe (1985), studying the compatibility of S.margrebowiei with hamsters, mice and gerbils repeated the same experiment. However, he sacrificed his animals on days 50, 60 and 70, and presented his data as number of eggs per gram of tissue. The organs used were the liver, ileum, colon and caecum.

Although Ogbe's (1985) data omits the actual weight of



each tissue, it is reasonable to assume that in hamsters, on average the liver would weigh 3.00g, ileum 1.80g, colon 1.00g and the caecum 0.5g<sub>†</sub> (Auld, 1990). This gives an approximate percentage value of 53.2% in liver and 46.8% of eggs in intestine, which differs markedly to that of Southgate and Knowles (1977) who recorded 80.9% in the intestine and only 18% in the liver. This marked difference probably results from the fact that Southgate and Knowles (1977) included days 30, and 40 in their results, periods during which few eggs would be found in any tissue (since the prepatent period of S.margrebowiei in hamsters is 28-30 days). The inclusion of data from the duodenum and jejunum in the latter study would also influence the final numbers recorded.

During normal passage of the parasite, it was noticed that large numbers of eggs were deposited in the spleen. It was therefore decided to include this organ and instead omit the caecum which proved to be very difficult to clean of faecal material. In the present study, small intestine refers to duodenum, jejunum and ileum. Since Ogbe (1985) also used mice as the definitive host although he used TFI mice and the paddle method of infection while BKTO mice were and the ring method used in our study, a direct comparison has been made.

Table 4.5 The number of eggs per gram of tissue X1000 recorded by A) Ogbe (1985) and B) the present study

A)

Days p.i	% W. rec	liver	ileum	colon	caecum
50	50	32.3	9.5	36.4	3.6
60	42	34.0	8.2	28.3	4.6

B)

Days p.i	% W. rec	liver	S.int	colon	spleen
50	48.2	48.3	54.9	44.0	73.0
60	41.2	71.2	69.5	75.1	134.7

A comparison of the two sets of data, shows the worm recovery was almost identical in our experiments. However, more eggs were recovered in our study compared with those of Ogbe (1985). Basically three reasons could explain these differences as follows, 1) sex, 2) breed of mouse and 3) size of worms. In our study 30 female, BKTO mice were used whereas in the case of Ogbe (1985) TFI mice (sex not indicated) were used. So the difference could be due to sex or strain variations or indeed both. However, the actual influence of the sex of the definitive host on the rate of infection is not clear. Purnell (1966) reported that male hamsters were more susceptible to infection with S.mansoni and S.haematobium whereas Agnew et al., (1988) studying the pathology of S.haematobium in CBA mice concluded that no differences in worm burdens were observed in male or female mice infected when they were 5, 8, 11 and 26 weeks old. More recently, Eloi-Santos et al., (1992) concluded that female CBA/J mice showed greater susceptibility to infection with



S.mansoni showing greater mortality and pathophysiology. These differences are thought to be associated with immunological, physiological and hormonal differences. Female mice had increased humoral responses, and vascular anatomical differences. Furthermore, male castration prior to infection showed similar pathology to that of female mice.

The actual size which female worms attained in the present study could also be an important factor in the differences in the number of eggs deposited in the tissues of BKTO mice and Ogbe's (1985) TFI mice. Ogbe (1985) recorded a maximum female size of 22.8mm from TFI mice, Southgate and Knowles (1977) reported a 33.8mm long female worm from a hamster, while the maximum length of female worms in the present study was 35.00mm recorded from a 60 day post-infected mouse. Locker (1983) pointed out that there is a positive correlation between the female length and the number of eggs contained within the uterus.

Hsu and Hsu (1960) have demonstrated that in the case of S.japonicum infections, the distribution of eggs in different organs is affected not only by the strain of the parasite, but also the species of host and the intensity of infection. Schistosome strains passaged for many generations in laboratory animals are also likely to give different results from those strains freshly isolated from a natural definitive host and passaged for the first time.

Table 4.6 shows the percentage of eggs deposited in the various tissues of hamsters and mice infected with different species of schistosomes. The most striking difference,

compared with previous workers, is the large number of eggs found in the spleen in the present study

Table 4.6 Percentage of eggs belonging to a range of schistosome species recovered from different tissues of the hamster and the mouse.

Species	Host	Liver	Intestine	Spleen
<u>S. leiperi</u> <sup>+</sup>	Hamster	79.5	20.2	<0.3
<u>S. bovis</u> <sup>+</sup>	Hamster	53.1	46.8	0
<u>S. curassoni</u> <sup>+</sup>	Hamster	84.5	15.1	<0.4
<u>S. haematobium</u> <sup>+</sup>	Hamster	95.3	4.7	0
<u>S. margrebowiei</u> <sup>+</sup>	Hamster	18.2	80.9	0.05
<u>S. margrebowiei</u> <sup>*</sup>	Hamster	53.2	46.8	0
<u>S. margrebowiei</u> <sup>*</sup>	TFI mouse	77.6	22.4	0
<u>S. margrebowiei</u> <sup>.</sup>	BKTO mouse	49.19	39.7	12.3

+ Southgate et al., (1977, 1981, 1985).

\* Ogbe (1985), Recalculated from egg/gram.

. The present study.

Previous authors showed that few eggs (<0.4%) occurred in the spleen. However, in the present study, after day 50 large numbers of eggs were recovered from the spleen of BKTO mice representing 12% of the total eggs recorded.

As the duration of the infection increases (Fig. 4.1 and 4.2), the number of eggs found in different tissues increased significantly ( $F = 5.21$  for liver, 12.9 for spleen, 23.24 for small intestine and 9.9 for colon). However, the number of eggs recovered from 55 day post-infected mice (Fig. 4.1 and 4.3) is slightly less than that of the 50 day post-infection group. This finding can be explained by referring to the mortality table (Table 4.4) which indicates that 4 mice died between day 51 and 54 accounting for 57.1% of total death. According to Southgate and Knowles (1977), the period



of peak egg production is around 50-60 days post infection when almost 100% of female worms are gravid and the highest uterine egg counts were recorded. So death was very likely due to very large numbers of eggs being deposited at this time, resulting in the most heavily infected animals succumbing, leaving behind surviving mice with lower worm burdens. The lower average number of worms recorded from the survivors supports this assumption. The lower average number of worms recovered from 55 days post-infection animals means that fewer eggs would be produced. This low egg production is balanced to a degree by the duration of infection, hence more eggs were recovered from the spleen and colon of 55 day post-infected mice than the 45 day post-infection ones. A similar trend can be observed in figure 4.3 where number of eggs per gram of tissue is presented. However, it is clear that more eggs per gram of tissue were deposited in the spleen of the infected animals in the present experiments than has been reported in the previous studies. Examination of the data for percentage of eggs deposited in particular tissues (Fig. 4.4) however, confirms the earlier findings of Southgate and Knowles (1977) and Ogbe (1985) that the liver and intestine are the main organs in which eggs are deposited.

Figures 4.5 and 4.6 clearly show that eggs are deposited in the spleens of infected mice leading to granuloma formation. The presence of giant cells and a large eosinophilia indicate an immunological response by the infected host. Butterworth et al., (1975) have shown that eosinophils are capable of killing schistosomes in vitro. The

spleen is a large lymphatic organ which contains a large numbers of lymphocytes, specialized vascular spaces, a meshwork of reticular cells and reticular fibres, and a rich supply of macrophages. The functions related to the spleen include, lymphocyte production, antibody production, destruction of damaged red blood cells and storage of blood. However, despite the importance of these functions the spleen is not essential to life and is surgically removed in some conditions.

Without carrying out studies concerning the measurement of hypertension, the size and number of granulomata in the liver and spleen and a detailed examination of the vascular system of each strain of mouse used and its relationship with the hepatic portal system (where the adult S.margrebowiei reside) it is very difficult to explain why large numbers of eggs were recovered from the spleens of BKTO mice. It is possible that the large number of eggs recovered throughout this study from different tissues could cause an early blockage of the portal venules, leading to hypertension and development of anastomoses directing the blood flow together with eggs via collateral vessels from the liver to other organs such as the spleen. The actual size and numbers of worms could also initiate hypertension. Since female S.margrebowiei measure 30mm in length and could block the blood flow leading to hypertension and modification of the vascular system earlier than smaller species of schistosomes.

In S.mansoni infected mice, eggs have been recovered from the lungs (Wilson, 1990). This is thought to be



associated with high numbers of eggs in the posterior mesenteric veins of the large intestine leading to the development of collateral vessels in the veins of the rectal plexus particularly between the superior rectal vein and middle inferior rectal vein. This results in the passage of large numbers of eggs directly to the lungs via these veins. A similar network could take eggs to the spleen of mice infected with S.margrebowiei.

Butterworth et al. (1989) reported an interesting observation regarding schistosomiasis in the Kambu district of Kenya. In general, S.mansoni, infections in rural Kenya are mild, causing hepatomegaly in 7-17% of infected children, but hepatosplenomegaly in only 0.2-0.5% of children. In Kambu, in contrast, it was found that while 17% of children had simple hepatomegaly, an additional 16% had hepatosplenomegaly! Moreover, the number of eggs found in the faeces of individuals in Kambu was far greater than those from other areas. The following reasons were given to explain this: the intensity of infection, duration of infection, host genetic differences, parasite strain differences, age at first infection and host nutritional status. Some of these reasons together with those given earlier could apply to our study and explain the presence of eggs in the spleen of infected mice.

As far as the host-parasite relationship is concerned, BKTO mice and S.margrebowiei are quite compatible. This conclusion is derived from the fact that few mice died during the course of this study and perhaps the best indication is

the ratio of male to female worms recovered. Here a ratio of 1:0.87 was observed which indicates normal development. It is generally regarded that in non-permissive hosts normal development is impaired such that male worms greatly outnumber females.



## CHAPTER FIVE

Longevity and infectivity of the cercariae of  
S.margrebowiei.

## CHAPTER FIVE

### Introduction:

The free-living cercariae of all schistosome species are non-feeding organisms which derive their energy from metabolism of endogenous food stores. They must therefore, find a suitable host before their energy reserves are depleted. Anderson and Whitfield (1975) suggested that the longevity of cercariae is directly dependent upon the size of their glycogen reserves and the rate of utilization. Cercarial metabolism of glycogen is an aerobic process requiring oxygen for oxidative phosphorylation and the yield of ATP. In the absence of oxygen, cercariae die within four hours (Olivier et al., 1953).

Reports show that the longevity of cercariae varies considerably. Kuntz and Stirewalt (1946) reported that the cercariae of S. mansoni lived up to 72 hours in spring water, whereas Schreiber and Schubert (1949) concluded that the cercariae of this species had a half-life of 8-16 hours and that all die within 18 hours. Lawson and Wilson (1980) however, give a more "moderate" time of 45-50 hours. Donnelly et al., (1984) reported that the cercariae of S. mattheei, S. haematobium and S. mansoni live for a maximum of 60, 50 and 48 hours respectively. These discrepancies could be due to a number of factors including, experimental procedure, strain of schistosome, used, the type of media and the number of cercariae in a given volume of the medium used. Experimental studies have shown that the quality of water has a major influence on longevity. For example, distilled water is



unsuitable (Frick and Hillyer, 1966), indicating that natural dissolved salts are essential for survival. Fresh copper-free, water is more suitable than the water in which the shedding snails were kept (Stirewalt and Fregeau, 1968), since cercariae are susceptible to bacteria and snail products which act as contaminants. A change in pH from 6.4 to 9.0 increases the longevity of cercariae from 3.5 to 25.0 hours respectively (Asch, 1975) which together with the fact that molluscs require an alkaline environment for healthy growth of the shell could explain why schistosome snails (indeed trematodes) are found in alkaline waters. Chernin and Bower (1971) confirmed that both miracidia and cercariae of trematodes survive and maintain their infectivity when kept in brackish water (as high as 25% sea water). However, the susceptibility of the snail host to pH is obviously of greater significance and importance. B.glabrata, can survive for 35 days in 20% sea water but only for 3 days in 25% sea water (Brumpt, 1941 and Chernin & Bower, 1971). In a series of experiments, Donnelly et al., (1984) realized that the longevity of cercariae in salinities  $\leq 5.25\text{‰}$  (parts per thousand) was longer than that in fresh water. The cercariae of S.mattheei for example lived for 120 hours in 1.75‰ salinity as opposed to 60 hours in fresh water.

The temperature of the waters in which the cercariae live directly affects their longevity and infectivity. Lawson and Wilson (1980) working on the cercariae of S.mansoni, concluded that in general the cercarial longevity decreased as the water temperature increased from 15 to 40°C. The

cercarial stage of schistosomes is poikilothermic and environmental temperature affects its ability to penetrate the definitive host. Initial studies by DeWitt (1965) on the effect of temperature (10-45°C) on the penetration of mice by cercariae of S.mansoni, concluded that the percentage of cercariae that successfully penetrated and matured ranged from 5 to 56%, and that the optimal temperature was 30-35°C

It has been reported that longevity can be prolonged by addition of glucose to the medium (Asch, 1975), this implies that the cercariae somehow utilize this exogenous glucose. Bruce et al., (1969), concluded that the metabolism of exogenous glucose although slow at first, increases 18 hours post-emergence as the levels of glycogen reserves decline.

External environmental factors, as mentioned above, have therefore an important influence on the longevity of the cercariae. However, it is also generally accepted that parasite-related factors also play a major role. Factors such as the number of miracidia used for infecting snails (intensity of infection), the temperature of the water in which the infected snails were maintained and the nutritional status of the snail host can affect not only the number of cercariae shed but also their longevity. This view was reinforced by Stirewalt and Fregeau (1965) who concluded that, the commonly encountered variability in survival, penetration and maturation of schistosomes " cannot be controlled by standardising the environmental conditions".

Factors influencing the longevity of cercariae have obvious implications in the epidemiology of the disease in



infested waters. However, in nature, due to fluctuations in environmental factors and the damaging effects of ultraviolet light together with the presence of natural predators, cercariae probably have a much shorter life-span (Olivier 1966) than ~~those~~ in laboratory situations. The crucial question regarding the life-span of cercariae is: how long do they retain their infectivity?

Olivier (1966) clearly showed that the infectivity of cercariae of S.mansoni declined with time. The percentage of cercariae successfully developing into adult worms in the definitive host, was 12.3% from a 0.6 hour old batch but decreased to 2.3% from a 12.6 hour old and 0.2% from a 30.6 hour old cercariae batch. Donnelly et al., (1984) concluded that no infection of the mammalian host occurred when the cercariae of S.mansoni, S.haematobium and S.mattheei respectively, were maintained in salinities greater than 10.5‰. The overall conclusion was that there was a progressive reduction in cercarial infectivity with increasing salinity. The maximum salinity which cercariae of these species of schistosomes could withstand without affecting their infectivity was 2.5-3‰.

During experimental work, it was noticed that if freshly emerged cercariae were kept in the refrigerator (water temperature 8-12° C), they remained alive for a longer period than if kept at normal culture temperature of 26-28°C and indeed a proportion remained alive even upto 7 days post emergence. An experiment was therefore designed to examine the effect of temperature on longevity and infectivity of

S.margrebowiei cercariae.



## Materials and methods:

### Life-span:

S.margrebowiei cercariae from a large number (50) of infected Bulinus natalensis were collected as described previously (Chapter 2). Five cercariae were individually transferred with a glass pipette into each 2x5 cm glass tube which contained 10 ml fresh (26-28°C) copper-free water. The exact time of transfer was recorded. All the tubes were placed in plastic "lunch packs" and covered with a glass top to avoid evaporation. The life-span of cercariae was recorded under three different conditions as follows:

- a) Kept in a 12h light dark cycle at snail room temperature (26-28°C).
- b) Kept in 24h darkness at snail room temperature.
- c) Kept in 24h darkness in a refrigerator at 8-12°C.

Cercariae were examined every 5 hours and longevity recorded. The criterion of viability was motility of the organism as judged by complete loss of overt movement even when the tube was gently shaken. Thus any moving cercaria was judged to be alive. The experiment was replicated using three separate sheddings of cercariae for each time interval, and 5 tubes were counted for each treatment group.

### Cercarial infectivity:

The infectivity of two different batches of cercariae were compared. Those kept in total darkness in the snail room and those kept in total darkness in the cold. Freshly emerged cercariae were poured into several "lunch packs" containing 300 ml of fresh warm copper-free water. All the containers

were covered, half were placed in the refrigerator and half in darkness in the snail room. When required, these containers were gently taken out and placed under light for 10 minutes. This was sufficient to stimulate the cercariae to swim to the surface. Live cercariae were collected, concentrated and used to infect mice.

Twenty five randomly selected female BKTO (6-8 weeks old) mice were selected and divided into two groups designated A (10 mice) and B (15 mice). Five mice in group A were infected with 0-3 hour old cercariae and 5 with 24 hour old cercariae both of which had been kept in the dark at snail room temperature.

Mice in group B were infected respectively with 24 , 48 and 72 hour old cercariae from the refrigerator (5 mice in each group).

All mice were infected with 100 cercariae using the ring method as described previously (Chapter Two). After 30 minutes, the water in the rings was collected and checked for presence of cercariae. Mice were sacrificed and perfused (Doenhoff et al., 1978) on day 50 post-infection and the number of worms recorded.

#### **Glycogen determination:**

Glycogen determination was carried out to measure the amount of glycogen in the cercariae of S.margrebowiei with time. The technique used was an adaptation of Montgomery, (1957) and Good et al., (1933).

Three test tubes (for each time interval) each with 500 live cercariae per ml of copper-free water were digested with 2ml



of 45% potassium hydroxide solution in a boiling water bath for 1 hour. The mixture was cooled to room temperature and 5ml of cold 95% ethanol added to each tube. The glycogen was collected by centrifugation at high speed for 30 minutes. The supernatant was discarded (any alcohol present can be expelled by heating) and the pellet resuspended in 5 ml cold alcohol again. After the second centrifugation, the pellet, was resuspended in 1 ml distilled water. Finally 0.05 ml of 80% phenol was added to each tube, followed (very slowly) by 2.5 ml of concentrated sulphuric acid. Test tubes were shaken well to mix and left for 1 hour to cool at room temperature. The yellow-orange colour produced in the reaction, was measured at 490nm using a CE 272, series 2 linear ultraviolet spectrophotometer. A blank was prepared by substituting distilled water for the glycogen solution. The amount of glycogen was determined by reference to a standard curve previously constructed for pure glycogen (Sigma, type IX: from bovine liver).

Unfortunately due to shortage of cercariae, the glycogen determination was performed on cercariae which were shed at different times to those used in the infection of mice.

**Results:**

**Life-span:**

Table 5.1 The percentage of cercariae alive in different conditions at 5 hourly intervals. A total of 75 cercariae were examined at each time interval.

Time (Hours)	Percentage cercariae alive		
	12hL/12hD	24hD	24hD( 8-12°C)
0	100	100	100
5	98.6	100	100
10	93.3	97.3	94.6
15	86.6	90.6	93.3
20	80.0	86.6	92.0
25	54.6	68.0	92.0
30	45.3	52.0	77.3
35	28.0	26.6	73.3
40	14.6	18.6	66.6
45	8.0	13.3	64.0
50	4.0	9.3	62.6
55	2.6	5.3	60.0
60	2.6	5.3	60.0
65	1.3	0.0	57.0
70	1.3	0.0	57.0



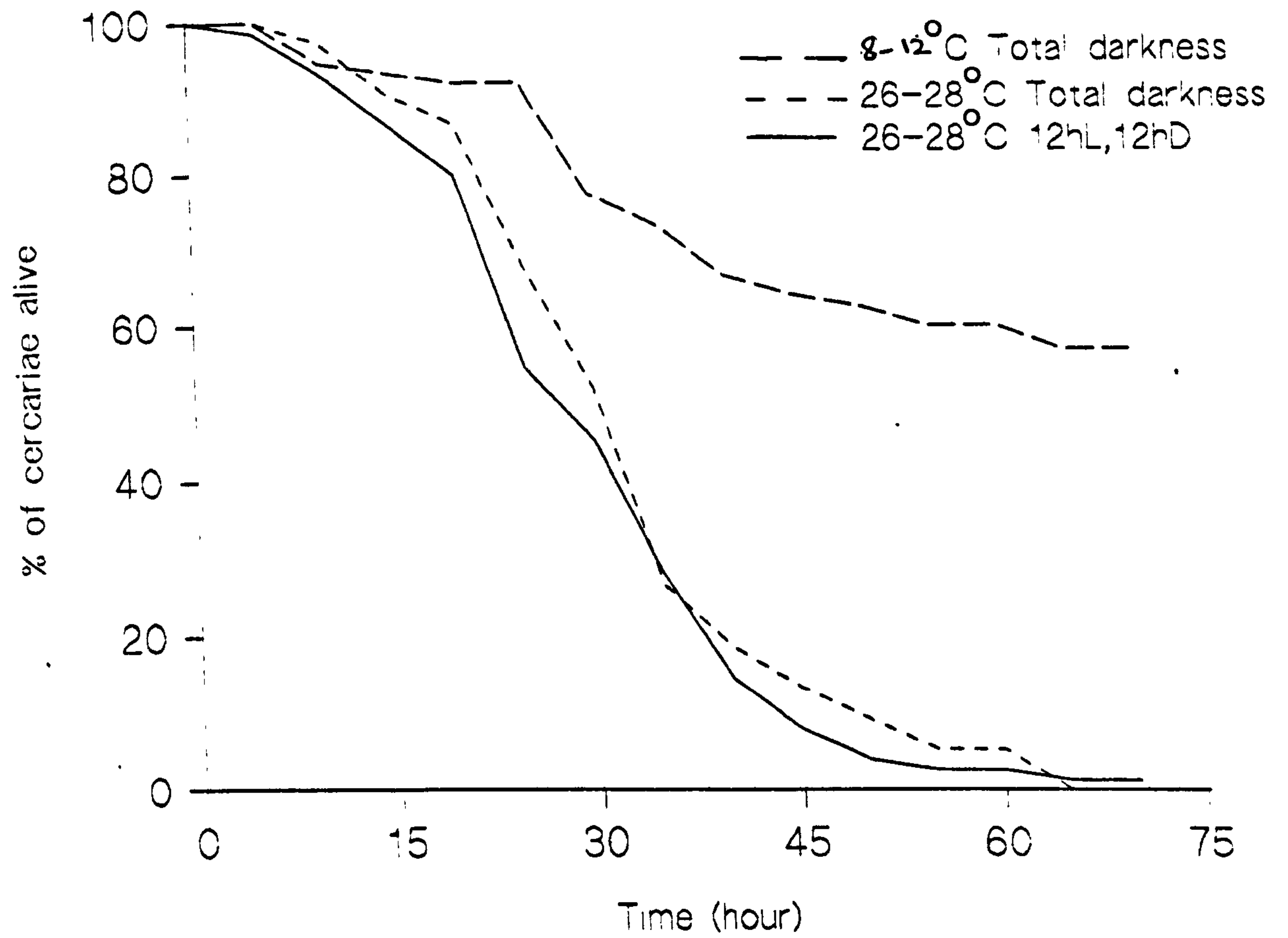


Figure 5.1 The life span of cercariae kept in three different conditions.

### Infectivity:

Upon examination of water in the rings after infection, it was found that in all cases 90-95% of the cercariae penetrated the abdominal skin of the host. However, not all the cercariae which penetrated matured to adult worms.

Table 5.2 shows the mean worm recovery with aging cercariae.

Age of cercariae (hours)	No. of mice	Mean worm burden( $\pm$ s.d.)	% worm burden reduction
0-4 (26-28°C)	5	42.4 $\pm$ 11.0	0.0
24 (26-28°C)	5	10.8 $\pm$ 3.3	74.5
24 ( 8-12°C)	5	31.2 $\pm$ 2.5	26.4
48 ( 8-12°C)	5	25.2 $\pm$ 6.2	40.4
72 ( 8-12°C)	5	19.8 $\pm$ 5.7	53.3

### Glycogen determination:

Table 5.3 The mean amount of glycogen per cercaria as it ages.

Age of cercariae (hours)	Mean amount of glycogen/cercaria (ng) ( $\pm$ s.d.)
0-4 (26-28°C)	23.16 $\pm$ 3.40
24 (26-28°C)	8.50 $\pm$ 1.39
24 ( 8-12°C)	16.16 $\pm$ 2.08
48 ( 8-12°C)	11.75 $\pm$ 3.19
72 ( 8-12°C)	8.16 $\pm$ 1.87



## Discussion:

Once the cercaria has escaped from the snail it has a limited time in which to find and penetrate a suitable host. In order to maximize the chance of infection, cercariae have adopted various host-finding and identification strategies which are specific to the particular species of host. The cercariae of S.mansoni for example, infects man in deep, clear, waters where sensing the chemical and behavioural signals from the skin surface is more easily achieved. In contrast the cercariae of S.spindale, have adapted to shallow, muddy, waters which interfere with chemical signals, so thermal signals instead are detected. The cercariae of S.japonicum on the other hand, does not respond to specific chemical, thermal, and water turbulence signals and finds its host in a passive manner (Haas et al., 1987). However, in most cases a combination of water turbulence, heat and chemical signals are used. To save energy, all cercariae exhibit intermittent swimming patterns comprising an active backward (tail first) swimming phase, and a passive floating slowly sinking phase. As cercariae age, there is a reduction in this active phase and they sink to the bottom of the vessel in which they are kept and eventually die.

The graph of longevity of cercariae (Fig. 5.1), clearly shows that there are no significant differences between the two groups kept in the snail room. Using a  $X^2$  test, the p values are as follows: 0.07, 0.42, 0.22 and 0.53 for time 24, 30, 50 and 60 hours respectively post emergence. However, when compared to those kept in the cold, significantly more

cercariae remained alive at low temperatures at any given time ( $p=1.00$ ). Since light had no significant effect on longevity, the infectivity of cercariae kept in the cold (dark) were compared to those kept in the dark in the snail room.

Although not shown in the results, during the course of this study it became clear that high density had an adverse effect on the longevity of cercariae, care was taken to avoid overcrowding.

The results of the infectivity of cercariae with age as measured by the number of adult worms recovered by day 50 post-infection (Table 5.2), demonstrates that cercariae lose their infectivity with age. This is in agreement with previous findings (Stirewalt and Fregeau, (1968), Olivier (1966), Ghandour and Webbe (1973) and Wilson and Lawson (1983)). A one way analysis of variance showed that this reduction was significant at the 0.05 level ( $F = 16.56$  which is higher than the critical value of 2.87). Furthermore, using Students' 't' Test it became clear that there was no significant difference in the infectivity of fresh cercariae and 24 hours old cold ones ( $p = 0.058$ ), whereas significant differences were observed in other treatments i.e fresh and 24 hour old cercariae ( $p = 0.0003$ ), fresh and 48 hours old cold cercariae ( $p = 0.016$ ) and fresh and 72 hours old cold cercariae ( $p = 0.0036$ ). The results from Table 5.3, concerning the amount of glycogen in the cercaria at different ages, indicates that infectivity is somehow related to the levels of glycogen available to the cercaria. Table



5.4 below, summarizes this finding in terms of percentage reduction in worm burden and glycogen levels when compared to the control group (fresh cercariae).

Table 5.4 A comparison of the relationship between infectivity with glycogen levels.

Age of cercariae (hours)	% reduction in worm burden	% reduction in glycogen level
0-4 (26-28°C)	0.0	0.0
24 (26-28°C)	74.5	63.2
24 (8-12°C)	26.4	30.2
48 (8-12°C)	40.5	49.2
72 (8-12°C)	53.3	64.7

The reduction of 63.2% in the glycogen level of a 24 hour old cercaria (compared with a newly emerged cercaria) compares favourably with that reported by Bruce *et al.*, (1969) who found that 18 hour old cercariae lost 75% of their glycogen and Lawson and Wilson (1980) who reported that 24 hour old cercariae lost around 80% of their glycogen reserves.

The most interesting observation made in the present study is that the age of the cercariae did not affect their ability to penetrate the host. This agrees with the findings of Lawson and Wilson (1980) on *S.mansoni* who, using both the tail immersion and ring methods of infection, reached the same conclusion. However, Oliver (1966) using the immersion method of infection, reported that the percentage of cercariae recovered from the infection tube, increased from 25.1% from a 0.6 hour old batch to 75.1% from a 30.6 hour old batch.

There is no doubt that the ring method of infection as used here, provides the cercariae with an easy route for penetration (shaved abdomen and a large number of cercariae in a given area) with an immediate contact with the host skin. The infective cercariae would therefore require less energy to find the host. From the results of the penetration and adult worm recovery experiments, it is clear that the mechanisms involved in the penetration of cercariae are somewhat different to those involved in their further migration and maturation. During the present experiments no attempts were made to examine the host skin or the lungs for the presence of dead schistosomula. However, previous work by Ghandour and Webbe (1973) showed that the percentage of dead schistosomula from the skin of mice increased with the age of cercariae. An obvious explanation for this observation is that by the time the parasite had reached the skin, it had exhausted all its energy reserves and therefore died before attaining adulthood. The fact that the glycogen reserves of cercariae decrease with age support this view. Furthermore, Coles (1973) and Lawson and Wilson (1980) have calculated that the tail of the cercaria contained half of its total glycogen content, and as the cercaria penetrates the skin, it uses both the tail and the body. The tail is lost during penetration leaving enough glycogen in the newly transformed schistosomulum to find its way to the lungs. In older cercariae, the level of reserve glycogen is markedly reduced, and the parasite is unable to migrate to the lungs and dies in the skin.



The penetration of the skin by cercariae is a rapid process (Stirewalt and Hackey, 1956) which is facilitated by secretions from the post and pre-acetabular glands (Stirewalt and Dorsey, 1974). The presence of the enzyme hyaluronidase in cercarial extracts was first reported by Levine et al., (1940). Stirewalt (1973) identified the main protease activity found in cercariae as a product of the pre-acetabular glands. Stirewalt and Kruidenier (1961) showed that the contents of the cercarial pre and post-acetabular glands, quickly became exhausted during penetration of the stratum corneum and entry into the keratogenous zone of the epidermis. With regard to the molecular weight of this protein, it is now believed that both Mckerrow's 28KD protein and Marikovskey's 30KD protein are the same and it is now referred to as a 28KD serine elastase (Fishelson et al., 1992). Significantly, Cohen et al., (1991) selected protease inhibitors which, by inhibiting the cercarial elastase, prevented cercarial penetration of the skin. However, age and cooling the cercariae did not affect the activity of this enzyme. The fact that during penetration the cercaria empties all the contents of its pre and post-acetabular glands (Stirewalt and Kruidenier, 1961) suggest that the schistosomula may possess other enzymes that enable them to burrow their way through tissues and into blood vessels. Keene et al., (1983) studied the enzymatic activity of young schistosomula, with respect to their ability to hydrolyse an extracellular matrix derived from vascular smooth muscle cells. Over the first 24 hours, enzymatic activity overlapped

and appeared identical with the cercarial elastase, but after 1 day in culture, a second, predominant and distinct schistosomulum proteolytic enzyme, was detected and characterized as a metalloenzyme which was thought to facilitate subsequent progress of the parasite through the vascular system.

Since most cercariae, regardless of their age, penetrate the host's skin and there are different enzymes involved in the penetration and migration phases, it can be concluded that the enzymatic activity of live cercariae is retained and the failure of schistosomula to mature is largely due to lack of energy.

The results of the present study indicate that when cercariae are kept in cold water, they retain their glycogen reserves for longer periods and thus lived longer. In the introduction to this chapter, other factors which prolong the life of cercariae were mentioned. Our findings indicate that glycogen is the main source of energy. It is involved not only in the physical movement of the cercaria but also in metabolic processes, ionic and osmotic regulations (Lawson and Wilson, 1980). When kept at low temperature, cercariae tend to sink to the bottom of the container and become motionless. In this way energy is conserved resulting in a prolongation of life.

Schistosomula which die in the tissues are destroyed by immune mechanisms of the host. Although a large number of older cercariae die in the skin of their host, the vital question is what percentage of the successful penetrators



migrate as far as the lungs and die there? The importance of knowing this becomes clear when one considers the consequences of irradiated-attenuated cercarial infection in experimental animals. It is well known that attenuated cercariae, stimulate high levels of protective immunity in experimental hosts as opposed to normal cercarial infections which require the presence of adults and the laying of eggs, (Radke and Sadun, 1963, Smithers and Terry, 1965b, Bickle et al., 1987 and Moloney et al., 1987). Bickle (1981) showed that survival of schistosomula within the host for 1-2 weeks was essential for induction of optimal resistance in mice to infection. Detailed studies on the nature of infection, the percentage of larvae dying within the skin, lung and portal system of experimental mice are needed. This information could explain whether older and therefore less infective cercariae could be used to promote a protective immune response without resulting in patent adult infection.

## **CHAPTER SIX**

**Cross-reactivity of antigens from different stages in the life-cycle of schistosomes.**



## CHAPTER SIX

### Introduction:

The success story of the control of schistosomiasis in China where the number of schistosome cases was reduced from 12 million (in 1950) to below 1 million in recent years, has not been repeated and indeed the new outbreaks of the disease in Senegal (Talla et al., 1990) and also new settlements in Kenya (Butterworth, 1992), clearly demonstrate that the disease is spreading. It is now generally accepted that total eradication of schistosomiasis is an impractical task. The aim of any control programme is therefore to reduce the transmission to levels below which intensities of infection are insufficient to allow the development of morbidity. Complete ~~control~~ of schistosomiasis, however, could be achieved by vaccination, and perhaps stronger efforts should be made to develop a vaccine to control/eradicate this parasite.

Two facts about the disease are very interesting. Firstly, the parasite does not increase in numbers within its final host and by itself causes no damage (most of the morbidity associated with schistosomiasis infection is due to the deposition of eggs in tissues); secondly, a slow development of immunity with age in patients continuously exposed to the parasite has been reported (Taylor & Bickle, 1986). According to Butterworth et al., (1987) the susceptibility to infection of young children is associated with high levels of "blocking antibodies" against the carbohydrate epitopes in eggs and schistosomula. The

"blocking antibodies" hypothesis postulates that during primary infections in man, antigens from trapped eggs are the main immunogens. These egg antigens, which include a major polysaccharide component (K3) (Dunne et al., 1987), bear carbohydrate epitopes that are also expressed on glycoproteins on the surface of <sup>the</sup>schistosomulum. Antibodies of an inappropriate isotype, elicited in response to egg polysaccharide antigen, may cross-react with the schistosomula of a challenge infection, and prevent the binding of anti-schistosomulum antibodies, of an appropriate effector isotype, with specificity for the same or closely adjacent epitopes. As the individual ages, the levels of these ineffective, "blocking" antibodies decline allowing schistosomicidal antibodies to become more effective. A vaccine that could boost this partial immunity should therefore reduce the number of adult worms present. As mentioned earlier the pathology of the disease is such that 100% protection may not be necessary.

The success of a live attenuated vaccine in protecting against parasitic bronchitis of cattle caused by Dictyocaulus viviparus (Jarrett et al., 1960), led to similar studies concerning the cercariae of schistosomes. Cercariae attenuated by exposure to irradiation from an X-ray source, cobalt 60 or ultraviolet light, have proved successful in inducing resistance in mice (Radke and Sadun, 1963, Bickle et al., 1979), rats (Smithers and Terry, 1965b, Moloney et al., 1987), and sheep and cattle (Taylor et al., 1977). Other interesting observations concerning this technique are as



follows: the level of resistance to subsequent challenge is influenced by the radiation dose and not the number of larvae or frequency or the route of administration of cercariae (Bickle et al., 1979). Furthermore the acquired resistance is species-specific (Bickle et al., 1985, Moloney and Webbe, 1987). However, it would be unacceptable to use such live preparations on man. Nevertheless, irradiated larvae are being used to immunise cattle against S.bovis in Sudan (Taylor, 1980, and Taylor and Bickle, 1986) and S.japonicum in China (in Taylor and Bickle, 1986).

Partial protection has also been achieved by the use of non-living antigens derived from different stages in the life-cycle of the parasite. The ever increasing list of these antigens (Colley and Colley, 1989) with molecular weights ranging from 22 to 200 KDa is encouraging. Among these, P28 or GST (glutathione S-transferase) has proven the most successful. P28 was derived from protein secreted by the excretory system of the adult worm, and two injections of 50 $\mu$ L in rats and one of 50ng in mice, led to a level of protection ranging between 50 to 70% in rats and 39 to 43% in mice (Balloul et al., 1987). During further studies, it became clear that even in the partially protected animals, there was a significant decrease in the size and volume of egg granulomas in the livers of infected animals. Furthermore in the monkey Patas patas, immunized with P28 against S.haematobium infection, a marked difference was observed between immunized and control animals in terms of urinary bladder lesions (Capron et al., 1987, Capron et al., 1992).

The immune effector mechanisms involved in vaccine-induced immunity include both the humoral and cellular immune responses. Dean et al., (1981), showed that resistance conferred by vaccination could be transferred to naive mice by parabiosis (the formation of an anastomosis, enabling the parabiotic partner to receive humoral and cellular components of the blood from its partner). Furthermore Sher et al., (1982) demonstrated that athymic nude mice, and mice depleted of B-lymphocytes, failed to develop resistance after vaccination. The importance of the humoral immune response was also demonstrated in a series of experiments where passive transfer of sera from vaccinated animals was shown to protect naive recipients from infection (Bickle et al., 1985, Mangold and Dean, 1986).

Complement although important in vaccine-induced immunity in rats (Vignally et al., 1988) is not involved in the process in mice. Sher et al., (1982) showed that normal levels of vaccine-induced resistance occurred in mice genetically deficient in the fifth component of complement and also those de complemented with COF (cobra venom factor).

The involvement of cell-mediated immunity and a major role for lymphokine-activated macrophages in vaccine-induced immunity became clear when an inbred mouse strain P/N-J (P) (which has a defect in macrophage function) failed to develop vaccine-induced immunity (James and Sher, 1983).

Antigenic cross-reactivity within the different stages of the schistosome life-cycle including antigens from the intermediate snail host is a common feature in



schistosomiasis. Von Lichtenberg et al., (1963), reported that mice immunized with eggs, also developed antibodies that reacted with the cercarial surface. Capron et al., (1965) using immunoelectrophoresis (IEP) reported four antigens in S.mansoni adult worms that reacted with an anti-B.glabrata anti-serum. Capron et al., (1969) also showed that human patients developed three precipitin arcs reactive with B.glabrata. Jackson and De Moor (1976), reported that the cercariae of S.haematobium, have antigens in common with their intermediate snail host B.africanus. They postulated that once inside the definitive host, the cercariae might induce an immune reaction to the snail antigen. Their results, indicated that this was indeed the case. Anti-snail antibodies were present in greater amounts in infected than non-infected individuals. Vieira and Kusel (1991), also reported the presence of antigen that cross-reacted with snail tissues or cercarial glycocalyx on the surface of freshly transformed schistosomula, cultured schistosomula, lung stage and adult worms. Furthermore significant anti-Keyhole Limpet Haemocyanin (KLH) antibodies were also reported in schistosome patients Markl et al., 1991) indicating that this cross-reactivity is wider than was previously thought (Dissous et al., 1986).

As mentioned earlier most antigens used to elicit an effective immune response by the host, are derived from non-living components of different stages in the life-cycle of schistosomes. The possibility of immunization using antigens shared between schistosomes and their snail host has been

studied by Roder et al., (1977), Farouk and Amira (1979), Stein and Basch (1979) and Diehl et al., (1987). The cross-reactivities between the snail host and different stages in the life-cycle of schistosomes reported here and encountered during the present study, led to an experiment designed to evaluate the potential of snail hepatopancreas as a vaccine. However, due the shortage of B.natalensis and S.margrebowiei derived antigens and sera, this study was carried out on B.glabrata and S.mansoni. Furthermore, cross-reactivities between snail hepatopancreas and Sm480 was tested. Sm480 is a 480 KDa glycosylated S.mansoni egg antigen (Sm480), which hydrolyses chymotrypsin-like substrates, gives partial protection to mice against S.mansoni challenge in passive transfer experiments (Curtis, 1991). Sm480 is currently under further investigation in our laboratory regarding its potential as a vaccine (Doenhoff personal communication).



## Materials and Methods:

The techniques relevant to this work are outlined below.

### Parasite species:

A Puerto Rican isolate of S.mansoni and its snail host, Biomphalaria glabrata have been maintained in the laboratory since 1990. S.margrebowiei was maintained as described previously (Chapter 2).

### Experimental animals:

Out-bred Taylor's Original (TO) and out-bred coloured mice were used for the laboratory maintenance of both parasites. TO mice were also used for experimental purposes. The rabbits used for the production of antisera were Half Lops bred on site.

### Infection of mice:

Infections were carried out using the ring method (Chapter 2), and mice were perfused when required (Chapter 4)

### Bleeding of mice:

For the collection of chronic, normal and immunized mouse sera, mice were bled under ether anaesthesia via the retro-orbital venous plexus with a drawnout capillary tube which had been rinsed in citrated saline. The blood was incubated for 1 hour at 37°C, kept at 4°C over night and the serum was separated from the clot by centrifugation at 1000g for 10 minutes and stored at -20°C.

### Egg antigen:

Forty mice were infected either with 400 cercariae of S.mansoni or S.margrebowiei. On day 42 post-infection, these animals were injected with 2.5 mg of hydrocortisone acetate

to reduce the granuloma around the eggs. All mice were sacrificed and portally perfused by day 45. The liver and intestine and in the case of S.margrebowiei, the spleen were removed, cleared of fatty tissue and faecal matter and mechanically homogenized. The resulting homogenate was suspended in one litre of 1.8% saline and 1g of pancreatic trypsin (Sigma Chemical Co.). This was incubated for 2 hours at 37°C with regular agitation. The suspension was passed through a series of sieves with mesh sizes 500, 300 and 180µm. This filtered suspension was finally washed repeatedly by centrifugation at 200g for 5 minutes (each time the supernatant was discarded and eggs resuspended in 1.8% saline), until the eggs were totally free of contaminating host tissue materials. The final two washes were carried out in PBS pH 7.2. The eggs were next homogenized and the homogenate centrifuged at 12,000g for 1 hour, the supernatant being aliquoted and stored at -20°C as soluble egg antigen (SEA). The protein content of each batch of SEA was determined using a kit prepared by Bio-rad U.K Ltd. based on the method of Lowry et al., (1951) and was approximately 12.5 mg/ml for S.mansoni and 7.5 mg/ml for S.margrebowiei.

#### Snail antigen:

Soluble hepatopancreas antigens (SHA) from Biomphalaria glabrata (1.5-2cm in diameter) were prepared by the removal of the hepatopancreas and mechanically homogenizing it in normal saline (200µl per three hepatopancreas). The homogenate was then centrifuged at 12,000 g for 15 minutes; the supernatant was aliquoted and stored at -20°C as either



infected SHA (I), or uninfected SHA (U). The protein content of each batch was determined by the method of Lowry et al., (1951) and was approximately 1.5 mg/ml for infected and 2.00 mg/ml for uninfected SHA. This difference was due to the bigger size of the hepatopancreas of the uninfected snails. Soluble hepatopancreas antigens from Bulinus natalensis snails were similarly (200 $\mu$ l saline per nine hepatopancreas<sup>es</sup>) prepared.

#### Immunological techniques:

The methods employed in the work presented here are described below. The recipes for the buffers used are presented in the appendix.

#### Immuno-electrophoresis:

The immuno-electrophoresis (IEP) method used was that described by Graber and Williams (1953). The separation of protein mixtures by electrophoresis is based on the principle that a molecule in solution at any pH other than its isoelectric point has a net average charge, which causes it to move in an applied electric field. Electrophoretic separation of the antigens which are placed in holes in a buffered agar plate is followed by diffusion of antibody placed in troughs cut beside the antigen hole and parallel to the direction of the electric field. The diffusion of the antibody towards antigens which have been dispersed lengthwise through the agar results in the formation of precipitin arcs between the trough and the line of antigen migration. The immuno-electrophoresis plates were prepared with 12 ml of 1.5% agar (agar Nobel Difco labs) in 0.075 M

barbitone buffer pH 8.6 (see apendix).

The holes and antibody troughs were cut using a template from Miles Laboratory Ltd, and the plates placed in the electrophoresis tank. Approximately 5-10  $\mu$ l of antigen was added to the holes and electrophoresis was performed for 3 hours with a constant potential difference of 5 volts/cm applied across the plate (30 mA/plate at a voltage of 42 V). After electrophoresis, the troughs were filled with the antiserum to be tested, and after a few hours when the serum had diffused into the agar, the plate was placed in a humidity chamber and incubated overnight at 37°C. The precipitin lines formed were examined over indirect light and photographed.

#### **Immunodiffusion:**

This method, developed by Ouchterlony (1958), is commonly used for the evaluation of whether the molecules share antigenicity with each other. This method involves the use of agar plates with wells for both antigen and antibodies. The agar plate was prepared as before. The antigen and antibodies were put into appropriate wells and left for a few hours to diffuse into the agar. The agar plate was next put in a humidity chamber and incubated at 37°C overnight. The resultant immunoprecipitin lines were photographed.

#### **Immunization of mice:**

24 male (4-6 weeks old) BKTO mice were divided into 4 groups. Each group was immunized as follows:

A: was immunized with hepatopancreas of infected (15-18 days



post infection), Biomphalaria glabrata containing sporocysts.

B: with hepatopancreas of uninfected B.glabrata.

C: with Freund's adjuvant.

D: non-immunized controls.

For the immunization of mice in groups A and B, the antigens were prepared as described above. Mice were first injected subcutaneously with a 0.2ml emulsion containing 50 $\mu$ g of protein of the antigen, diluted in 50 : 50 saline and Freund's Complete adjuvant. After two weeks, four subsequent boosts (every 10 days) were administered with a 0.1ml emulsion containing 25 $\mu$ m of protein of the antigen, diluted in 50 : 50 saline and incomplete Freund's adjuvant.

Group C, received a 50 : 50 mixture of saline and complete Freund's adjuvant (0.2ml) for the primary injection and 4 subsequent boosts with a 50 : 50 mixture of saline and incomplete adjuvant (0.1ml). Before infection all mice were bled<sup>and</sup> the sera analyzed for cross reactivity responses in IEP, Immunodiffusion and Western blot. After the last boost, all mice were exposed to 200 cercariae of S.mansoni by the ring method. On day 42 post-infection, mice were perfused and worms recovered, the livers from mice were digested in 4% potassium hydroxide for egg counts as described in Chapter four.

Preparation of monospecific anti-Keyhole Limpet (Megathura crenulata) haemocyanin sera in a rabbit:

A rabbit was subcutaneously injected with a mixture of haemocyanin (KLH, Sigma) dissolved in saline (1mg/ml) and 1ml Freund's Complete adjuvant. After two weeks, subsequent

(weekly) boosts (1ml) were given in Incomplete Freund's adjuvant. After 5 injections, a serum sample was taken and tested in immunoelectrophoresis with dissolved haemocyanin for the intensity and degree of monospecificity of the response. The rabbit was next serially bled from the ear until 150ml of the serum was collected. Finally the rabbit was exsanguinated by cardiac puncture and the serum retrieved was pooled with that from the serial bleeds. The pooled rabbit serum was aliquoted into 5ml amounts and stored at -20°C.

#### Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

Electrophoretic analysis of complex mixtures of proteins using polyacrylamide gel as a supporting material in the presence of sodium dodecyl sulphate (SDS), is commonly used and by varying the concentration of the polyacrylamide gel, different resolution ranges of molecular weights is obtained. In the presence of SDS, proteins bind the SDS, and become negatively charged and have a similar charge : weight ratio. So when SDS-coated proteins are placed in an electric field, their spatial separation will depend only upon their size and shape.

The SDS-PAGE method used here was an adaptation of that described by Studier (1973) which incorporated the method of Laemmli (1970). SDS-PAGE was run on a mini-Protean 11 dual cell (Bio-Rad) which allows rapid separation of the proteins in relatively small gels. Glass plate sandwiches were assembled using a 4-screw clamp assembly, then transferred to



the alignment bar of the separate casting stand. The clamp screws were loosened, the plates and the spacers were aligned, the screws were re-tightened, and the glass plate sandwich assembly was snapped into one of the two casting slots of the casting stand. A resolving gel (12% acrylamide) was made up from 3.8ml of the 30% acrylamide stock, 2.5ml resolving buffer and 3.7ml of distilled water (see appendix). The mixture was then de-gassed for 10 minutes since oxygen causes polymerization of the gel, and once de-gassed the mixture was slightly warmed with running hot water. Then 100 $\mu$ l of SDS 10% stock, 100 $\mu$ l of ammonium persulphate and 10 $\mu$ l of N,N,N',N'-tetramethylethylene diamine (TEMED) were added. The gel was poured into the assembled mould and a few drops of butanol were added which levels the gel surface during polymerization. After polymerization the butanol was washed away with distilled water and the surface of the gel was dried using a Whatman No1 filter paper. The stacking gel was then made up (3% Acrylamide) from 0.5ml stock acrylamide, 2.5ml of stacking buffer and 2ml of distilled water (see appendix). The gel was de-gassed and warmed as before and then 50 $\mu$ l of SDS were added along with 75 $\mu$ l of ammonium persulphate and 10 $\mu$ l TEMED. The gel was quickly dispensed and layered above the resolving gel and 0.75mm thick teflon comb was lowered into it to form the sample wells. Once set the comb was removed leaving lanes or a continuous trough into which the antigens would be loaded.

Preparation of native samples was done by adding 1 $\mu$ l of 36mg/ml iodoacetamide for every 10 $\mu$ l sample and keeping the

mixture in the dark for 30 minutes. Then for every 20 $\mu$ l of sample, 5 $\mu$ l of loading buffer was added and this was boiled for 2 minutes.

The bottom chamber of the electrophoresis tank was filled with bottom tank buffer and the upper chamber between the plates was filled with run buffer (see appendix). 10 $\mu$ l of the samples were then loaded using a 100 $\mu$ l Hamilton syringe (Scientific Supplies Ltd) into lanes. Molecular weight standards (Pharmacia), were applied to the first or end lane of each gel for reference. The markers were 14kDa, cytochrome c; 20kDa, soyabean trypsin inhibitor; 30kDa, carbonic anhydrase; 43kDa, ovalbumin; 67kDa, bovine serum albumin and 94kDa, phosphorylase B. The apparatus was connected to a BIO-RAD power pack which was set to deliver 200volts for 45 minutes, or until the bromophenol blue in the sample buffer had ran off the bottom of gel. After electrophoresis, the stacking gel was removed and discarded.

#### Western blotting:

The Western blotting technique used was that of Pekkala-Flagan & Ruoslahti (1982), which was a modification of the method of Towbin et al., (1979). After electrophoresis, and the separation of proteins, a sheet of nitrocellulose paper (NCP) (Anderman & Co. Ltd.) was cut 1cm longer on each side than the size of the gel, wetted in distilled water and equilibrated in transfer buffer (see appendix) for 30 minutes. Two pieces of Whatman 3mm filter paper were cut to a size slightly larger than the size of NCP and soaked in transfer buffer. A blot 'sandwich' was assembled in the



following order: perforated perspex support, 'Scotch Brite' absorbent pad, Whatman paper, SDS-PAGE gel, NCP, Whatman paper, 'Scotch brite' pad, perspext support. The 'sandwich' was assembled under transfer buffer, making sure that no air bubbles were trapped.

An electroblot tank (Bio-rad) was filled with transfer buffer and chilled to 6°C using a coil containing circulating coolant, connected to a cooler [LKB]. The assembled sandwich was clamped shut and transferred in the electroblot tank such that the gel was cathodal to the NCP, which in turn was anodal to the gel. The tank was connected to a power supply set to deliver 35 volts and 0.55 amps for 3 hours. When blotting was complete the unit was disassembled to leave the gel resting on the NCP. The lane containing the marker was cut out and silver stained. This was done by dissolving 200mg of trisodium citrate and 80mg of ferrous sulphate in 9.9ml of distilled water in a universal bottle. The marker lane was put into the mixture. Finally 100µl of 20% silver nitrate was added. The bottle was shaken and once the markers were stained clearly, the paper was removed and washed in distilled water. The rest of the NCP paper was covered with tween transblotting solution (TTBS) (see appendix) containing 1% dried milk (Marvel) for 1 hour to prevent non-specific binding of immunoglobulins to the sites on the NCP not bound by antigen. The NCP paper was then washed three times with TTBS for 15 minutes and if required, each lane was cut out and treated as follows: each strip was incubated with an appropriate (1/250 or 1/500 dilution) primary antibody for 90

minutes with constant agitation. The strips were next washed three times in TTBS, and the appropriate peroxidase labelled secondary antibody (1/1000 dilution) was added. After 3 hours the NCP was washed as before and finally the substrate solution (see appendix) was added. The protein bands were left to develop in darkness for a few minutes and the blot was given a final wash in distilled water. The blots were then photographed and stored at  $-20^{\circ}\text{C}$ .



## Results:

### Cross-reactivities:

In immunoelectrophoresis (IEP), two different anti-Sm480 (worm stage) rabbit sera reacted with the soluble hepatopancreas antigen (SHA) of infected (I) and uninfected (U) Biomphalaria glabrata. The infected SHA, although precipitated an extra arc, failed to react with one batch of anti-Sm480 rabbit serum (Fig. 6.2a). Anti-K3 (S.mansoni egg antigen) rabbit sera also reacted with infected SHA (result not shown) and uninfected SHA. Most batches of SHA precipitated a cathodal migrating arc, however, in a few cases an anodal arc was also formed (Fig. 6.2b). Although anti-Sm480 rabbit sera reacted with both S.mansoni SEA and uninfected SHA, the arcs were visibly different (Fig. 6.2c) and furthermore, whereas the egg antigen precipitated by the rabbit sera hydrolysed a chromogenic substrate (N-acetyl-DL-Phenylalanine  $\beta$ -Naphthyl Ester and Fast Blue B salt) used to detect chymotrypsin-like enzymes, the snail antigen did not, (results not shown).

IEP studies also indicated that anti-S.mansoni rabbit sera reacted with SHA from uninfected Bulinus natalensis snails (Fig. 6.3a), these included anti-K3 and anti-Sm480 (egg stage).

Immunodiffusion studies using Keyhole Limpet Haemocyanin (KLH, Sigma) dissolved in normal saline, Barbitone pH 7.2 and Barbitone pH 9.6 showed no cross-reactivities with a large battery of rabbit sera raised against different S.mansoni antigens, (results not shown). However, IEP studies revealed

that when KLH was dissolved in barbitone buffer pH 9.6, an anodal precipitate was formed with anti-KLH rabbit sera (Fig. 6.3b), moreover, cercarial transformation fluid (CTF), (the medium in which S.mansoni cercariae were mechanically transformed to schistosomula) also reacted with anti-KLH rabbit sera forming a cathodal precipitant (Fig. 6.3b, well 2).

Western blot analysis which is more sensitive revealed that there were strong cross-reactivities between uninfected SHA of B.glabrata, and various batches of anti-Sm480 rabbit sera (lanes 4,5,7,8,9 and 10) which gave similar results, anti-K3 rabbit sera (lane 3) with 3 prominent protein bands with molecular weights of 75-85 kDa, anti-KLH rabbit sera (lane 2) with a strong response and anti-TCA CTF rabbit sera (lane 1). Normal rabbit serum (lane 6) did not recognize this antigen (Fig. 6.4a). KLH dissolved in barbitone pH 9.6 was recognized by rabbit sera raised against KLH, trichloroacetic acid treated CTF (TCA CTF) and K3. All three recognized proteins with molecular weights of 67-94 kDa. Both normal and anti-Sm480 rabbit sera did not recognize KLH (Fig. 6.4 b). SHA from B.natalensis was also recognized by rabbit anti-sera raised against S.mansoni derived antigens as follows, K3, Sm480 and TCA CTF. By direct comparison of lanes 1 with 5, 2 with 7, 3 with 8 and 4 with 6, it becomes evident that B.glabrata and B.natalensis share many common antigens, which are recognized by anti-K3, anti-Sm480 and anti-TCA CTF rabbit sera respectively. Most of these shared antigens have molecular weights of 67-110 kD (Fig. 6.4 c).



### Immunization experiments:

Using IEP, the reactivity of sera from mice immunized with infected and or uninfected SHA of B.glabrata, were tested with uninfected (U) SHA and infected (I) SHA. Sera from mice immunized with uninfected SHA precipitated two clear arcs with both infected and uninfected antigens, whereas sera from mice immunized with infected SHA only precipitated one arc. No cross-reactivity was observed with normal mouse serum (Fig. 6.5 a). Immunodiffusion studies indicated that the serum of each mouse immunized with either infected SHA or uninfected SHA reacted in a similar manner with both infected and uninfected SHA, furthermore these two antigens did not cross-react with normal mouse serum or with sera from mice immunized with Freund's adjuvant alone (Figs. 6.5 b and c.)

Western blot analysis also showed that sera from both infected and uninfected SHA immunized mice, recognized infected and uninfected SHA in a similar manner with two prominent protein bands with molecular weights of 50 and 80kDa and that sera from Freund's adjuvant alone immunized mice did not recognized SHA and nor did the normal mouse serum (Figs. 6.6. A and B).

The results of immunization as indicated by the number of worms recovered and the number of eggs deposited in the liver of mice were as follows:

Table 6.1. Immunization of mice with snail soluble hepatopancreas antigens.

Immunogen	No of mice	Mean number of worms recovered	Mean number of eggs in the liver x 10 <sup>3</sup>
C	5	84.2 ± 18.5	34.9 ± 13.1
F	5	88.0 ± 17.1	39.6 ± 21.9
U	6	91.3 ± 30.5	42.3 ± 16.7
I	4	58.2 ± 14.5	29.2 ± 8.1

C= Control group

F= Immunized with Freund's adjuvant alone

U= Immunized with uninfected hepatopancreas.

I= Immunized with infected hepatopancreas.

Originally, 6 mice were treated in each group, however, 1 died as a result of infection and 3 died during bleeding under anaesthetic. The analysis of variance, comparing the results in table 6.1 indicates that there is no significant difference in the number of worms or eggs between these groups. The observed F values of 2.03 and 0.59 for worms and eggs respectively were less than the critical value of 3.24. However, comparing the mean worm returns and mean egg deposition of immunized mice as a percentage of the control group, revealed that when mice were immunized with Freund's adjuvant alone and or with uninfected SHA, there was an



increase in both the number of worms recovered and the number of eggs deposited as opposed to a large decrease in the group of mice immunized with the infected SHA.

Table 6.2 Percentage difference in the mean numbers of worms and eggs in immunized mice compared with that of the control group.

Immunogen used	worm recovery	eggs deposited
F	4.5% Increase	13.5% Increase
U	8.4% Increase	21.2% Increase
I	30.8% Decrease	16.3% Decrease

F= Mice immunized with Freund's adjuvant alone.

U= Mice immunized with Uninfected hepatopancreas ..

I= Mice immunized with Infected hepatopancreas..

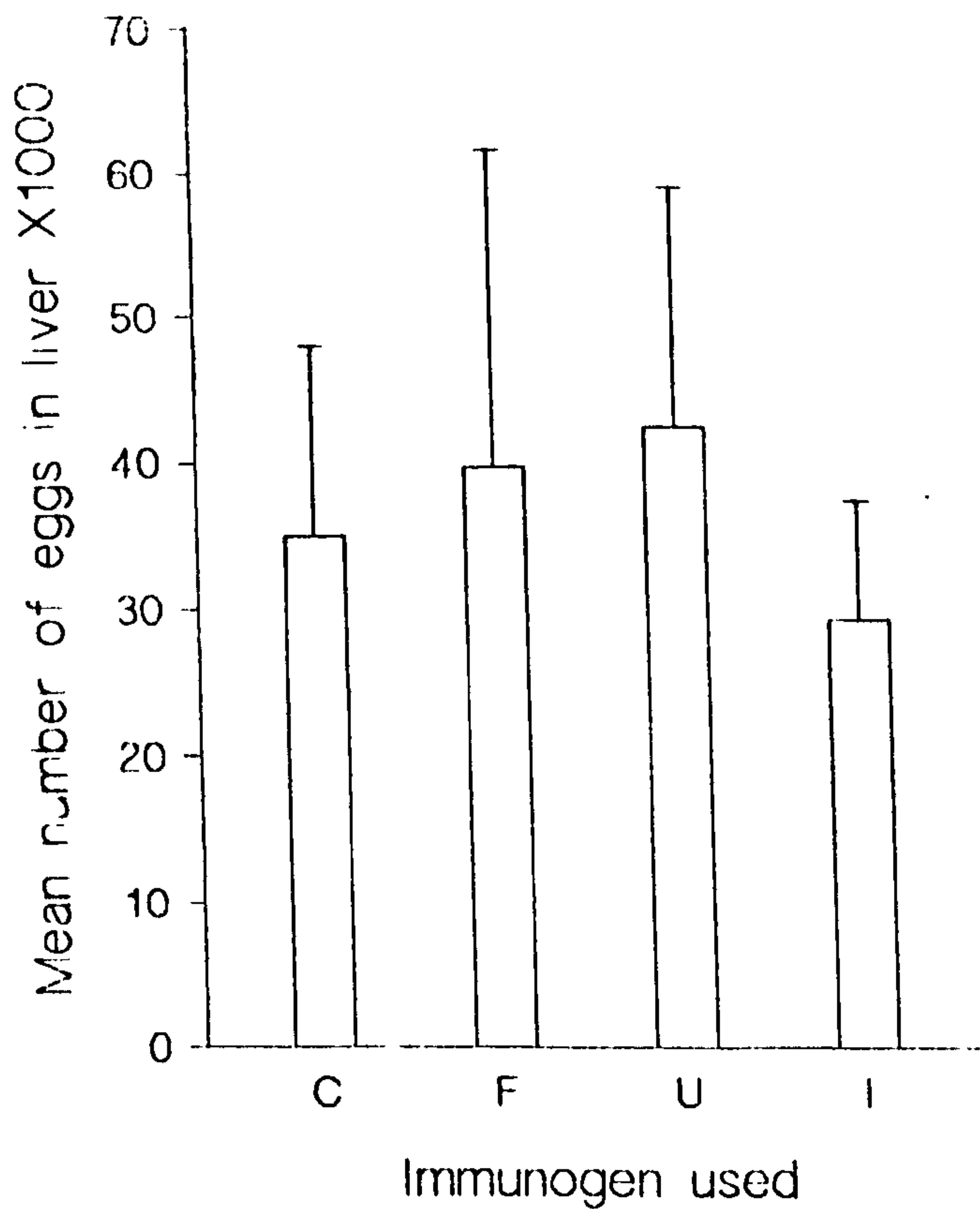
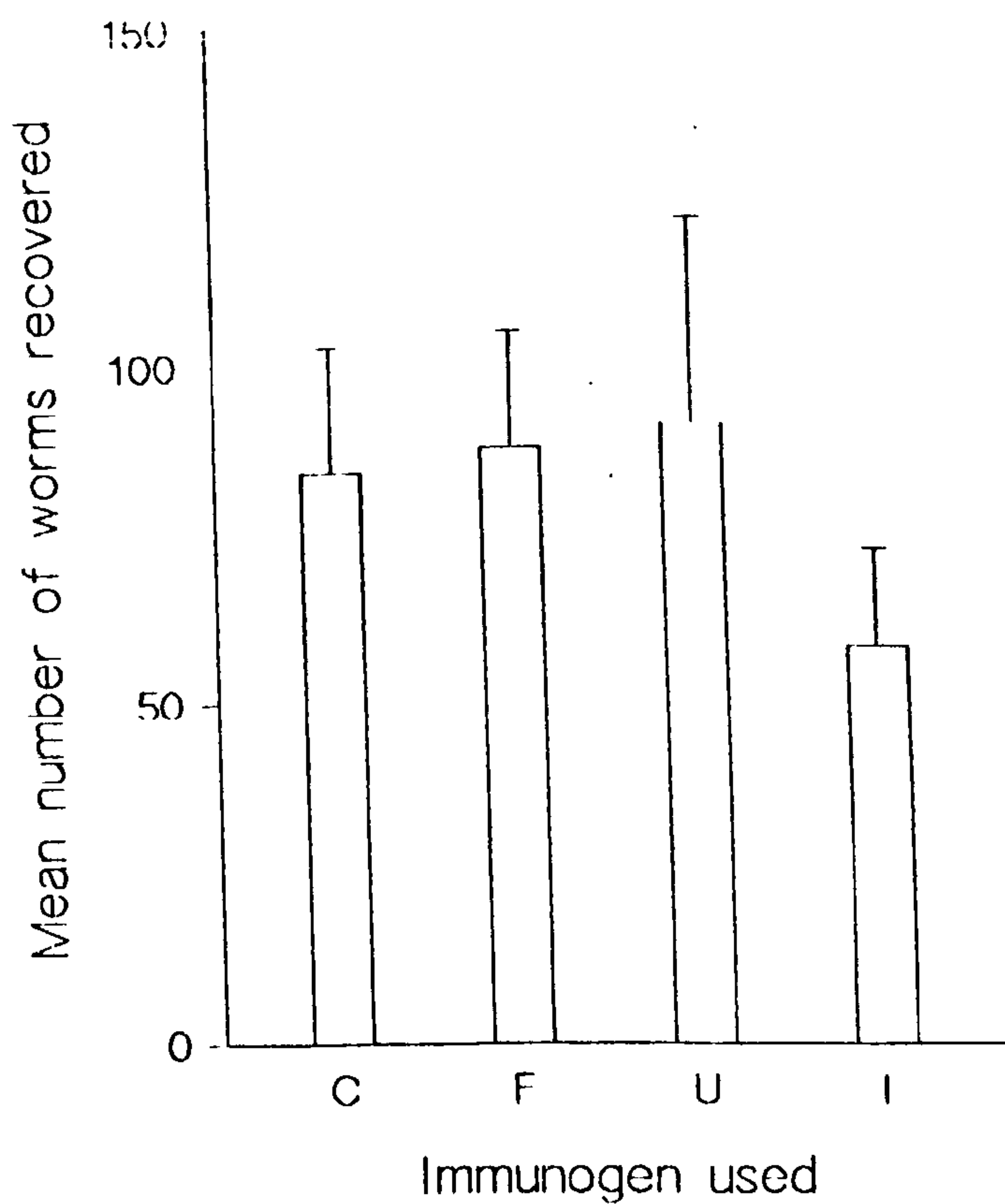


Figure 6.1 A bar chart representation of the immunization experiments indicating the general wide overlaps observed in both sets of data and the apparent reduction of worms and eggs in mice immunized with infected material.

C = Control

F = Freund's adjuvant

U = Uninfected hepatopancreas

I = Infected hepatopancreas

Bar = Standard deviation of the mean



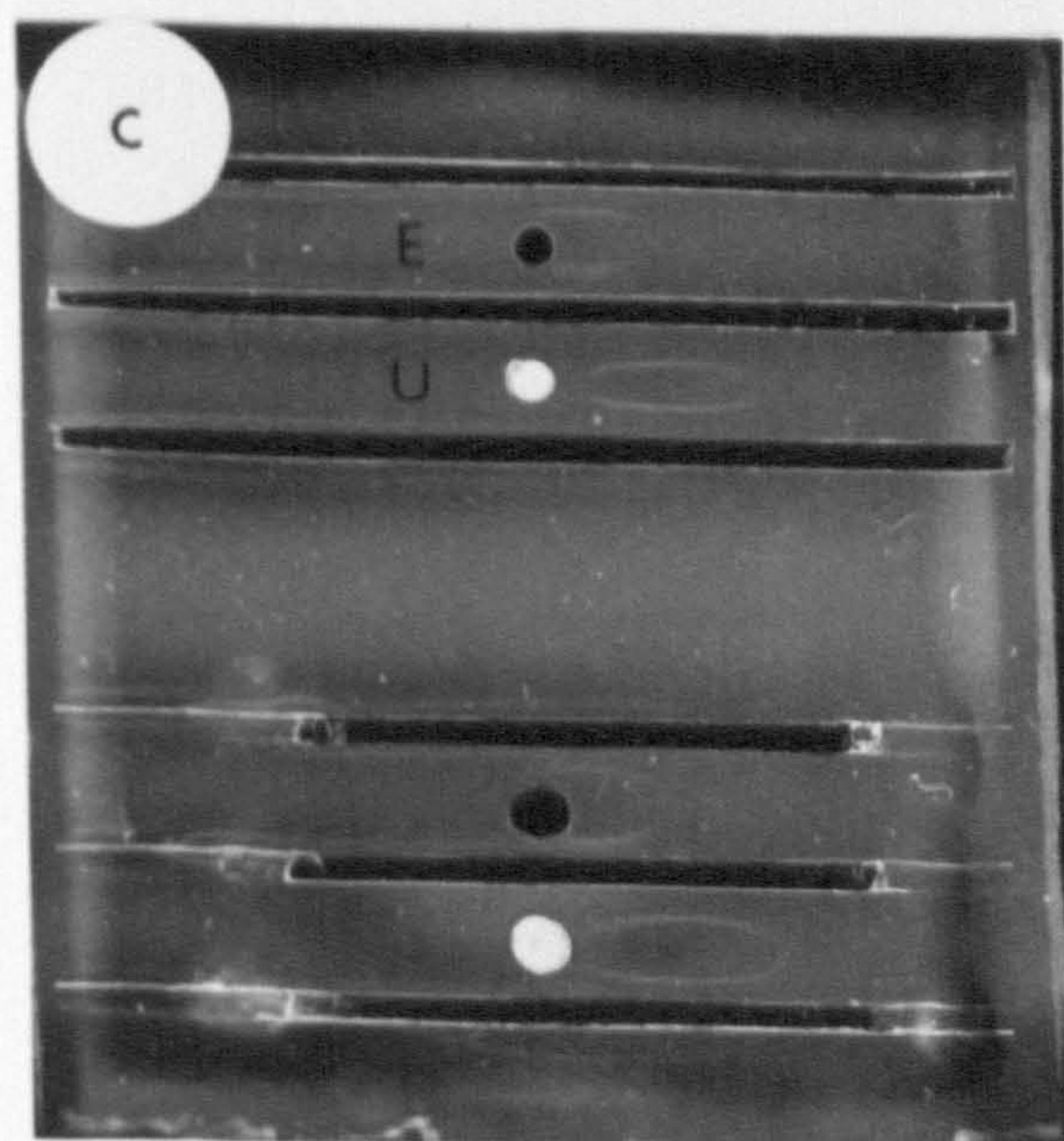
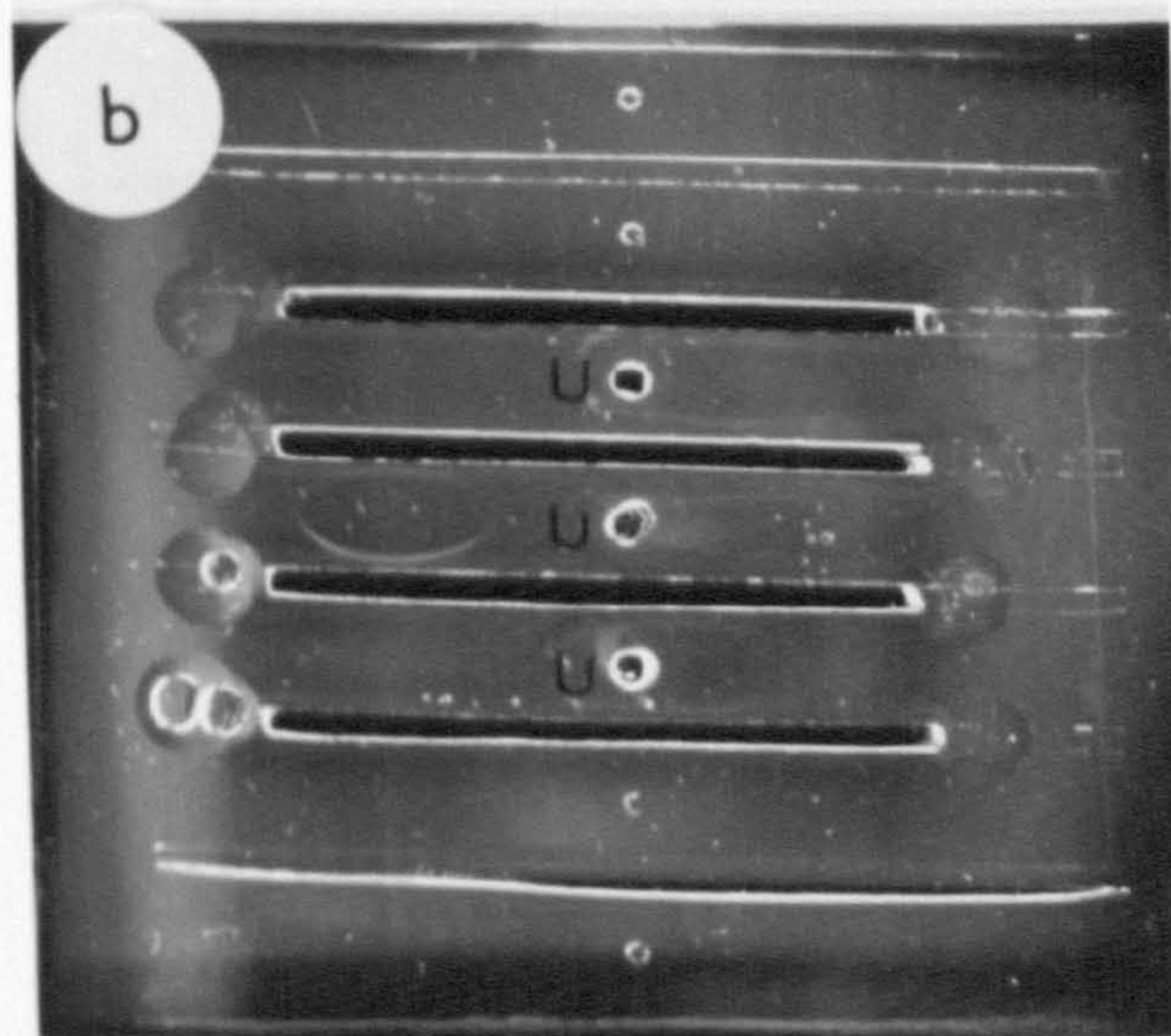
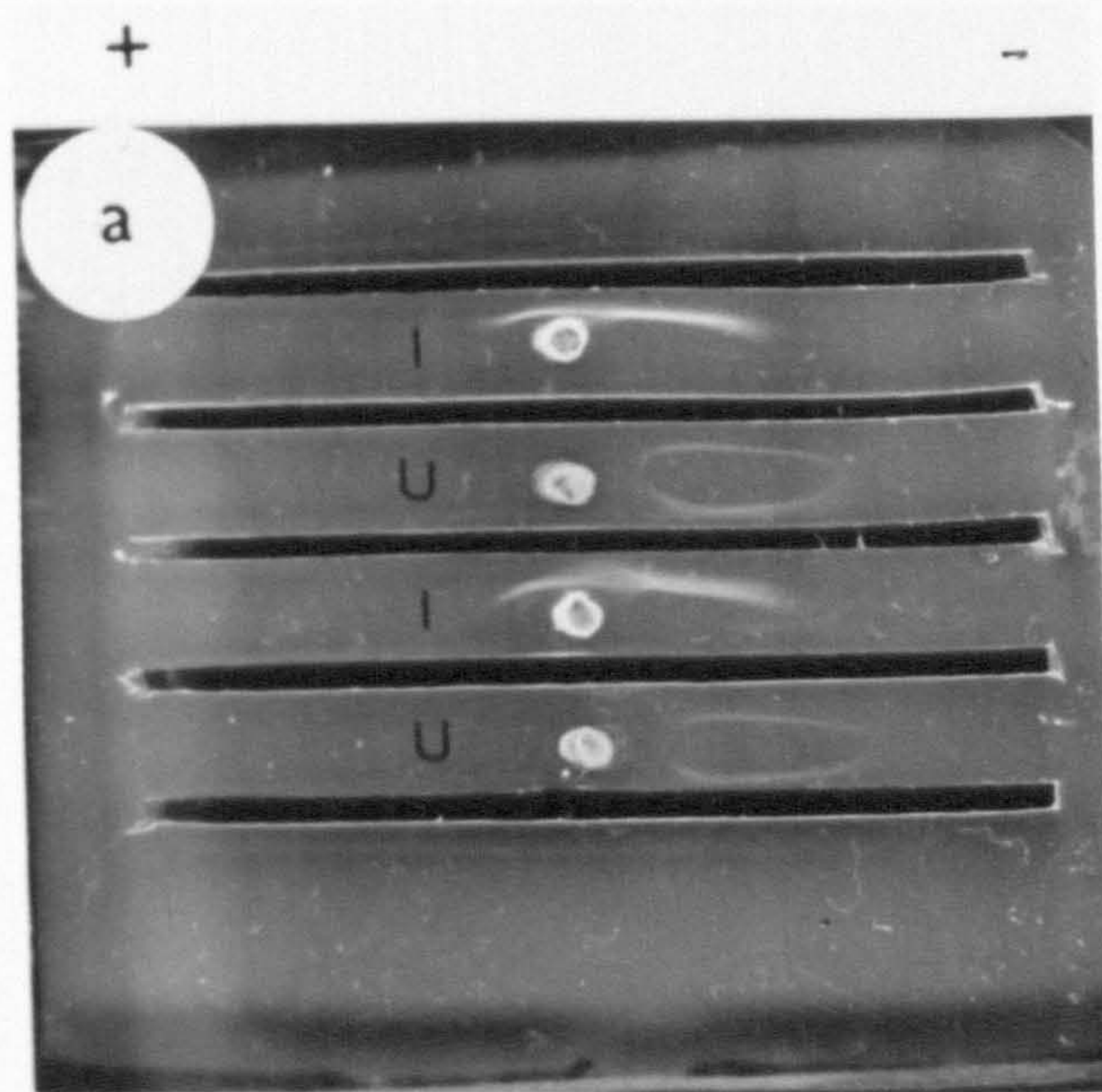
Figure, 6.2. IEP, reactivity of various rabbit sera with soluble hepatopancreas antigen (SHA) from infected (I) and uninfected (U) Biomphalaria glabrata snails.

a) Reactivity of SHA from infected (I) and uninfected (U), B.glabrata (wells) two different batches of anti-Sm480 rabbit sera (troughs).

b) Reactivity of SHA from uninfected (U) B.glabrata (wells) with an anti-K3 (a component of S.mansoni SEA) rabbit sera (all troughs).

c) Reactivity of S.mansoni SEA (E), compared with that of SHA from uninfected B.glabrata (U) with an anti-Sm480 (from worm) rabbit sera (all troughs). The bottom half of the photograph is the same using larger wells.







Figure, 6.3. IEP reactivity of various rabbit sera with SHA from uninfected Bulinus natalensis, Haemocyanin (from Keyhole Limpet (KLH, Sigma) and Cercarial Transformation Fluid (CTF: concentrated medium in which S.mansoni cercariae have been mechanically transformed to schistosomula)

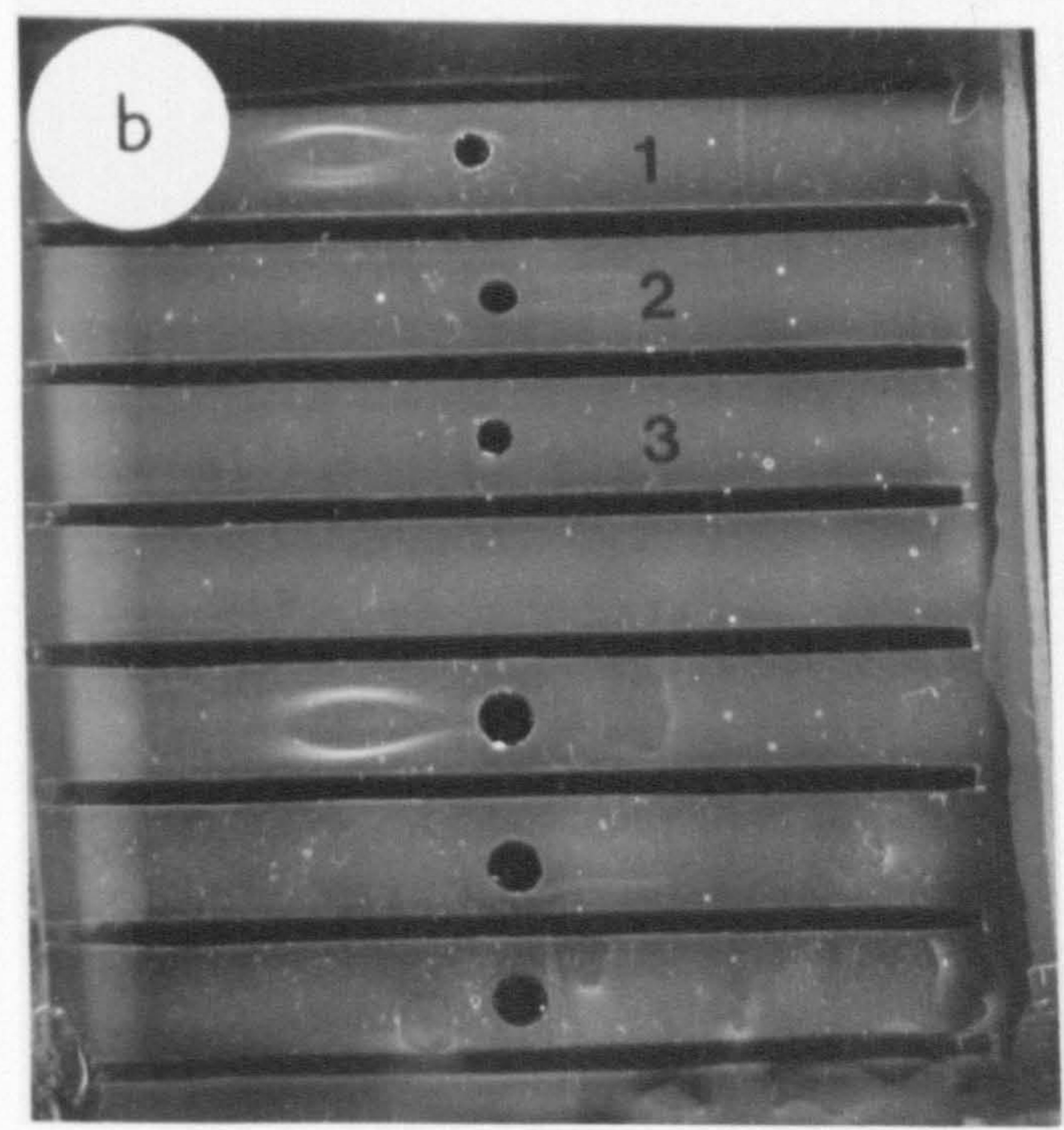
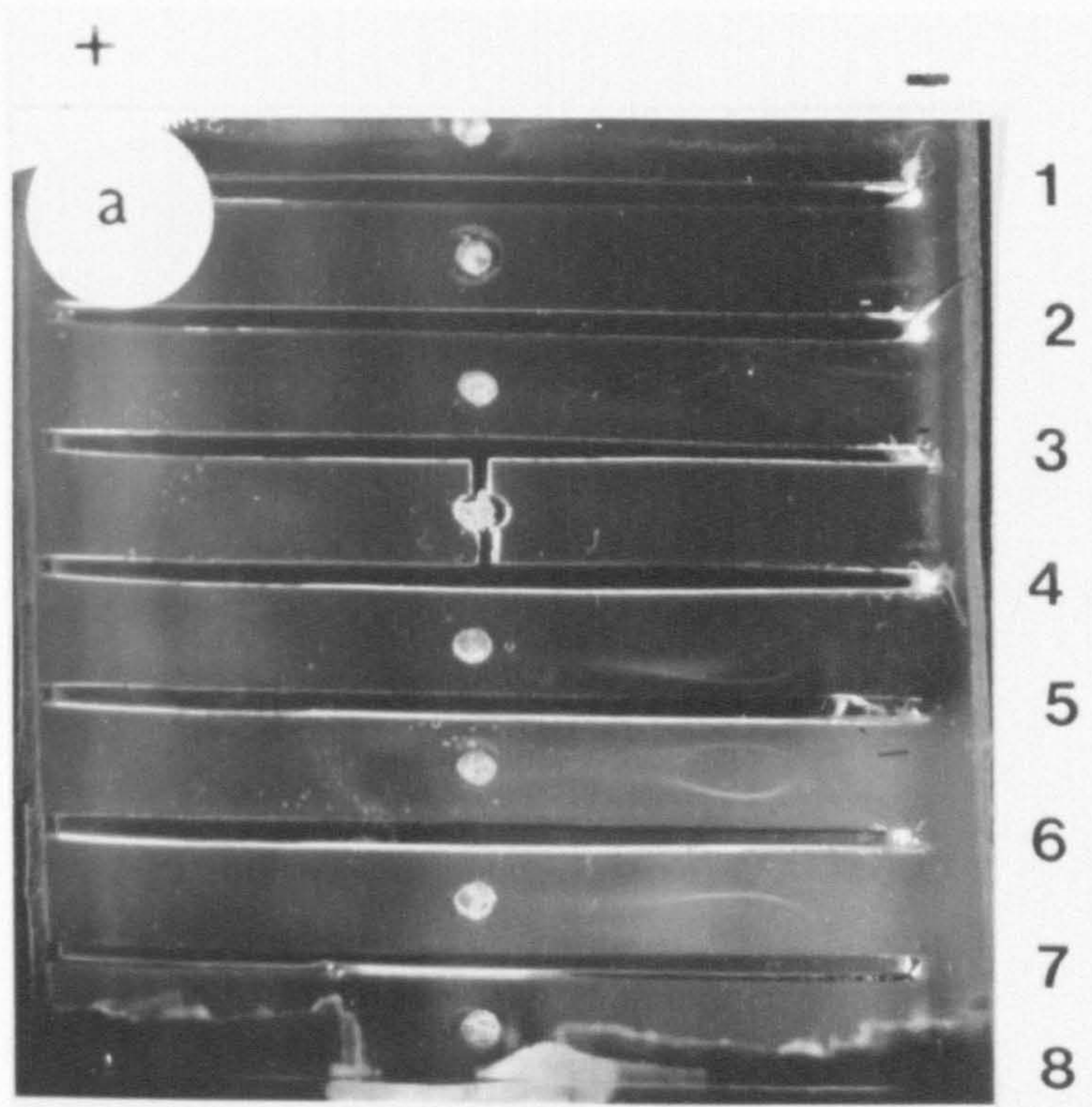
a) Reactivity of SHA from uninfected B.natalensis (all wells) with the following rabbit sera (all raised against S.mansoni derived antigens)

- 1) Anti-egg homogenate.
- 2) Anti-Trichloroacetic acid (TCA) treated hepatopancreas of B.glabrata.
- 3) Anti-Sm480 (from the egg stage).
- 4) Anti-Sm480 (from the worm stage).
- 5) Anti-K3 (a component of S.mansoni SEA).
- 6) Anti-Sm480 (from the egg stage).
- 7) Anti-Sm480 (from the worm stage).
- 8) Anti-Keyhole Limpet haemocyanin (Sigma).

N.B. Different batches of Sm480 rabbit sera are used.

b) Reactivity of KLH dissolved in barbitone (pH 9.6) well 1, CTF well 2 and KLH dissolved in barbitone (pH 7.2) well 3, with anti-KLH rabbit sera in all troughs.







Figure, 6.4. Western blot analysis, indicating a wide range of cross-reactivities.

A) Western blot of SHA from uninfected B.glabrata probed with the following rabbit sera:

- 1) Anti-Trichloroacetic Acid treated Cercarial Transformation Fluid (TCA CTF).
- 2) Anti-KLH.
- 3) Anti-K3.
- 4) Anti-Sm480.
- 5) Anti-Sm480.
- 6) Normal rabbit sera.
- 7) Anti-Sm480.
- 8) Anti-Sm480.
- 9) Anti-Sm480.
- 10) Anti-Sm480.

N.B Different batches of Sm480 used.

B) Western blot of Haemocyanin of Keyhole Limpet (2mg in 2ml barbitone, pH 9.6) probed with the following rabbit sera:

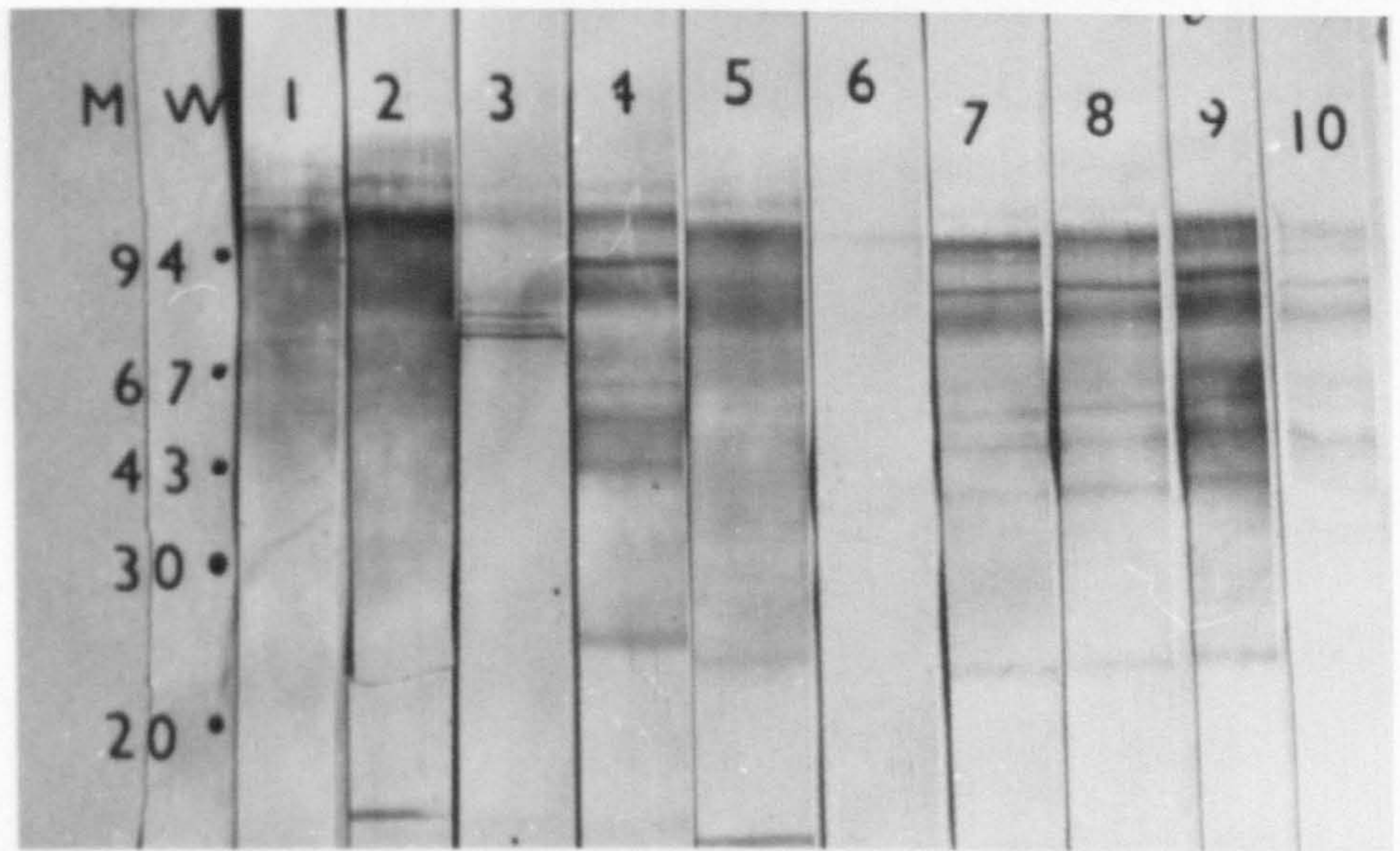
- 1) Normal rabbit sera.
- 2) Anti-KLH.
- 3) Anti-TCA CTF.
- 4) Anti-Sm480.
- 5) Anti-K3.

C) Western blot, comparing hepatopancreas of B.glabrata (lanes, 1,2,3 and 4) and B.natalensis (lanes, 5,6,7 and 8) with the following rabbit sera:

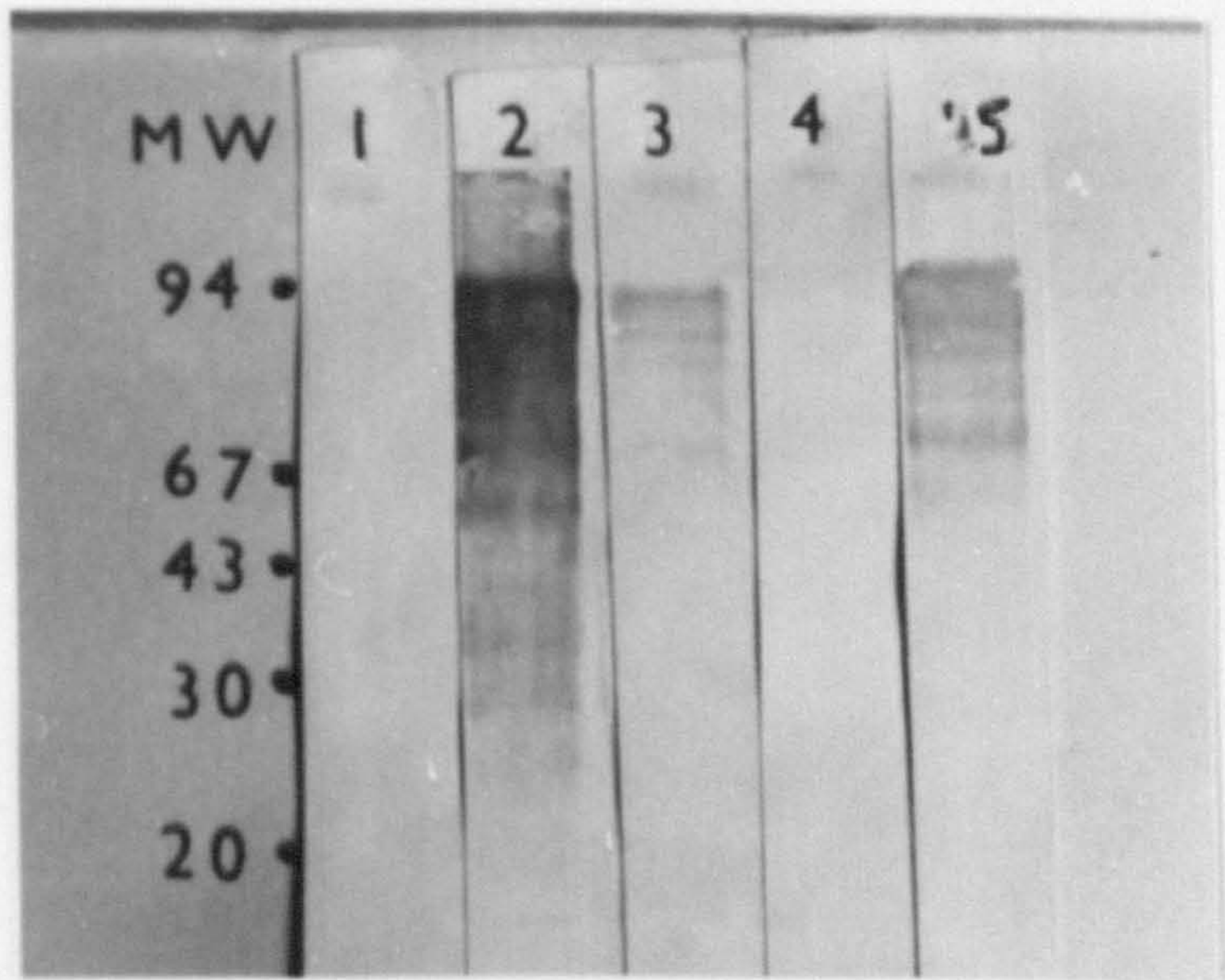
- 1) Anti-K3.
- 2) Anti-Sm480.
- 3) Anti-TCA CTF.
- 4) Normal rabbit sera.
- 5) Anti-K3.
- 6) Normal rabbit sera.
- 7) Anti- Sm480.
- 8) Anti-TCA CTF.



A



B



C

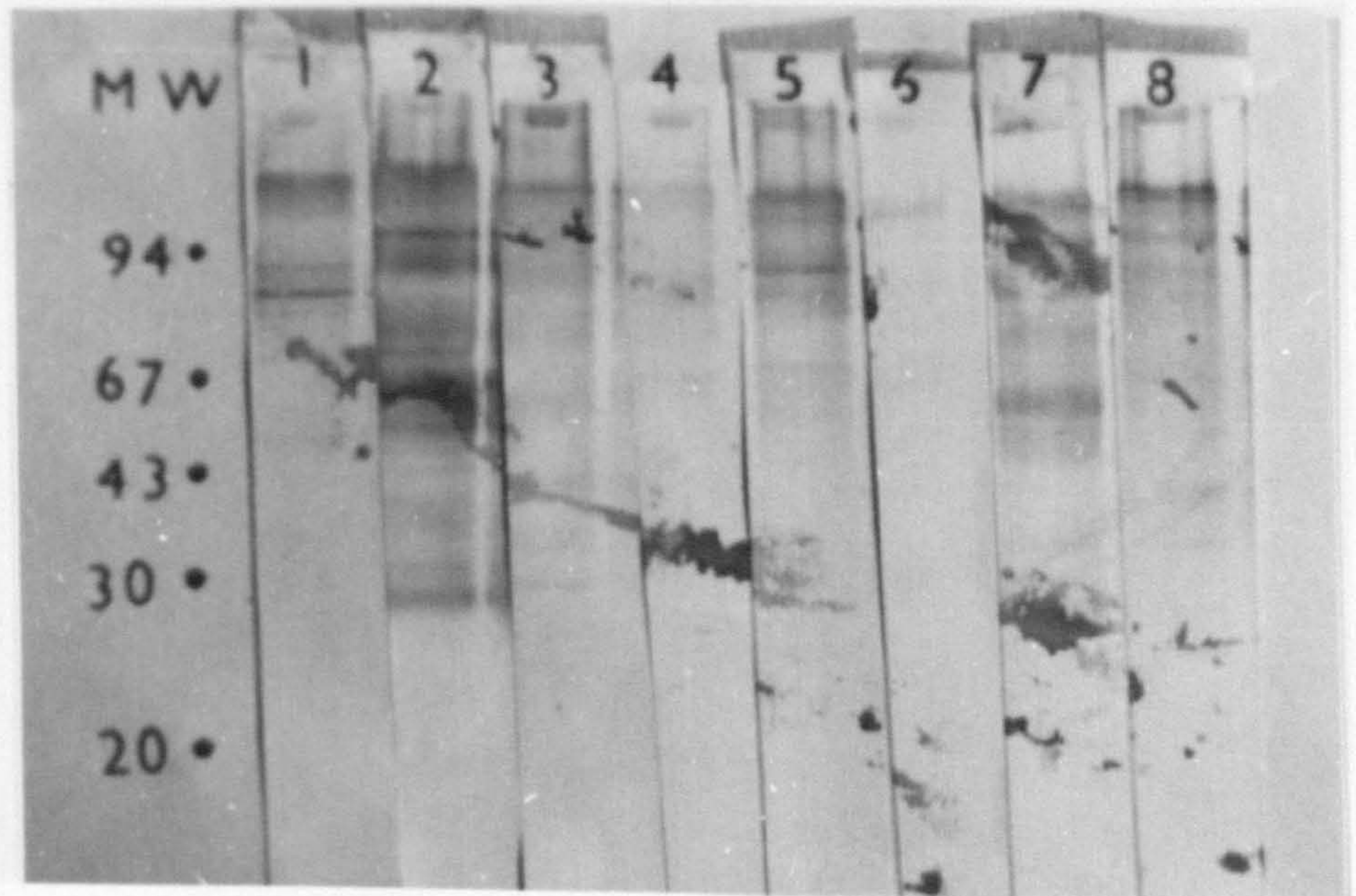




Figure 6.5. IEP and Immunodiffusion reactivities with immunized mice.

a) IEP reactivity of hepatopancreas of infected (I) and uninfected (U) Biomphalaria glabrata snails (in wells) with the following mouse sera:

- 1) Normal mouse sera.
- 2) Mice immunized with uninfected hepatopancreas of B.glabrata.
- 3) Mice immunized with infected hepatopancreas of B.glabrata.
- 4) Mice immunized with Freund's adjuvant.

b) Immunodiffusion cross-reactivity of snail hepatopancreas with individual immunized mouse.

A) Uninfected hepatopancreas (centre well) with individual mouse sera (numbered 1-6) immunized with Freund's adjuvant alone.

B) Infected hepatopancreas (centre well), with the same sera as used in A. (Sera are placed in an anti-clockwise order).

C) Uninfected hepatopancreas (centre well) with individual mouse sera immunized with infected hepatopancreas.

D) Infected hepatopancreas (centre well) with the same sera as used in C.

c) Immunodiffusion cross-reactivity of snail hepatopancreas with various mouse sera.

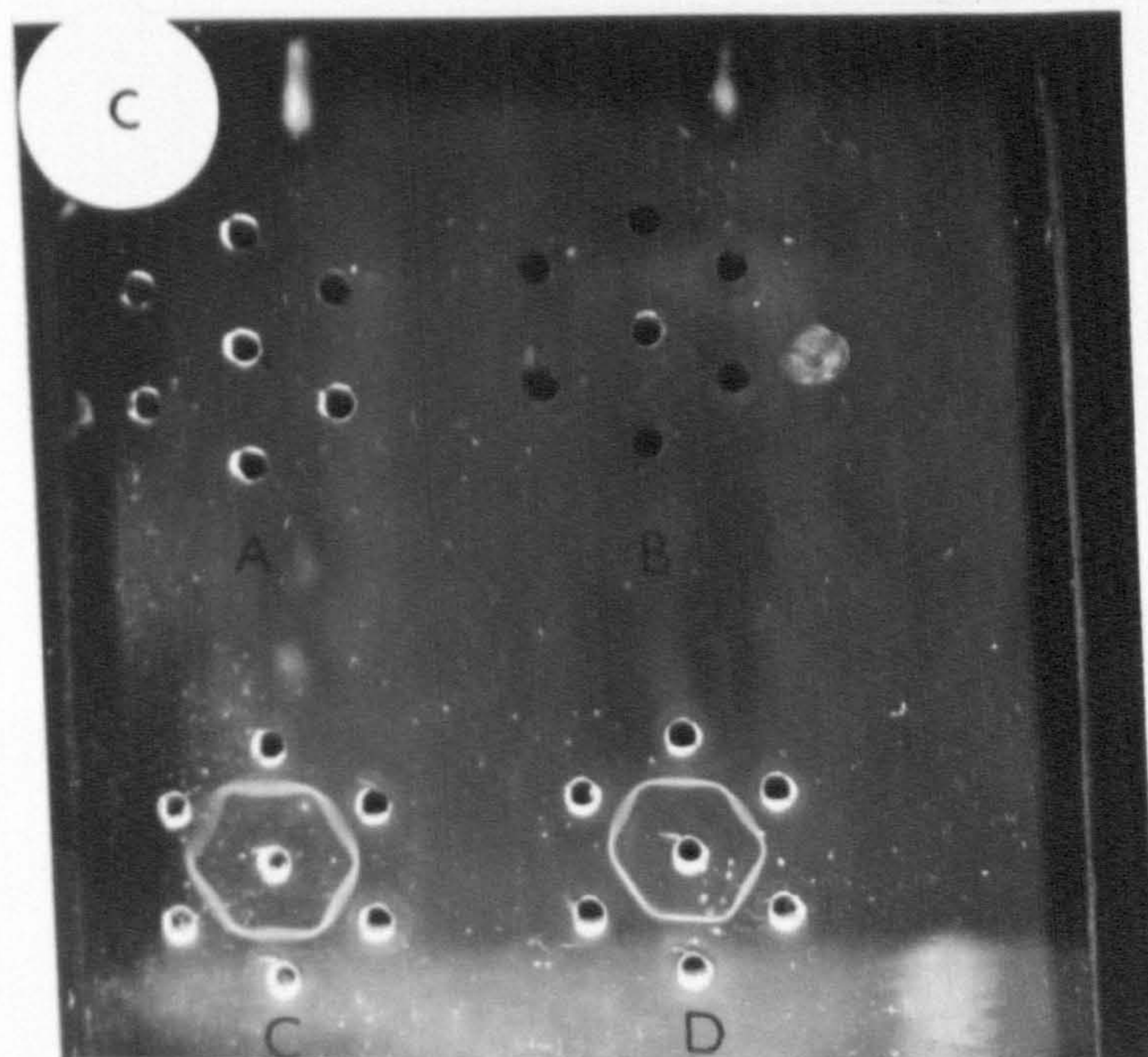
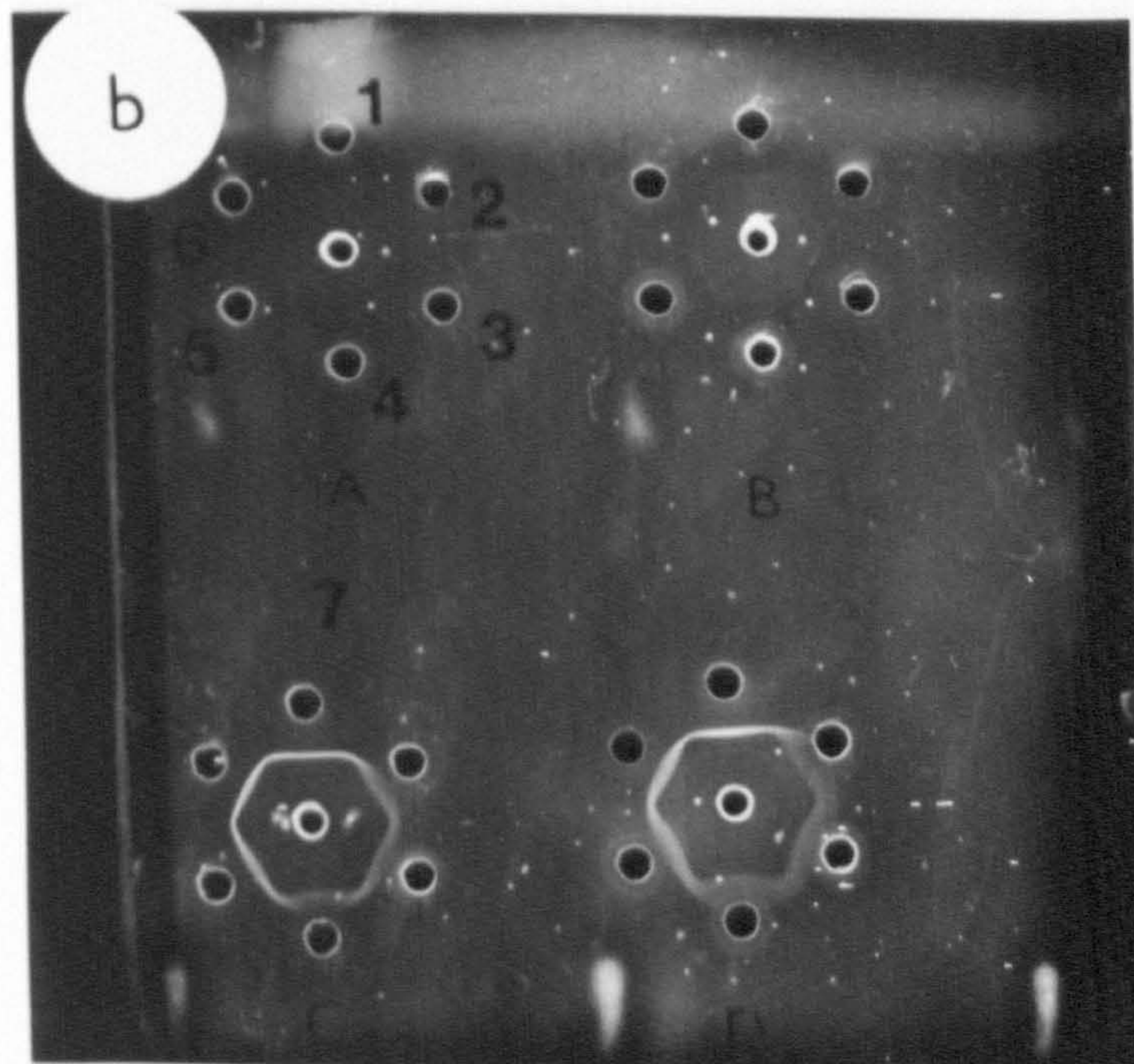
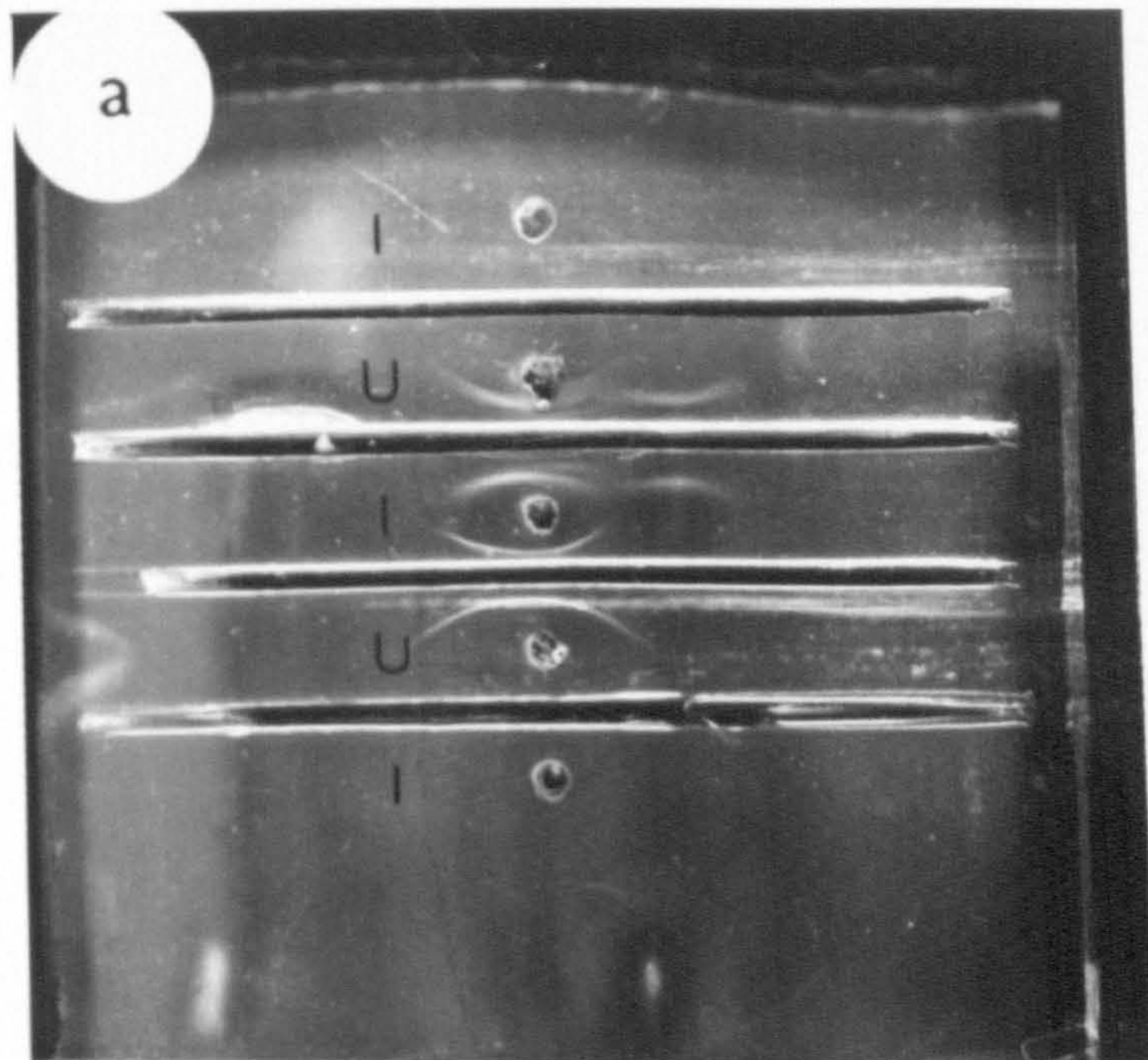
A) Uninfected hepatopancreas (centre well), with sera from the control (normal mouse sera) group.

B) Infected hepatopancreas (centre well), with the same sera as used in A.

C) Uninfected hepatopancreas (centre well), with sera from individual mouse immunized with uninfected hepatopancreas..

D) Infected hepatopancreas (centre well), with the same sera as used in C.







Figure, 6.6. Western blot analysis of soluble hepatopancreas antigen (SHA) of both infected and uninfected snails with various mouse serum.

A) Western blot analysis as follows:

Lane 1 : SHA from Infected snails probed with sera from mice immunized with uninfected SHA.

Lane 2 : SHA from uninfected snails probed with sera from mice immunized with uninfected SHA.

Lane 3 : SHA from uninfected snails probed with sera from mice immunized with infected SHA.

Lane 4 : SHA from infected snails probed with sera from mice immunized with infected SHA.

Lane 5 : SHA from uninfected snails probed with normal mouse sera.

Lane 6 : SHA from infected snails probed with normal mouse sera.

Lane 7 : SHA antigens from uninfected snails probed with sera from mice immunized with Freund's adjuvant alone.

Lane 8 : SHA from infected snails probed with sera from mice immunized with Freund's adjuvant alone.

B) Western blot analysis repeated using less concentrated primary antibodies 1/500 as opposed to 1/250.







## Discussion:

Schistosomes have a long history of association with their intermediate and definitive hosts during which the parasites have developed a highly successful host-parasite relationship. Schistosomes like other trematodes, develop only in specific molluscan hosts, indicating evolutionary as well as physiological dependence by the fluke and tolerance by the hosts (Damian, 1964). Although a large proportion of the snail viscera is occupied by the multiplying sporocysts, the snail exhibits little phagocytic and fibrotic reaction.

However, in resistant snails, the onset of parasitic encapsulation reaction is very rapid and within 48 hours the process is completed (Newton, 1952, and Richards, 1975). From these observations it can be concluded that the miracidium or early mother sporocyst are the first to be involved in the mechanism of immune evasion. Indeed it has been shown that early stage of schistosome larvae and their snail hosts share similar carbohydrate determinant (Yoshino et al., 1977). Using ultrastructural immunocytochemistry, Yoshino and Cheng (1978) found that newly hatched miracidia of S.mansoni possessed surface membrane-associated determinants that were antigenically similar to macromolecular components of B.glabrata haemolymph. Furthermore, Yoshino and Bayne (1983) using an immunofluorescence method have shown that antibodies to snails haemolymph cross-reacted with miracidial epidermal and ciliary membranes as well as the surface membrane of the intercellular ridges and with the surface tegument of the sporocyst. It is thought that the sharing of snail host

antigens by the miracidia or sporocyst represents a possible mechanism by which these parasites are able to avoid host immune recognition in susceptible snails. The same principle applies to the emerging cercariae and the snail tissue surrounding them. Through their intimate and continuous contact with host tissues, the cercariae would acquire some antigens from the snail.

The possibility of some of these antigens being passed on to the mammalian host, could be one explanation for the existence of common antigens between trematodes and their snail hosts as demonstrated by Capron (1969), Capron et al., (1965), Heyneman et al., (1971) and Kemp et al., (1974). However, Vieira and Kusel (1991) showed that although, both anti-snail and anti-glycocalyx antisera bind to freshly transformed schistosomula and to 12 days old worms cultured in vitro, no binding occurred when worms were left to mature in vivo. They concluded that these antigens are possibly masked in vivo or indeed lost during the natural development of the worms. Nevertheless, snail-like antigens have been found in S.mansoni adult worms (Devine and Kemp, 1984 and Rasmussen et al., 1985).

The results of the present study indicate that monospecific sera raised against various antigens of different stages of S.mansoni, including adults, cercariae and eggs also cross-react with hepatopancreas of Biomphalaria glabrata, the natural intermediate host of S.mansoni, Bulinus natalensis which is resistant to the miracidia of S.mansoni, and commercial KLH (Figs. 6.2, 6.3 and 6.4). The IEP studies



reveal an obvious difference in the precipitin arc formation of infected and uninfected SHA. The infected material exhibited an extra arc which is thought to be due to cross-reactivity of antigens of cercariae and sporocysts with a given rabbit antisera (Fig. 6.2a).

Although a standard procedure was followed during the preparation of snail antigen, some batches of uninfected SHA formed an anodal precipitin arc with anti-K3 rabbit sera. This arc, referred to as anti-K3 contaminant arc (which also resembles an albumin arc), could not be reproduced by various treatments of SHA (heating, freezing and thawing and acid or alkaline additions). It is possible that the extra anodal arc is formed by some parasites of snails (Fig. 6.2b).

Monospecific anti-Sm480 (worm stage) rabbit sera also cross-reacted with uninfected SHA, Figure 6.2c (Sm480 is an enzyme which is inhibited by protease inhibitors indicating that it belongs to the serine proteases, possibly chymotrypsin-like, Curtis, 1991). Previous studies by Curtis, (1991) and ongoing investigations by Doenhoff (personal communication) have indicated that Sm480 is a protective antigen and that antisera against it detects antigen(s) in all stages of the life-cycle of S.mansoni.

Western blot (Figs. 6.4 A,B and C) analysis confirmed and widened the range of cross-reactivities observed in IEP studies. Uninfected SHA of B.glabrata and B.natalensis were recognized by rabbit antisera raised against a variety of S.mansoni antigens.

Against anti-Sm480 rabbit sera, a protein with a molecular

weight of 80-90 kDa was evident. This is thought to be the protective epitope, referred to by Dissous et al., (1986), which they described as oligosaccharide associated with a 90kDa component in uninfected B.glabrata. This antigen which is also shared by the adult S.mansoni worm is expressed as a molecule with a relative mass of 38kDa (Dissous et al., 1986). Further studies by these authors indicate that KLH also possesses this antigen. KLH is a copper-containing protein which is used as a potent immunogen in many research laboratories. In most molluscs, haemocyanin serves as an extracellular oxygen carrier in blood. However, it is absent in the snail vector of schistosomes where an extracellular haemoglobin is present instead. Moreover, the amino acid sequence of gastropod haemocyanin and haemoglobin are not related. Nevertheless according to Markl et al., (1991), 51 out of 68 sera from Egyptian patients with schistosomiasis showed significant anti-KLH antibody titres in ELISA. Furthermore Grzych et al., 1987 (In Markl et al., 1991) have shown that rats could be protected against a challenge with helminth cercariae by immunization with KLH. Results obtained by Dissous et al., (1986) and Markl et al., (1991) clearly suggest the presence of a major antigenic epitope in the human parasite which is not only present in the fresh water snail but also in the marine mollusc.

Immunodiffusion studies carried out in the present study did not show any cross-reactivity between KLH and a large number of rabbit sera raised against S.mansoni antigens. However, in IEP, only CTF and KLH (dissolved in <sup>barbitone</sup> pH 9.6)



reacted with anti-KLH rabbit sera (Fig. 6.3b). CTF which consists of the contents of the acetabular glands and the glycocalyx of the cercariae is rich in 30-28kDa serine elastase (Fishelson et al., 1992) and K3 (Dunne, 1990). The immunization capability of KLH could thus be related to this cross-reactivity, since cercarial elastases are known to be involved in the penetration process (Cohen et al., 1991). In Western blot analysis where KLH was probed, cross-reactivities were recorded with anti-KLH, anti-TCA CTF and anti-K3 rabbit sera (Fig. 6.4B). However, the expected 28-30kDa band was not evident in the carbohydrate-depleted TCA CTF, and instead protein bands 70-90kDa were prominent. Nevertheless, it was shown that KLH cross-reacts with anti-S.mansoni rabbit sera, including eggs and the contents of the acetabular glands and the glycocalyx (CTF) of the cercariae. The fact that KLH that had been dissolved in low pH showed no reactivity is explained by the quaternary structure of KLH. According to Markl et al., (1991) KLH dissociates into subunits at pH 9.6 and reassembles at pH 7.5. It is possible that in its assembled form, this protein is too heavy to migrate in IEP.

The existence of these shared antigens, has led to the possibility of using extracts of the snail host for the diagnosis of schistosomiasis and/or vaccination against it.

As early as 1919-1920, Fairley utilized an extract of B.boissyi to diagnose schistosomiasis in humans and monkeys by the complement fixation tests. Kagan (1955) also showed that sera of rabbits immunized with hepatopancreas of

infected B.glabrata or Lymnaea species were positive in the cercarienhullen reaction. This reaction is obtained when anti-S.mansoni antibodies bind to the cercarial glycocalyx. A positive reaction is characterized by the apparent thickening of the glycocalyx. Cheieffi et al., (1982), used whole B.glabrata soluble antigens after Triton X-100 extraction, and reported higher titers of anti-B.glabrata antibodies in the serum of man and mice infected with S.mansoni when tested by passive haemagglutination. This confirmed the earlier findings of Jackson and De Moor (1976).

Attempts to vaccinate against trematodes using antigens derived from snails have been mostly unsuccessful. Kozer (1974) reported a 55% reduction of Faciola in rats immunized with extracts of Galba truncatula its (snail host). Roder et al., (1977) working on Trichobilharzia ocellata, were unsuccessful in immunizing ducks with mucus, haemolymph and hepatopancreas of Lymnaea stagnalis, Farouk and Amira (1979) immunized mice with hepatopancreas of B.alexandrina and reported only a 15-20% reduction in worm burden. Stein and Basch, (1979) who used antigens from permanent B.glabrata embryo cell lines reported no significant reduction in worm burdens. The most successful attempt is that reported by Diehl et al., (1987). Mice were immunized with hepatopancreas homogenate of B.alexandrina using AL(OH)<sub>3</sub> as adjuvant and were challenged by 1) subcutaneous injection of S.mansoni cercariae, 2) percutaneous invasion of cercariae and 3) intravenous injection of schistosomula. The results indicated a 50% reduction in worm burdens in the subcutaneous injection



group when compared to the control group. No significant reduction was observed in the other groups. A similar immunization procedure was used in the present study, however the antigen preparation and the adjuvants used were different. The IEP and immunodiffusion studies, indicate that infected and uninfected SHA reacted in a similar way to sera from mice immunized with either infected or uninfected SHA (Fig. 6.5.a,b and c). However, whereas sera from uninfected SHA mice precipitated two arcs with both antigens, sera from infected SHA mice only precipitated one arc (Fig. 6.5 a), this indicates that sera from mice immunized with infected material somehow inhibits the expression of other antigens in IEP. As expected, there was no cross-reactivity with normal or Freund's alone immunized mice sera and SHA (Fig. 6.5. a,b and c). The Western blot analysis (Fig. 6.6 A and B), confirmed that sera from normal or Freund's adjuvant alone immunized mice, did not recognize SHA. Furthermore, there was a similar response from both immunized groups of mice with uninfected and infected SHA. Figure 6.6 B, clearly shows the two prominent protein bands of 50 and 80kDa. These results indicate that both immunogens are antigenically similar. This could be explained by the fact that upon preparation of 15 days post-infected SHA, it was noticed that the hepatopancreas only possessed between 3-7 sporocysts, which might be too small (antigen material) to be detected in IEP, immunodiffusion and Western blot analysis.

The one way Anova statistical analysis carried out on the results in Table 6.1, indicate that immunization has no

significant influence on the reduction of worms or eggs. Although not significant at the  $P < 0.05$  level, in mice immunized with uninfected SHA, there was an increase in both the worm burden and egg deposition. This could be due to chance or could imply that immunization with uninfected SHA leads to suppression of cellular immunity, or indeed leads to the production of blocking antibodies which would bind to and protect the invading cercariae. The apparent reduction of worm burden and egg deposition (Fig. 6.1 a and b) in mice immunized with infected SHA indicates that there is a degree of protection involved and would justify further studies to evaluate the potential of infected material as possible vaccines.



## CHAPTER SEVEN

Evaluation of the potential of S.margrebowiei antigens for the diagnosis of human urinary schistosomiasis.

## CHAPTER SEVEN

### Introduction:

A successful strategy for the control of schistosomiasis, requires an accurate estimate of the number of infected cases in an area and regular accurate data regarding the effectiveness of the particular control programme. Current parasitological, diagnostic techniques which are based on the detection of eggs in the faeces (S.mansoni) Kato-Katz faecal smear method or the urine (S.haematobium) nucleopore membrane filtration method are time consuming, inefficient and sometimes inaccurate. Furthermore, successful control programmes in Japan, Puerto Rico, and St Lucia, have reduced the prevalence of infections to such low levels that current techniques often fail to detect low numbers of eggs and are also not cost-effective (Mott & Dixon, 1982). Regarding S.haematobium infections, diagnosis is even more inaccurate mainly due to the large fluctuation in urine egg counts as reported by Stimmel and Scott (1956), Scott (1959) and McCullough and Bradley (1973). However, as yet no new diagnostic tests are available to detect S.haematobium infections. There are two main reasons for this apparent failure; a) unlike other species of schistosomes, S.haematobium has proved to be very difficult to culture in the laboratory. Hence antigenic material has to be collected from endemic areas, and b) most often these antigens are contaminated with material from other parasites and even other species of schistosomes due to the occurrence of mixed infections in many S.haematobium endemic areas



(Pitchford 1961, Southgate et al., 1976, Wright and Ross 1980). Because of these difficulties, investigators have relied on antigenic material derived from heterologous but phylogenetically related species of schistosomes for the immunodiagnosis of S.haematobium. Animal schistosomes belonging to the terminal spine egg group are the obvious candidates. S.bovis, in particular is thought to be the best possible choice since it has the closest phylogenetic relationship with S.haematobium.<sup>Thus</sup> Taylor (1970) showed that S.bovis can hybridize with S.haematobium and produce fertile offspring. Moreover Le Roux (1961) had suggested that exposure to the cercariae of S.bovis could account for the absence of S.haematobium in places where the snail host for S.haematobium was abundant, for example in Sicily and Corsica.

Immunodiagnostic techniques involve skin tests where an antigen is injected subcutaneously and a swelling occurs due to an immediate hypersensitivity reaction. The most promising results have been obtained with a purified proteolytic enzyme from adult worms administered intradermally (Senft & Maddison, 1975). However, these methods often lack specificity since cross reactions occur with other trematodes as well as with other species of schistosomes. Another method for the direct diagnosis of infection with schistosomes is the detection of circulating antigens. These antigens which are the secretory and excretory products of the adult worms, include gut-associated schistosome proteoglycan (GASP) which are circulating anodal antigens (CAA) and circulating

cathodal antigens (CCA). Antibody detection is another method for diagnosing infection. For detection of antibodies a suitable target antigen is required and the Enzyme-linked immunosorbent assay (ELISA) method is often used. Mott & Dixon (1982) working with several antigens for the immunodiagnosis of schistosomiasis, concluded that antigens derived from eggs in general yield better results than those from worms. The aim of this preliminary study was to evaluate the potential for using S.margrebowiei soluble egg antigen (SEA), (this species is closely related to S.haematobium), for diagnosis of S.haematobium infections. Samples of S.margrebowiei SEA were prepared and sent to London for further investigation.



## Materials and Methods:

### Egg antigen:

Soluble egg antigens from both S.margrebowiei and S.mansoni eggs were prepared as described in chapter Six. Cation exchange fraction 6 of S.mansoni SEA (CEF6) and deep frozen (old) batches of S.bovis and S.haematobium SEA were provided by Dr. Doenhoff.

### Immunodiffusion:

Immunodiffusion was carried out as described in chapter Six.

### Sodium dodecyl sulphate polyacrylamide electrophoresis:

Carried out as described in chapter Six.

### Detection of Leucine aminopeptidase (LAP) activity:

Two chromogenic substrates were used to detect LAP activity in the SEA of S.margrebowiei.

1) The substrate L-leucine  $\beta$ -naphthylamide (Sigma) was used to visualize LAP activity in immunoprecipitate arcs formed by immunodiffusion or immunoelectrophoresis. Five mg of L-leucine  $\beta$ -naphthylamide were dissolved in 2 ml dimethylformamide (Sigma) with 10 mg Fast Blue B. This substrate solution was then diluted to 30mls in 0.05M PBS, pH 7.6. Agar plates which had been prepared earlier and washed in isotonic saline were incubated at 37°C with this solution for 1 hour.

2) The substrate L-leucine p-nitroanilide (10 $\mu$ M), was used to detect LAP activity in antigen solutions. Twenty five mg of leucine p-nitroanilide was dissolved in 2ml Ethylene glycol monomethyl ether (Sigma), 3ml of PBS pH 7.6 and 5ml distilled

water. An equal volume of this solution was added to the SEA of S.margrebowiei and the mixture incubated at 37°C for 30 min. The resulting colour (Yellow) was read at 410 nm using a CE 303 spectrophotometer (Cecil Instruments Ltd. Cambridge). S.mansoni SEA was also used as a positive working control.

**Enzyme-linked immunosorbent assay (ELISA):**

This was carried out by J.Lillywhite of Imperial College London, and P.L. Chiodini and Tahawy of the Hospital for Tropical Diseases, London.

Sera from 27 patients infected with S.mansoni and 26 infected with S.haematobium were studied in ELISA to determine the diagnostic sensitivity and specificity of S.mansoni SEA, CEF6 and S.margrebowiei SEA.



## Results:

### LAP activity:

When sera from mice infected with S.margrebowiei of 16 weeks duration, or longer, were individually screened in immunoelectrophoresis (IEP) with S.margrebowiei SEA, considerable homogeneity in the individual immunoelectrophoretic patterns was observed (Fig. 7.1 A). However, considerable heterogeneity was observed in S.mansoni pattern (Fig.7.1 B).

Using the Fast Blue B and the chromogenic substrate L-leucine  $\beta$ -naphthylamide, the precipitin lines of S.margrebowiei did not stain whereas those of S.mansoni did, indicating LAP activity (Fig.7.1 C). However, LAP activity was demonstrated in both S.margrebowiei and S.mansoni SEA using the chromogenic substrate leucine p-nitroanilide. A yellow colour change was observed in the SEAs of both species but the reaction was considerably weaker in S.margrebowiei. When the colour change was read spectrophotometrically at 410nm, a figure of 1.94 (amount of p-nitroanilide liberated) was recorded for S.mansoni SEA (1/100 dilution) as opposed to 0.2 for the same concentration of S.margrebowiei SEA. Furthermore protein, assay indicated that S.mansoni SEA had a protein concentration of 12.52mg/ml and S.margrebowiei 7.64mg/ml. From these findings it can be calculated that the LAP level per mg of protein was 15.5 for S.mansoni SEA and only 2.6 for S.margrebowiei SEA. A summary of these findings is given in Table 7.1.

Table 7.1 A comparison of LAP levels in the soluble egg antigen of S.margrebowiei and S.mansoni.

SEA Antigen	Nitroanilide liberated $\times 10^2$ (OD 410n)	Protein (mg/ml)	LAP/mg protein
<u>S.mansoni</u>	194	12.52	15.49
<u>S.margrebowiei</u>	20	7.64	2.61



Figure 7.1

A) One-dimensional Immunoelectrophoretic (IEP) spectrotype of S.margrebowiei SEA with different chronically infected S.margrebowiei mouse sera.

B) One-dimensional IEP of different batches of S.mansoni SEA with different chronically infected S.mansoni mouse sera.

C) Plate B washed in isotonic saline and stained with Fast Blue B using L-leucine  $\beta$ -naphthylamide as substrate. Note the stained cathodal precipitin lines indicating LAP activity. No staining occurred with the S.margrebowiei plate (result not shown).







### Cross-reactivity:

The immunodiffusion of different SEAs (Fig.7.2 A), indicates that only S.margrebowiei SEA reacted with chronically infected S.margrebowiei mouse sera (D, wells 2,4).Furthermore, only S.mansoni SEA (C) reacted with an anti-TCA CTF (Trichloroacetic acid treated S.mansoni cercarial transformation fluid) rabbit serum well 6 and one anti-K3 rabbit serum well 1.

Figure 7.2B, A, shows S.margrebowiei SEA reacting with sera from chronically S.margrebowiei mice wells 1 and 3 and not with sera from S.mansoni infected mice well 2, and figure 7.2 B, B shows that S.mansoni SEA also reacts with S.mansoni infected mouse sera wells 1 and 3 and not with S.margrebowiei infected mice well 2.

Western blot analysis probing S.margrebowiei and S.mansoni SEAs (Figure 7.2 C, indicates that there is a strong degree of cross-reactivity between S.mansoni and S.margrebowiei (lanes 1 and B in particular) not observed in immunodiffusion.

Figure 7.2

A) Immunodiffusion: The reactivity of the following sera:

- 1) An anti K3 rabbit
- 2) Chronically infected S.margrebowiei mouse.
- 3) Normal mouse sera
- 4) Chronically infected S.margrebowiei mouse.
- 5) An anti-K3 rabbit
- 6) An anti-TCA treated CTF rabbit

placed in wells in a clockwise direction were tested with the following SEA placed in the central well,

- A) S.bovis
- B) S.haematobium
- C) S.mansoni
- D) S.margrebowiei

B) Immunodiffusion: The reactivity of the following sera:

- A:
- 1) Chronically infected S.margrebowiei mouse.
  - 2) Chronically infected S.mansoni mouse.
  - 3) Chronically infected S.margrebowiei mouse.

placed in the outer wells were tested with S.margrebowiei SEA.

- B:
- 1) Chronically infected S.mansoni mouse.
  - 2) Chronically infected S.margrebowiei mouse.
  - 3) Chronically infected S.mansoni mouse.

placed in the outer wells were tested with S.mansoni SEA.

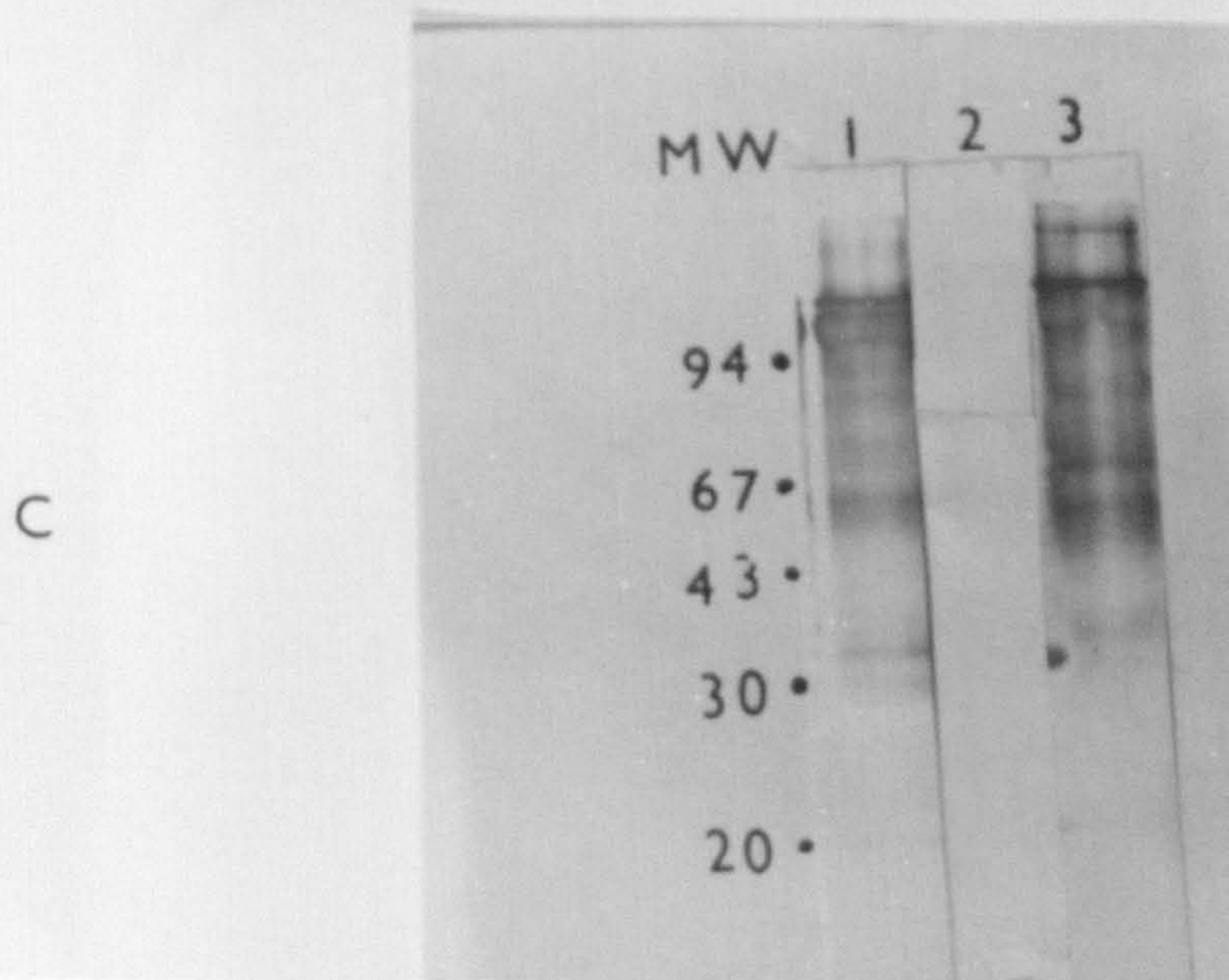
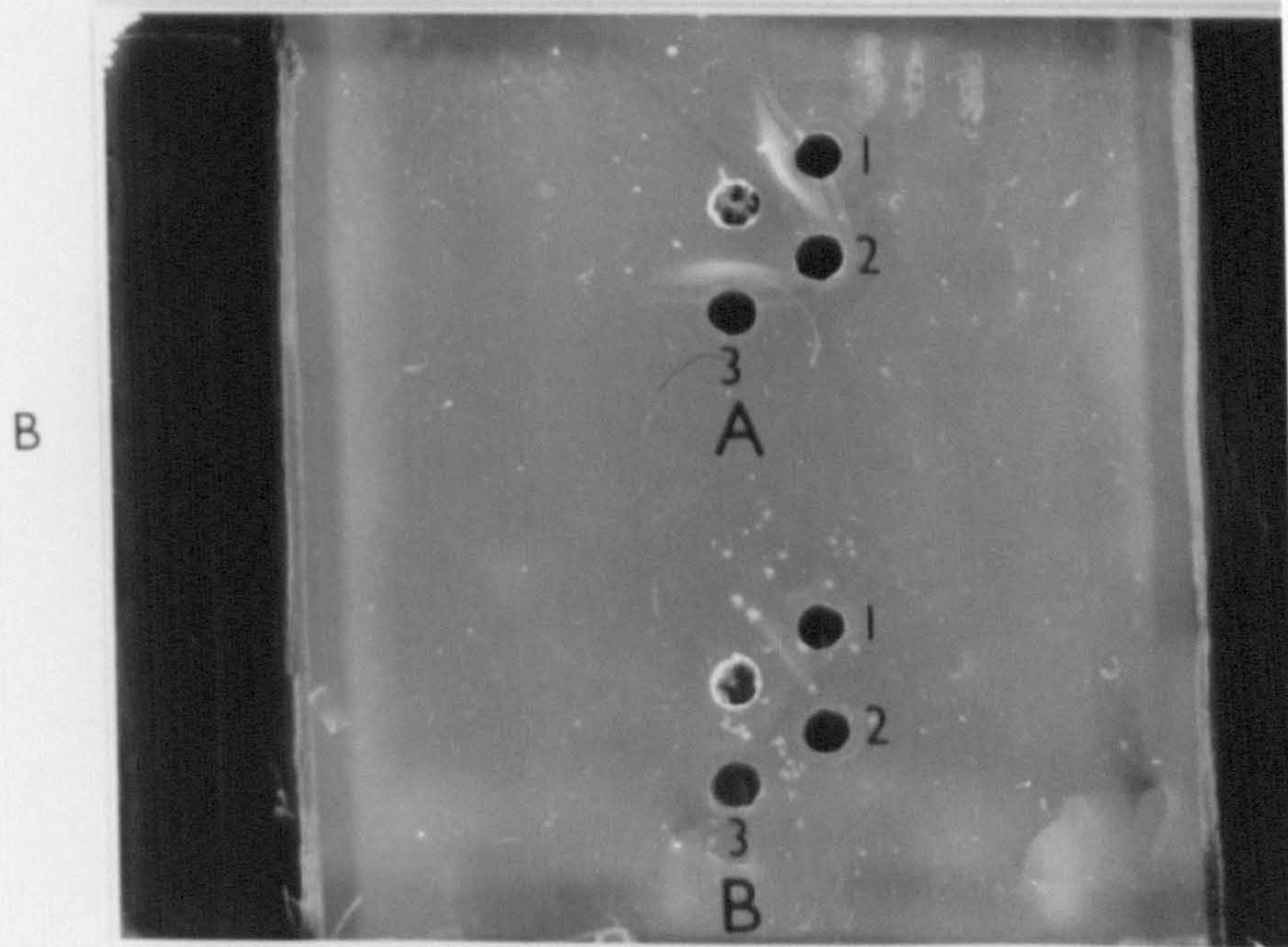
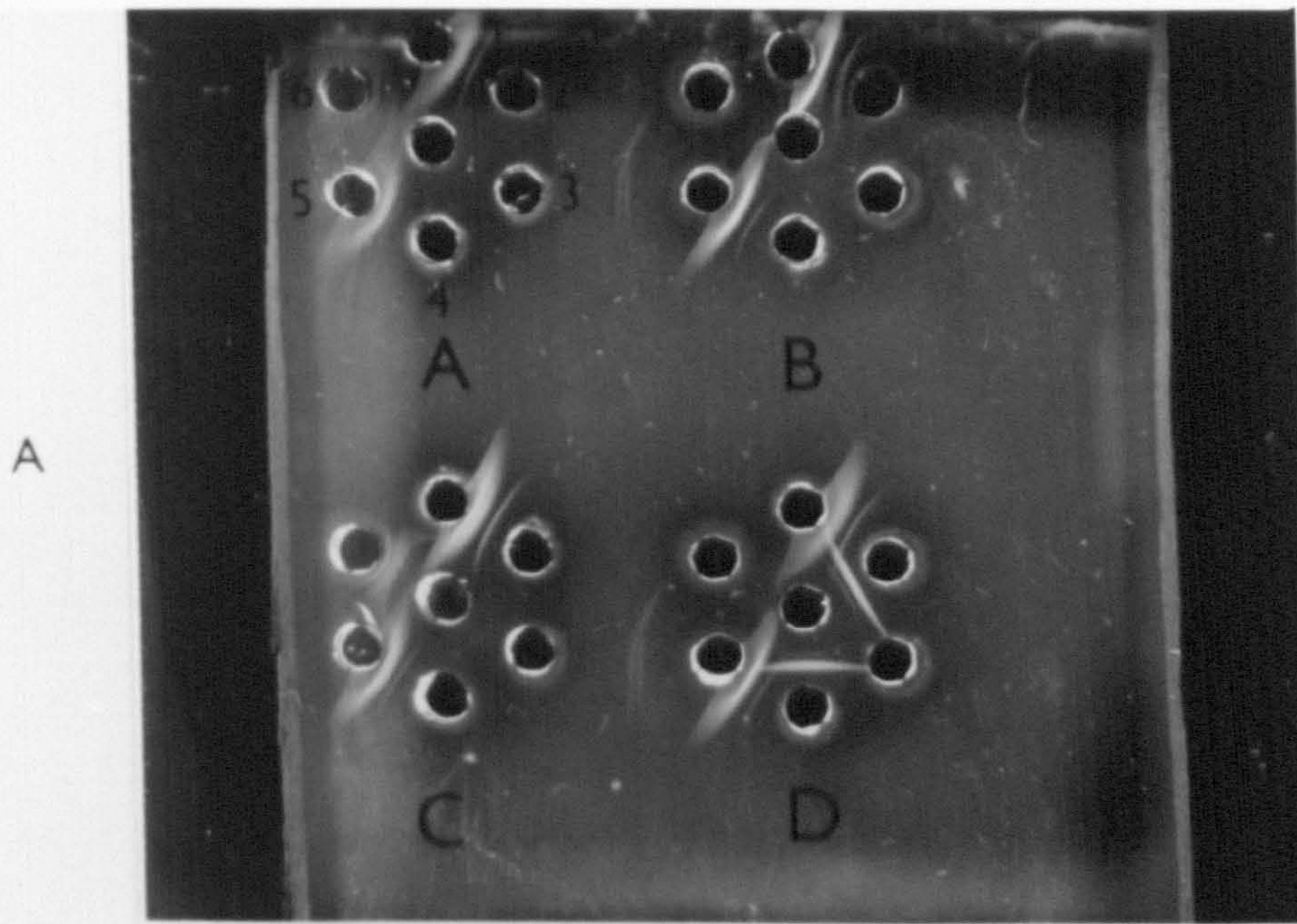
C) Western blot of the following SEA:

- A) S.margrebowiei.
- B) S.margrebowiei.
- C) S.mansoni.

probed with the following mouse sera:

- A) Chronically infected S.margrebowiei mouse.
- B) Normal mouse.
- C) Chronically infected S.margrebowiei mouse.







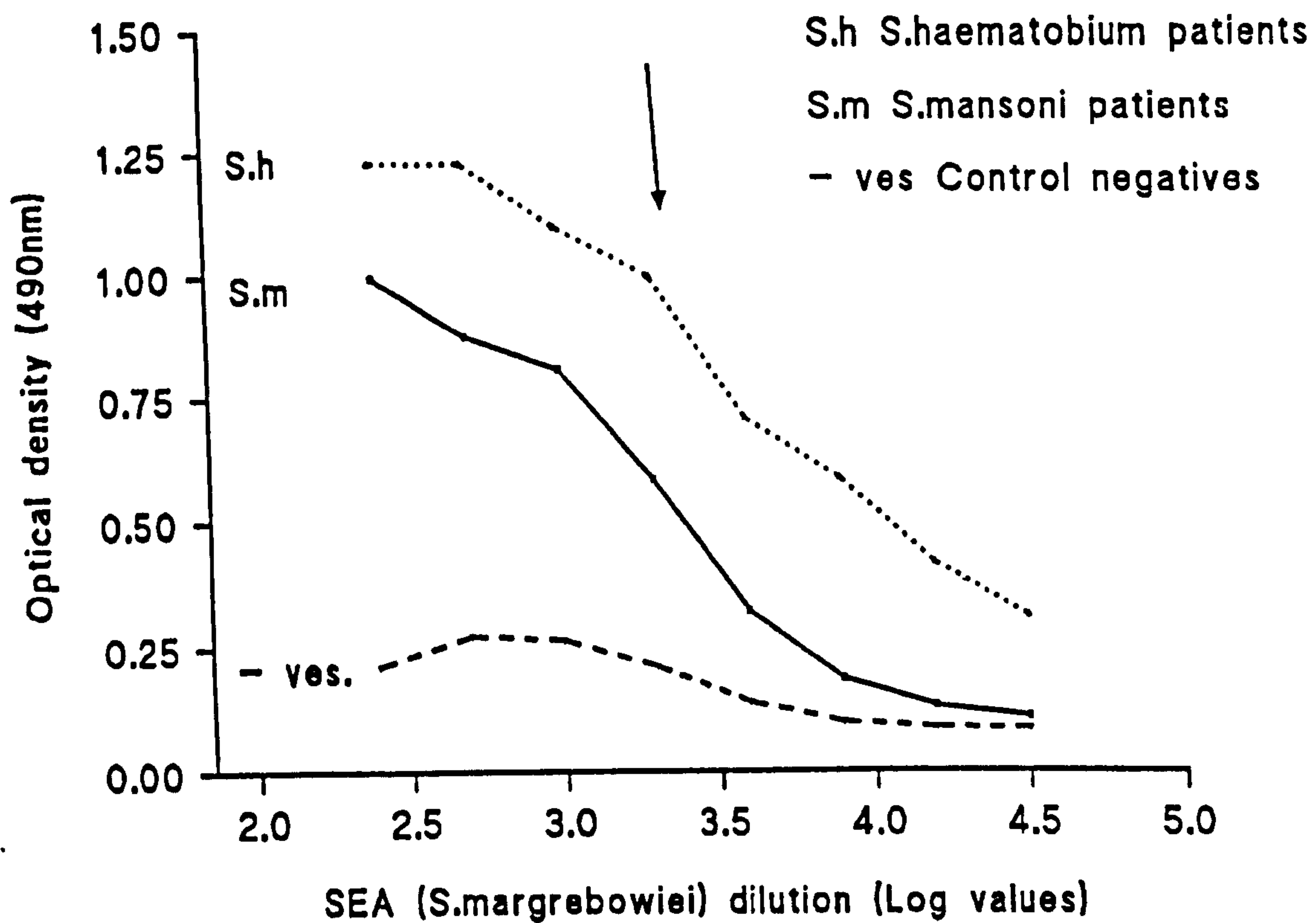


Figure 7.3 The determination of optimum S.margrebowiei soluble egg antigen (SEA) dilution for Enzyme-linked immunosorbent assay (ELISA) with human sera. Arrow indicates the optimum S.margrebowiei SEA dilution (1/4000) for the maximum discrimination between S.haematobium, S.mansoni and negative control sera.

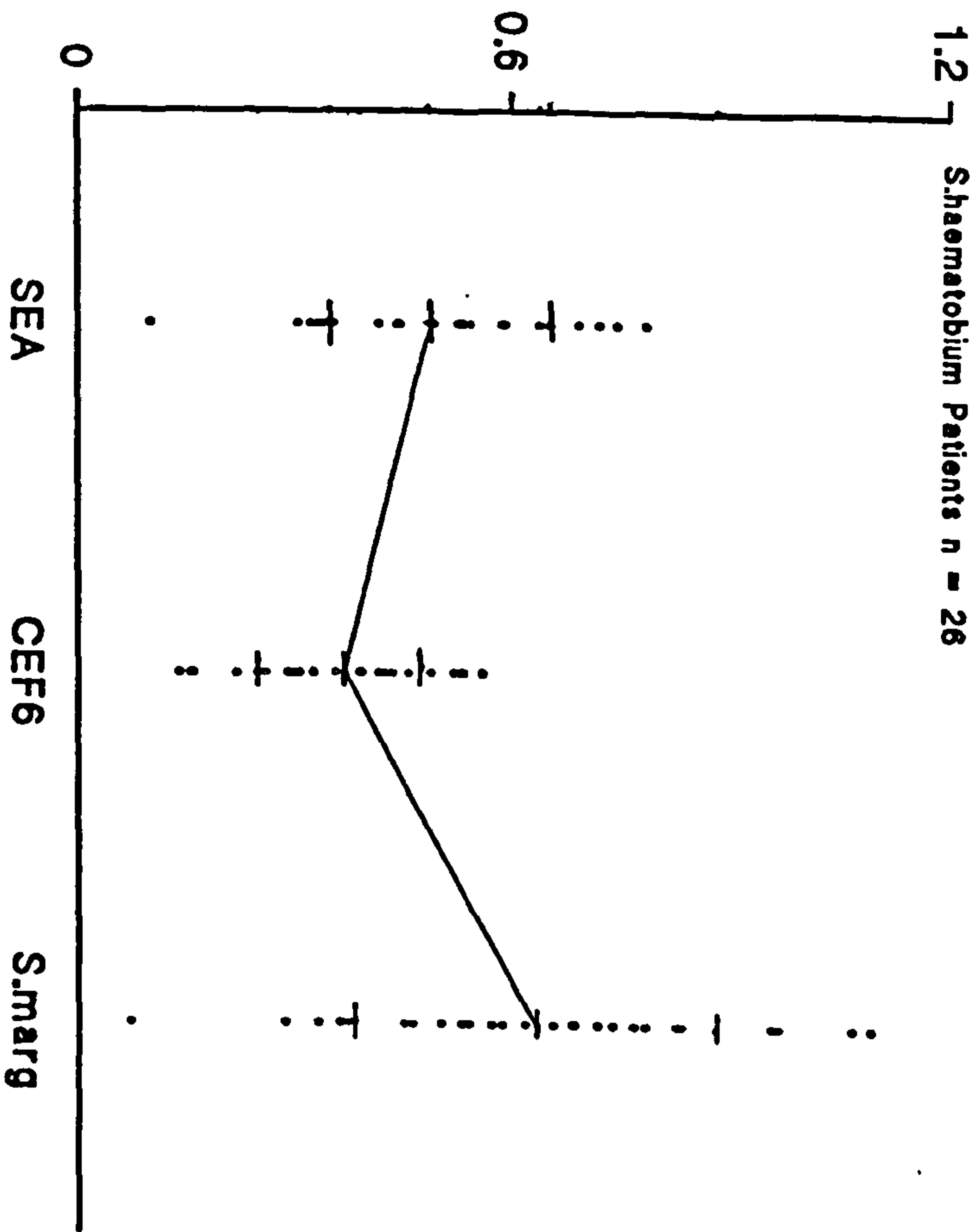


Figure 7.4 Species specificity of S.mansoni SEA, CEF6, and S.margrebowiei SEA by ELISA with human sera from parasitologically positive S.haematobium and S.mansoni patients.

Lines join the means of the data and bars represent the standard deviation of the mean.

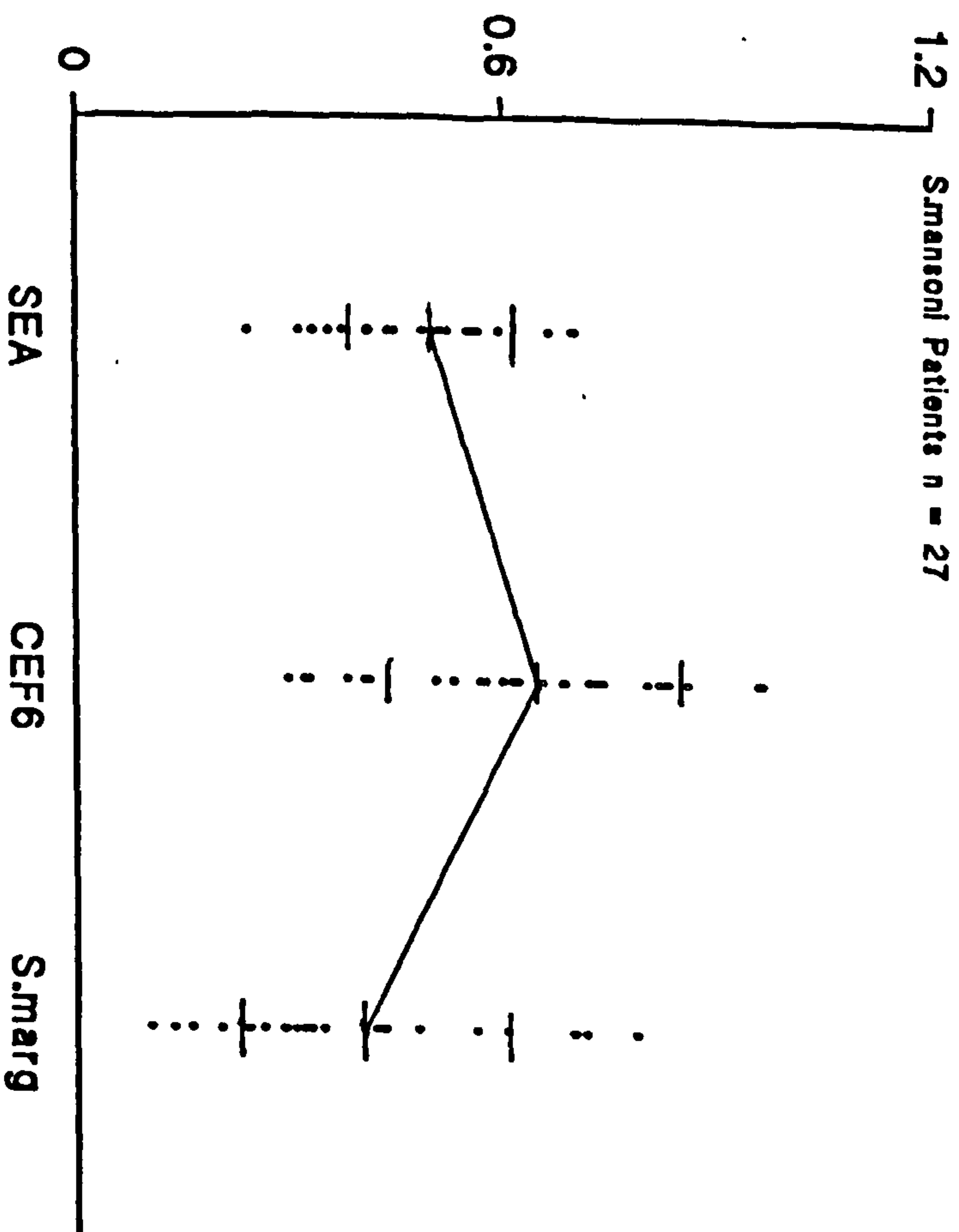
Student's t-test analysis of results comparing S.haematobium and S.mansoni sera, using S.margrebowiei SEA indicated a significant difference ( $t= 2.7852, p < 0.01$ ).

Optical density at 490nm.



Species specificity of S.mansonii SEA, CEF6, and S.margrebowiei SEA by ELISA with sera from Shaeematobium patients.

Optical density at 490nm.



Species specificity of S.mansonii SEA, CEF6, and S.margrebowiei SEA by ELISA with sera from Smansonii patients.



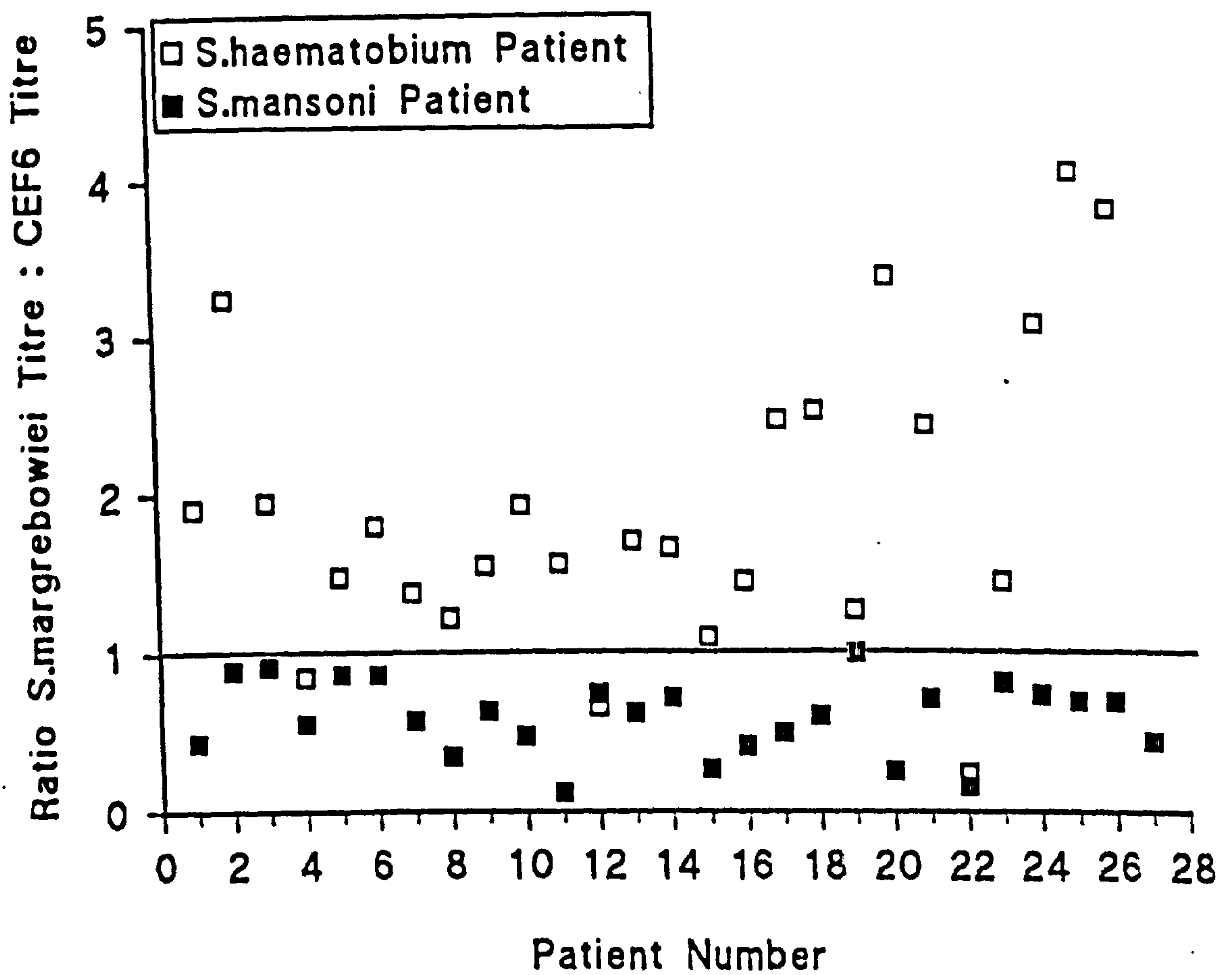


Figure 7.5 A comparison of of the ratio of S.margrebowiei SEA titre : CEF6 titre for individuals infected with S.mansoni or S.haematobium.

## Discussion:

Leucine aminopeptidases (LAP) from schistosomes were first reported in S.rodhaini in the dorsal and lateral cuticle of the adult male worm (Fripp, 1967). Coles (1970) also demonstrated LAP from S.mansoni by electrophoresis. It was found to be a single, non-migrating fraction in adult male worm homogenate. Auriault et al., (1982) reported that these LAPs were active at neutral pH and metal dependent. LAP activity was also observed histochemically from the egg stage (Bogitsh, 1983). Xu and Dresden (1986) located LAP activity in extracts of eggs, miracidia, cercariae, adult worms, SEA and hatching fluid of S.mansoni. Their results, indicated a possible role for LAP in the hatching mechanism of eggs. This was realized when bestatin an inhibitor of LAP was used. This compound, inhibited hatching, supporting Kusel's (1970) hypothesis that a "hatching factor" or that proteolytic enzyme(s) could be involved in the hatching process. An increase in NaCl concentration, also inhibited LAP activity, and is an explanation of why eggs fail to hatch in saline. Enzymes are known to be biologically active following immunoprecipitation with antibodies (Vriel, 1963), therefore staining with a suitable chromogenic substrate should locate this activity. Using this principle, Doenhoff et al., (1988) reported LAP activity in the eggs of S.japonicum and S.mansoni. LAP has also been visualized in the immunoprecipitate of S.haematobium and S.bovis (Tricker and Doenhoff, unpublished) using L-leucine  $\beta$ -naphthylamide, they also found that antisera raised by infection with LAP of one



schistosome species, were not immunologically cross-reactive with LAPs of other species (a small degree of cross-reactivity was recorded in the related of S.haematobium and S.bovis ), moreover, there was also a degree of stage specificity in that egg LAP was not detected in the adult nor other larval stages of schistosomes. The result of the present study, using L-leucine  $\beta$ -naphthylamide indicated that although LAP was present in the SEA of S.mansoni, it was absent in S.margrebowiei SEA (Fig. 7.1). However, when L-Leucine p-nitroanalide substrate was used, results contradicted this conclusion and confirmed that both SEAs exhibited LAP activities. The failure of the IEP to demonstrate LAP activity, could be due to presence of large amount of antibodies in the precipitant, hence inhibiting the enzymatic activity of LAP. The protein assay results showed that the S.mansoni SEA used in the present study had approximately twice the protein concentrations of S.margrebowiei and six times the amount of LAP. This explains the higher readings for S.mansoni SEA at 410nm (Table 7.1). Earlier findings of Tricker and Doenhoff (unpublished data) regarding the species and stage specificity of LAP and possible cross-reactivity among sibling species, has lead to investigations on the possible serodiagnostic properties of this enzyme, LAP in S.margrebowiei SEA (a closely related species to S.haematobium) if cross-reactive with S.haematobium, could be used as a possible substitute for the diagnosis of S.haematobium patients.

The immunodiffusion studies on the possible cross-

reactivity among various SEAs and chronically infected S.margrebowiei mouse sera gave negative results. However it should be noted that both S.bovis and S.haematobium SEAs used in the present study (Fig. 7.2 A) were very old batches, which had been thawed and frozen regularly, this could have denatured the proteins or activated the proteases in SEA hence reducing the antigenicity of the SEA. Since fresh materials were not available, these assumptions could not be tested. Alternatively, it is possible that there was no cross-reactivity or the immunodiffusion technique was not sensitive<sup>enough</sup> to detect any cross-reactivity. The evidence for this came from results shown in Fig. 7.2B and Fig.7.2C. Although no cross-reactivity was seen between S.mansoni and S.margrebowiei, the Western blot analysis, suggested that there was a strong degree of cross-reactivity between the two. Uninfected mice did not form precipitates with any SEA (Fig.7.2 B) nor was there any cross-reactivity seen in the Western blot (Fig. 7.2C lanes C and F). To test this further and to investigate the potential of S.margrebowiei SEA in diagnosis of S.haematobium patients, aliquots of S.margrebowiei, S.mansoni SEAs and CEF6 were sent to London for ELIZA analysis.

Using S.haematobium, S.mansoni, and normal human sera with dilution of 1/300, and S.margrebowiei SEA with dilutions of 1/250, 1/500, 1/1000, 1/2000, 1/4000, 1/8000, 1/16000, and 1/32000, it was found that S.margrebowiei SEA discriminated between the above three sera, with S.haematobium giving the highest readings in every case. The maximum discrimination



between the sera was achieved when 1/4000 dilution was used (Fig. 7.3). These results indicated that there was cross-reactivity between S.margrebowiei SEA and sera from S.haematobium and S.mansoni patients used here, and that generally, S.haematobium sera were more reactive to S.margrebowiei SEA than S.mansoni which in turn was more reactive than normal negative sera. In parallel studies, the diagnostic potential of S.margrebowiei SEA was assessed using S.haematobium and S.mansoni (Fig. 7.4) human sera. The results showed a great overlap of ELISA reactivities. S.haematobium and S.mansoni infection sera had similar distribution of reactivity against S.mansoni SEA (The mean value for S.haematobium infection sera =  $0.49 \pm 0.16$  and for S.mansoni infection sera =  $0.49 \pm 0.12$ ). As expected, S.mansoni sera were more reactive against CEF6 than S.haematobium (the mean value for S.mansoni sera =  $0.63 \pm 0.19$  and for S.haematobium sera =  $0.36 \pm .12$ ). Interestingly, S.haematobium sera were found to be more reactive against S.margrebowiei SEA than S.mansoni (the mean value for S.haematobium sera =  $0.63 \pm 0.25$  and for S.mansoni sera =  $0.40 \pm 0.18$ ). Comparison between S.mansoni and S.haematobium patients using the three antigens were as follows:

<u>S.mansoni</u> SEA	No significant difference	
CEF6	t = 4.5583	P < 0.001
<u>S.margrebowiei</u> SEA	t = 2.7852	P < 0.01

So S.margrebowiei SEA gave significantly higher values for S.haematobium patients when compared with S.mansoni patients.

The specificity of S.margrebowiei SEA could become higher if the ratio of antibody titres determined using S.margrebowiei SEA over those determined using CEF6 were looked at .

The ratio of antibody titres determined using S.margrebowiei SEA over those determined using S.mansoni CEF6 were calculated for each individual. The results are shown in Figure 7.5, 88.5% of patients infected with S.haematobium had S.margrebowiei SEA : CEF6 value of greater than 1, and 92.5% patients infected with S.mansoni had S.margrebowieiSEA : CEF6 value of equal or less than 1. This we believe provides a meansby which S.haematobium and S.mansoni infections could be discriminated.



## **CHAPTER EIGHT**

**General conclusions.**

## CHAPTER EIGHT

Throughout their long association with their intermediate and definitive hosts, schistosomes have evolved sophisticated mechanisms enabling them to survive in an immunologically hostile environment. Furthermore, the observation that S.mansoni living within mice which have been deprived of their T cells are much less able to excrete their eggs compared with those in immunologically intact controls (Doenhoff et al., 1978, Doenhoff et al., 1985), indicates that the parasites use the host defense system to help in the completion of their life-cycle.

Today chemotherapy is the most cost effective means of controlling schistosomiasis both in single individuals and in large scale populations (Archer, 1985), and Praziquantel (PZQ) and Oxamniquine (OX) are the chemicals of choice. While chemotherapy is likely to continue to play a leading role in control of the disease in the foreseeable future, since cured individuals are always at risk from reinfection and there is always the possibility of resistance developing to these drugs, the search for a vaccine must be the ultimate goal in the control of this debilitating and ever increasing disease.

A review of the literature on schistosomiasis shows that studies on S.mansoni, exceed those carried out on any other species of the genus. One reason for this is the apparent ease of culturing Biomphalaria glabrata the intermediate snail host for S.mansoni in laboratory conditions. This thesis on the biology of Schistosoma margrebowiei (a sibling



species of S.haematobium belonging to the group of schistosomes with terminal spined eggs) is an attempt to redress some aspects of this imbalance.

To carry out large scale studies on S.margrebowiei, the available method of maintaining Bulinus natalensis snails had to be improved. In Chapter two a new method of maintenance yielding rapid and continuous growth of B.natalensis has been developed. Maintenance of snails in aerated 15 litre aquarium tanks, coupled with the infection of snails (2-4mm in length) en mass has proven to be a successful method of culturing the parasite. The results of this aspect of study confirm the earlier findings of Loker et al., (1987), Evans, (1985), and Niemann and Lewis, (1990), that the size of the snails at exposure has a marked effect on their susceptibility to infection.

Studies on the morphology and ultrastructure of S.margrebowiei in particular its larval stages (Chapter three), have shown that it resembles closely other species of schistosomes. It is therefore impossible to identify or classify species of the genus on the basis of the morphology of their larval stages.

Upon pairing, females of S.margrebowiei are carried by the males worms to the venules of the anterior mesenteric veins where they lay eggs, initiating the pathology associated with the disease. The results <sup>reported in</sup> Chapter four indicate that apart from the involvement of expected tissues, such as the liver and intestine, 10-15% of the total eggs recovered were deposited in the spleen of infected BKTO mice.

This suggests that the splenomegaly typically seen in schistosomiasis is due not only to a passive congestion of the portal circulation (splenic vein) and hyperplasia of splenic cellular elements, but arises also as a direct result of the deposition of eggs in this organ.

The cercariae of S.margrebowiei, can live up to 70 hours at 26-28°C in fresh copper-free water. In Chapter five, it has been shown that life expectancy is prolonged from three days to a week when cercariae are maintained in water temperatures of 0-4°C. However, the majority (90%) of cercariae older than 1 day, although capable of penetration fail to develop into adults. Thus these cercariae were clearly attenuated. Previous studies on irradiated-attenuated cercariae by, Radke and Sadun, (1963), Smithers and Terry, (1965), Taylor et al., (1977), Bickle et al., (1979), Ford et al., (1984), and Moloney et al., (1987) have shown that a significant level of resistance to infection with schistosomes is induced by administration of these irradiated-attenuated larvae. It would be interesting to undertake further studies to evaluate the potential for using such naturally attenuated cercariae for immunization purposes.

Although immunological cross-reactivities between various species of schistosomes and their particular intermediate hosts have been established for a long time (Capron et al., 1965 and 1968), very few workers have attempted to utilize snail constituents as a means of immunizing mice against Schistosoma species infection. The



unlimited availability and cheapness of snails would make molluscan tissue an ideal vaccine candidate. Results from Chapter seven have shown that homogenates of the digestive gland of B.glabrata and B.natalensis cross-react with various anti- S.mansoni rabbit sera, indicating that snail material from at least two species could be potentially used. A pilot study on the immunization of mice was carried out using soluble hepatopancreas antigen (SHA) of B.glabrata and followed by subsequent S.mansoni challenge. Although mice immunized with infected SHA, showed 30 and 16% decreases in worms and eggs respectively, statistical analysis indicates that SHA did not confer significant protection against S.mansoni infection. This does not mean, however, that snail tissue has no immunoprophylactic potential. Studies on the value of SHA in the passive transfer of anti-SHA sera with PZQ would be potentially interesting.

Although serological detection of specific antibodies does not differentiate between active and past infections, it does offer high sensitivity and reproducibility compared to antigen detection methods. Collaborative results in Chapter seven using the sera from actual human infections indicate that Schistosoma margrebowiei soluble egg antigen (SEA) gives higher optical densities with S.haematobium sera, than S.mansoni sera. It is therefore possible to discriminate S.haematobium infection from S.mansoni using S.margrebowiei SEA. The specificity of this antigen in the serodiagnosis of S.haematobium could be improved by purifying this crude egg homogenate. Naturally while this would cost more, genetic

engineering might offer a cheaper alternative.

The direction of future work on S.margrebowiei, resulting from the observations presented here have been outlined in the relevant preceding chapters. For effective disease control, a reliable, cost-effective and reproducible serodiagnostic technique is required. The identification of the value of S.margrebowiei SEA in the serodiagnosis of S.haematobium patients, we believe could achieve this goal. This thesis also demonstrates the value and importance of non-human animal schistosomes as potential models for the human species, and in particular those that are difficult to culture in laboratory conditions. There is thus considerable scope for further studies on this species of schistosome. For example investigation of the production of DNA changes in infected mice and the effects of such changes on tumour formation similar, to those shown in S.mansoni in our laboratory (Badawi et al., In press) could help to explain the connection between the incidence of human bladder cancer and infection with S.haematobium.



## **APPENDIX**

## Appendix

### 1) Barbitone buffer pH 8.6

27.6g barbitone  
154.5g barbitone sodium  
10L distilled water

### 2) Perfusion fluid

8.6g sodium chloride  
15g trisodium citrate  
2000 units heparin  
0.2g merthiolate  
1L distilled water

### 3) Phosphate-buffered saline pH 7.2 (PBS)

42.5g sodium chloride  
1.95g sodium dihydrogen orthophosphate  
5.34g disodium hydrogen orthophosphate  
5.0L distilled water.

### 4) SDS-PAGE and Western blotting

#### a) Resolving Buffer

3M Tris-Cl pH 8.8 filtered through a 0.22 $\mu$ m filter

#### b) Stacking Buffer

0.25M Tris-Cl pH 6.8 filtered through a 0.22 $\mu$ m filter

#### c) Lower Reservoir Buffer

50mM Tris-Cl pH 8.8



d) Run Buffer

0.25M Tris, 1.92M glycine, 1% SDS pH 8.4

e) Electroblothing Buffer

25mM Tris, 190mM glycine, 20% methanol

f) Transblotting Solution (TBS)

20mM Tris, 0.9% NaCl pH 7.2

g) Tween Transblotting Solution (TTBS)

20mM Tris, 0.9% NaCl, 0.1% Tween 20 pH 7.2

5) Silver Stain

200mg Trisodium citrate

80mg Ferrous sulphate

9.9ml distilled water

6) Substrate solution

20ml TBS

20mg 4-chloro-1-naphthol in 4ml methanol

10 $\mu$ l H<sub>2</sub>O<sub>2</sub>

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