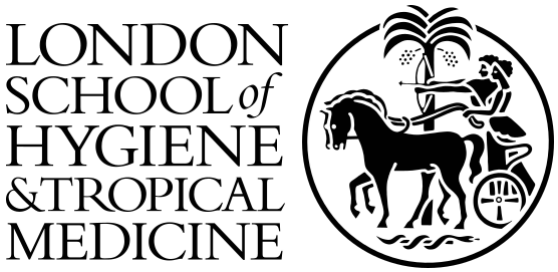

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STUDIES ON SOME FACTORS AFFECTING THE DEVELOPMENT OF SCHISTOSOMES IN THEIR MOLLUSCAN HOSTS

A thesis submitted for the
Degree of Doctor of Philosophy in the
University of London (Faculty of Science)

by

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A B S T R A C T

After a brief historical account of research in schistosomiasis and a statement of the nomenclature of Schistosoma and its molluscan host, an attempt has been made in the first part of the thesis to synthesize the available information about the influence of factors of the external environment, the parasite and the host upon the development of schistosomes in snails. Several gaps in our knowledge of the subject are pointed out, especially with regard to bulinid snails and members of the Schistosoma haematobium group.

In the second part a general description is first given of the histology of the head-foot, tentacle, mantle and digestive gland of Bulinus (Physopsis) africanus and Bulinus (Bulinus) truncatus. With the head-foot and mantle special attention was paid to the morphology, behaviour and origin of amoebocytes and a lymphoid area is described in the mantle which, it is suggested, is the source of the amoebocytes. A combination of histological, histochemical and electron microscopical techniques has shown that the digestive gland contains the cells, called "calcium cells" in other molluscs, which are very actively secretory and digestive cells that are probably mostly absorptive.

It is then shown that a refractory strain of B. truncatus was as attrative for the miracidia of S. matthoei as were two susceptible races of B (P) africanus. Comparative histological observations have shown that the surface of B. truncatus offered a very effective barrier against the penetration of the miracidia. Electron microscope observations have revealed a modification of the surface of the epidermal plate of the apical papilla of the miracidium that may be the means of attachment of the organism to the host surface. It is suggested that the apparatus is probably better adapted for attachment on the surface of the normal host, B (P) africanus than on B. truncatus. Histological observations have shown that the few miracidia that penetrated B. truncatus were destroyed by an amoebocytic reaction. The tissues of B (P) africanus did not react to the miracidia, mother sporocysts or daughter sporocysts, but there was a strong amoebocytic reaction against cercariae. It is suggested that a schistosome infection will probably only succeed if the host fails to recognize the early stages as foreign material. It is also shown that the thickness of the tissue in which miracidia settle determines their survival. The pathological effects of schistosome infectio n on the digestive gland are briefly described.

Finally the results of a combination of histochemical and electron microscope observations have strongly suggested that the body wall of the daughter sporocyst of S. mattheei and S. bovis plays a fundamental role in actively transferring carbohydrate, in the form of glucose, from the tissues of the host snail B (P) africanus, to the cercarine developing inside the parasite.

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PURPOSE AND DESIGN

The thesis is concerned with factors which influence the development of trematode parasites of the genus Schistosoma in molluscan hosts, but before discussing this subject an introductory account is given of the main landmarks in the knowledge of schistosomiasis together with a statement of the nomenclature of the parasites and their snail hosts.

In dealing with the factors affecting the development of schistosomes the thesis is divided into two parts. Part I is a synthesis of information from previous work. The only factors considered here are those which affect the location and penetration of snail hosts by schistosome miracidia and those which influence the subsequent development of the parasite inside the host. The thesis is not concerned with wider ecological factors which influence the distribution and population structure of snail hosts. Part II of the thesis records observations on some of the factors which influence the development of S. matthoei and S. bovis in the snail hosts Bulinus (Physopsis) africanus and Bulinus (Bulinus) truncatus.

These studies cover:-

(1) the internal environment in which the parasites live - a histological and histochemical study of the tissues of the head-foot, mantle and digestive gland of Physopsis africanus and Bulinus truncatus;

(2) the mechanisms of resistance of the snails to infection with the schistosome parasites;

(3) the metabolism of the daughter sporocyst - a histological, histochemical and electron microscope study of the mature daughter sporocyst.

C H A P T E R 1

HISTORICAL INTRODUCTION

The disease now called schistosomiasis, or bilharziasis, has affected man and animals for many centuries. Several Egyptian papyri 3,000 to 4,000 years old refer to haematuria and give prescriptions for its treatment, (Malek, 1961). In 1910 Ruffer confirmed the antiquity of the disease when he discovered eggs of Schintosoma haematobium in Egyptian mummies dating from the 13th century, B.C.

But the cause of the disease was not known until 1851 when Theodor Bilharz, an anatomist of Cairo Medical School, discovered paired trematodes in the mesenteric veins of an Egyptian. He described the parasite and named it Distomum haematobium (Bilharz, 1852). He then showed that the urine of patients with haematuria contained the eggs of the trematode parasite. Bilharz found that although most of the female worms contained terminal-spined eggs there were a few which had lateral-spined ones. In 1864 Harley noted that in South Africa haematuria was caused by a schistosome which produced only terminal-spined eggs. He called this form Bilharzia capensis to

distinguish it from the Egyptian parasite which was generally believed to produce both terminal-spined and lateral-spined eggs.

That there was more than one species of the blood fluke was clearly shown by Sonsino. In 1876 he described Schistosoma bovis which he had recovered from the mesenteric veins of cattle at the Cairo abattoir; (Weinland had by this time created a new genus, Schistosoma, for the Distomum haematobium of Bilharz). The discovery of S. bovis must have strengthened the views of those, especially Manson and Sonsino, who believed that in Egypt two species of Schistosoma infected man. But Looss, the leading helminthologist of that time, strongly argued for one species. He believed that the lateral-spined eggs were produced by unfertilized females. Reports of the occurrence of only lateral-spined eggs in the faeces of patients in the West Indies and in Uganda greatly strengthened the case for the separation of the parasites with lateral-spined eggs from those with terminal spines. Consequently Sambon (1907) described and created the species mansoni for the parasite with lateral-spined eggs. But the validity of the species was not accepted by all helminthologists until Leiper in 1915 finally showed that not only did S. haematobium and S. mansoni produce different types of eggs, but they also developed in snail hosts belonging to two quite distinct genera as intermediate hosts.

It took helminthologists more than fifty years to discover the life cycle of Schistosoma. Two factors seem to have been responsible for this delay. The first is that Bilharz's discovery of 1851 came before the life cycle of any trematode had been worked out. Reinhard (1957), in his historical review of the discovery of the life cycle of Fasciola hepatica, has shown that by the middle of the 19th century parasitologists and naturalists had come to believe that the cercariae and rediae so commonly found in gastropod molluscs were developmental stages of flukes. That molluscs served as intermediate hosts of trematodes was proved by Leuckart and Thomas in 1882. These workers, one in Germany and the other in England, independently discovered that Lymnaea truncatula was the intermediate host of Fasciola hepatica.

The other factor responsible for the delay in discovering the life history of Schistosoma was the authority of Looss. Many unsuccessful attempts were made to find the molluscan or arthropodan intermediate host of the blood fluke. The failure of the efforts of such eminent helminthologists as Cobbold, Lortet, Sonsino, Vialleton and Looss himself forced Looss to abandon the intermediate host hypothesis. He proposed instead that Schistosoma had a direct life cycle; miracidia penetrated man when

he came into contact with infected waters. Once inside the human body the miracidium travelled to the liver where it changed into a sporocyst which gave rise to the adult worms. Looss (1908) bitterly attacked the intermediate host hypothesis in his paper entitled, "What is Schistosomum mansoni?" Leiper (1918) says that much effort was wasted over a period of 20 years by workers trying to verify Looss's theory of direct human infection by miracidia. This, Leiper says, was done in submission to Looss's "great authority in helminthological matters and his skill in dialectics." But there were men like Manson, Blanchard and Sambon who never abandoned the belief that a mollusc was necessary for the development of larval stages of blood flukes.

This long and heated controversy was brought to an end by the investigations carried out by Japanese workers and by Leiper. A new species, S. japonicum, was described by Katsurada in 1904. The presence of trematode eggs in the organs of people who had died of "Katayama disease" had led a number of Japanese physicians to believe that there was a causal relationship between the eggs and the disease. In 1903 Kasai, working in a district called Katayama, found the trematode eggs in the faeces of a patient. Fujinami in the following year recovered an

adult female from the mesenteric veins of man. In the same year, 1904, Katsurada, unable to obtain material from man, collected and described paired adults from dogs. By 1909 Fujinami had shown that cattle, horses, dogs, as well as man, were natural hosts of the schistosome. During the years 1912-1914 Miyagawa, Miyairi and Suzuki, between them, worked out the complete life cycle of the fluke. They first showed experimentally that cercariae shed by the snail now called Oncomelania nosophora infected mammals through the skin and developed into S. japonicum adults. They then found that miracidia which hatched from the eggs of the schistosome penetrated into Oncomelania and developed, through two generations of sporocyst, into cercariae. In 1914 Leiper and Atkinson visited China and Japan. Although they failed to find the intermediate host of S. japonicum in China, they confirmed the findings of the Japanese workers (Leiper and Atkinson, 1915).

During the first World War large numbers of British troops were stationed in Egypt, especially in the Nile Delta where schistosomiasis was both widespread and severe in the peasant population. The British War Office was therefore anxious to discover the mode of transmission of the disease so that the troops could be protected. In 1915 Leiper was sent to Egypt in charge of a mission

whose two-fold task was to discover the way in which schistosomiasis was transmitted and then to advise on what preventive measures should be instituted. Leiper quickly found that the human blood flukes were not exceptional, but like the other digenetic trematodes they had molluscan intermediate hosts; bulinid snails acted as intermediate hosts of S. haematobium while species of Biomphalaria were hosts of S. mansoni. By 1883 it was known that the development of a digenetic trematode inside a molluscan host took one of two routes: a miracidium penetrated into the snail in whose tissue it became a sporocyst which gave rise either to more sporocysts or to rediae; the second generation (daughter) sporocysts and rediae then produced cercariae (Thomas, 1883). Leiper demonstrated that miracidia of both S. haematobium and S. mansoni penetrated the soft parts of the snail host and these became sporocysts. These gave rise to a second generation (daughter) sporocysts which migrated to the digestive gland where they, in turn, gave rise to cercariae. Miyairi and Suzuki had shown this to be the mode of development of S. japonicum in Oncomelania. Subsequent observations on the route of development of the molluscan stages of other schistosomes have shown that the production

of cercariae by daughter sporocysts is the only route of development in the family Schistosomatidae. Leiper also made a very careful study of the development of the two Egyptian schistosomes in experimental mammalian hosts - monkeys and rats.

The next important landmark, after the discovery of the cause and mode of transmission of schistosomiasis, was the introduction of drugs for the treatment of the disease. Emetine, which had been discovered by Rogers in 1913 for amoebic dysentery, was first employed for the treatment of schistosomiasis by Tsamis (1913) and Diamantis (1916). But much more important was the independent discovery of the curative value of antimony compounds in 1917 by McDonagh and Christopherson. Antimony compounds are still the best drugs for the treatment of all three types of human schistosomiasis. However, they are toxic and often difficult to administer. During the last 20 to 25 years search has also been made for other schistosomacides. A number of xanthone derivatives have shown promise during the screening stages, but at the present time only lucanthone hydrochloride (Miracil D) is of any clinical value. The recent development by Ciba of Ambilhar, a nitrothiazol, has received much publicity, but the drug has not yet been adequately tested in the field (World Health Organization, 1966).

During the last 15 years Bueding and his co-workers have studied in great detail the carbohydrate metabolism of S. mansoni. They have shown that although the parasite utilizes some oxygen, the major source of energy is anaerobic metabolism which proceeds via the Embden-Meyerhof scheme of phosphorylating glycolysis. They have found that trivalent antimonials kill the parasite by inhibiting the phosphorylating enzyme, phosphofructokinase. Bueding has also demonstrated that alkyldibenzylamines interfere with the active transport of glucose into S. mansoni adults without inhibiting any of the enzymes involved in the carbohydrate metabolism of the parasite (see Standen, 1963).

Compared with other trematode diseases, schistosomiasis has been very extensively studied. Even before the life cycle of schistosomes was known, important clinical and pathological observations were made on the disease due to S. haematobium in Egypt, S. japonicum in Japan and S. mansoni in South America. Fairley's experimental studies on monkeys infected with S. haematobium and S. mansoni and his clinical observations in 1920 laid the foundations upon which much of the later work has been based. In 1924 Faust and Meleney made an important contribution in their studies on the disease due to

S. japonicum in China. During the last 40 years many hundreds of papers on the pathology of schistosomiasis have been published and a good deal is therefore now known about the pathological manifestations corresponding to the period when the worms are developing, the early period of egg-laying and the later chronic stage when eggs tend to be trapped in the tissues of the patient. The World Health Organization Expert Committee on Bilharziasis (1965) have summarized what is known of the pathology of the disease, but have pointed out many gaps which need filling.

However, although a great deal is known about the clinical manifestations of schistosomiasis, the importance of the disease as a public health problem has not always been adequately assessed; this is particularly true for the disease due to S. haematobium. This conflict of opinion is well illustrated by the discussion which has followed the paper by Forsyth and Bradley (1964) and Forsyth and Macdonald (1965) on so-called irreversible urological changes in children infected with S. haematobium in Tanzania (see Nelson in the discussion of the paper by Webbe and Jordan, 1966; also Lucas, Adeniyi-Jones, Cockshot and Gilles, 1966).

One important, if not the most important, reason for the uncertainty about the public health importance of schistosomiasis is the paucity of information on acquired immunity to/disease. As long ago as 1910 Yoshimoto demonstrated the presence of a complement-fixing antibody in man infected with S. japonicum; other Japanese had demonstrated the presence of antibody against the parasite in sera of calves in 1909. Since that time a vast literature on serological tests for schistosomiasis has accumulated. Kagan and Pellegrino (1961) have provided a good critical review of all these methods except the fluorescent antibody technique which Sadun and his co-workers have recently introduced (Sadun, 1963).

Although there is plenty of evidence (as is shown by the various serological reactions) for the presence of antibodies in circulation in the blood of both man and animals, the significance of these antibodies in relation to schistosome infections is not known. Fujinami in 1916 was the first to draw attention to the fact that people exposed to S. japonicum for the first time became much more seriously ill than those constantly exposed to infection. It is now generally accepted that in endemic areas schistosomiasis is more severe in children than in adults. This suggests that man develops some degree

of resistance to the disease. But the only experimental evidence in support of this comes from the work of Fisher (1934) who exposed 6 Congo men from a highly endemic area to hundreds of cercariae of S. intercalatum and found that they were resistant to infection. It was the studies of Vogel and Minning (1953) on S. japonicum in rhesus monkeys that stimulated the large volume of work that has been published (especially by Sadun and his associates working on S. mansoni) during the last and present decades. Smithers (1962) has critically reviewed the literature on this subject; he concluded that, from largely circumstantial evidence, man can gradually become resistant to Schistosoma infection, mice and hamsters become partially resistant to re-infection, while rhesus monkeys under certain conditions become completely resistant. The mechanism of resistance is not known, although both humoral and cellular are believed to be involved.

An interesting suggestion was made by Le Roux (1961) that in endemic areas the exposure of man to cercariae of bovine schistosomes may immunize him against the human species, and vice versa. This idea has been more fully developed by Nelson (1964).

During the last 50 years almost all research on the snail side of schistosomiasis has been concerned with two questions: discovering actual and potential intermediate hosts in various countries and finding means of destroying the snail vectors so as to interrupt transmission of the disease. In Africa a very large number of species of planorbid snails were reported as natural hosts of human and animals schistosomes. Most of these "species" were, however, mainly based on shell characters and this led to much confusion which has been generally resolved by Mandahl-Barth (1957), who has systematized the African snail hosts on the basis of their soft anatomy and shell characters. In the Far East Abbott (1948) had already shown that all forms of Oncomelania could be reduced to 4 species. An important result of the attempts to infect potential snail hosts was the discovery of the existence of geographical strains of both schistosomes and snails (Vogel, 1941; review by Wright, 1962[a]). Hsu and Hsu, working on S. japonicum, and Saoud (1966) on S. mansoni have shown that geographical races of schistosomes also differ in their behaviour in mammalian hosts. (See Hsu and Hsu, 1962, for studies on strains of S. japonicum)

Almost as soon as the role of molluscs in the transmission of schistosomiasis had been discovered, the importance of destroying the intermediate hosts was recognised. In 1915 Leiper recommended, among other things, the drying of canals in order to destroy snails by dessication. (At that time it was not known that some snails can survive for long periods in dry mud). Leiper also suggested the use of ammonium sulphate for killing snails. Thus, from the beginning, the two main approaches to snail control were discovered; the use of molluscicides and the alteration of snail habitats. The latter method has been used on a large scale in China and the Philippines. From China, considerable success has been reported by Mao Shou-Pai (1962) - see also Discussion following Shou-Pai's paper (Ciba Foundation Symposium on Bilharziasis, 1962, p. 222 ff). But where alteration of snail habitats cannot be integrated into agricultural and development schemes this approach is prohibitingly costly, and this applies to most of the endemic areas in African countries at the present time.

More attention was therefore paid to molluscicides. The first really effective molluscicide to be used was copper sulphate. Chandler discovered the molluscicidal properties of copper sulphate in 1920 and in 1924 Khalil

demonstrated that quite high dilutions of the compound were lethal to species of Bulinus and Biomphalaria. But, as with other aspects of work on schistosomiasis, a large-scale search for really effective molluscicides had to await the stimulus of the Second World War. Since the end of the war, many hundreds of chemical compounds have been screened for molluscicidal properties. There are now 6 really satisfactory molluscicides - satisfactory in their toxicity to molluscs, relative harmlessness to other aquatic fauna and to flora, and in being comparatively cheap. These are: sodium pentachlorophenate, which was introduced in 1948; Baylucide (Bayer 73); ICI.24223 and WL.8008, which is said to be 80 times more toxic to snails than sodium pentachlorophenate and 8 times more toxic than Baylucide (World Health Organization, 1965). Many of these molluscicides are being employed in control projects in Africa, South America and the Far East. The World Health Organization manual entitled, "Snail Control in the Prevention of Bilharziasis" (1965) is a valuable summary of snail control techniques.

In the last 10 years great progress has been made in the ecology - especially the population dynamics - of snail vectors of S. japonicum, S. mansoni and S. haematobium. These studies have revealed seasonal

fluctuations in snail populations and a corresponding pattern of transmission of the parasites (World Health Organization, 1965). Recent attempts by Hairston (1962) and Macdonald (1965) to obtain mathematical models of the epidemiology of the disease have been a valuable stimulus to work in this field (World Health Organization, 1965).

Finally, the development of the concept of schistosomiasis as a zoonosis must be mentioned. Although the importance of domestic animals in the maintenance of S. japonicum was recognized early this century, the possibility that animals might play an important part in the transmission of human schistosomiasis in Africa and South America was not seriously considered until 1952 when Kuntz discovered S. mansoni in a gerbil in Egypt. A number of species of ungulates, rodents, carnivores, primates and insectivores have since been found naturally infected with schistosomes of medical importance in Africa and South America. Although none of these animals has been definitely shown to play an essential role as a maintenance host of infection, the high infection rates of S. mansoni in the baboon (Papio doguera) in parts of East Africa may be of epidemiological significance. (See Nelson 1960, 1964, and Nelson, Teesdale and Highton, 1962).

NOMENCLATURE OF SCHISTOSOMES AND THEIR SNAIL HOSTS

1. SCHISTOSOMES

According to Yamaguti (1958) the family Schistosomatidae Looss, 1899 contains at least 75 species belonging to 14 genera. Four of these genera - Schistosoma, Schistosomatium, Heterobilharzia and Divitellobilharzia - are exclusively parasites of mammals; one genus, Ornithobilharzia, contains species which infect both mammals and birds. The rest of the family parasitize birds only. The genus Schistosoma contains all the species which infect man and domestic animals. It is for this reason that the genus has been studied much more extensively than any other member of Schistosomatidae.

In the older literature the generic name, Bilharzia, was more commonly used than Schistosoma. Bilharzia was erected by Cobbold in 1859 for Bilharz's Distomum haematobium. Weinland had, however, created Schistosoma for the same parasite in 1858. Both Bilharzia and Schistosoma were used indiscriminately and in 1954 to avoid confusion the International Commission on Zoological

(a)	S. haematobium group: (cont'd)	S. spindale Montgomery, 1906.
		S. incognitum Chandler, 1926.
		S. indicum Montgomery, 1906.
		S. leiperi Le Roux, 1955.
(b)	S. japonicum group:	S. japonicum Katsurada, 1904.
		S. margrebowiei Le Roux, 1933.
(c)	S. mansoni group:	S. mansoni Sambon, 1907.
		S. rodhaini Brumpt, 1931.

Others which have not yet been adequately studied include the following:-

S. curassoni Brumpt, 1931.

S. nasale Rao, 1933.

S. suis Rao and Ayyar, 1935.

S. edwardiense Thurston, 1964.

S. hippopotami Thurston, 1963.

Experimental studies on S. mattheei and S. bovis are reported in Part II of this thesis. S. mattheei was discovered by Veglia and Le Roux (1929) on a sheep

Nomenclature ruled in favour of Schistosoma on grounds of historical priority.

In 1958 Le Roux suggested that the Genus Schistosoma should be split into 4 genera - Schistosoma, Sinobilharzia, Rhodobilharzia and Afrobilharzia. This classification does not offer any obvious advantages over the older ones and it has not been generally accepted by helminthologists. Grotillat (1962) has proposed a new genus, Proschistosoma, for S. curassoni Brumpt, 1931 chiefly on the grounds that in the snail host the parasite multiplied by external budding. This has not yet been confirmed by other workers and Proschistosoma is therefore regarded as a genus inquirenda.

Over 20 species of Schistosoma have been described but the validity of a number of these is questionable. The following are well recognized species:-

(a) S. haematobium group: S. haematobium (Bilharz, 1852) Weinland, 1858.

S. bovis Sonsino, 1876.

S. intercalatum Fisher, 1934.

S. mattheei Veglia & Le Roux, 1929

farm owned by a Mr. Matthod. Le Roux (1929) studied the pathology of the infection and showed that the parasite was responsible for heavy losses of sheep in this area. It was later reported in man, the baboon, wild ungulates, sheep, goats and cattle in Rhodesia (Blackie, 1932), and in cattle, sheep and baboons in East Africa (Nelson, 1960; Dinnik and Dinnik, 1965). Pitchford (1959) has shown that S. mattheei is widespread in South Africa with high infection rates in both man and cattle. Pitchford (1965) and Dinnik and Dinnik (1965) have studied the morphology and distribution of S. mattheei, S. haematobium and S. bovis and agree that S. mattheei is a distinct species.

S. intercalatum is apparently indistinguishable from S. mattheei morphologically, but it has been reported only from the Congo. In Africa S. mattheei is mainly a southern parasite and S. bovis a northern one; in East Africa they overlap in their distribution.

Schistosoma bovis was first briefly described by Sonsino in 1876 and more fully by Khalil in 1924. The life history of this parasite has been fully studied by Lengy (1962). S. bovis is mainly, if not entirely, a parasite of cattle, sheep, goats, and camels in Africa, the Middle East and Southern Europe. There have been a

few reports of the infection in man, but the identity has not been fully established because the morphology of the eggs is the only criterion for distinguishing this species from others in its group of the genus.

2. SNAIL HOSTS

All the intermediate hosts of Schistosoma belong to two orders, Pulmonata and Pectinibranchiata, of the class Gastropoda. The intermediate hosts of S. japonicum are members of the family Aminicolidae of the Pectinibranchiata. Abbott (1948) has shown that all the natural intermediate hosts of S. japonicum belong to one genus, Oncomelania: these are O. formosana, O. hupensis, O. nosophora and O. quadrasi. Species of Pomatiopsis, which is very similar to Oncomelania in its anatomy, life history and ecology (Malek, 1961), have been infected with S. japonicum in the laboratory.

In the Pulmonata two families, Planorbidae and Lymnaeidae, have natural snail hosts of schistosomes, and one species (Ferrissia tenuis) of a third family Ancylidae, has been experimentally infected with S. haematobium (Gadgil and Shah, 1956); but the role of this snail in the transmission of the parasite has not yet been fully established. In the Lymnaeidae, Lymnaea luteola transmits S. incognitum.

The majority of the snail hosts of Schistosoma, however, belong to the Planorbidae. In Africa at least 5 genera and 136 species were described for the subfamily Bulininae: Mandahl-Barth (1957) has shown that all those can be reduced to 21 species and 6 subspecies which belong to a single genus, Bulinus, comprising two subgenera, Bulinus and Physopsis. He has similarly reduced all the forms which serve as intermediate hosts of S. mansoni and S. rodhaini from at least 5 genera and 45 species, to 19 species and subspecies belonging to one genus, Biomphalaria, of the family Planorbinae. Hubendick (1955) showed that Australorbis and Tropicorbis, snail hosts of S. mansoni in South America and the West Indies, were anatomically indistinguishable from Biomphalaria. An application by Wright (1962b) has resulted in a ruling by the International Commission on Zoological Nomenclature (1965) which means that all the hosts of S. mansoni belong to Biomphalaria which has precedence over other synonyms. However, for convenience, the older names Biomphalaria, Australorbis and Tropicorbis are retained in this thesis. For African snails the terminology of Mandahl-Barth (1957) is adopted here because although malacologists still disagree over identification of species and especially subspecies of Bulinus and Biomphalaria, Mandahl-Barth's

monograph is the only systematic work on these snail hosts that is available. Indoplanorbis exustus, a member of the Bulininae, is the host of S. indicum and S. spindale in Asia. Planorbarius metidjensis, which belongs to a third planorbid subfamily, Helisomatinae, has been experimentally infected with S. haematobium (Bettencourt, Borges and de Seabra, 1921) and S. mansoni (Barbosa, Barbosa and Morais, 1959). The classification of the Planorbinae by Baker (1945) has been retained here because, although Hubendick (1955) has shown that in some cases it does not reflect phylogenetic relationships, it is the most familiar in the literature on snail hosts of Schistosoma.

The snails studied in Part II of this thesis are Bulinus (Physopsis) africanus (Krauss) which originally came from Nelspruit and which Wright (personal communication) now regards as B (P) globosus on the basis of the electrophoretic patterns of the proteins of its eggs, and Bulinus (Bulinus) truncatus (Audouin) from Egypt and Iran.

STUDIES ON FACTORS AFFECTING THE DEVELOPMENT OF SCHISTOSOMES IN THEIR MOLLUSCAN HOSTS

PART I

A REVIEW OF PREVIOUS WORK

C H A P T E R 2

A REVIEW OF PREVIOUS WORK

INTRODUCTION

In November 1965 the British Society for Parasitology held a Symposium on "Factors influencing the development and behaviour of parasites in their arthropodan and mollusean hosts." This subject "was chosen for discussion at this Symposium because the council felt that little attention had been paid in the past to the factors influencing host-parasite relationships in invertebrate hosts. It was felt that discussion of these factors might also provide a greater understanding of the way in which parasites are disseminated." It was this consideration that had led the council to propose, through the World Federation of Parasitologists, that the subject of host-parasite relations in invertebrates should be included in the proposed "Animal Parasitism and Human Welfare" section of the International Biological Programme of the International Union of Biological Sciences. (Second Symposium of the British Society for Parasitology, 1964, p.vii).

The World Health Organization has estimated that between 180 and 200 million people are infected with schistosomiasis (World Health Organization, 1965). But this is not the only snail-transmitted infection affecting man. Opisthorchis, Clonorchis sinensis, Fasciolopsis

buski and Paragonimus westermani are all important trematode parasites affecting many millions of people in the Far East. Molluscs are also important intermediate hosts of the nematode Angiostrongylus whose larvae wander in the central nervous system of man causing eosinophilic meningitis (Alicata, 1964). In the veterinary field trematodes, especially liver fluke, are of great economic importance.

In this field of trematode-borne diseases a great deal of attention has been paid to the clinical aspects of the diseases, their treatment and, especially in schistosomiasis, the control of the disease using molluscicides. But relatively little attention has been given to the intimate relationships of the trematodes and their molluscan hosts. The observations that have been made are scattered through the literature and no previous attempt has been made to bring the subject together. I have therefore considered that an essential part of this thesis should be a critical review of the factors influencing the host-parasite relationships of schistosomes and snails.

Host-parasite relationships, like other biological systems, consist of such closely and intricately interconnected events that attempts to discuss parts of the system in isolation are always arbitrary. It is proposed

to restrict the review to that part of the host-parasite relationship which begins when schistosome miracidia and host snails occur in the same ecological field and ends when the cercariae leave the infected snail. The review, therefore, covers factors which affect the penetration of miracidia into snail hosts, the subsequent development of the sporocyst and the production of cercariae. Wider ecological and epidemiological factors affecting the population dynamics of the parasite and its host are outside the scope of the thesis.

The factors affecting the mollusc-schistosome relationships are reviewed under three main headings:-

A. The External Environment, including temperature, light, gravity and water velocity.

B. The Parasite, including the age of the miracidia, the density of the miracidia population, means of penetration and the race of the parasite.

C. The Host, including attraction for miracidia, infection with other parasites, the age, race, , mechanisms of resistance and physiology and biochemistry of the internal environment.

A. EXTERNAL ENVIRONMENTAL FACTORS

Three factors in the environment of the hosts and parasites are generally considered important in the development of schistosomes in molluscs. These are temperature, light and gravity. A fourth factor, the speed of water, may be equally important, but it has not been adequately studied.

1. TEMPERATURE

There is evidence that temperature affects (a) the penetration of schistosome miracidia into snails and (b) the subsequent development of the parasite.

(a) Effect on Penetration of Miracidia

De Witt (1955) appears to be the only investigator who has attempted to discover the effect of temperature on the penetration of schistosome miracidia into snails. He exposed groups of Australorbis glabratus to miracidia of S. mansoni at 10°, 25°, 35° and 40°C. The snails were then maintained at

26-28°C. during the incubation period. Using shedding of cercariae and dissection of snails as tests for infection, De-Witt found that none of the snails exposed at 0°C. became infected. Higher proportions of the snails exposed at 35°C. were infected than of snails exposed at 25°C. The highest infection rate was in the group exposed at 40°C. But the high mortality in the group makes it difficult to be certain that the high rates in the few survivors represent such high rates for the group exposed at 40°C. as a whole.

De-Witt interpreted the increase in infection rates with increase in temperature as indicating an increase in the penetration rates of miracidia. He concluded that the ability of S. mansoni miracidia to penetrate the snail host was probably directly related to the environmental temperature and that the optimum temperature might be the maximum temperature that the snail host could tolerate. But the way in which the exposure temperature affected the rate of infection of snails is not clear. De-Witt noted that whereas the miracidia swam very sluggishly at 0°C. they were very active at 40°C. It is thero-

fore possible that by increasing the swimming activity of miracidia, a rise in temperature might have increased the chances of penetration by increasing the number of contacts between the miracidia and the snails. The rise in temperature could also have raised the penetrating efficiency of the miracidia, or it might even have altered the skin of the snail in such a way as to render it more easily penetrated. Another possibility is that the exposure temperature might have affected the viability of the parasites without affecting the penetration rate: Chao and Ball (1962), for example, have shown that exposure of mosquitoes to a temperature of 4°C. within 48 hours of exposure to infection with Plasmodium relictum had an adverse effect on the early developmental stages of the parasites - the oocysts developed, but the sporozoites were not viable.

Gordon (1956), in a review of De-Witt's paper, suggested that the experiments should be repeated using African S. mansoni and the appropriate snail hosts, but to date the subject has not been studied further. It is therefore not known whether the temperature at the time of exposure influences the

infection rates of other strains and species of schistosomes in their snail hosts. Nor is it known for certain that the exposure temperature affects the penetration of snails by miracidia.

(b) Effects on Development

The temperature of the environment in which infected snails live affects the development of schistosome parasites in two ways. It influences (i) the rate of development of the parasite, and (ii) the rate of infection of the snail hosts.

(i) Rate of Development

The first evidence of the effect of temperature on the speed of development of schistosomes in snail hosts came from the studies of Manson-Bahr and Fairley (1920) in Egypt; they observed that the natural rates of infection of Bulinus with S. haematobium and Biomphalaria with S. mansoni over a period of one year and found, from snail dissections, that in early spring most infections consisted of immature sporocysts and cercariae. The highest incidence of mature infections was in the autumn months. It would seem

therefore that the development of sporocysts and the production of cercariae (already present in snails in autumn) was arrested by the low winter temperatures. At the same time they studied other trematodes and their snail hosts, paying particular attention to an amphistomate cercaria (probably of Paramphistomum cervi - see El-Gindy and Rushdi, 1962) which parasitized Bulinus truncatus. They found that the pattern of seasonal development in this was similar to that of the schistosomes.

Sopakar (1921) studied the incidence of Schistosoma spindale in the Indian planorbid snail, Indoplanorbis exustus over a period of two years. He demonstrated clear seasonal fluctuations in the proportion of snails shedding cercariae. The highest infection rates were in autumn and the lowest in winter. This work has often been regarded as evidence for arrested or retarded development of the schistosome in the host snail (De-Witt, 1955; Standen, 1949).

The clearest evidence of the effect of temperature on the development of schistosomes in molluscs was provided by Gordon, Davey and Fenston (1934) from Sierra Leone. This work is considered at some length because it is a classic in the field of

experimental infection of snails with schistosomes. They first worked out the prepatent periods of S. mansoni in Biomphalaria pfeifferi and Schistosoma haematobium in Bulinus (Physopsis) globosus in aquaria maintained at 26-28°C. Then they studied the effect of lowering the temperature to 20-22°C. and 14-15°C. and of raising them to 32-33°C., 34-35°C. and 37°C. All the snails were laboratory bred. After exposure to large numbers of miracidia at room temperature, snails were transferred to aquaria maintained at the test temperature. The rate of development of the parasites was followed by dissecting snails at intervals of time and by examining others for the shedding of cercariae. Lowering the temperature to 20-22°C. increased the prepatent period of S. mansoni from an average of 22 days at 26-28°C. to an average of 35 days. At 20-22°C. sporocysts (presumably daughter sporocysts) did not appear until the 12th day; compared with 7-9 days at 26-28°C. The snails kept at 14-15°C. had not shed cercariae by the 67th day when most of them started dying; those snails were known to be infected because sporocysts were seen of members of the group 56 days after exposure.

Raising the temperature to 32-33°C. shortened the incubation period of S. mansoni to about 15 days. Most of the snails maintained at 34-35°C. died before the development of the parasite could be followed beyond 10 days. But the authors believed the rise in temperature beyond 32-33°C. retarded the growth of the parasite because the few survivors of the group kept at 35°C. started shedding cercariae 16 to 17 days after exposure. It is likely, however, that this only represented a normal variation in the prepatent period. Gordon et al. themselves noted that at 26-28°C. discharge of cercariae started from the 19th day onward, with 22 days as the average prepatent period.

The findings with S. haematobium in B. (P.) globosus were similar to those with S. mansoni in B. pfeifferi. A rise in temperature of from 26-28°C. to 32-33°C. was accompanied by a shortening of the incubation period from about 36 days to an average of 23-24 days. At 33-34°C. and 35°C. the prepatent periods averaged 27-28 days. Snails maintained at 20-22°C. did not start shedding cercariae until 66 days had elapsed after exposure. The results of the dissection of snails of this group were inconclusive, but suggest little or no development occurred at this temperature.

This work clearly established the direct relationship between environmental temperature and the rate of development of schistosome parasites as expressed by the incubation period. I have not included the data of Gordon et al. on the development of sporocysts because, in general, dissection of snails for schistosome sporocysts is not an easy or very reliable method of following development and, in particular, because their observations on the development of schistosomes beyond the first generation sporocysts do not agree with later work. Their data suggested that temperature affected the rate of development of both mother and daughter sporocysts as well as of cercariae.

Stirewalt (1954) studied the effect of maintenance temperature on the development of S. mansoni in A. glabratus. She found the prepatent period decreased from 35-56 days at 23-25°C., to 22-23 days at 26-28°C. and 18 days at 31-33°C. Wagner and Moore (1959) have confirmed the direct relationship of maintenance temperature on the speed of development of S. mansoni in A. glabratus. They reported prepatent periods of 31-33 days at 24°C., 26 days at 25.5°C., 24 days at 27°C. and 20 days at 28.5°C.

Malek (1961) has reported finding that the prepatent period of S. haematobium in B. truncatus decreased by an average of 16 days when snails were kept at 26-28°C. compared with those at 20-22°C.

The most recent study of the subject is that of Pitchford and Visser (1965) in South Africa. In an effort to discover the role of temperature changes on the transmission of S. matthoei, S. haematobium and S. mansoni, the authors studied the development of the parasites in the appropriate snail hosts over a period of 2 years. At fortnightly intervals batches of 30 snails were each exposed to the miracidia of the appropriate schistosome and subsequently kept in an outdoor shed. This ensured that the infected snails experienced the natural temperature fluctuations throughout the year. In winter the prepatent periods for all 3 parasites were at least 4 times as long as they were in summer. The prepatent period for S. matthoei in a B. (Physopsis) sp. increased from 4-5 weeks in summer to 19-20 weeks in winter, and that of S. haematobium in the same B. (Physopsis) sp. from 5-6 weeks to 29-30 weeks. Similarly the prepatent period of S. mansoni in B. pfeifferi changed from 4-5 weeks to 20-21 weeks.

There can be little doubt from those studies that there is a direct relationship between the rate of development of schistosomes and the temperature of the environment in which the host snails live. This direct relationship is what one might expect with parasites in poikilothermic animals, especially aquatic ones. However, rather surprisingly, there is evidence that the development of some other trematodes may be independent of temperature. McCoy (1928) studied the rates of infection in snails from a North American artificial lake. He found that while the numbers of snails with mature infections of Cercaria lamata fluctuated seasonally, those carrying the mature infections of Plagiorchis ameluronsis remained fairly constant throughout the two years of study. Another example comes from the studies of Miller and Northup (1926) who noted that infection rates in the marine snail, Nassa obsoleta, with larval trematodes had no relationship with temperature fluctuations; they suggested that in this case the infection rates of the mollusc must be related to the habits of the (unknown) definitive hosts.

(ii) Infection Rates

The earlier studies of Manson-Bahr and Fairley and Sopakar are often quoted as evidence for the influence of temperature on the infection rates of snails with schistosome parasites. However, recent work on the dynamics of the transmission of schistosomiasis has shown that climatic factors other than temperature can be much more important. For example, Webbe (1962), working in an area of East Africa where temperatures are fairly constant throughout the year, has shown that the infection rates of B (P) nasutus with S. haematobium mainly depend on the age structure of the snail population.

There is, however, experimental evidence indicating that temperature affects not only the speed of development of the parasite in the snail, but also the numbers of snails in which schistosome parasites reach maturity. Standen (1952) exposed A. glabratus to S. mansoni miracidia at 20°C. and then maintained the snails in a room whose temperature varied from 25°C. to 30°C. He found that a rise in temperature of the room resulted in a rise in the percentage of snails infected. He concluded that the best results were obtained with maintenance temperatures of 26-28°C.

Stirewalt (1954), working with S. mansoni and A. glabratus, has also demonstrated a direct relationship between temperature and the percentage of snails infected. Employing one miracidium per snail she found that 27% of the snails kept at 23-25°C. and 52% of those kept at 31-33°C. discharged cercariae after the appropriate prepatent period. She obtained a similar reduction in the infection rates of snails kept at 33-35°C. compared with those kept at 26-28°C.

Pitchford and Visser (1965), in the study referred to above, found that the infection rates of B. pfeifferi with S. mansoni were influenced by environmental temperature. The highest rates were obtained with snails exposed during the spring and mid-summer, and the lowest rates with snails exposed in winter; the rates with snails exposed in late summer fell between the two values. S. mattheei and S. haematobium differed from S. mansoni in that they did not show any dependence on temperature in their infectivity to B (Physopsis) sp. Very similar rates of infection of this snail with the two parasites were obtained throughout the year. Lengy (1962), working in Israel, found that although low temperatures retarded the development of S. bovis in B. truncatus

temperature had little effect on the percentage of exposed snails that became infected. He says, "Snails became infected equally well at 14°C. and 31°C."

These studies indicate that, as far as temperature is concerned, there are important differences between the relationships of S. mansoni and its Biomphalaria hosts and the terminal-spined schistosomes, S. haematobium, S. mattheei and S. bovis, and their bulinid hosts. While temperature definitely influences the numbers of Biomphalaria which become infected with S. mansoni, it apparently has no effect on the numbers of bulinid snails which become infected with the terminal-spined schistosomes. The way in which temperature affects the rate of infection of Biomphalaria with S. mansoni is not known. It would be interesting to know, for example, whether the defense mechanisms of Biomphalaria against S. mansoni become more efficient at lower temperatures. Another possibility is that S. mansoni may be unable to stand low temperatures and infected snails may therefore lose their infections.

2. LIGHT

As long ago as 1924 Faust and Meloney suggested that S. japonicum miracidia were positively phototactic. Takahashi, Mori and Shigeta (1961) have re-examined the response of S. japonicum miracidia to light. In their study they employed a long and shallow vessel which was placed horizontally in a constant temperature bath and lighted at both ends. They found that the response to light depended upon the light intensity and temperature to which the miracidia were subjected. At temperatures above 10°C. and with light intensities above 3,000 Lux miracidia moved away from the light. At temperatures of up to 28°C. the miracidia moved towards light of intensities between 10 and 100 Lux.

Chernin and Dunavan (1962) have studied the effect of light on the miracidia of Puerto Rican S. mansoni. They employed 3 methods. In the first, miracidia were placed in glass capillary tubes whose ends were then sealed off. The tubes were placed horizontally on the stage of a dissecting microscope and one half of each tube was covered with black material. Counts of miracidia at intervals showed that between 70 and 100% of the miracidia were in the lighted half of each tube. In the second method, miracidia were placed in a flask which was covered

with black material and which carried a side arm. Samples from the lighted side arm contained much higher concentrations of miracidia than those from the bottom or top of the flask. In the third method, four vertical cylinders were used: the first cylinder was completely covered with black tape, the second had the top few inches uncovered, the third had the bottom few inches uncovered and the fourth was left completely uncovered. Counts of miracidia in samples from the top and bottom of each cylinder showed that miracidia concentrated at the top in all the cylinders, but the concentration of miracidia increased in the lighted bottom of the third cylinder.

McClelland (personal communication) has investigated the response of miracidia of a Tanzanian strain of S. haematobium to light, using the cylinder method of Chernin and Dunavan and he found that they were indifferent to light.

The inadequacy of the information about the response of schistosome miracidia to light is evident. It would therefore be unwise to make a general statement of the behaviour of the miracidia in relation to light; but the available evidence indicates that at best the organisms are only weakly attracted by light of ordinary intensities. Schistosome miracidia, unlike those of many

other digenetic trematodes, do not have eyespots and a weak response to light is probably to be expected. Light probably plays only a small part, if any, in the location and penetration of snail hosts by schistosome miracidia.

3. GRAVITY

Faust and Meleney (1924) showed that miracidia of S. japonicum tended to congregate in the top layers of water. By taking samples from different layers of water in 250 ml. cylinders, they found that the majority of the miracidia concentrated in the top 3 or 4 cm. Later Faust and Hoffman (1934) found that miracidia of S. mansoni from Puerto Rico behaved in the same way as S. japonicum. In a study in which the temperature and light intensity were controlled Takahashi, Mori and Shigata (1961) found that at ordinary temperatures and light intensities the miracidia of S. japonicum congregated at the top of tall water colums and in their experiments on the influence of the dispersion of the host and parasite on the ability of S. mansoni miracidia to infect A. glabratus Chernin and Dunavan (1962) showed that with this species the highest concentrations were also always at the top of the containers.

Other workers, however, have found different distribution behaviour in schistosome miracidia. McClelland (personal communication) has studied the vertical distribution of S. haematobium miracidia of Tanzanian origin and found that there were nearly twice as many miracidia at the bottom as at the top of glass cylinders. Wright (1962a) also noted the tendency of S. haematobium miracidia to remain at the bottom of vessels (this strain was thought to have come from Iraq). In contrast with these findings of McClelland and Wright, Faust (1924) stated that the miracidia of an Egyptian strain of S. haematobium distributed themselves more or less evenly throughout vertical water columns. The variation in the distribution behaviour is not confined to S. haematobium miracidia. I have noticed that the miracidia of a strain of S. mansoni from St. Lucia (West Indies) had a very striking tendency to concentrate at the bottom of uniformly lighted urine jars and other vessels; this is in marked contrast to the observations of Faust and Hoffman (1934) and of Chernin and Dunavan (1962).

As a result of the studies of Faust and Maloney (1920) and of Faust and Hoffman (1934), schistosome miracidia have been generally believed to be negatively geotactic, but there is no direct evidence that gravity per se influences the distribution of these organisms. The

variations found in the distribution behaviour of S. mansoni and S. haematobium miracidia, indeed, suggests that the vertical distribution of the larvae may be independent of gravity and could be the result of some less obvious factor. But even if gravity influences the vertical distribution of the miracidia it probably has only a limited effect on the transmission of the parasites in nature. Chernin and Dunavan (1962) demonstrated that with Puerto Rican S. mansoni and A. glabratus higher infection rates were obtained in snails confined to the top 2 cm. of a 20 cm. long column of water than in those confined to the bottom 2 cm. But the difference was not as great as might have been expected: they obtained infection rates of 42% at the top and 29% at the bottom using a single miracidium in 100 ml. of water for exposure. These authors further showed that miracidia travelled at least 86 cm. horizontally and 33 cm. vertically to find and infect snails. McClelland and Jordan (1962) reported that the bottom-dwelling snail, Biomphalaria choanomphala, was responsible for the transmission of S. mansoni in Lake Victoria, in East Africa. Although the level at which the snail gets infected is not known, the observations of McClelland and Jordan and the experiments of Chernin and Dunavan suggest that miracidia will travel considerable

distances to infect snails.

4. WATER MOVEMENT

Swift-flowing rivers and lakes with a great deal of wave action are inimical to snails, but many hosts of Schistosoma inhabit slow-flowing streams and irrigation channels. For example, Teesdale (1962) and Kinoti (1964b) have reported that B. (P) africanus, an important host of S. haematobium, S. mattheei and S. bovis, is mainly a riverine snail and in a review of recent work on the transmission of schistosomiasis in East Africa, Webbe (1964) has emphasized the fact that perennial and seasonal water courses and irrigation systems are the main habitats of the intermediate hosts of S. mansoni. (Webbe & Jordan, 1966).

In spite of the importance of flowing water systems very little is known about the effect of the velocity of water on the infection of snails with schistosomes. Over 20 years ago, Faust and Meleney (1924) stated that miracidia of S. japonicum exhibited "negative rheotropism", but the significance of the effect of water velocity on the infection of snails with schistosomes has been recognised only recently. The work of Webbe/(Webbe & Jordan, 1966) is important in this respect; he devised a water flow system in which he exposed Biomphalaria sudanica tanganyicensis

to varying numbers of S. mansoni miracidia. High infection rates were obtained when he exposed the snails to more than 1,250 miracidia at velocities between 0.5 and 3.5 feet per second and reduction of the number of the miracidia to one tenth did not affect the rates of infection if the water velocity was between 0.5 and 1.5 foot per second. It was also shown that miracidia swam against the water current to infect 15.4% of the snails which had been placed one foot above the point at which the miracidia were introduced. Webbe concluded that "water velocity, over the range studied, enhanced the scanning capacity of the miracidia". His results demonstrated that water velocity, within the range studied, did not stop S. mansoni miracidia finding snail hosts both above and below the point where they were introduced, but further evidence is necessary before it can be accepted that the scanning capacity of the miracidia was increased. It is interesting to note that Yasuraoka (1953) observed that the miracidia of Fasciola hepatica were carried downstream at water velocities above 3.0 cm. per second, but that at a velocity of 1.5 cm. per second they were able to swim against the current. These are, of course, much lower velocities than those investigated by Webbe.

Undoubtedly there are many other features of the external environment such as the hydrogenion concentration and the oxygen and carbon dioxide tensions which will influence the behaviour of schistosome miracidia. Apart from the work of Fairley (1962) who showed that the hydrogen ion concentration in water determined the survival of the miracidia of Schistosomatium douthitti, little is known about this aspect of the host-parasite relationships.

PARASITE FACTORS

In this section it is proposed to discuss first the factors which affect the location and penetration of the host and then the factors which influence the development of the parasite inside the host snail.

1. AGE OF MIRACIDIA

The relation between the age of miracidia and their infectivity is poorly understood. In the laboratory it is usual to expose snails to as young miracidia as possible and to leave the host and parasites together for 6-12 hours; this practice has probably arisen from statements in the literature about the life span of schistosome miracidia. A wide ran e of longevity has been reported. Faust and Meleney (1924) stated that S. japonicum miracidia lived for 60-72 hours: Faust later reported that in tap water miracidia of the same parasite lived for 24-30 hours (Faust, 1924). Porter (1938) found that Schistosoma haematobium survived for 8-10 hours at 30°C. and less than 6 hours at 10-15°C. Miracidia of S. mansoni are said to live for as long as 40 hours (Lampe, 1927) and for as short as 9 hours (Maldonado and Acosta-Matienzo, 1941).

However, Maldonado and Acosta-Matienzo (1948) appear to be the only workers who have actually studied the influence of the age of miracidia on their infectivity to snails. These authors observed the penetration of S. mansoni, which had hatched 0-8 hours previously, into A. glabratus. They found that infectivity, which they defined as the percentage of miracidia present at the beginning of the exposure that successfully penetrated, decreased with the increase in the age of the miracidia. Infectivity varied from 72-80% with miracidia 0-3 hours old to 62.3-42.5% with miracidia aged 4-7 hours. But infectivity would appear not to have been a simple function of time. Whereas with miracidia which were only a few minutes old infectivity was 73.3%, it was 80% with miracidia that were one hour old and 4 out of 5 miracidia that were alive at 8 hours successfully penetrated snails.

A study by McClelland (1961) has provided some evidence suggesting that the infectivity of miracidia may be limited to the first two or three hours of their life. He pipetted 10 miracidia of S. haematobium, not more than 40 minutes old, into 4 ml. of water in each of 60 specimen tubes. After the first hour he replaced the snails with a fresh batch and repeated this after another hour. He found that 42 of the first group of 60 snails

became infected; only 1 of the next group and none of the third group became infected although active miracidia were present. Long exposures may be unnecessary: in a previous study I have found that 95-100% of B (P) africanus became infected when exposed to 6-12 S. bovis miracidia for only 2-4 hours (Kinoti, 1964a).

Axmann (1947) has shown that miracidia of S. japonicum and S. haematobium contain quite large deposits of glycogen. It is reasonable to suppose that all schistosome miracidia, like other free-living larvae of helminths, contain deposits of glycogen and fat as sources of energy (Rogers, 1962). It may be expected then that the swimming activity of miracidia will slow down with time as the sources of energy become depleted. Reduced swimming activity would be expected to curtail the chances of infection of snail hosts through reduced contacts between host snails and miracidia. If, as is claimed by many authors (e.g. Longy, 1962), penetration involves vigorous pushing, twisting and revolving movements on the part of the miracidia then the process probably requires relatively large quantities of energy. Depletion of the food stores as the miracidia age may therefore affect the ability of miracidia to penetrate snail hosts. The rate of ageing is probably directly related to the rate of

the carbohydrate metabolism of miracidia: this is suggested by the fact that in S. mansoni the longevity is directly dependent on the temperature of the environment (Purnell, 1966).

2. DENSITY OF MIRACIDIA

"Density of miracidia" is used here to mean the number of miracidia per unit volume of water.

Schreiber and Schubert (1949) exposed A. glabratus individually to different numbers of S. mansoni miracidia in 3 ml. of water. They found that 14%, 55%, 70% and 85% of the snails exposed to 1, 3, 7 and 12 miracidia respectively, became infected; they used the shedding of cercariae as criterion of infection.

Standen (1952) compared the infection rates obtained by individual and mass exposures of A. glabratus to S. mansoni miracidia. He found an increase in infection rates as the density of miracidia was increased. In this study his figures for 1950 show that about 82% of the snails exposed individually to 6 miracidia in 2 ml. of water became infected, compared with 72% and 67% of those exposed en masse to 6 miracidia per 10 and 500 ml. of water respectively.

The findings of Schreiber and Schubert and of Standen have been confirmed by Chernin and Dumayan (1962) who demonstrated that the rate of infection of A. glabratus was directly related to the density of S. mansoni miracidia used. For example, they recovered daughter sporocysts in 87% of the snails exposed to 5 miracidia and 42% of those exposed to 1 miracidium in beakers containing 1.5 ml. of water. The observations of McClelland (1965) on Biomphalaria sudanica and S. mansoni suggest that the rate of infection of the snail is, like A. glabratus, directly related to the density of miracidia.

McClelland (1965) is the only investigator who appears to have studied the effect of the density of the miracidia of a schistosome with terminal-spined eggs on snail infection rates. He exposed B (P) nasutus to S. haematobium miracidia individually and in groups of 25 snails. Individual exposures were carried out in small specimen tubes containing 4 ml. of water and the group exposures in aquarium tanks containing 5 litres and 20 litres of water. In the series where individuals exposed were employed 1, 5 and 10 miracidia produced 0, 30 and 40% infection rates. The other groups similarly showed a rise in infection rate as the density of miracidia increased.

An incidental observation by McCullough (1959) is interesting in this connection. He worked with two Ghana strains of S. haematobium: one from Pokoasi district where B (P) globosus was its natural snail host and the other from the/district where B. truncatus rohlfsi was host. Using about 200 miracidia per snail he failed to infect B (P) globosus with the Ke parasite or B. truncatus with the Pokoasi parasite. When he raised the number of miracidia per snail to more than 2,000 he was able to infect 4 out of 34 B. truncatus and a similar proportion of B (P) globosus with the other strain of the parasite. Snails were exposed in petri dishes and an increase in numbers of miracidia must have meant a rise in the density of miracidia. Although the experiment was designed for another purpose I found that when bulinid snails were exposed to S. bovis (usually in 2 ml. of water) 67.5%, 95% and 100% of them became infected at a dose rate, respectively, of 4-6, 6 and 8-9 miracidia (Kinoti, 1964a).

Under laboratory conditions of exposure the rate of infection of snails appears to depend on the density of miracidia, but the way in which the density influences the rates of infection is not known. One/possible way is that an increase in density increases the chances of infection by raising the number of contacts between miracidia

and snails. It is also possible that not all miracidia are capable of penetrating and developing in snails; the presence of larger numbers of miracidia would therefore increase the chances of successful infections. Another possibility, raised by McCullough's results, is that a minimum number of miracidia is quired to overcome the resistance of the host snails: if this minimum number of miracidia varies with individual snails then an increase in the density of miracidia would result in an increased infection rate.

Recently, Moore (1964) has published results which appear to be in conflict with the findings of previous workers on the relation of snail infection rates to the density of miracidia. Working with A. glabratus and S. mansoni he obtained an infection rate of 56.7% in snails exposed en masse to miracidia in a 3 gallon tank and only 23.9% in those exposed individually to 10 miracidia each in small volumes of water; but the published data are inadequate for assessing the significance of the observation.

Moore's results suggest that exposure of snails to miracidia in large volumes of water may be a much more efficient use of miracidia than the usual laboratory practice of confining snails and miracidia in small volumes of water. The ability of miracidia to find snail hosts in large volumes of water has probably been underestimated. In support of this McClelland (1965) has shown that very low densities of miracidia will produce surprisingly high infection rates in snails. I have re-analysed his results in the following table:-

Snail	Miracidia density	Infection rate(%)	Method of exposure
1. B (P) nasutus	2.25	40	small tubes
	0.04	48	large tank
	0.02	24	" "
	0.01	0	" "
2. B. sudanica	2.25	57	small tubes
	0.45	64	large tank
	0.30	33	" "
	0.25	12	" "
	0.05	36	" "
	0.012	17	" "

Similarly the figures given by Standen (1952) for his observations in 1950 show that even with miracidial densities as low as 0.012 and 0.6, he got infection rates

of 67% and 72%. A similar analysis of the results of Chernin and Dunavan (1962) also shows that exposing A. flabratus to low concentrations of miracidia in large volumes of water, yields relatively larger infection rates than exposing snails to large numbers of miracidia in small volumes of water. They pointed out that the scanning capacity of miracidia is much greater than it is generally realized.

Those observations on the scanning capacity of the miracidia are of obvious importance in relation to the problem of transmission and control in endemic areas. It seems likely that transmission will continue in the presence of very low densities of miracidia. Therefore control which is based on the reduction of the worm load in the human population or on sanitary measures may have little effect on the transmission unless it is wholly effective in stopping the passage of schistosome eggs into snail habitats. This point should be considered in mathematical formulations, such as that by Macdonald (1965), of the transmission of schistosomiasis.

3. RACE OF PARASITE

The terms "race" and "strain" are in common use in bacteriology, helminthology, protozoology and population genetics, but both within and outside each of these disciplines the terms do not have a uniform meaning. In this thesis the term "strain" will be used to mean a particular isolate of a parasite or snail species; "race" will be used to mean a particular isolate or population of a parasite or snail species that is known to differ in one or more characters from all other isolates or populations of the same species. Hence a race is a strain that is known to differ from all other strains of the same species in some particular character. In this section infectivity to snail hosts is the character under discussion; in Section C of this thesis susceptibility to schistosome infection is the character that is considered.

In 1941 Vogel showed that the miracidia of S. mansoni from different geographical areas differed in their ability to infect A. glabratus; in this snail a West African strain produced higher infection rates in the snail than a South American strain. This difference was best seen when snails were exposed to one miracidium each. During the last 25 years, polymorphism in schistosome species has been studied a great deal. It is now a

well-established fact that intra-specific variations in infectivity to the snail hosts occur in at least two species of Schistosoma - S. mansoni and S. japonicum. Most of the early work on geographical races of S. mansoni was carried out in America especially by workers at the National Institutes of Health. For example, Files and Cram (1949) exposed A. glabratus from Puerto Rico, Venezuela and Brazil, B. pfeifferi from Liberia and B. alexandrina from Egypt to miracidia of S. mansoni from Puerto Rico, Venezuela and Egypt. Those snails were also exposed to miracidia of a cross between the Brazilian and Puerto Rican strains of S. mansoni. The authors found that miracidia from different endemic areas produced different infection rates in the snail hosts. For example, the Puerto Rican, Venezuelan and Egyptian miracidia infected 46%, 44% and 9% of the Puerto Rican snails, respectively. Files (1951) extended these observations to cover S. mansoni from Puerto Rico, Venezuela, Surinam, Egypt and seven crosses of those geographical strains. She found clear differences in their infectivity to snail hosts from different regions, e.g. A. glabratus from Puerto Rico, Dominican Republic, Venezuela, Surinam and Brazil and B. pfeifferi from Liberia and B. alexandrina from Egypt. The Puerto

Rican A. glabratus gave infection rates of 54%, 12% and 6% when exposed to S. mansoni from Puerto Rico, Egypt and Brazil, respectively. Cram (1953) has reviewed the literature up to 1952. Later studies such as those of Kagan and Goiger (1965) and Saoud (1965a) have provided further evidence for the existence of geographical races of S. mansoni as evidenced by differential infectivity to snail hosts.

There is also good evidence for geographical variation within S. japonicum. Hunter, Ritchie and Otori (1952) found that S. japonicum from Japan produced much higher infection rates in the Japanese snail, O. nosophora (44.4%) than in the Formosan O. formosana (0.8%). They concluded that the Japanese and Formosan strains of S. japonicum must be different because the Formosan variety must be more infective to O. formosana for the snail to serve as its natural intermediate host, as it undoubtedly did in Formosa. Further evidence was soon produced by De-Witt (1954) who clearly demonstrated differences in the infectivity of S. japonicum from China, Japan and Formosa to O. hupensis, O. nosophora, O. formosana and Pomatiopsis lapidaria. For instance, 34%, 13% and 0% of O. hupensis were infected when exposed to miracidia from China, Japan and Formosa, respectively.

Hsu and Hsu (1960) exposed O. hupensis, O.formosana and O. noseophora to S. japonicum from the Philippines and found that 20%, 9,6% and 0% of the Chinese, Formosan and Japanese snails became infected and concluded that each geographical strain of S. japonicum infects best the Oncomelania of its own endemic area. Thus there are four geographical races of S. japonicum: the Japanese, Chinese, Formosan and Philippine.

There is evidence for the existence of geographical races of S. haematobium also, though it is not as good as for S. japonicum and S. mansoni. Difficulties of culturing bulinid snails, infecting them with S. haematobium and the lack of satisfactory experimental hosts in the laboratory account for the most part for the poorer evidence. Cowper (1917) failed to infect any of a few specimens of an Egyptian strain of B. truncatus with S. haematobium from South Africa. In another trial he could not infect any of 22 specimens of B. truncatus from Egypt with S. haematobium which he had obtained from two Nigerian patients (Cowper, 1953). However, in view of the well-known difficulties of infecting bulinid snails with S. haematobium, the failure to infect snails in experiments carried out on a limited scale cannot be regarded as evidence of geographical variation in the

infectivity of the parasite. Barlow and Abdel-Azim (1948) reported that South African B.(P) africanus was susceptible to Egyptian S. haematobium, but B. tropicus from South Africa was refractory. In the same way this cannot be considered satisfactory evidence for strain differences in S. haematobium because B. tropicus has never been satisfactorily shown to be susceptible to S. haematobium, or any other schistosome in the laboratory, or in the field. /The claims of Porter (1938) that B. tropicus was an intermediate host of S. haematobium and S. mansoni in South Africa were based on a total of three specimens of the snail and have not been substantiated by other workers in South Africa. Teesdale (1962) reported finding on one occasion S. bovis in B. tropicus in Kenya, but this observation also needs confirmation./

The best evidence available is probably that obtained by McCullough (1959). Working in Ghana, McCullough demonstrated that there were two distinct strains of S. haematobium in two neighbouring districts: one strain was transmitted by B. truncatus rohlfsi in the Ko district and the other by B.(P) globosus in Pokoasi district. With up to 200 miracidia per snail he was unable to infect B. t. rohlfsi with the Pokoasi

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strain or B. (P.) globosus with the Ko strain. When the number of miracidia per snail was raised to over 2,000, a few snails became infected with the foreign strain, but only 200 miracidia per snail resulted in 100% infection rates in the natural hosts.

Witenberg and Sallternik (1957) reported that Israeli B. truncatus was refractory to S. haematobium obtained from Moroccan and Yemen immigrants. These authors also found that 4% and 30% of the Israeli B. truncatus were susceptible to S. haematobium from Iraq and Egypt, but the numbers of snails exposed and mortality rates are not given. Wright (1962)[a] has studied the compatibility between S. haematobium and bulinid snails from several African and Middle East countries. He reported differences in the strains, but his results are difficult to compare with those of other workers because although consideration was given to infection rate, cercarial yield and loss of infection, the "successful results were scored on a purely subjective basis".

Le Roux (1958) suggested that the South African Physopsis-borne parasite was not S. haematobium because he had on numerous occasions failed to infect snails of the truncatus group with it. Wright (1962)[a] supported Le Roux's suggestion that the South African parasite

should be called S. capense (Harley, 1864) because he also had, after repeated attempts, failed to infect snails of the truncatus group with the parasite normally using snails of the africanus group, and vice versa. However, Barlow and Abdel-Azim (1948) successfully infected South African B.(P.) africanus with Egyptian S. haematobium. And recently, Pitchford and Visser, (1965) have successfully infected a South African snail of the truncatus group with S. haematobium from South Africa. Further, McCullough (1959) showed that, with sufficient numbers of miracidia, B. truncatus could be infected with a strain which normally uses B.(P.) globosus (a member of the africanus group) as host and that B.(P.) globosus could be infected with a strain which normally develops in B. truncatus. The evidence from infectivity experiments does not therefore justify the separation of S. capense from S. haematobium.

Geographical strains of schistosomes display differences not only in their infectivity to snail hosts, but also in their behaviour patterns in laboratory animals. Ifsu and Ifsu have studied the characteristics of the Chinese, Japanese, Formosan and Philippine strains of S. japonicum and found differences in the susceptibility of laboratory animals, number and location of testes in

males, frequency of hermaphroditic males, size and shape of eggs, prepatent period, distribution of eggs in host viscera, virulence, the circumoval reaction and response to drugs. (See Hsu and Hsu, 1962, for references). Saoud (1966) has studied some of these characteristics in S. mansoni. Using mice and hamsters as hosts he found differences in the prepatent period, virulence, distribution of eggs in host viscera, number of supernumerary testes and frequency of hermaphroditic males. The differences are probably of fundamental importance in determining the epidemiology of schistosomiasis in different regions. For example, it is the low infectivity of the Formosa race of S. japonicum for man which accounts for the absence of human infection in Formosa, in spite of high infection rates in animals (see Hsu and Hsu, 1962).

Williams and Swanson (1963) have reported that as few as four passages of Formosan S. japonicum in the Japanese snail, O. nosophora, were sufficient to make the parasite completely uninfective to the natural Formosan host, O. formosana. Saoud (1965) found that the infectivity of an Egyptian strain of S. mansoni to B. alexandrina was greatly diminished after passage

in A. glabratus for 15 years. Recently Smithers, in the discussion of a paper by Webbe and Jordan (1966), has reported that the pathogenicity of the cercariae of a Puerto Rican strain of S. mansoni varied over a period of months. He thought this variation was related to the condition of the host snails, but this needs experimental confirmation.

The evidence for the differences in the infectivity of geographical races of schistosomes serves as a warning against treating, in the words of Mayr (1964), "each species as if it were a separate act of creation, monotypic and uniform". Recent work on population genetics has demonstrated enormous intrapopulational and interpopulational variations. The reports of Williams and Swanson, Saoud and Smithers, cited above, are a warning against a concept of schistosome strains as monotypic and uniform. Some variation in a particular character, such as infectivity, is to be expected not only between geographical races and populations, but also between populations of the same geographical race. In the paper quoted above, Mayr was concerned to show that despite the enormous intrapopulational and interpopulational variations, the species concept is still very much valid. To quote Mayr again, "In spite of superficial differences,

the populations of a species seem to share the same homeostatic systems and the same physiological constants as a consequence of the same basic genotype. This ties the totality of local gene pools together into a single genetic system with great stability and strong cohesion." All this has two important implications with regard to schistosomes. First, variation in a single character does not necessarily mean differences in other characteristics. Thus, for example, differences in the infectivity of two populations of miracidia to a given snail host do not necessarily mean that the two schistosome populations are different in their response to schistosomacides, or in their pathological manifestations in mammalian hosts. Secondly, as Mayr goes on to show, speciation, at the species level at any rate, is very strongly resisted by the factors which maintain the gene pool of a species. Consequently, creation of new schistosome species on the basis of infectivity to snails should be treated with great caution.

4. MEANS OF ENTRY INTO SNAIL TISSUE

(a) Miracidium

In 1883 Thomas described the penetration of miracidia of Fasciola hepatica into Lymnaea truncatula

as a process which involved much vigorous, boring action, possibly aided by histolytic substances secreted by the larvae. Most authors agree that the penetration of trematode miracidia into molluscan hosts is accomplished as a result of combined chemical ("digestive") and mechanical action. But the origin and nature of the chemical substance or substances and the relative importance of histolysis and mechanical action have been and still are a matter of much discussion and controversy.

The anatomy of the schistosome miracidium is fairly simple. The body of the organism, except for the anterior tip (the anterior papilla), is covered by a ciliated epithelium. Next to this epithelium lies a syncytial sub-epithelial layer. The cavity enclosed by this layer contains a granular material in which are embedded a number of organs and cells. Three flask-shaped structures, a mass of nervous tissue immediately posterior to them and an excretory system of two pairs of flame cells and their ducts are the only organs seen with the light microscope; germinal cells, which occupy most of the space posterior to the neural mass, are the only other objects seen under the light microscope.

The three flask-shaped structures appear glandular and are therefore called glands. They are arranged so that one lies in the middle, with one of the other two on each side. What are regarded as their ducts open on the apical papilla. Many names have been given to these organs according to what the various authors thought were their functions. In this thesis the terminology of Hyman (1951) will be used because of its wide usage in zoology. She adopted the name "apical gland" for the central organ and "penetration glands" for the lateral pair.

In what is probably the best study of the process of penetration of a trematode miracidium, Dawes (1960) has shown by histological evidence that the miracidium of Fasciola hepatica digests its way into the tissues of L. truncatula. Dawes found that the effect of the substances produced by the larva was to loosen the epithelial cells at the point of attachment of the miracidium and then to lyse the cells. He expressed the view that "the secretion of the apical gland was the most important agent in penetration of the snail."

Longy (1962) felt that the apical gland of S. bovis miracidia secreted an histolytic substance, but he did not indicate how he had arrived at this opinion. Wajdi (1963) stated that the contents of the apical gland

of S. mansoni miracidia disappeared after a few minutes of attachment of the miracidium to the snail host. However, Maldonado and Acosta-Matienzo (1947) reported finding the apical gland intact in recently penetrated miracidia of S. mansoni which they dissected out of snail tissue. Earlier investigators regarded the apical glands as a non-functional, rudimentary gut. For example, Cort (1919) in the first complete description of the morphology of the miracidia of S. japonicum and S. mansoni figured the apical gland as a "rudimentary digestive sac". He did not suggest any function for the sac, although he expressed the opinion that the penetration (his "cephalic") glands produced secretions which aided the penetration of the miracidium "either by dissolving the tissue or by neutralizing the secretions of this (intermediate) host."

Opinion also differs on the function of the penetration glands. Maldonado and Acosta-Matienzo (1947) supported the suggestion by Cort (1919) that those glands produced histolytic substances. Lengy (1962) did not study the function of the glands, but seems to have agreed with Wootton (1957) who suggested that in Allocreadium alloneotenicum, the penetration glands secreted a substance which formed a cuticle round the young sporocyst in the cephalopod host. But it is not clear how Wootton formed

his opinion from an observation of the penetration of the miracidium under the microscope.

A number of histochemical tests on schistosome miracidia have been reported, but they have not thrown much light on the nature of the contents of the glands. During a study of the passage of several larval nematodes and schistosome cercariae through mammalian tissue, Lewert and Lee (1954) applied the periodic acid-Schiff (P.A.S.) test to miracidia of S. mansoni. They found P.A.S.-positive material in both the penetration and apical glands of free-living miracidia and also in miracidia still inside their egg shells. Miracidia in the process of penetration were freeze-dried and tested. The apical gland of these miracidia did not stain, but penetration glands retained most of their P.A.S.-positive material. Because the penetration glands of the cercariae were also found to contain P.A.S.-positive material, Lewert and Lee concluded that the glands of the cercariae and miracidia contained the same or similar substances. The secretions of the penetration glands of the cercariae progressively reduced the reaction of P.A.S. with the connective tissue of mice and increased the water-soluble content of the tissue. Lewert and Lee interpreted these changes as depolymerization of the ground substance (glycoprotein)

of the connective tissue. They suggested that the P.A.S.-positive material in the glands of the miracidia produced similar changes in snail tissue.

Wajdi (1963) found that the contents of the penetration glands, but not of the apical gland, of S. mansoni miracidia, stained with P.A.S. Using Best's carmine method she did not find any glycogen in the glands and concluded that the contents of the glands were mucoid. She also stained sections of recently penetrated and free-living miracidia by Mallory's triple method and concluded that the penetration glands contained a mucoid substance. She said that 48 hours after penetration, the penetration glands were still intact. Wajdi suggested that the mucoid substance present in the penetration glands served two functions. It helped the miracidium adhere to the snail host during penetration and also lubricated the passage of the larva into snail tissue. However, Mallory's triple stain is not a histochemical test and its reliability in identification of mucoid substance is open to serious doubt; Wajdi also used alcian blue, which is a more reliable test for mucin, but she seems to have got better results with Mallory's method.

Peplar (1958) reported an acetylcholinesterase in an "almost circular area near the middle of the miracidium" of S. mansoni. He thought, probably correctly, that this area was "associated with the central nervous system" of the miracidium. He did not find any other kind of esterase or lipase. Fripp (1964) reported the occurrence of non-specific esterase throughout the body of S. rodhaini miracidia, with a "strongy staining area soon about the centre of the anterior half". This area seems to correspond to the neural mass which would be expected to have a high acetylcholinesterase activity. Fripp also found alkaline and acid phosphatases and β-glucuronidase throughout the body of the miracidia of S. mansoni and S. rodhaini.

It is apparent then that no enzymes have been identified in the penetration or apical glands of the schistosome miracidia which have been studied, and from the slightly conflicting evidence of Lewert and Lee and Wajdi, both the apical and the penetration glands contain some polysaccharide. But whether this is the main or the sole content of these glands is not known, nor are its exact nature and function.

(b) Daughter Sporocysts

Recent workers such as Pan (1965) and Lengy (1962) agree that schistosome miracidia settle close to their point of entry into the tissues of the head-foot or the mantle of the snail host. Here the miracidium metamorphoses into a sporocyst which produces a number of daughter sporocysts. Most of the daughter sporocysts soon find their way to the inter-tubular spaces of the digestive gland of the host. The sporocysts settle here and produce large numbers of cercariae.

It is not clear, however, whether daughter sporocysts actively migrate to the digestive gland or whether they are carried there in the circulating blood of the host. Maldonado and Acosta-Matienzo (1947) believed that the sporocysts were swept into the tissues of the digestive gland by the circulating blood. Pan (1965) found a majority of S. mansoni in the rectal and renal ridges. He concluded that this was the route of migration of daughter sporocysts and expressed the opinion that the sporocysts migrated actively because, under the microscope, they were capable of some movement. Lengy (1962) also noticed slight side to side movements in daughter sporocysts of S. bovis, but he saw little forward progress. However, Pan (1965) described the presence of fine spines

in the cuticle of the sporocysts of S. mansoni and stated that those spines aided the forward movement of the sporocysts by giving them a grip on the host tissues. But, on the other hand, Lengy (1962) did not see any such spines in S. bovis sporocysts.

It is now well-known that the digestive gland of snails is the main organ for the storage of polysaccharide and fat (see under "Host Factors"). It is possible, therefore, that the high concentration of nutrients might act as a stimulus for the migration and settling of daughter sporocysts in this organ. But the question of the stimulus for migration and settling has not been studied.

In the laboratory it is a common experience that most of the snails infected with schistosomes die within a few weeks of the beginning of cercarial discharge. Faust (1920) and Pan (1965) have shown that in the snail hosts A. glabratus and B (P) africanus, the enlarging daughter sporocysts of S. mansoni and S. haematobium cause much congestion of the tubules of the digestive gland: many of the tubules become blocked and eventually their epithelial lining atrophies completely. Cercariae also cause considerable damage in most of the snail body where

where they provoke intense tissue reactions as they migrate. The damage to snail tissue soon leads to the death of the host. This is not peculiar to the schistosomes: many other trematode infections have serious pathological effects which eventually lead to the death of the molluscan host, thus terminating the life of the parasites (see Wright, 1966, for a general review).

C. HOST FACTORS

In this section it is proposed to discuss the effect of the host on the host-parasite relationships. The following factors are considered: (1) attraction for miracidia; (2) infection with other parasites; (3) the age; (4) the race; (5) the defense mechanisms; and (6) the internal environment in terms of the histology, physiology and biochemistry of the snail host.

1. ATTRACTION FOR MIRACIDIA

In his search for intermediate hosts of Schistosoma haematobium in Egypt, Looss studied the behaviour of the miracidia of the schistosome in the presence of a variety of species of molluscs: he failed to see any attraction of the miracidia by any of the molluscs, and this was an important reason why, by 1896, he insisted that man served as both the intermediate and the definitive host of the schistosome. However, Leiper (1915) was not convinced that there was no attraction and he used the attraction of miracidia as an important test in screening possible intermediate hosts of S. haematobium. He noted a "definite

attraction by Planorbis boissyi (Biomphalaria alexandrina), Bulinus spp., B. (Py.) forskali and Lymnaea truncatula", but other species of snails "were entirely ignored by the miracidia" of S. haematobium. Similar observations were made by Faust and Meleney (1924), who reported that the miracidia of S. japonicum became "positively tactic to the snail (Oncomelania) swimming full speed and attacking the body of the animal at the first place available." They thought that the attractant was a chemical substance which was present in the mucous secretion of the snail because they saw miracidia actively following the trail of snails. Faust and Hoffman (1934) reported that A. glabratus exerted the same kind of attraction for the miracidia of S. mansoni and many other workers reported similar attraction with other species (Gordon, Davey and Poaston, 1934; Adams, 1934; Porter, 1938; Cram, Jones and Wright, 1944; Stunkard, 1946; MacInnis, 1965; Wajdi, 1966). There is, however, a conflict of opinion and those observations have not always been confirmed. For example, Abdel-Malek (1950) reported that S. mansoni miracidia behaved in the same way towards the normal snail host, B. alexandrina (B. boissyi), snail shells and gravel and Stirewalt (1951) working with S. mansoni and A. glabratus, found no suggestion of attraction of

of the miracidia; also Sudds (1960) studied the behaviour of the miracidia of S. mansoni, Schistosomatium douthitti, Trichobilharzia elvae and T. physellae in the presence of 26 species and varieties of gastropods, including susceptible and refractory snails, and found no evidence of attraction. He concluded that "miracidia were not attracted to normal hosts by a specific chemotactic stimulus, but contact was a chance phenomenon involving abnormal hosts as well." Barbosa and Carneiro (1965) also doubted whether snails attract miracidia after finding that S. mansoni miracidia were able to penetrate tadpoles of Phyllomedusa

Faust and Maloney (1924) suggested that miracidia were attracted only by snails in which they could develop, but there is disagreement about the specificity of attraction even among workers who have observed miracidia attracted by snails. Adams (1934) working in Mauritius, reported that the miracidia of S. haematobium "energetically attacked" B. forskalii (now called B. cernicum) but showed no interest in Lymnaea, Physa, Melanoides and Gyraulus. Gordon, Davey and Feaston (1934) stated that although B. (P) globosus attracted S. mansoni miracidia and B. pfeifferi attracted S. haematobium miracidia, each parasite was attracted much more strongly by its appropriate host. However, other

writers found that a wide range of snails attracted schistosome miracidia: Adams (1932) reported that S. haematobium miracidia swarmed round Lymnaea, Physa, Melania and Paludina in addition to B. forskalii; Stunkard (1946) found that most of 22 species of gastropod molluscs of North America attracted miracidia of S. haematobium, S. japonicum and S. mansoni; and Cram, Jones and Wright (1944) reported that S. mansoni miracidia were attracted by A. glabratus, Planorbarius corneus and 9 species and subspecies of Helisoma.

The main reason for the disagreement about the existence and specificity of attraction of miracidia by snails has been the lack of an objective test for attraction: it is not certain that "attraction" means the same thing to different workers. Kloetzel (1950) attempted to correct the situation by a quantitative estimation of the swarming of S. mansoni miracidia round A. glabratus. In the first of three series of experiments he compared the concentrations of miracidia close to the snail host and some distance from it: he found higher concentrations close to the snail. The second series was similar to the first except for the addition of macerated tissues of the snail host to the contents of the petri dishes in which observations were made. He found that the macerate

had the effect of lowering the concentration ratios from about 4 : 1 to 2 : 1. In the third series of experiments, he placed a total of seven living and crushed snails and empty snail shells in various positions in the petri dish and found that the highest concentrations of miracidia were in the samples taken close to the living snails and the lowest in those taken near empty shells. Chernin and Dunavan (1962) have shown that the miracidia of S. mansoni tend to concentrate round the perimeter of containers; they found 3 to 7 times as many miracidia at the perimeter as at the centre of petri dishes. This distribution of miracidia might have influenced the results of Kloetzel, especially in his third series of experiments in which living snails appear to have been closest to the perimeter of the petri dish. Chernin and Dunavan (1968), indeed, found no difference between the concentrations of S. mansoni in samples of water taken close to freely moving specimens of A. glabratus and those taken some distance from the snails. With the aid of the flying-spot microscope of Young and Roberts (1951), Davenport, Wright and Causley (1962) compared the swimming behaviour of S. mansoni miracidia in clean water and in extracts of whole A. glabratus. Photographs of the televised image of the miracidia showed that the

larvae changed their direction of swimming much more rapidly in the snail extract than in clean water; other workers on attraction have noted a similar increase in the swimming activity of schistosome miracidia when they came close to snail hosts.

Faust and Maleney (1934) stated that attraction for the miracidia of S. japonicum was effective only within a very short distance of the snail host. Etges and Becker (1963) have obtained results which not only give some support for the theory of attraction, but also indicate that snails may attract miracidia at some distance. They designed a cast-iron apparatus which consisted of a central chamber and four smaller side chambers; each side chamber communicated with the central chamber through a side arm. A snail, whole or crushed, was placed in each of two side chambers and a sham snail, made out of aquarium cement, in each of the other two side chambers and the apparatus was left to stand for about an hour. 100 - 200 S. mansoni miracidia were then introduced into the central chamber and after another hour the miracidia in each chamber were counted. Statistically significant differences were noted between the numbers of miracidia which entered the side chambers with A. glabratus and those with sham snails; more miracidia entered the

chambers with real snails. However, in 6 experiments with Bulinus snails, slightly more S. mansoni miracidia entered the chambers which had sham snails than those with real snails and in a single experiment with Helisoma anceps more miracidia entered the chambers which had sham snails. It was concluded that the "miracidia of S. mansoni show moderate, but definite, attraction toward Australorbis glabratus, but not Helisoma anceps or Bulinus spp." As a result of the work, Etges and Decker were of the opinion that in the location of snail hosts light and gravity were more important stimuli than attraction by the snail.

MacInnis (1965) has studied in considerable detail the behaviour of S. mansoni miracidia in the presence of chemical stimulants and of A. glabratus. His results showed that a number of short-chain fatty acids (especially butyric acid), amino acids and a sialic acid increased (a) the rate of swimming of the miracidia considerably, (b) the number of contacts miracidia made with the source of the stimulus (agar blocks) and (c) the amount of time each miracidium spent within 0 - 5 mm. of the source of the stimulus. He defined chemical attraction as "changes in the orientation of miracidia in response to chemicals diffusing from a snail or test

object, resulting in manoevers which bring the miracidia to the source of the stimulus and tend to keep them there." This is a good working definition and A. glabratus and the various chemicals may be said to have been shown to be attractive to S. mansoni miracidia. MacInnis found that the miracidia of Schistosomatium douthitti were only slightly attracted by valeric, butyric and sialic acids, which were the best attractants for S. mansoni: and miracidia of Fasciola hepatica did not respond to butyric acid, the only chemical tested against this trematode. These observations suggest that attraction of miracidia is broadly specific.

The controversy about the existence and specificity of attraction of miracidia applies to all digenetic trematodes and their snail hosts and not only to schistosomes and their hosts (see MacInnis, 1965, for references). It is, however, generally agreed that whether there is attraction or not, random swimming is the most important factor which brings miracidia close to the snail host (La Rue, 1951; Wright, 1959). The factors which stimulate the discharge of the contents of the penetration glands of miracidia have not been studied, but from the work of MacInnis (1965) it would appear that they are not identical with attractants of miracidia. The only function that

attractive chemical substances could serve, therefore, miracidia would be to keep/close to the snail, thus increasing the chances of contact between the larvae and the snail host.

2. INFECTION WITH OTHER PARASITES

(a) External Parasites

Oligochaete annelids of the genus Chaetogaster are commonly found attached to the exposed soft parts of several species of molluscs, but it is not clear whether they live as external parasites or as commensals of the molluscs. In the aquarium in the London School of Hygiene and Tropical Medicine we have noted that heavy infestation with the worms usually leads to much loss of the infested snails, especially bulinid species; the worms appear to irritate the snails so much that heavily infested snails spend most of their time withdrawn into their shells and therefore probably die of starvation. Coelho (1957) found S. mansoni miracidia in the gut of specimens of a Chaetogaster on A. glabratus which he had failed to infect with the schistosome. Khalil (1961) found that very large numbers of Fasciola gigantica miracidia were necessary in order to infect the normal snail host, Lymnaea natalensis, which were infested with Chaetogaster limnaei: he found up to 15 miracidia in the gut of some of the worms.

Wajdi (1964b) described the active ingestion of S. mansoni miracidia by a Chaetogaster species which infested A. glabratus. These observations suggest that Chaetogaster can protect snails against infection with schistosomes.

(b) Internal Parasites

It is well known that in nature snails are frequently infected with more than one species of trematode parasite. For example, Cort, McMullen and Brackett (1937) in field observations recorded double and triple infections in Stagnicola emarginata angulata. They also found that some of the 17 species of cercariae which they studied frequently occurred together while others rarely or never did so. They therefore suggested that some trematode species were mutually antagonistic while in other cases infection with one species might predispose a snail to infection with other trematode parasites. In an experimental study Joo Li, Basch and Umathevy (1967) found that the rediae of two echinostomes, Echinoutoma audyi and Echinoparyphium dunni actively attacked and completely eliminated a Trichobilharzia species, a strigoid cercaria and a xiphidiocercariae from Lymnaea rubiginosa. In a similar investigation Joo Li (1966) has shown that rediae of an Echinostoma sp. were respon-

sible for considerable reduction in numbers of daughter sporocysts of S. mansoni in A. glabratus. The rediae were most effective in their removal of the schistosome when snails were exposed simultaneously to the miracidia of both flukes and when the echinostome was superimposed on S. mansoni infection; little clearance of the schistosome was obtained when S. mansoni was superimposed on echinostome infection. However, total removal of the schistosome was never achieved (a) because the distribution of the sporocysts of S. mansoni and the rediae of Echinostoma in the organs of the host snail, especially in the digestive glands and ovotestes, did not overlap completely: the anterior part of the digestive gland, for example, was occupied only by the schistosome sporocysts whose development and production of cercariae was not interfered with, and (b) because the destructive ability of the rediae was limited to a short period, 4 - 5 weeks: subsequent generations of rediae steadily lost their ability to attack and remove schistosome sporocysts.

It has been suggested that infection with other trematodes makes otherwise refractory snails susceptible to infection with schistosomes (Richards, 1965). Experiments with small numbers of Tropicorbis spp. already harbouring strigoid cercariae have shown that S. mansoni

can be superimposed on other trematode infections (Cram and Files, 1946; Richards, 1961 and 1963), but this cannot be regarded as good evidence that infection with other trematodes predisposes refractory snails to infection with schistosomes. On the contrary, except for a few unconfirmed observations by Porter (1938) combined infections of schistosomes with other trematodes have not been recorded, even though Biomphalaria and Bulinus are commonly infected with other trematodes (Gordon, Davey and Pearston, 1934; El-Kindy and Rushdi, 1962; Kinoti, unpublished observations, 1963-4). Although the apparent competition between schistosomes and trematodes for snail hosts is not sufficiently keen to eliminate schistosomes in nature, the studies initiated by Joe Li and his associates on echinostomes and schistosomes appear to be a hopeful line of search for biological agents for the control of schistosomiasis.

3. RACE

Much of the experimental work that revealed the existence of geographical races of schistosomes provided, at the same time, evidence for geographical races of the snail hosts. This is particularly true of S. mansoni and A. glabratus. But for clarity the evidences for races of hosts and parasites are considered separately in this thesis.

The compatibility between geographical strains of African Biomphalaria and S. mansoni has not been studied, but differences in susceptibility may be expected since there are sufficient morphological variations, especially in B. pfeifferi, to justify the recognition of several subspecies (Mandahl-Barth, 1957). Moreover, A. glabratus is now recognized as a species of Biomphalaria and there is good evidence for differences in susceptibility of geographical races of this snail.

Capron, Deblock, Biguet, Clay, Adenis and Vernes (1965) found that a Corsican strain of Bulinus truncatus was more susceptible to an Algerian strain of S. haematobium than were Egyptian and Tchad strains of the snail: when snails were exposed to 5 miracidia each 58.33%, 48.79% and 17.59% of the Corsican, Tchad and Egyptian strain, respectively, became infected. Wright and Ross (1965) have demonstrated interpopulational differences in the electrophoretic patterns of the egg proteins of some bulinid snails, but the relationship between egg proteins and the susceptibility of snails to infection has not been studied.

There is some evidence for differences in susceptibility of geographical races of Oncomelania to S. japonicum. Moose and Williams (1963) reported differences in the susceptibility of O. formosana to a

Taiwan strain of F. japonicum. They found that, with 4 miracidia per snail, 18% of snails from Changhua became infected, but snails from Kaohsiung were refractory. Raising the number of miracidia per snail to 10 resulted in infection rates of 56.2% and 1.8% in the Changhua and Kaohsiung snails, respectively. Later, Moose and Williams (1964) compared the susceptibility of Oncomelania from a third area, Ilan, with that of snails from Kaohsiung. Using 10 miracidia per snail, the authors found that a Japanese strain of S. japonicum infected 5.6% of snails from Ilan and none of those from Kaohsiung. Exposure to the same number of miracidia of a Philippine strain of the parasite yielded infection rates of 5.0% and 0% in snails from Ilan and Kaohsiung respectively.

But the best evidence for geographical strains of snail hosts was provided by workers on A. glabratus and S. mansoni. Files and Cram (1949) demonstrated differences in the susceptibility of A. glabratus from Puerto Rico, Venezuela and Brazil to S. mansoni from Puerto Rico, Venezuela and Egypt. For example, the Venezuelan strain of the parasite infected 44%, 25% and 0% of the snails from Puerto Rico, Venezuela and Brazil, respectively. With the Egyptian S. mansoni, infection rates of 9%, 7% and 0% were obtained in the Puerto Rican, Venezuelan and Brazilian snails respectively.

Files (1951), in a study already referred to under the race of parasite, demonstrated differences in the susceptibility of A. glabratus from Puerto Rico, Venezuela, Surinam and two areas (Recife and Salvador) in Brazil. She found, for instance, that an Egyptian strain of S. mansoni infected 12%, 8%, 14% and 0% of snails from Puerto Rico, Venezuela, Surinam, Recife and Salvador. 45%, 30% and 7% of snails from Puerto Rico, Recife and Salvador were found to be susceptible to a strain of S. mansoni from Puerto Rico.

Barbosa and Barreto (1960) showed that A. glabratus from two parts of Brazil (Salvador and Pernambuco) differed markedly in their susceptibility to S. mansoni from Pernambuco. They found that exposure of snails to 10 miracidia each gave infection rates of 1.7% and 85.9% in the snails from Salvador and Pernambuco, respectively. These authors noted that equal numbers of miracidia penetrated the two races of the snail; most of the miracidia were, however, destroyed by a cellular reaction in the Salvador, but not the Pernambuco snails.

Paraense and Correa (1965a) exposed snails from 23 A. glabratus populations and 4 crosses between some of the populations to S. mansoni from a patient

who had been infected on a single occasion at Belo Horizonte, Brazil. Although some of the exposures involved rather small numbers of snails, the experiments of Paraense and Correa clearly showed variations in infection rates of from 0% to 100%. Those authors found that the susceptibility of a snail population was independent of its distance from the location of the parasite. Thus, for example, snails from Salvador were completely refractory to infection when exposed to up to 50 miracidia each, whereas exposure to 10 miracidia each produced over 90% infection rates in strains of the snail from Puerto Rico and St. Kitts.

McQuay (1953) investigated a suggestion by Stunkard (1946) that susceptibility might be genetically determined. In a series of studies Cram and her associates (Cram et al. 1945, 1946, 1947) had shown that Tropicorbis havanensis from Baton Rouge, Louisiana, was slightly susceptible to S. mansoni from Puerto Rico: McQuay studied the possibility of raising, by selection, the susceptibility of this snail to a Puerto Rican strain of S. mansoni. He established several lines of descent from a single susceptible snail; each snail of every line of descent was kept in a separate container in order to avoid cross-fertilization. He found that the progeny

of individual susceptible and refractory snails were equally susceptible to infection.

Shortly afterwards, however, Newton (1953) showed that, in A. glabratus, susceptibility was a heritable character; he raised hybrids of a pigmented and an albino A. glabratus and demonstrated that susceptibility was inherited through the three generations he studied.

Kagan and Geiger (1965) have studied the susceptibility of three strains of A. glabratus, one from Puerto Rico and two from Brazil, to three strains of S. mansoni. These workers exposed snails at intervals for periods of 1 - 3 years and found monthly fluctuations in the percentages of snails infected in each host-parasite combination. However, each host-parasite combination when considered over a whole year, remained distinct from every other, as can be seen from the graphs of Kagan and Geiger. This work, therefore, seems to lend further support to Newton's evidence that the susceptibility of a snail population is a genotypic and not a phenotypic character.

There is then good evidence that there are numerous races of snail hosts, just as there are numerous races of schistosome parasites. This variability of

both the infectivity of the parasite and the susceptibility of the host must complicate host-parasite relationships greatly. There is evidence that infection with schistosomes inhibits, or at least greatly reduces, the reproductive capacity of snail hosts (Pan, 1965; Etges and Grosso, 1965). Infection would, therefore, tend to reduce the susceptibility of a snail population, but in endemic areas this tendency would be checked by variations in the susceptibility of the host and in the infectivity of the parasite, as well as by ecological factors. The net product of the interaction of all these factors would be the evolution, not only of races of parasite and snail, but also of a host-parasite relationship unique to a particular endemic area. Theoretically the best relationship is one in which least harm is done to the snail population and which at the same time allows maximum survival for the parasite.

4. AGE

Only contradictory evidence is available on the influence of the age of snail hosts and their susceptibility to infection with schistosomes: this is true for both normal and abnormal host-parasite combinations.

Moore, Thillot, Carney and Moloney (1953) demonstrated that the susceptibility of an Egyptian strain of Bulinus truncatus to infection with an Egyptian strain of S. haematobium varied with the age of the snail. They exposed snails to 5 - 10 miracidia each and found that the infection rates declined from about 50% in snails aged one week to 2.6 - 14.2% in those aged 3½ - 5 weeks at the time of exposure. Newton (1953), working with Puerto Rican strains of A. glabratus and S. mansoni, showed that an exposure of 10 miracidia per snail resulted in infection rates of 93% in snails whose shell diameters were 7 - 9 mm. and 66% in those with shell diameters of 12 - 15 mm; the size of snail is not always a reliable measure of the age of a snail, but the snails which Newton studied had been bred in the laboratory and the size of the shell was known to be a measure of the age of a snail. However, other workers found that the age of a snail did not influence its susceptibility to its usual schistosome parasite. Abdel-Malek (1950) obtained similar infection rates (20 - 32%) when he exposed Biomphalaria alexandrina aged 2 - 3 weeks, 6 weeks, 3, 4½ to 5 months to the miracidia of its normal strain of S. mansoni. Similarly, Moose (1963) found no age differences in the susceptibility of Oncomelania nosophora

to its normal strain of S. japonicum.

The evidence on the effect of the age of a snail host on its susceptibility to unusual strains of schistosomes is also conflicting. Newton (1953) reported that a Brazilian strain of A. glabratus was completely refractory to infection with S. mansoni from Puerto Rico when snails older than 36 days were exposed to up to 50 miracidia, but up to 39% of the snails became infected when specimens aged 1 - 24 days were exposed to 10 miracidia each. Cram and Filos (1944) exposed Tropicorbis havanensis to S. mansoni miracidia and found that 7 out of 19 young specimens became infected whereas only 3 out of 8 adults did so. These results indicated that younger snails were more susceptible than older ones, but it is not possible to arrive at a definite conclusion because of the small numbers of snails studied and especially because some of the snails were exposed to a few miracidia on one occasion while others were exposed to numerous miracidia on several occasions. McQuay (1953) studied T. havanensis and S. mansoni and found that adult snails were more susceptible to infection than young ones: 14.5%, 7.1%, 3.5% and 1.7% of the "adults, large juveniles, medium-sized juveniles, and very small juveniles", respectively, became infected. Abdel-Malek (1950) failed

to infect groups of an Egyptian strain of Biomphalaria alexandrina (B. boissyi) aged 2, 3, 4, 5 - 6, 7, 8 and 12 weeks and 4½ - 5 months with S. mansoni from Puerto Rico. However, he exposed each snail to only 4 - 6 miracidia and this might not have made allowance for variation in the infectivity of miracidia.

Kendall (1950) and Kendall and Parfitt (1959) have clearly demonstrated that a number of refractory species of Lymnaea could be infected with Fasciola only when very young snails, 2 - 6 days, were exposed to the miracidia of the fluke; older snails were completely refractory to infection. They also showed that normal snail hosts were equally susceptible at all ages. Kendall (1964), in his review of the factors influencing the development and behaviour of trematodes in molluscan hosts, concluded that age had little influence on the susceptibility of a snail with its habitual trematode parasite, but young snails were more susceptible to infection with unusual parasites than were older snails. However, as far as schistosomes and their snail hosts are concerned, the evidence is clearly contradictory: some workers found that with both normal and unusual snail hosts, younger specimens were more susceptible to infection than older ones; one author reported that older snails were more susceptible, while yet other workers found that age did not influence susceptibility.

5. RESISTANCE TO INFECTION

(a) Normal Snail Hosts

(i) Natural Resistance

In the laboratory usually only a fraction of the snails exposed to schistosome miracidia become infected. Thompson (1954) has shown that all members of a group of A. glabratus could eventually be infected by repeatedly exposing uninfected individuals to S. mansoni miracidia. This suggests that individual snails may be naturally immune to infection with some individual miracidia. It is generally agreed that the tissues of snail hosts do not react to their habitual trematode parasites (Salt, 1963); it is reasonable, therefore, to suppose that lack of infection in normal snail hosts is due to the failure of miracidia to penetrate.

(ii) Acquired Resistance

Faust (1924) claimed that massive invasion by schistosome miracidia made normal snail hosts resistant for a few hours to further penetration. There is evidence that some invertebrates such as insects can actively acquire immunity of short duration to bacteria (Stephens, 1963), but Faust's claim that snails can develop immunity has not yet been substantiated.

Micholson (1964) demonstrated the immobilizing effect of aqueous extracts of infected digestive gland and ovotestes of A. glabratus on S. mansoni miracidia. He found that in one hour extracts of snails with mature S. mansoni infections immobilized 97.9% of the miracidia, whereas extracts of uninfected snails immobilized 17.8%. With 6 - 8-days infections (before sporocysts migrated to the digestive gland) 36.2% of the miracidia were immobilized compared with 17.2% using uninfected snails. With more mature infections (9 - 15 days), 97.3% of the miracidia were immobilized. He also found that 9 of 10 extracts of infected A. glabratus miracidia immobilized 100% of Fasciola hepatica. (In most of the experiments there were wide variations in the rates of immobilization and only the mean percentages are given above).

These observations suggest that the immobilizing substance or substances are not very specific. Micholson (1963) has studied the specificity of the substance or substances by testing S. mansoni miracidia against aqueous extracts of A. glabratus which had been infected with an acid-fast bacillus, echinostome metacercaria, and the nematode Daubaylia potomaca as well as extracts of snails into which either S. mansoni, plastic balls, or bovine albumin had been injected. Significant immobilization rates were

obtained only with extracts of the snails infected with D. notomaca and of those inoculated with S. mansoni eggs. These results suggest some degree of specificity in that only infections with helminth parasites produced significant immobilization. But it is not clear from these studies whether the immobilizing factor or factors were produced by the host, or by the parasite since he extracted the host and parasite together.

There is evidence that infected snails are readily re-infected with the homologous schistosome. Faust and Meleney (1934) demonstrated histologically that S. japonicum miracidia will penetrate Oncomelania nosophora which were already shedding cercariae of the parasite. Chernin (1966) has also found that S. mansoni miracidia penetrated A. glabratus which were already shedding cercariae of the schistosome. Kagan and Geiger (1964) have shown that complete development of S. mansoni can take place in A. glabratus already infected with the schistosome. They did this by exposing snails individually to a single miracidium, determining the sex of the infection in each snail by infecting mice with cercariae from the snails, re-exposing each snail to a single miracidium, and finally determining the sex of infection in each snail. They found that some of the snails had bisexual infections thus showing that snails which already had mature infections could be re-infected. Similarly Barbosa and Coelho (1956) showed that infected

A. glabratus could be re-infected with S. mansoni. They also noted cellular infiltration around some of the miracidia which penetrated snails which were shedding cercariae, but not in those which had lost their infection. However, the presence of host cells around a few miracidia should be interpreted with great care because Pan (1963, 1965) has shown that amoebocytes often collect over large areas in infected A. glabratus: Pan called those collections of amoebocytes "generalized tissue reaction" as distinct from "focal reactions" which developed around individual cercariae or collections of cercariae.

There have been a number of reports of both A. glabratus and bulinid snails losing infection after they had been shedding cercariae for some time (Barbosa, 1965; Stirewalt, 1934; Wright, 1962[a]), but the factors which cause such "self-cure" are not known. Usually it is snails which are poor hosts that lose their infections (Barbosa, 1965; Wright, 1962[a]) and this suggests that the snails actively eliminate the parasites from their tissues. However, all the evidence available suggests that snail hosts do not react to their habitual schistosome parasites: cellular infiltration around miracidia and sporocysts has been found reported in refractory or only slightly susceptible snails (see below under "Abnormal Hosts"), but not in normal hosts.

(b) Abnormal Snail Hosts

Newton (1953) was the first to demonstrate the effectiveness of cellular response, on the part of the snail host, against schistosome sporocysts. He exposed a susceptible (Puerto Rican) and a refractory (Brazilian) strain of A. glabratus to 30 to 50 S. mansoni miracidia per snail and followed, by a histological study, the fate of the sporocysts. He found that in most of the snails from Brazil the parasites were soon surrounded by concentric layers of host cells and were apparently destroyed and removed from the tissues of the host within 24 to 48 hours when areas of cellular reaction were common, but no parasites were seen. In the susceptible strain, parasites developed normally, without any cellular reactio n around them. Brooks (1953) carried out a similar study on a highly susceptible strain of A. glabratus and a slightly susceptible strain of Tropicorbis havanensis: only 10% of the latter snail were susceptible to the strain of S. mansoni which he studied. He found that equal numbers of the S. mansoni miracidia penetrated the two snail hosts, but whereas in A. glabratus the parasite developed normally in some of the T. havanensis, the sporocysts were surrounded by host cells in 12 hours

and were apparently destroyed and removed within 7 days. In 14 of the 38 specimens of T. lavanensis which Brooks studied had both normally developing sporocysts and sporocysts with intense cellular reaction around them, side by side in the tissues of the same snail hosts. Newton (1954) reported similar results in a second generation (F2) hybrid of a completely refractory and a highly susceptible strain of A. glabratus. He found that 54 hours after exposure of the snails to S. mansoni miracidia, 3 snails contained sporocysts which were developing normally as well as some which were surrounded by an intense cellular reaction; in 5 other snails all the sporocysts were surrounded by reactions, while no reactions to parasites were found in 14 other snails. Cellular reactions against S. mansoni have been reported by other workers on other refractory or only slightly susceptible strains: A. glabratus (Barbosa and Barreto, 1960), and A. tenagophilus (Coelho, 1957, 1962).

The response of bulinid and other snails to schistosomes has not been studied to the same extent as that of the hosts of S. mansoni. Wajdi (1964a) did not find any cellular response to an Aden strain of S. haematobium in strains of Bulinus forskalii, B. beccari and B. reticulatus. In contrast, in a series of obser-

vations carried out in East Africa in 1964, I found intense cellular reactions around sporocysts of Schistosoma haematobium in 3 out of 14 specimens of a Kenya strain of B. tropicus within 12 hours of exposing the snails to the miracidia. No sporocysts or evidence of reaction were found in the other 11 snails. The response of bulinid snails to schistosomes of S. haematobium group is examined further in Part III of this thesis.

There is some confusion over the types of host cells that collect round parasites. Newton (1952) simply called them "host cells", Brooks (1953) identified fusiform cells, which had spindle-shaped nuclei, in the early stages of the reaction and "collagenous fibres" and "fibreblast-like cells" in later stages. Coelho (1957, 1962) and Barbosa and Barreto (1960) identified the cells involved in the host reactions as amoebocytes. Tripp (1961) has shown that in A. glabratus, as in other molluscs, amoebocytes are actively phagocytic: he injected yeast, bacteria, chicken erythrocytes and carmine particles into A. glabratus and found that the amoebocytes rapidly took up these particles and digested the digestible material. They removed, by migration to outer epithelia, those particles that were indigestible, or in the case of carmine, simply stored the particles within their

cytoplasm. When he injected the snails with particles - polystyrene balls and pollen grains - which were too large for phagocytosis, he found that within 16 to 24 hours amoebocytes spread themselves, in concentric layers, around the particles; fibroblasts finally formed a capsule surrounding the amoebocytes. When he introduced pieces of A. glabratus tissues which had been fixed in formalin, and fresh tissues of Planorbarius corneus, he found that only fibroblasts formed capsules around the tissues. He suggested that fibroblasts migrated from the surrounding tissues, but the difference between amoebocytes and fibroblasts is not very clear. The presence of cells with thin and elongate nuclei and cytoplasm in fibrous capsules is not good evidence for migration of fibroblasts: in insects haemocytes, which are equivalent to amoebocytes in molluscs, encapsulate parasites by first stretching themselves over the surface of the invading organisms and then producing fibrous material with which they become interwoven (see Salt, 1963, for review).

There is evidence that schistosome miracidia will penetrate snails in which they cannot undergo complete development (Stunkard, 1946; Files, 1951; Richards, 1963; Cram, Jones and Wright, 1944). Bonex and Lamy (1959) compared the effects of extracts of refractory and

susceptible snails on S. mansoni miracidia. They found that aqueous extracts of whole Planorbarius corneus and a Brazilian strain of A. glabratus immobilized all the miracidia placed in them in 15 to 45 minutes, but extracts of a susceptible strain of A. glabratus from the Carribean had no effect. However, interpretation of their results is complicated by their finding that extracts of a refractory strain of A. glabratus from Pointe'- a'Pitre did not have any effect on the miracidia. Michelson (1964) has also produced conflicting evidence: he found that aqueous extracts of the digestive gland of one strain of Helisoma caribaeum and Bulinus truncatus immobilized significantly higher percentages of S. mansoni miracidia than did extracts of the normal host, A. glabratus, but extracts of other refractory snails, 7 strains of H. caribaeum, P. corneus and Lymnaea palustris, had no significant effect on the miracidia. But even if these results were more consistent, it would be impossible to know whether the factors that immobilized the miracidia were from the tissues of the snails or from external sources, especially the alimentary tract, since extracts of the whole snail were used.

In conclusion, it would appear that snails like other invertebrates do not develop an acquired resistance against metazoan parasites (Huff, 1940). The defence mechanism against schistosomes is mainly cellular and operates only when these parasites invade snails that are not their usual hosts.

6. THE INTERNAL ENVIRONMENT

(a) Histology and Histochemistry

The only complete description of the histology of a molluscan host of Schistosoma is that of Fan (1958), who has studied A. glabratus, in great detail. Three of his observations are relevant to this discussion. These are (i) the possibility of the occurrence of lymphoid tissue in snails (b) the histology of the digestive gland and (c) the occurrence of masses of mucus cells in the foot of the snail. Fan described an area between the saccular portion of the renal organ and the pericardium as haemopoietic; he suggested that in this area fibroblasts transform into amoebocytes whose importance in the defence mechanisms of snails against schistosomes has been reviewed above. This is an interesting observation because the origin of amoebocytes is not known in any

other mollusc (Fretter and Graham, 1962). He identified three types of cell - in the epithelium of the digestive gland and vesicular cells in the connective tissue on which the epithelium rests. He also described mucus cells which open outside the epithelium of the sole of the foot of the snail: these cells are of interest because the mucous secretion of snail hosts is generally believed to contain a substance or substances that are attractive to schistosome miracidia (see "Attraction for Miracidia" above). But some of Fan's observations appear to need confirmation because they differ in important respects from those of other workers on the histology of molluscs. For example, he claims that the digestive cells were derived from the fibroblasts of the connective tissue this seems most unlikely but if true it would be unique in the animal kingdom. Another example is that he suggests that the polysaccharide content of the digestive gland is stored in the vesicular cells of the connective tissue; other investigators of the molluscan digestive gland have found that the epithelial cells are the main storage sites for glycogen (see, for example, Hurst, 1927, and Sumner, 1965).

The only other published work on the histology of intermediate hosts of schistosomes is that of Faust (1920). He studied the histology of infected and uninfected digestive gland of A. glabratus and B (P) africanus (which he called, respectively, Planorbis guadeloupensis and Physopsis africana) and identified two cell types: the "lime" cells and the liver or digestive cells. He found deposits of glycogen, protein and fat in these cells, but it is not clear how he identified protein and fat deposits because Best's carmine was the only histochemical technique he used. Using Best's carmine, von Brand and Files (1947) also identified glycogen in the digestive gland of A. glabratus, but they did not specify the cells in which the polysaccharide occurred.

Muller (1965) had made a general survey of the occurrence in A. glabratus of acid and alkaline phosphatases and esterases, including a "true" lipase and an acetylcholinesterase. The possible importance of some of these enzymes to schistosome parasites is discussed in Chapter 5 of this thesis.

(b) Physiology and Biochemistry

von Brand and Files (1947) studied the effect of S. mansoni infection on the physiology of A. glabratus

by comparing the fat and polysaccharide contents and the oxygen consumption of infected and uninfected snails. They found that in the infected snails there was a reduction in the polysaccharide content, but they did not find any significant difference between infected and uninfected snails in their fat storage or oxygen consumption. However, Faust (1930) reported a decrease in the amount of food (presumably glycogen, fat and proteins) stored in the digestive gland of infected A. glabratus and B.(P) africanus. But the two sets of observations are not strictly comparable because the chemical analyses of von Brand and Filos were quantitative, whereas Faust's histological and histochemical observations could only be qualitative. Moreover, the age of the schistosome infections was not stated by these authors; also the analyses of von Brand and Files included the fat and polysaccharide of both the host and the parasite.

In a general survey of freshwater gastropods, von Brand, Saernstein and Mehlman (1950) investigated the anerobic and aerobic carbohydrate metabolism and the resistance to anaerobiosis in several species of Planorbidae, including Australorbis glabratus, Biomphalaria pfeifferi, B. alexandrina and Tropicorbis spp. and of operculates including Oncomelania nosophora. Their results did not

show any differences between hosts of Schistosoma and other snails, except possibly for the lower lactic acid accumulation in the tissues of the Planorbidae and the operculates in anaerobic conditions: however, most members of the Planorbidae and the operculates are not hosts of schistosomes. von Brand and Mehlman (1951) later provided evidence suggesting that propionic and acetic acids were the end products of anaerobic carbohydrate metabolism in A. glabratus. Olivier, von Brand and Mehlman (1953) have found that A. glabratus infected with S. mansoni was more susceptible to lack of oxygen than uninfected specimens. They also found that although anaerobiosis reduced the number of cercariae shed, keeping infected snails under anaerobic conditions for 6 hours, or even 16 hours, did not affect the ability of the cercariae to penetrate and mature in mice. That the rate of anaerobic metabolism bears no obvious relation to the ability of a snail to serve as a host of a schistosome is further shown by the finding that two strains of A. glabratus, both hosts of S. mansoni, had significantly different levels of anaerobic metabolism (Olivier, von Brand and Mehlman, 1953).

Targett (1961) has compared the occurrence and composition of the respiratory pigments as well as the occurrence of free and bound amino acids in hosts of Schistosoma and other snails. Using electrophoretic and spectrophotometric procedures, he found that haemoglobin was the main and probably the only protein present in the blood of A. glabratus, B (P) globosus, B (P) nasutus, and B. truncatus: Wright and Ross (1963) have confirmed this in mature A. glabratus. Targett found that the haemoglobins of the intermediate hosts of Schistosoma had the same structure and electrophoretic mobility as those of Planorbarius corneus and Bulinus tropicus. The haemocyanins of the blood of O. nosophora and of Lymnaea stagnalis were very similar in structure and mobility, but Jones (1964) has seriously questioned the occurrence of haemocyanin in L. stagnalis. Targett's chromatographic analyses of the amino acids of which the respiratory pigments are made, showed no differences between the hosts of Schistosoma and other snails. Similarly he found no differences in the free amino acids which occurred in the blood of A. glabratus, P. corneus and L. stagnalis. However, he found that free methionine occurred in the anterior (? headfoot and mantle) of A. glabratus, but not in P. corneus or

L. stagnalis. He further found that in infected A. glabratus methionine occurred in cercariae but not in the anterior tissues of the snail: he suggested therefore that methionine might be a specific requirement of the schistosomes. However, most of the free amino acids occurred in such small amounts in all snails that the failure to detect methionine in infected snails may not be significant.

Two attempts to cultivate the molluscan phases of S. mansoni in vitro have not thrown much light on the metabolite requirements of schistosomes in their snail hosts. (Chernin, 1964; Targett and Robinson, 1964): indeed, Targett and Robinson found that the blood and whole extracts of Helix pomatia and Lymnaea stagnalis were much better than those of the normal host, A. glabratus, as media for the metamorphosis of the miracidia and the initiation of development in the sporocysts of S. mansoni.

Acetivation of the intermediate hosts of schistosomes is an interesting aspect of their physiology, especially in relation to the life of the schistosome parasites. Barbosa and Coelho (1953) found that when A. glabratus with mature infections of S. mansoni were kept out of water they lost their infection; but when snails with young mother and daughter sporocysts were

subjected to dessication, the infections survived for up to 90 days (Barbosa and Coelho, 1959). The field observations of Barbosa and Barbosa (1958) have provided some evidence of the synchronization of the dormancy of S. mansoni with that of the host, A. glabratus: the authors found that snails which had been in aestivation for one to four months did not start shedding cercariae until they had been in the laboratory for 10 to 13 days. Hira and Muller (1966) have shown experimentally that aestivation for 30 days delayed the development of S. haematobium in B (P) globosus by an average of 23 days. They have also shown that the development was not completely inhibited; they found that after 30 days of aestivation, 3 specimens contained mother sporocysts at the stage of development comparable to sporocysts 12 days old in snails which had not been aestivating. Li (1953) presented evidence which suggested that the development of S. japonicum had been arrested or slowed down during the aestivation of the host, O. formosana; but those snails went into dormancy during the winter and the effects of the low temperature and aestivation cannot be separated. Webbe (1962) found that aestivating B (P) nasutus carried S. haematobium, but the effect of the dormancy of this snail on the rate of the development is not known.

This review of the factors affecting the development of schistosomes in their molluscan hosts is not exhaustive: the purpose of the review was to examine only those factors thought to have a direct influence on the life of the schistosome parasites. The review shows that although the subject has been under investigation for at least 40 years, there are still many gaps in our knowledge of this aspect of the host-parasite relationships of schistosome infections. This is especially true of the parasites of the S. haematobium group and their bulinid hosts. In Part II of this thesis are recorded observations on some factors affecting the development of S. mattheei, S. bovis and members of the S. haematobium group in two of their snail hosts, B (P) africanus and B. truncatus.

STUDIES ON FACTORS AFFECTING THE DEVELOPMENT OF SCHISTOSOMES IN THEIR MOLLUSCAN HOSTS

P A R T II

C H A P T E R 3

THE INTERNAL ENVIRONMENT

Some Histological, Histochemical and Electron Microscopical Observations on Bulinus (Physopsis) africanus and Bulinus (Bulinus) truncatus.

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(iv) Discussion.

4. SUMMARY AND CONCLUSIONS.

1. INTRODUCTION

The original aim of this work was to study the defence mechanisms of bulinid snails against schistosomes of the Schistosoma haematobium group, but right at the beginning it was obvious that there was a need for a knowledge of the tissues in which the parasites live. Apart from the work of Faust (1920) on the digestive gland of Bulinus (Physopsis) africanus, no studies of the histology of the bulinid snails have been published in spite of the importance of Bulinus as hosts of Schistosoma. A study was undertaken, therefore, of the histology of B (P) africanus and Bulinus (Bulinus) truncatus which, with closely related species, are the most important intermediate hosts of S. haematobium, S. mattheei and S. bovis. Only tissues of organs that have an obvious bearing on the development of schistosomes in the snails were studied. These are: the headfoot, the tentacle, the mantle and the digestive gland. The first three organs are the sites into which schistosome miracidia penetrate and where sporocyst development takes place. In other molluscs the blood cells, the amoebocytes, are generally believed to originate in the mantle; the organ is, therefore, of great interest in a study of the defence mechanisms of the snails. Although there is much controversy over

many of its physiological functions, it is generally agreed that the digestive gland plays an essential role in the nutrition of molluscs. The organ must therefore be of fundamental importance in the nutrition of all the developmental stages of schistosomes, especially of the daughter sporocysts and the vast numbers of cercariae which they produce inside the digestive gland.

The objective of this study was to identify all the histological elements of the four organs, paying particular attention to (a) any elements which secrete substances that might attract schistosome miracidia to the snail hosts, (b) the origin and distribution of amoebocytes, (c) the importance of the digestive gland in digestion and the storage of food substances, particularly glycogen.

Histological, histochemical and electron microscopical techniques were employed.

Except for the section on the digestive gland, where there is a discussion at the end of the description of the whole organ, the relation of the findings in Bulinus with previous work in other molluscs and the importance of these findings in the development of schistosomes are discussed as each organ or tissue is described, usually in a concluding paragraph.

2. MATERIAL AND METHODS

(a) Biological material

Most of the work recorded in this chapter was carried out on B (P) africanus and B (B) truncatus, but some histological observations were also made on B (B) tropicus. Two strains each of B (P) africanus and B (B) truncatus and one strain of B. tropicus were maintained in the aquarium at the London School of Hygiene and Tropical Medicine and were used in this work. The strains of B (P) africanus came from Mwanza in Tanzania and Nelspruit in South Africa, and those of B. truncatus from Egypt and Iran. B. tropicus was from Arusha (Tanzania).

Considerable difficulty was encountered in the breeding and maintenance of the snails. When they were first introduced into the aquarium, in July 1964, the snails started breeding very well, but most of them died during the autumn and winter months; only a few specimens of the two strains of B (P) africanus and the Iranian strain of B. truncatus survived the winter. During the following spring and summer months the survivors laid large numbers of egg masses, but only a small proportion of the young snails which hatched reached maturity. At this time the snails were kept in glass tanks and fed on

lightly boiled lettuce. During the latter part of the spring and over most of the summer, a unicellular green alga (or algae) grew on the walls of the tanks. It appeared to form very good food for the young snails; both young and older snails were found browsing on it almost continuously. This fact was taken advantage of to produce large numbers of snails, especially of B (P) africanus, whose young survived very poorly on lettuce. The bottoms of large glazed clay pans were covered with a thin layer of a mixture of fine river mud and sand; the pans were filled with aquarium water and left to stand near the large windows of the aquarium. When some growth of the alga had appeared a few snails from the breeding stock were introduced. These soon laid many egg masses and within two months large numbers of snails were available for study and for the maintenance of schistosomes. However, in the following autumn and winter months, most of the snails died, but each year more and more snails survived probably as a result of some degree of adaptation to the conditions of the aquarium.

The cause of mortality in the snails in late autumn and in winter was not established, but it was probably connected with light. When sunlight was available

for long periods each day (in spring and summer), mortality of the snails was negligible, and in winter most of the snails that survived were always in tanks close to the windows. In the spring and summer, snails survived equally well in tanks with algae growth and in those without any evidence of algae, or any other plant life. This suggests that light influenced the physiology of the snails directly and not through their diet. Water could not have been a cause of death of the snails: all the water used in the breeding and maintenance of the snails had been treated in the same way. Ordinary tap water was passed through a domestic water filter and stored for at least three weeks in plastic tanks with a capacity of several gallons and in which the water weed Eloidea was grown on a substratum of river mud and sand. /Water that had been treated in this way is referred to as "aquarium water" throughout this thesis7. Temperature was probably not an important factor in the death of snails either, because the winter fluctuation of 15 to 20°C. was within the range known to be well tolerated by bulinid snails in nature. Unsuccessful attempts were made to reduce the mortality in the winter months by the use of fluorescent strips which the manufacturers claimed to produce "daylight". Similarly, attempts to grow the

unicellular green alga in fluorescent and also [illegible] tungsten light were not successful. Thus, although a greater measure of success was achieved in breeding and maintaining the bulinid snails than has been reported outside the tropics, the production of adequate numbers of snails for experimental purposes had to depend on the weather.

Except for the special techniques described above, the snails were maintained in glass tanks which usually contained a plant weed (Ceratophyllum) and the water flea, Daphnia. Frequent changing of the water was avoided and tanks were cleaned out only when snails started dying: some tanks were usually left undisturbed for several months and although some of them looked very dirty, these tanks often contained the most healthy of the snail colonies.

(a)(i) Histological methods

Preliminary trials showed that for ordinary histology the fluid of Zenker was a better fixative than that of Bouin; most of the work was therefore done on material that had been fixed in Zenker's fluid, but Bouin's fluid was also used sometimes. When fixed in Helley's fluid, the digestive cells had a life-like appearance and the fixative was therefore occasionally

used, especially for material to be stained with Delafield's haematoxylin. The shells of snails to be fixed were gently cracked between two microscope slides. The snails were placed in water in a petri dish and freed from the pieces of shell and then immersed in the fixative; some of the fixative was injected into the mantle cavity. All these operations were carried out under a dissecting microscope; after a little practice it was easy to completely free a snail of its shell in one or two minutes without damaging its soft tissues in any way. Fixation in the fluids of Zenker and Helley was considered complete after 4 hours, while material was usually fixed in Bouin's fluid overnight.

After fixation the stomach of every snail was routinely dissected out in order to remove the sand present in the gizzard and crop. In order to avoid excessive hardening of the material, the last stages of dehydration were carried out in methyl benzoate instead of absolute alcohol. Snails were embedded in paraffin wax under pressure. Sections, 4 to 6 microns thick, were cut on a Cambridge rocking microtome; for ordinary histology they were stained with the haematoxyline of Erlich and Heidenhain and counterstained, when necessary, with eosin and mounted in Canada balsam. Standard histo-

logical procedures as given by Pantin (1959) and Carleton and Drury (1957) were followed, so they are not given in detail here.

Four special histological techniques were employed in a study of the nature of the connective tissue. These are: (a) the trichrome method of Gomori, (b) the orcein-aniline blue-orange G stain for the differentiation of collagen and elastic fibres, (c) the orcein method for elastic fibres and (d) Foot's silver impregnation method for collagen and reticular fibres. The first two methods were carried out on material which had been fixed in Bouin's fluid, following the procedures given by Gurr (1956). The procedures of Carleton and Drury (1957) were followed for the last two methods. In the tests for elastic fibres sections of rat aorta were used as positive controls.

(b)(ii) <u>Histochemical methods</u>

Snails for histochemistry were removed from their shells as described under histological methods and plunged into the appropriate fixative.

1. <u>The Methyl Green-Pyronin Y Method for Ribonucleic Acid (RNA) and Deoxyribonucleic Acid (DNA)</u>

Specimens of <u>B (P) africanus</u> were fixed for 30 minutes in Carnoy's fluid and then dehydrated and

embedded in paraffin wax (m.p. 58°C.) Sections, 5 microns thick, were stained in a buffered mixture of methyl green (of G. T. Gurr) and Pyronin Y (of E. Gurr). The staining mixture was made as follows: a solution of 0.5 gm. of methyl green per 100 ml. of sodium acetate buffer (pH 4.4) was extracted repeatedly in a separating funnel until the chloroform layer became colourless; Pyronin Y was then dissolved in the solution to give a concentration of 0.2 gm. per 100 ml.

After staining for 15 to 20 minutes at room temperature the sections were dehydrated in t-butyl alcohol cleared in xylol and mounted in the synthetic medium "Clearmount" of E. Gurr.

To confirm that pyronin-positive material was RNA, alternate slides were incubated for 1 hour at 37°C. in aqueous solution (0.5 mg/ml) of the enzyme ribonuclease (British Drug House, Biochemical grade) and in distilled water. Glass distilled water was used for both dissolving the enzyme and for incubating the control slides.

Details of the procedures followed here are given by Barka and Anderson (1963).

2. Best's Carmine for Glycogen

Snails were fixed overnight in the ice-cold (0 to 4°C.) picro-alcohol formalin of Rossman (Barka and Anderson, 1963). Before staining the paraffin sections, 5 microns thick, were coated with celloidin to avoid loss of glycogen. It was found that drying slides in the air made the celloidin coating too hard for adequate penetration of the stain; the coating was therefore hardened in 80% ethyl alcohol. The sections were stained in a freshly prepared Best's carmine, differentiated, dehydrated and cleared as in Pearse (1960) and then mounted in "Clearmount".

To ascertain that the material staining with carmine was glycogen, alternate slides were treated with a 1% solution of malt diastase (British Drug House) in aqueous 1% solution of sodium chloride at a temperature of 35 to 37°C; control slides were left in the salt solution at the same temperature and for the same length of time. Another group of slides was treated with filtered human saliva and the control slides with the salt solution.

3. The Benzidine Method for Haemoglobin

Snails were fixed over-night in the buffered neutral formalin of Lillie. Paraffin sections of the

digestive gland were stained in an acidic solution of benzidine and nitroprussido in methyl alcohol, washed in oxonic ether, dehydrated, cleared and then mounted in "Clearmount". Details of this method are given in Pearse (1960).

Thick smears of mouse blood served as positive controls. The slides were dried in the air, fixed for 1 hour in the buffered neutral formalin and then processed together with the sections of snail tissue.

4. Tests for Calcium

Three tests - the haematoxylin, alizarin red S and nuclear fast red - were used in attempts to identify calcium salts in the digestive gland of Bulinus. These methods were recommended by McGhee-Russell (1958) in his critical evaluation of the available histochemical tests for calcium.

All the snails to be studied by the three methods were fixed for 4 hours in a mixture of equal parts of absolute ethyl alcohol and slightly alkaline 40% formaldehyde solution; the alkaline formaldehyde solution was obtained by shaking it with lithium carbonate and then filtering the supernatant liquid if necessary.

Paraffin sections were stained in a 2% solution of the anthraquinone dye alizarin red S (G. T. Gurr) whose pH was adjusted to 4.2 with dilute ammonia. Other sections

were treated with a 5% solution of haematoxylin in strong ammonia solution (S.G. 0.88). After extraction of impurities by washing three times in distilled water, another anthraquinone dye, nuclear fast red (the Fast Red Salt D of G. T. Gurr was used) was dissolved in distilled water to give a saturated solution with which sections of snail tissue were flooded.

In all three methods the procedures recommended by McGhee-Russell were strictly adhered to, so they are not given in detail here. The efficacy of the staining mixtures was tested on ground shells of snails and on commercial calcium chloride and calcium carbonate.

5. The Alcian Blue Method for Mucin

Snails were fixed overnight in Bouin's fluid and processed as for ordinary histology. Paraffin sections were then stained in a 0.1% solution of Alcian Blue in 3% acetic acid. The Alcian Blue 8 GX of the Imperial Chemical Industries was used in the tests; neutral red and sometimes Erlich's haemalum was used as counterstains. After dehydration and clearing in xylol the sections were mounted in Canada balsam. (See Pearse, 1960, for details of the method).

6, The Hexamine-Silver Method of Gomori for Uric acid and Urates

After removal from their shells, snails were immersed in absolute alcohol and after about 30 minutes the mantles were dissected free by cutting along their points of attachment to the body: a similar technique was used with mantles for histology. The mantles were fixed for 24 hours in fresh absolute alcohol.

Paraffin sections were incubated at 37°C. in the hexamine-silver mixture of Gomori at pH 7.0. (Details of the method are in Pearse, 1960).

7.— Esterases and Alkaline phosphatase

Observations on the occurrence of these enzymes in the digestive gland were made during the work recorded in Chapter 5 where details of the methods are given.

(b)(iii) Electron Microscopy

Observations with the light microscopy showed no differences between the cells of uninfected digestive glands and of those with young schistosome infections: for example, there was no difference in the basophilia or glycogen content of the secretory cells. Within two days before or after the shedding of cercariae had

started snails were therefore fixed for observations on the fine structure of both the parasites and the cells of the host. Details of the techniques are given in Chapter 5.

General Notes

1. Except for the test for uric acid and urates, where only 3 mantles were studied, each histochemical test was performed on at least 6 specimens, usually in two or more experiments. All the histochemical observations were made on B (P) africanus and a few on B. truncatus as well.

2. The histological studies were done on a much larger number of snails, including those which were specifically processed for histology and those which were being studied for other purposes (see Chapter 4). Light microscope observations did not show any differences in the histology of B (P) africanus and B. truncatus: therefore the generic name Bulinus is used in this chapter to reduce unnecessary repetition of the specific names.

3. All pH values given in this chapter were determined with a Pye (Cambridge) glass electrode pH meter.

3. RESULTS

(a) The Head-foot

The term "head-foot" is used to describe the head and foot because there is no morphological differentiation between these two regions. The anterior part of the organ performs functions which are associated with the head in other animals: externally and on the dorsal aspect it carries a pair of tentacles, a pair of simple eyes and, on the ventral side, is the mouth. A dissection of this part of the mouth reveals a large buccal mass to which several large muscles are attached. Posteriorly the buccal mass leads into the oesophagus from the dorsal side of which a pair of salivary glands arise and run posteriorly on each side. A nerve ring, which consists of ganglia interconnected by commisures, encircles the anterior end of the oesophagus. Nerves from the ganglia run into all parts of the body. A penial complex is the only other organ found in the anterior part of the head-foot. It consists of the preputium, penis and its sheath, and vas deferens and large muscles which, presumably, operate the complex. The preputium opens to the outside just behind the left tentacle. The posterior two thirds of the head-foot do not carry any

organs internally or externally: locomotion appeared to be the only function of this part of the organ.

It is not proposed to describe the anatomy and histology of the reproductory and nervous systems, or of the alimentary canal, except the digestive gland, because these tissues are not thought to have a direct effect on the life of schistosome parasites. A brief description of the histology of the head-foot is given in this section.

(a)(i) Epitholium

The head-foot of Bulinus is covered by an epidermal epitholium which rests on a sheet of connective tissue. The epitholium of the sole of the head-foot consists of tall columnar cells which have long cilia. In haematoxylin and eosin sections these cells have a lightly eosinophilic cytoplasm and basal nuclei which are elongate and vesicular.

The rest of the surface of the head-foot is covered by an epitholium of low cuboidal to nearly squamous cells. The cells are not ciliated. The nuclei are nearly spherical, vesicular and basal.

(a)(ii) Connective tissue

Sections of the head-foot of Bulinus appear as a sheet of connective tissue enclosed by the epidermal epitholium. The sheet had blood spaces of irregular shapes, sizes and distribution. Mucus and vesicular cells, muscle fibres and nerves are embedded in the connective tissue; amoebocytes and pigment cells occur in the blood spaces. The connective tissue proper was found to comprise collagen fibres and fibroblasts

(a)(ii)(A) Fibres

The stain of Gurr (1956) for the differentiation of connective tissue showed only the presence of collagen. Tests with orcein confirmed this because they failed to reveal any elastic tissue in the head-foot. In these tests with orcein, sections of rat aorta stained brown with a tinge of violet showing that they were elastic tissue (Carleton and Drury, 1957). The silver impregnation technique of Foot showed the fibres which comprise the connective sheet very clearly. Each fibre consists of a number of very fine fibrils. Some of the finer fibres appeared to branch and may be reticular fibres, but the distinction between reticular and collagen fibres

is a matter of controversy among histologists: reticular fibres are generally considered to be young collagen fibres and are therefore called pre-collagen (Pearse, 1960; Bloom and Fawcett, 1962).

(a)(ii)(b)

Fibroblasts

These cells lie flat against the collagen fibres. In sections therefore they appear long and thin: the nucleus is usually at least four times as long as wide and measures about 6 microns long and 2 microns across, and it has no obvious nucleolus. The cytoplasm is also long, delicate, and difficult to see. In ordinary histological preparations no inclusions were seen except in a few cases of very old snails in which a few of the fibroblasts contained black granules very similar to those found in the pigment cells.

The collagen fibres and fibroblasts form a matrix (Fig. 2) in which three types of cell - amoebocytes, mucus gland cells and vesicular cells - as well as muscle fibres and nerves are distributed. Before describing these elements it may be noted here that the density or compactness of the connective tissue varies a great deal from one part of the snail to another. It is very loose

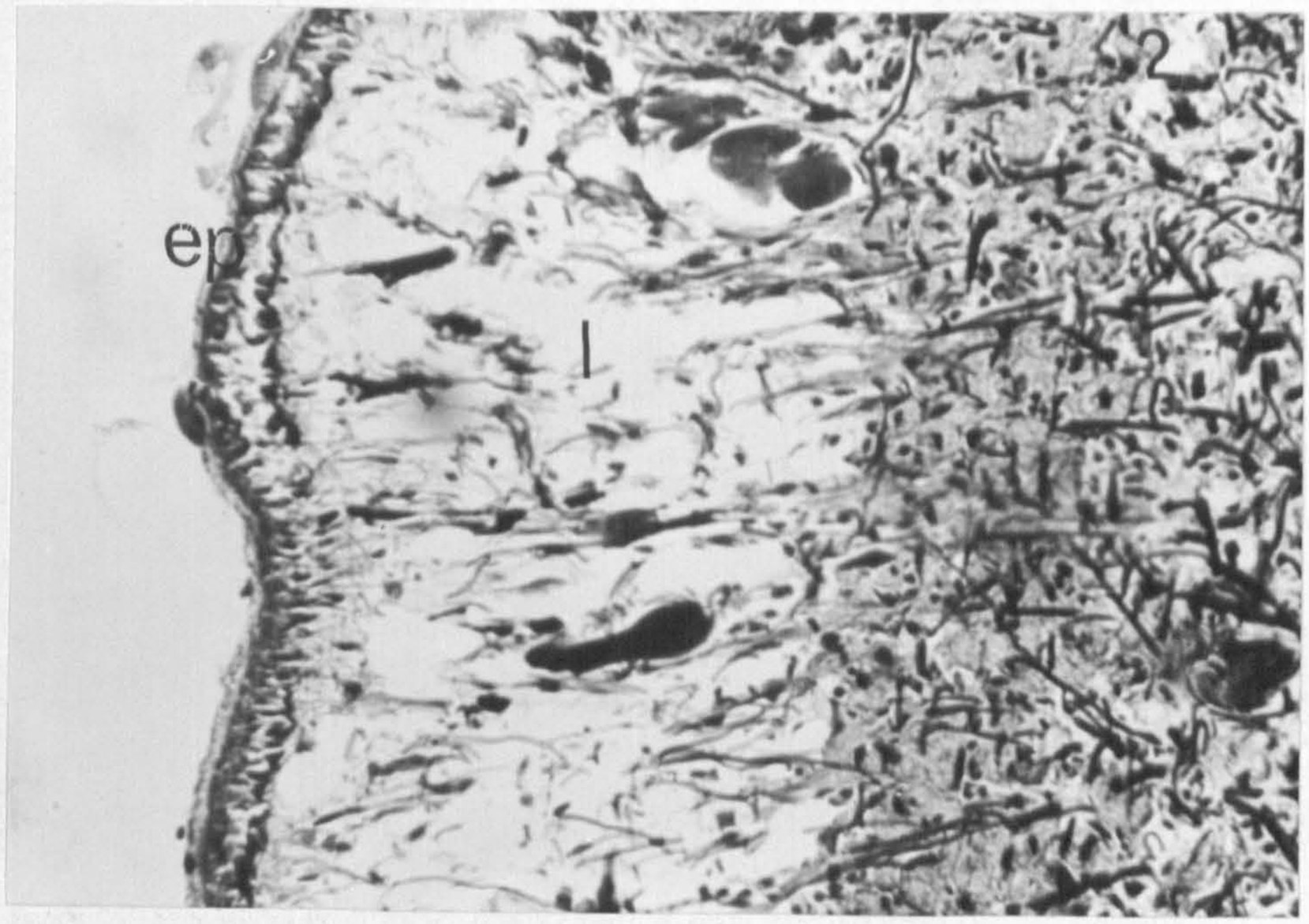


Fig. 1. Section of head-foot of Bulinus to show loose tissue (1) on ventral side and denser tissue (2) more dorsally. ep = epithelium. (Heidenhain). x 220.

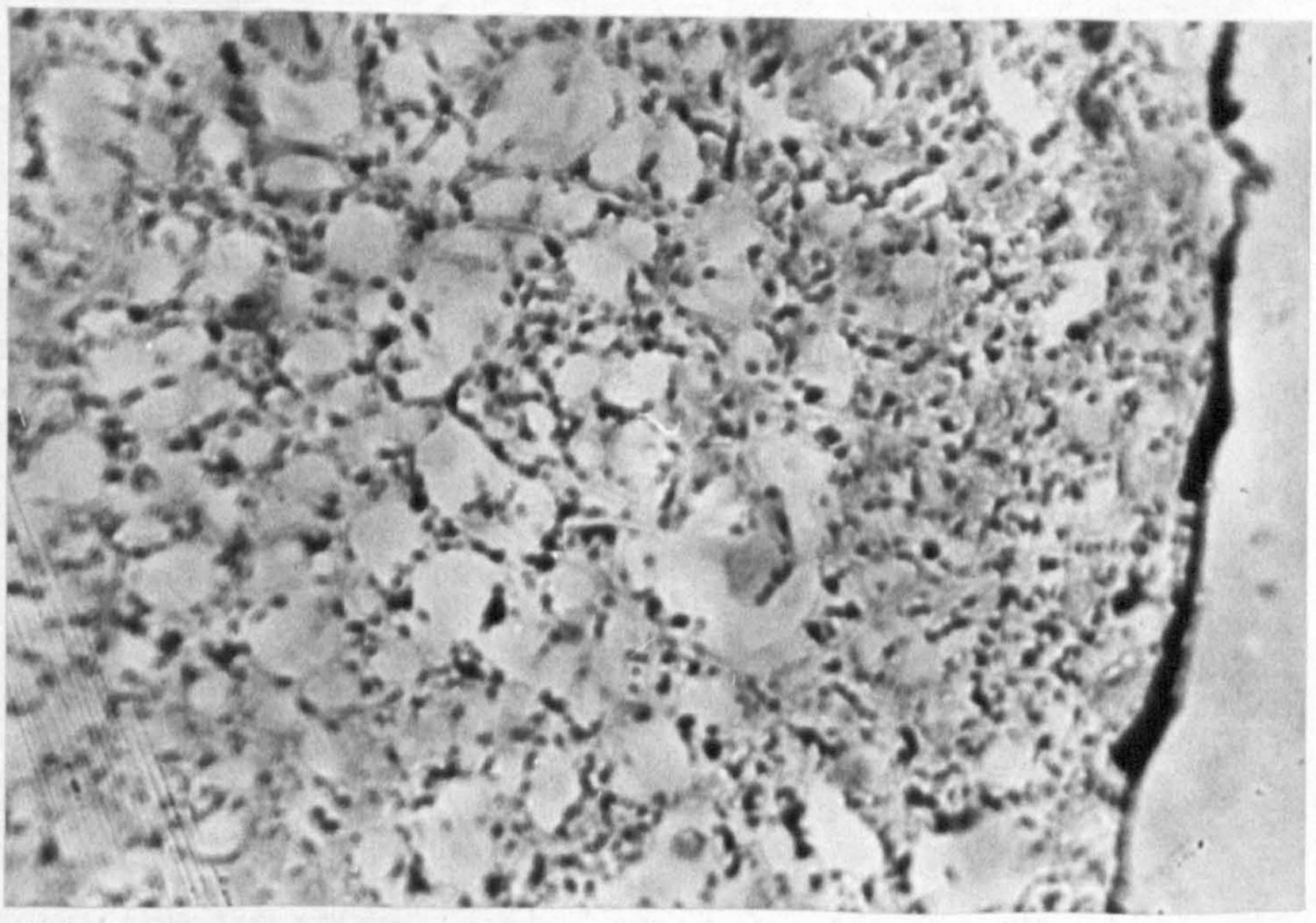


Fig. 2. Section of mantle of Bulinus to show matrix formed by connective tissue. Collagen fibres (in section) stained black. (Foot's silver method) about x 200.

(a)(iii) Mucus Cells

Two types of mucus secreting cells were found in the head-foot of Bulinus. Both types were considered mucus cells because they readily stained with alcian blue, which selectively stains acid mucopolysaccharides (Pearse, 1960).

In haematoxylin and eosin preparations the most prominent feature of the head-foot is the mass of large and very deeply basophilic mucus gland cells that are situated in the ventral half of the organ. The cells are distributed along most of the length of the head-foot, but the highest concentration is around the middle. These are probably proper goblet cells that have assumed a sub-epithelial position in order to allow the enlargement that is needed for the production of large quantities of mucus. Like other gastropods which move about a great deal (Fretter and Graham, 1962), Bulinus probably requires large quantities of mucus on its foot. The basophilic cytoplasm of these mucus cells tapers into a thin and long process which opens outside the epithelium of the sole of the head-foot (Fig. 3). The cells have large nuclei (about 8 by 6 microns) and prominent nucleoli that are characteristic of secretory cells.

The second kind of mucus cell was found on the dorsal side of the head-foot. These cells are much smaller

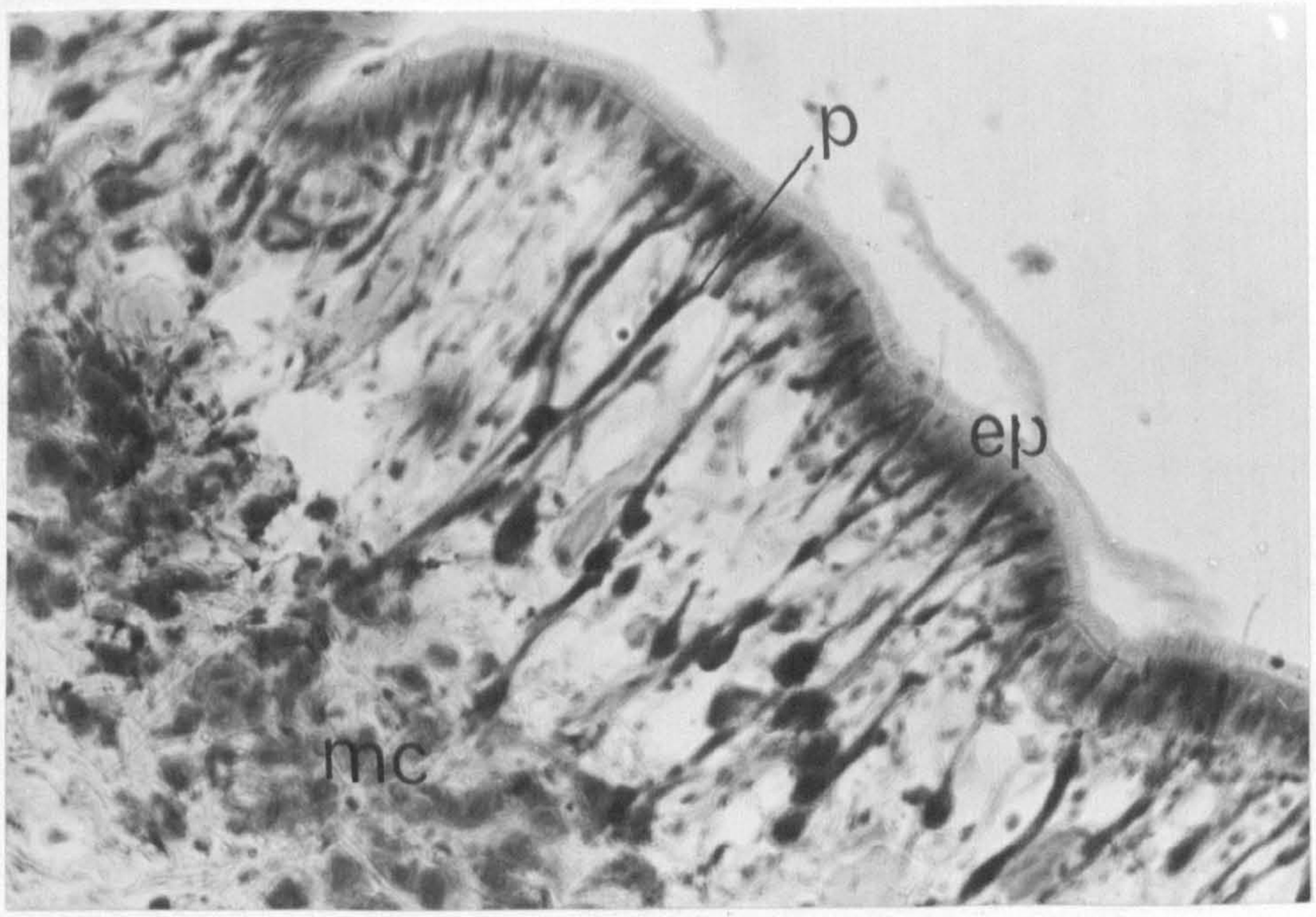


Fig. 3. Section of head-foot of Eulimus to show masses of mucus cells (mc) and their long processes (p) opening outside the ciliated epithelium (ep) of the sole. (H/E)

x 670.

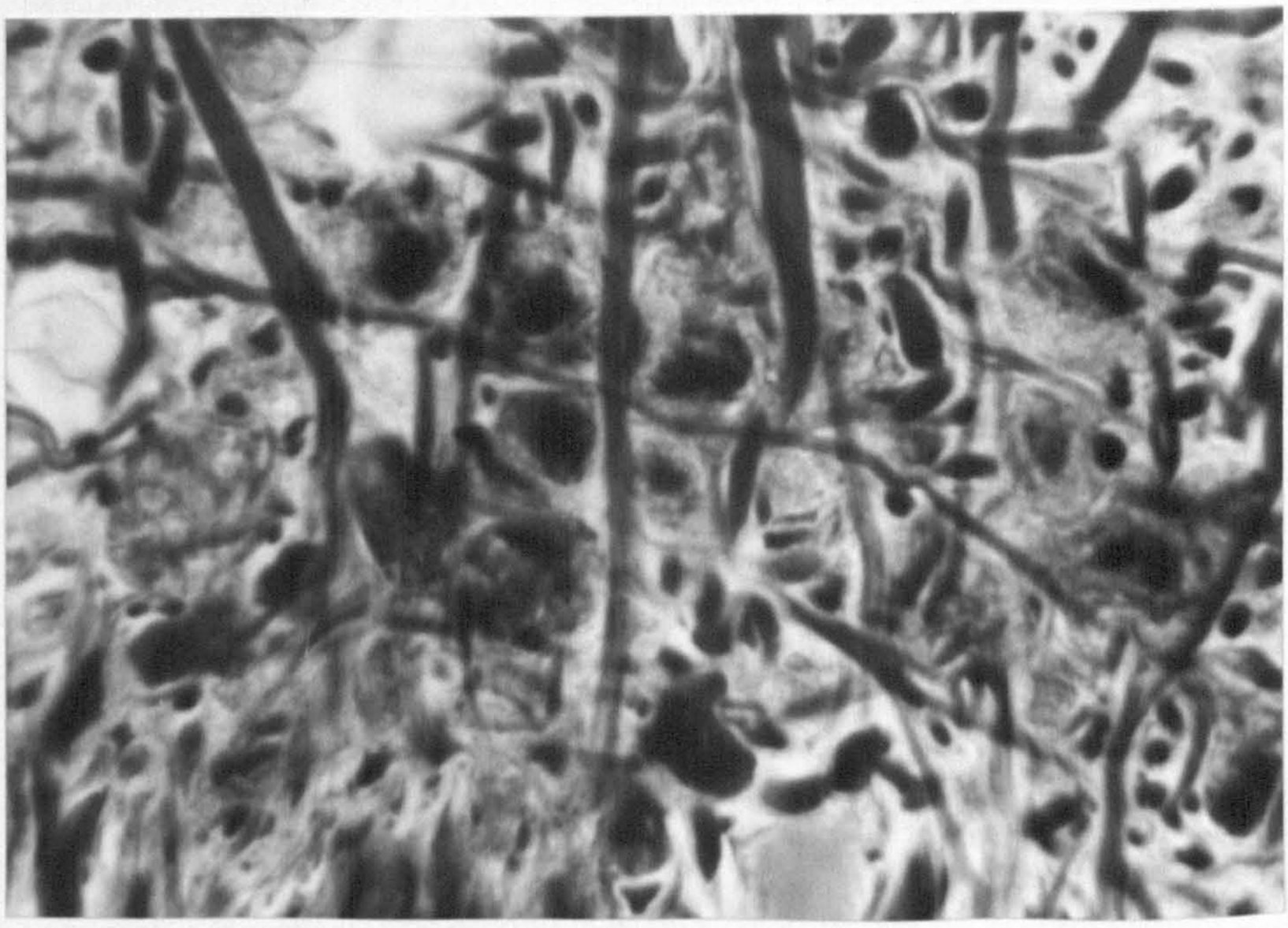


Fig. 4. Section of head-foot of Eulimus to show the three-dimensional arrangement of muscle fibres. Smaller black spots are transverse section of the fibres. (Heidenham) about x 200.

than those on the ventral side. They were found only a short distance below the epithelium - they do not extend deep into the substance of the head-foot - and they have only a short and wide process. In haematoxylin and eosin sections they stain much more lightly and the cytoplasm appears more vacuolated than in the other type of mucus cells. It is not possible to say whether the secretions of these two cell types differ qualitatively or only in the quantities produced, but both types produce one or more acid mucopolysaccharide.

In Chapter 4 it is shown that both B (P) africanus and B. truncatus rapidly pass into water a substance, or substances, that attract the miracidia of S. mattheei. Mucous secretions of the mucus cells are the most obvious substances that Bulinus snails pass into their surroundings. It seems likely, therefore, that these secretions contain attractants of the schistosome miracidia. Faust and Meleney (1924) suggested that the mucous secretions of Oncomelania nosophora were attractive to the miracidia of S. japonicum, but the importance of these secretions as attractants of schistosomes has not yet been investigated.

(a)(iv) Muscular System

The muscular tissue of the head-foot is arranged in a complicated way, but two distinct systems were

recognized. The first system comprises the fibres of the columellar muscle and the second consists of muscle fibres intrinsic to the head-foot.

The columellar muscle originates in the columella of the shell. On entering the head-foot the muscle divides into two large bundles: one runs towards the anterior and the other towards the posterior part of the organ. Each of the bundles fans out until fibres of the columellar muscle reach every part of the head-foot.

The fibres of the intrinsic system run singly and in all directions: in sections stained with Heidenhain's haematoxylin longitudinal, transverse and dorso-ventral fibres were readily recognized (Fig. 4). The fibres are arranged much more closely in the dorsal than in the ventral parts of the head-foot; a distribution which corresponds to the compactness of the connective tissue.

Only histologically smooth fibres were found in both the extrinsic and the intrinsic muscle systems. The thickness of the fibres of both systems vary, but the columella muscles are generally thicker than those of the intrinsic system. In haematoxylin and eosin preparations fibres of both systems are strongly eosinophilic; the nuclei are oval to elongate and vary in size with the fibres.

This arrangement of the muscular tissue is probably common to all gastropod molluscs which have a spirally coiled shell. In these molluscs the fibres of the columellar muscle are responsible for the retraction of the head-foot into the shell; contraction of the intrinsic fibres produces waves which travel down the sole of the head-foot and bring about crawling of the animals (Frotter and Graham, 1962). The arrangement of the muscle fibres is of interest because it was found that the density of the tissues of the head-foot was important in the survival of S. mattheei miracidia, which penetrate the head-foot (see Chapter 4).

(a)(v) Nervous System

The innervation of Bulinus was not studied in detail, but in haematoxylin and eosin preparations sections of nerves were readily identified. They stained reddish-blue or purple and their wavy appearance due to the neurofibrils is clearly seen under the high power of the microscope.

(a)(vi) Vesicular Cells

Pan (1958) described "vesicular" cells in A. glabratus. Similar cells were found in the head-foot of Bulinus. In the head-foot, these cells are confined

to the anterior third of the organ where large numbers of them are packed close together, especially in the lips.

The cells are mostly ovoid and their cytoplasm stains very lightly with haematoxylin. The cytoplasm has a very fine reticulate appearance. In a few of the cells there were large vacuoles, the cytoplasm forming a thin layer round the nucleus: in these cells fine cytoplasmic strands traversed the vacuoles to connect the cell membrane and the central collection of cytoplasm. A very distinctive characteristic of the vesicular cells is their thick cell membranes which stain with eosin. The cells have a relatively small nucleus with large and closely arranged chromatin granules. In routine histological preparations the structure of the nucleus was obscured by the deep-staining of the chromatin particles, but in very lightly stained sections a single, small and slightly eccentric nucleolus was seen.

It was found that the vesicular cells stained very lightly with alcian blue. They lack basophilia and the relatively small nucleus and nucleolus. These observations suggest that the cells are not secretory. Pan (1958) suggested that in A. glabratus these cells serve as stores for glycogen, but in Bulinus only small quantities

of the polysaccharide were found in these cells and this cannot be their main function. The distribution of the cells in the region of the lips, in the radular carrier and in the walls of the mouth to which the jaws are attached, lends some support to the suggestion that in the Pulmonata the vesicular cells perform a supportive function comparable to that of the chondrocytes of the vertebrate cartilage (see Carriker and Dilstad, 1946).

(a)(vii) Amoebocytes and Pigment Cells

These cells occur in the blood spaces of the head-foot of Bulinus. Their distribution, morphology and origin are discussed in the section on the mantle; their functions are considered in Chapter 4.

(b) The Tentacles

Histologically the tentacle resembles an evagination of the head-foot, but the organization of its tissues is specialized for tactile purposes. In the living snail the tentacles are in constant movement, feeling ahead as the animal moves about, which suggests that the tentacle is chiefly a tactile organ.

Figure 5 shows, in a longitudinal section, the general organization of the tentacle. For most of

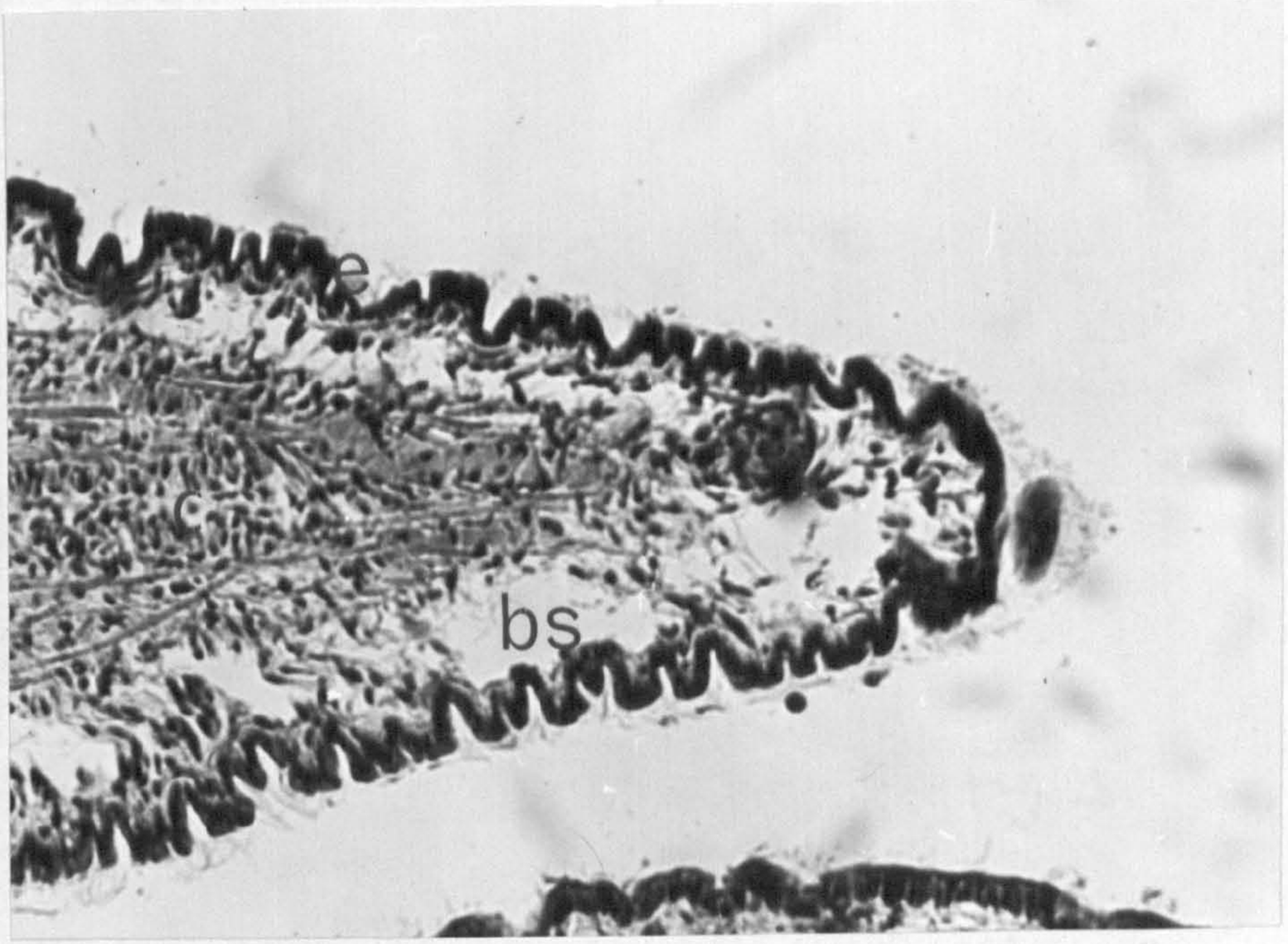


Fig. 5. Longitudinal section of tentacle of Dulinus to show central core (c), blood spaces (bs) and ciliated epithelium (e). (x 220) (H/E)

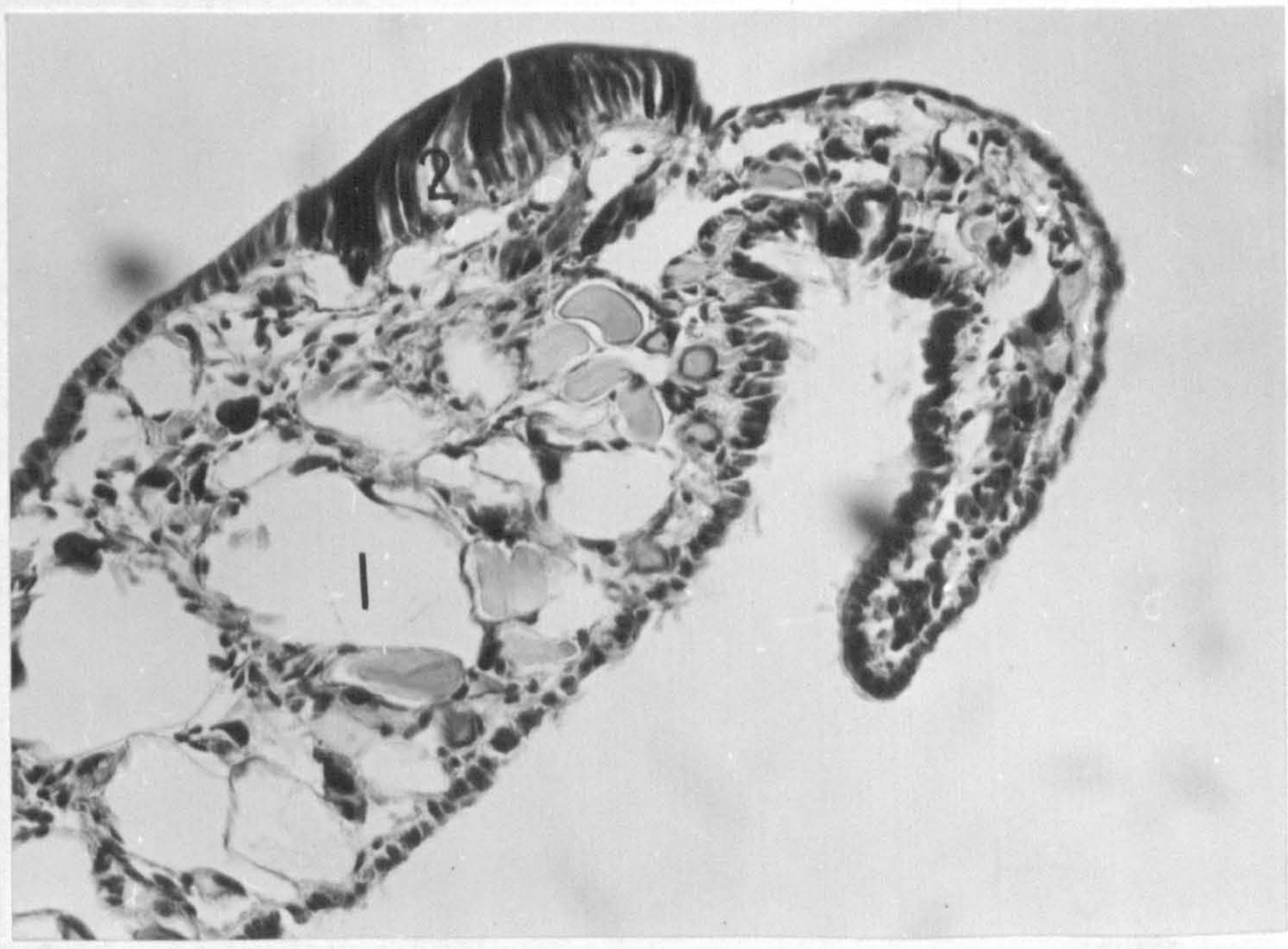


Fig. 6. Longitudinal section of mantle skirt of Dulinus to show general organization of its tissues. Note large blood spaces (1) and pseudostratified epithelium (2). (H/E)

x 220.

its length the tentacle looks rather like a cylinder whose walls are highly corrugated and inside which stands a core or rod. The corrugated skin consists of columnar cells which stand on a sheet of connective tissue and a few longitudinal muscle fibres. The epithelial cells carry long cilia that look thicker, but fewer than those of the sole of the foot. Frotter and Graham (1962) describe the tactile cilia of the cephalic tentacles of prosobranchus as immobile. But in Bulinus at least some of the cilia are not immobile because when tentacles of the animal were severed and observed under the microscope, ciliary movement was readily seen all over the surface of the appendages. The nuclei of the epithelial cells are ovoid or pear-shaped and lie with the narrow end towards the base of the cell.

The central portion or core of the tentacle consists of longitudinal muscle fibres and nerve trunks and the dense connective tissue which binds them together. A large nerve trunk enters the tentacle at the base and gives off branches to the periphery as it runs to the tip. The smooth muscle fibres run singly and are not organized into bundles. Scattered connective tissue fibres and fibroblasts connect the core to the skin across the large blood space, or sinus, that surrounds the core. The basal part of the tentacle is compact and muscular,

circular fibres being especially prominent.

Amoebocytes were found scattered throughout the connective tissue of the tentacles, especially towards the base where they were found in large masses. Small mucus cells occur in the subepithelial tissue at the base of the tentacles. They are the same type as those of the dorsal part of the head-foot.

Early workers on the development of Schistosoma in molluscs appear to have believed that the cephalic tentacles were the most important sites for the development of schistosomes. For example, Lutz (1919), working on A. glabratus and S. mansoni, reported that only miracidia which penetrated the tentacles survived and developed into sporocysts. Although it is now known that the head-foot proper is a far more important site, some schistosome species do develop in the tentacles, though not in preference to the head-foot (Gordon, Foaston & Davoy, 1934; Maldonado and Acosta-Matienzo, 1947; Olivier and Mao, 1949; Pan, 1965). The bulinid tentacle is probably not an important site for the development of S. mattheei. Although no parasites were ever seen in the tentacles of

B (P) africanus or B. truncatus in the work reported in Chapter 4 of this thesis its loose tissues and the large blood spaces must make it a very suitable habitat for the schistosome sporocysts when the miracidia penetrate into the tentacle.

(c) The Mantle

In the Gastropoda, the mantle serves several functions: it lays down and maintains the shell; the heart, main blood vessels and renal organ are situated in it; and in the pulmonates it serves as a lung. In this section the histology of the mantle and the organs situated in it is described briefly, attention being paid particularly to the distribution and possible origin of the amoebocytes in the tissues of the mantle. For convenience of description the mantle is divided into three parts: the mantle skirt, the roof of the mantle cavity and the tunica propria.

(c)(1) The Mantle Skirt

This is the part that lies anterior to the line of attachment of the mantle to the neck of the snail. When the snail is extended and moving about, the mantle skirt often lies outside the shell and it is for this reason that it is important as a site of penetration by schistosome miracidia.

In Bulinus the skirt has two parts, a thin anterior and a thicker and more fleshy posterior part. These two parts are separated by a shallow groove that runs parallel to the free edge of the skirt (Fig. 6).

The dorsal epithelium of the anterior part of the skirt consists of low cuboidal to almost flat cells which stand on a thin sheet of connective tissue. Immediately behind the groove the dorsal epithelium becomes a column of very tall cells which give the epithelium the appearance of stratification: in Australorbis this part of the epithelium has been described as pseudostratified (Pan, 1958). The cytoplasm of these tall columnar cells is deeply basophilic. It was found that the basophilia was due to ribonucleic acid (RNA) which stains very deeply with Pyronin Y. It is thought, therefore, that the pseudostratified epithelium may be involved in the synthesis of the protein matrix of the shell. The height of these cells decreases gradually posteriorly; the rest of the dorsal epithelium is cuboidal. As the snail grows older, the epithelial cells get heavily loaded with a black pigment, which completely obscures the structure of the cells. The origin of the pigment is not known, but it is likely that it is produced by the cells themselves because only fully loaded cells were

seen in all the snails examined; this suggests that the pigment appeared in the cytoplasm rapidly and was not the result of accumulation over a long period of time. The ventral epithelium of the skirt consists of columnar cells which are ciliated over the thinner, anterior portion.

As in the head-foot, the dorsal and ventral epithelia enclose a meshwork of connective tissue and muscle fibres. But the meshwork is very much looser, the muscle fibres much more scattered and the blood spaces much larger than in the head-foot. The muscle fibres are smooth and the connective tissue consists of collagen fibres and fibroblasts. The skirt is well supplied with nerves which, as in the head-foot, are easily recognized by their staining with haematoxylin and the wavy appearance due to the neurofibrils. Basophilic cells which stain with alcian blue are very common in this part of the mantle. These cells occur in a variety of sizes and differ in the intensity of their basophilia, but they all appeared to be subepithelial mucus cells, which open outside the ventral epithelium through vide processes. Vesicular cells, which are indistinguishable from those of the head-foot, occur singly not in masses as in the head-foot. Large numbers of amoebocytes and

some pigment cells occur in the mantle tissue. The amoebocytes and pigment cells are described in the following section.

(c)(ii) The Roof of the Mantle Cavity

In pulmonate snails the mantle cavity serves as an air sac and the roof of the cavity is the most important respiratory surface. In Bulinus an accessory gill, the pseudobranch, plays some role in gaseous exchanges, but the roof of the mantle cavity is still the main respiratory surface. The connective tissues of the roof are therefore very loose and have numerous large blood spaces and blood vessels. The heart and associated blood vessels, and the renal organ, are embedded in the roof. In addition, a lymphoid tissue has been found in this part of the mantle of Bulinus.

(c)(ii)(A)

Epithelium

As in the skirt, a dorsal and a ventral epithelium enclose the connective tissue in which the various histological elements and organs are embedded. The dorsal epithelium consists of low cuboided cells. This is continuous with the epithelium of the mantle skirt and

the cells contain very heavy deposits of the black pigment. Most of the cells of the ventral epithelium are also cuboidal, but there are specialized areas where other types of epithelial cells were found. In D. truncatus the roof has two folds or ridges which project into the mantle cavity: one along the rectum and the other along the renal organ. In D(P)africanus there is an additional ridge which lies between the renal organ and the rectum. All these ridges are projections of the connective tissue of the mantle. The sides of the ridges are covered by the normal epithelial cells which change into tall columnar cells with long cilia at the crests. Low cuboidal to flat cells form the epithelium over the area of the lymphoid tissue.

(c)(ii)(b)

Connective tissue, Muscle fibres & Nerve fibres

As in other parts of the mantle, the connective tissue comprises collagen fibres and fibroblasts. The tissue is very loose, having numerous large blood spaces. The muscle fibres were only found along the dorsal wall; they were all longitudinal. A few nerve fibres were found in this part of the mantle which appeared not to be as well supplied with nerves as the mantle skirt.

(c)(ii)(C)

Amoebocytes

Several unsuccessful attempts were made to get amoebocytes in smears of blood drawn from the heart of both B (P) africanus and B. truncatus. When small pieces of the mantle of these snails were cultured in the salt solution of Chernin (1963) by a hanging drop method, large numbers of amoebocytes migrated out of the tissue and were available for study. However, examination of the fresh cultures showed a much wider variation in size, shape and the inclusions of these cells than was the case in histological sections of the snails: it was concluded therefore that a study of amoebocytes grown in culture would not help in the identification of the types of amoebocyte present in Bulinus. For these reasons the description given here is based entirely on observations on amoebocytes in sections of B (P) africanus and B. truncatus.

In Bulinus only one type of amoebocyte was found. The cell has two constant characteristics (1) in the nucleus, coarse chromatin particles are arranged alllalong the nuclear membrane and unevenly but closely distributed throughout the nucleoplasm and (2) the cytoplasm is very thin, stains very lightly with both eosin

and haematoxylin and in ordinary light microscopy it contains no granules or other inclusions. The size and shape of the amoebocytes vary considerably, apparently depending on the amount of foreign material present in the tissues of the snail. In snails with no signs of bacterial infection the majority of the amoebocytes resemble the mammalian small lymphocyte. They have a relatively large, slightly ovoid or spherical nucleus, which may also be bean-shaped or deeply bilobed. The large diameter of such cells measures about 3 to 4 microns. In these cells the cytoplasm is hyaline and forms a thin rim, one or two microns across, round the nucleus. Amoebocytes with more cytoplasm, which was usually thrown into pseudopodia, were seen in healthy snails, but it is in snails with signs of bacterial infection that much larger cells occur. In such snails, amoebocytes measuring up to 13 microns and with nuclei up to 8 microns long are very common. Both the nucleus and cytoplasm of the larger cells are mostly elongate, but cells with bean-shaped or bilobed nuclei were common. The cytoplasm of these cells is usually thrown into pseudopodia, one large or several smaller pointed pseudopodia suggestive of active movement.

Figures 8 and 9 are electron photomicrographs showing the lymphocyte-like amoebocyte and a more elongate cell whose cytoplasm is thrown into a large and a smaller pseudopodium. The amoebocytes appear to have poorly developed mitochondria. The electron photomicrographs show a number of other inclusions in the cytoplasm, including crystalloid structures in the lymphocyte-like amoebocyte: similar crystalloid structures have been described in the eosinophilic polymorphonuclear leucocyte of the rabbit (Hirsch, 1965). However, the electron microscope observations are preliminary and the fine structure of the amoebocytes has not been adequately studied.

Amoebocytes were found in blood spaces in every part of Bulinus. However, they are not evenly distributed: in healthy snails the highest concentrations of the cells were in the connective tissue spaces of the mantle, with smaller numbers in the head-foot, tentacles and the intertubular connective tissue of the digestive gland. In the head-foot most of the cells are found in the loose connective tissue immediately inside the ciliated epithelium of the sole of the foot. In snails with evidence of bacterial infection, large masses of amoebocytes collect in the region of invasion, but this

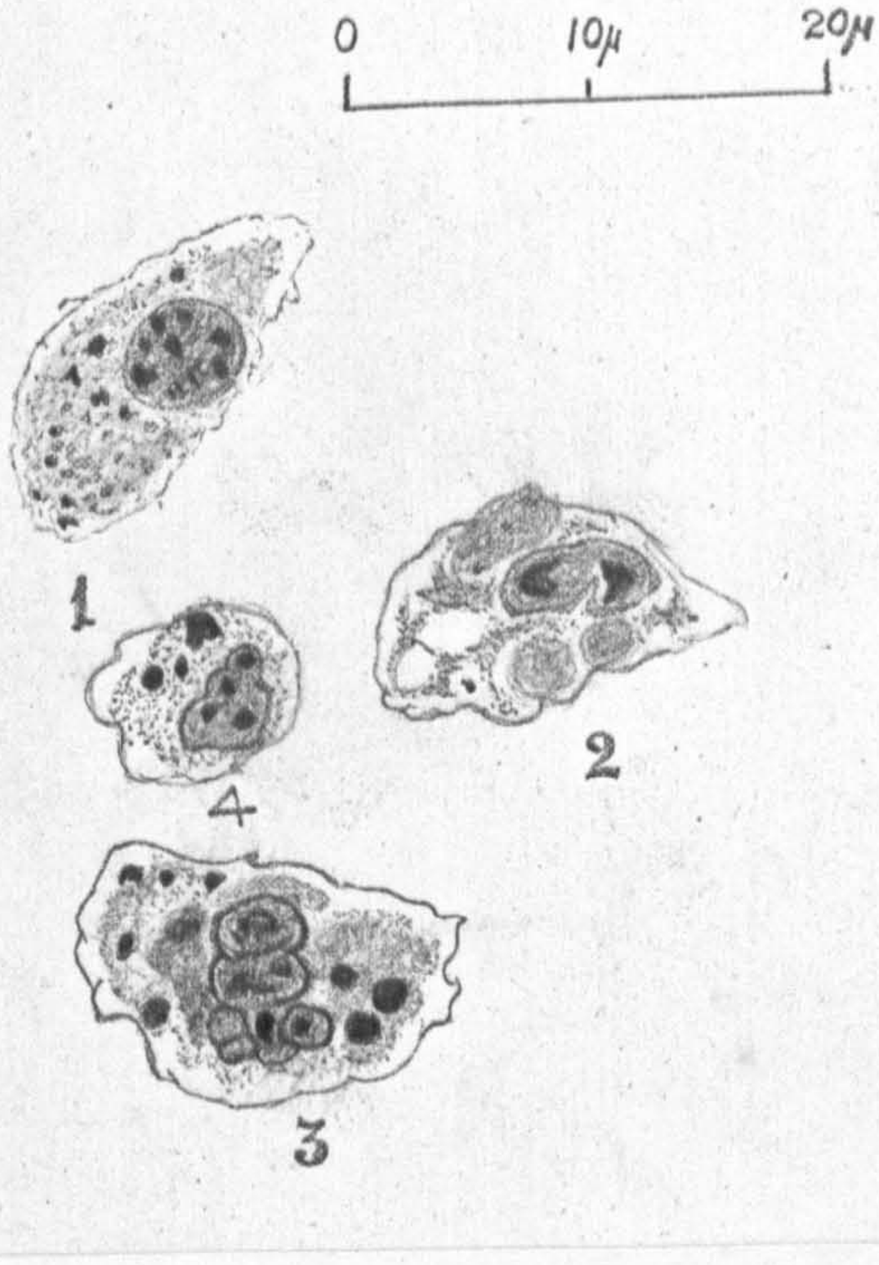


Fig. 7. Camera lucida drawings of pigment cells of B (P) africanus. Note vacuoles, diffuse pigment, pigment granules and different shapes of nucleus.

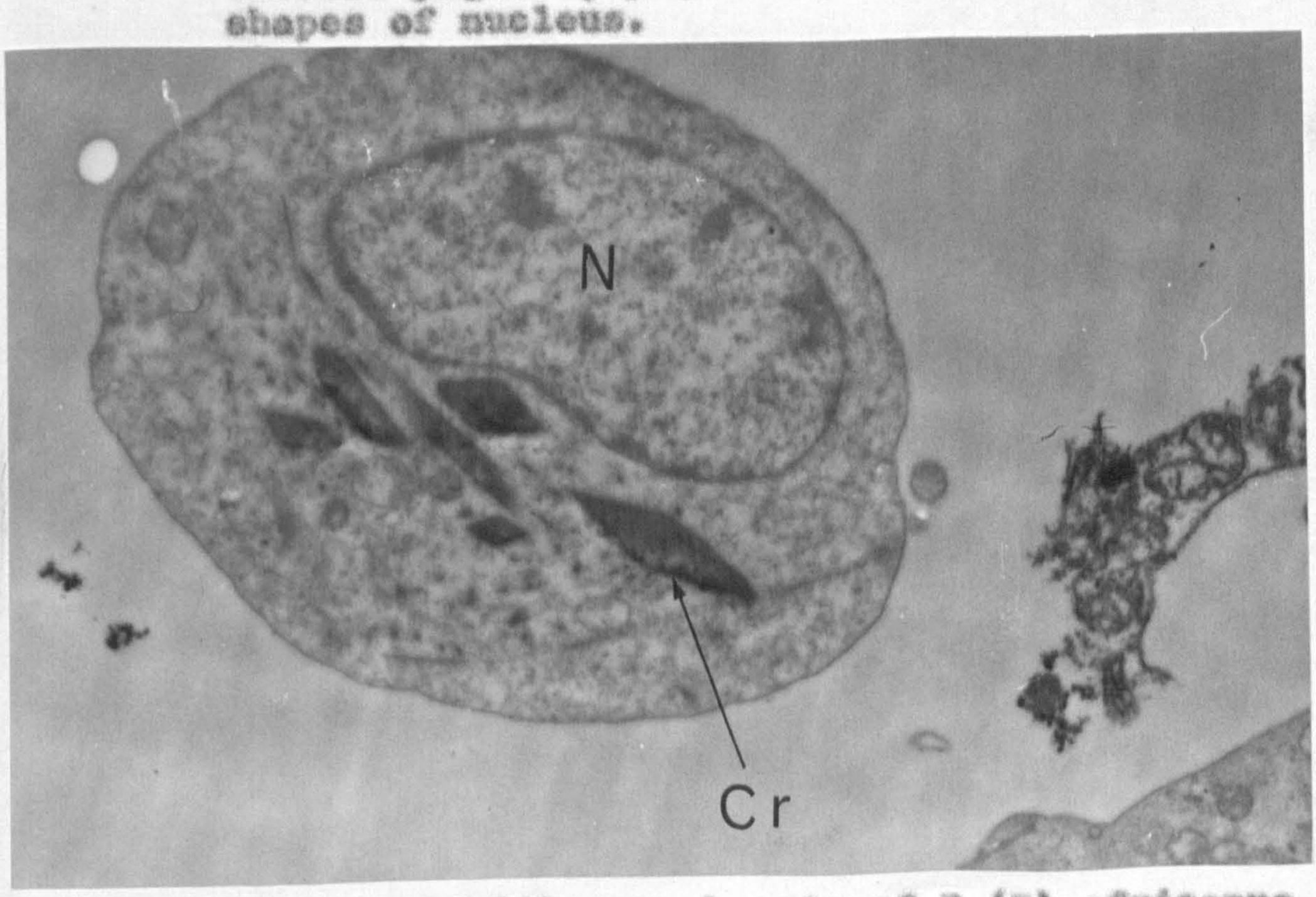
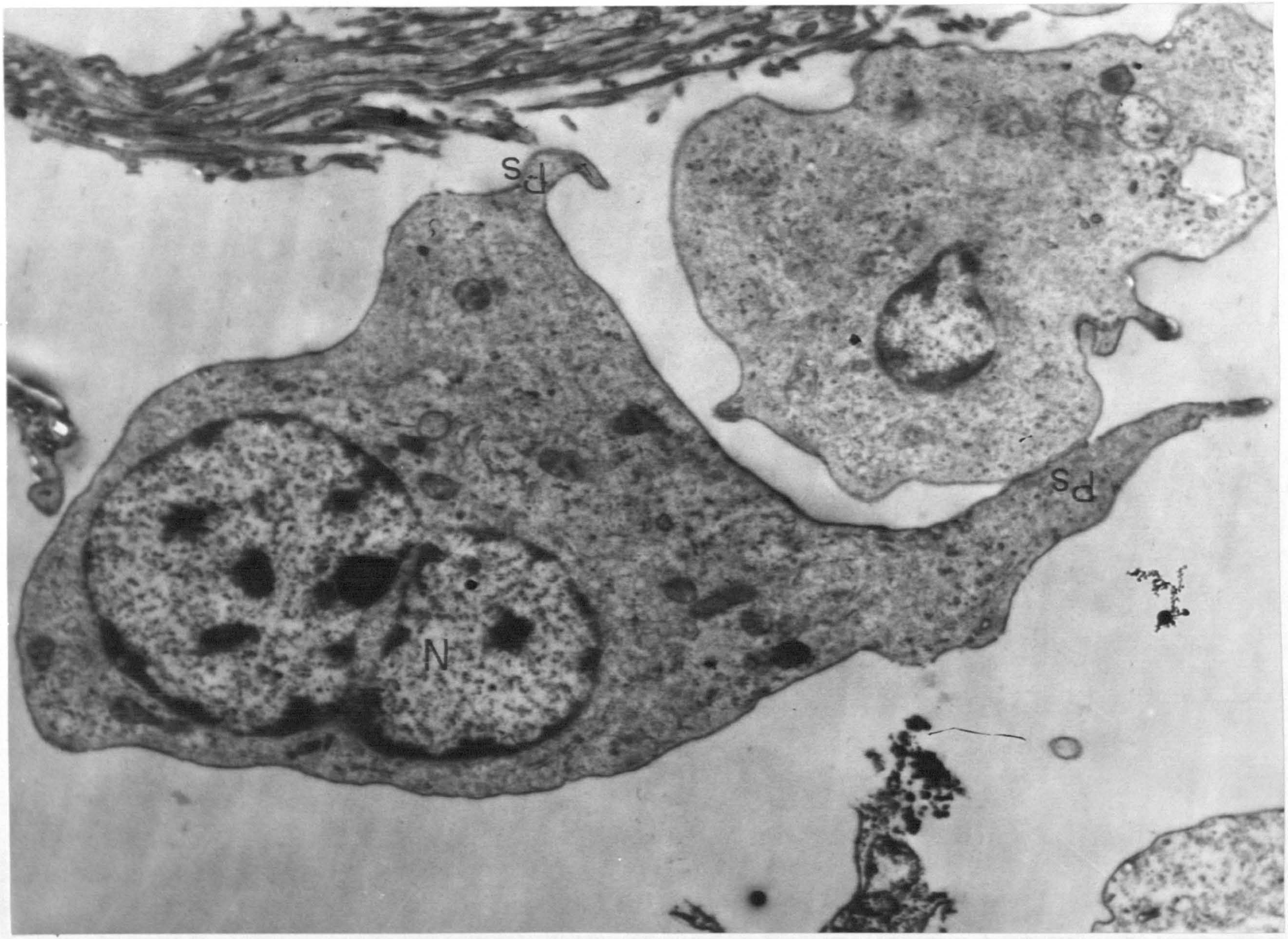


Fig. 8. Lymphocyte-like amoebocyte of B (P) africanus
N = nucleus; Cr = crystalloid structures (x 14,000)

Fig. 9. Extended amoebocyte of B (P) africanus
N = bilobed nucleus
Ps = pseudopodium.

(x 16,000)


PS
PS
N

does not appear to seriously affect the numbers of cells in the mantle or head-foot. In both normal and infected snails the amoebocytes are nearly uniformly distributed in the mantle, though large numbers of the cells occur on the walls of the saccular part of the kidney. In the head-foot they usually occur in masses in the loose connective tissue.

Many types of amoebocyte have been described in molluscs, but different workers used different criteria to distinguish the types and the whole subject is in some confusion (see Haughton, 1934; and Wagge, 1955, for reviews). In some molluscs clearly distinct kinds of amoebocytes have been described. For example, George and Ferguson (1950) found lymphoid cells, granular macrophages and eosinophilic granular amoebocytes in the blood of three species of prosobranch gastropods. These cells were in suspension in the blood of snails and differed in size, the presence and staining properties of granules in their cytoplasm and in their phagocytic activity. In other cases, however, size was the main criterion for the recognition of different types of amoebocytes (see Wagge, 1955). The reviews of Wagge and Haughton show also that many functions have been assigned to the amoebocytes of molluscs. These include defence against infection, digestion and transport of food substances,

excretion and the formation and repair of the shell. In some cases it has even been claimed that the molluscs were almost entirely dependent on their amoebocytes for the supply of digestive enzymes and the transport of nutrients through the tissues.

It has been pointed out above that amoebocytes of Bulinus vary considerably in shape and size both in the same individual and between different individuals. The variation in shape is very gradual and the variation in the shape is of the kind that would be expected in cells that are capable of active amoeboid movement. For these reasons I consider that the amoebocytes of Bulinus are of only one kind which varies in size and shape according to the needs of a snail to be rid of foreign material. Fan (1958) arrived at a similar conclusion about the amoebocytes of A. glabratus.

The amoebocytes of Bulinus appear to originate in the lymphoid tissue or nodo which is described below. The role of these cells in the defences of the snail against schistosome infections is considered in Chapter 4.

(c)(ii)(D)

Pigment Cells

The most constant feature of these cells was the presence of dirty yellow to dark brown inclusions in their cytoplasm. The amount of the pigment present varied from cell to cell: in some cells only a few small particles were seen as cytoplasmic vacuoles, while other cells were very heavily loaded with pigment. The cells vary considerably in size and shape. Pigment cells measuring from 8 to 20 microns and whose diameter varied from 5 to 10 microns were often found in the same specimen of Bulinus. The kind of variation that was commonly seen is illustrated in Figure 7b. But apart from the generally larger size, the presence of pigment and a little more basophilic cytoplasm, these cells resemble amoebocytes closely: the cytoplasm is often irregularly spherical, or it may be drawn out to form one large pseudopodium or several smaller ones. The nucleus is often spherical, bilobed, or ovoid in the smaller cells and irregularly shaped, ovoid, or bean-shaped in the larger ones. Further, the arrangement of chromatin particles in the nuclei of these cells is the same as that of amoebocytes.

A few pigment cells were found in the connective tissue all over the body of the snail, but the highest

concentrations of the cells were in the pseudobranch, the mantle and the rectal ridge area. In snails with evidence of bacterial infection, vast numbers of pigment cells occur in these regions, sometimes obscuring all other tissue over large areas. In infected snails increase in the numbers of amoebocytes was accompanied by a similar increase in the number of pigment cells.

Thus, in Bulinus, there is a close similarity between amoebocytes and pigment cells in their morphology and behaviour in infected snails. It appears therefore that the pigment cells are, in fact, amoebocytes containing phagocytosed material that is either indigestible, or in the process of digestion. In other molluscs the relationship between pigment and other blood cells has not been established. For example, workers on the bacterial diseases of oysters apparently use the terms amoebocyte and pigment cell interchangeably (see Tripp, 1963, for references), but on the other hand Pan (1953) regarded the amoebocytes and pigment cells of A. glabratus as different types of cells. Tripp (1961) suggested that in infected A. glabratus the amoebocytes phagocytosed bacteria and then carried them to outer epithelia whence the pathogens were eliminated to the external environment. In Bulinus the pseudobranch, parts of the mantle and the

rectal ridge probably serve as "dumping" areas where amoebocytes carrying the remains of bacteria and other matter removed from the snail tissues accumulate as pigment cells.

(c)(ii)(E)

Lymphoid Tissue

In Bulinus a lymphoid area has been found in the roof part of the mantle. Under the low power of the microscope a section of this area looks strikingly similar to the primary lymphoid nodule of mammals. It is an ovoid or kidney-shaped area which is basophilic, with large blood spaces in the middle and round the periphery (Fig. 11). It is situated along the border of the pericardial cavity, on the far side of the kidney: in transverse sections of the mantle the pericardial cavity and the heart lie between the lymphoid nodule and the saccular part of the kidney (Fig. 10). In some specimens of Bulinus, however, the nodules were very large and extended to the walls of the kidney, while in a few they encircled most of the pericardial cavity. In sections of young adult B (P) africanus (with a shell height of about 2 cm.) the lymphoid nodule measures about 300 x 180 microns; and from serial sections of the mantles of young adult snails its length was estimated at 300 to 500 microns.

Three types of nucleus were seen in the lymphoid tissue: nuclei of the same size and with the chromatin arrangement of ordinary amoebocytes and fibroblasts; the third nuclei are much larger, spherical or irregularly shaped and with fewer chromatin granules both along the nuclear membrane and in the nucleoplasm. The nuclei of these larger cells measure about 7 microns in diameter. Amoebocytes occur in the central and peripheral blood spaces (Figs 11)12)

But no germinal centre could be identified with certainty. In a few cases the chromatin material of all the cells of a part of the lymphoid tissue appeared to have been aggregated into lumps c. suggestive of mitotic division. Such areas were found side by side with normal looking areas, in the same section of the lymphoid tissue. This suggests that the "lumping" was not an artefact. However, no figures that could be definitely identified as mitotic were seen in ordinary histological sections, or in serial sections of a few mantles of B (P) africanus, which had been specially prepared for chromosomes. These mantles had been fixed in Sanfelices fluid and the sections were stained with iron haematoxylin. In the areas where fibroblasts, amoebocytes and the large cell with a very open nucleus were found there were also nuclei which gave the impression of undergoing binary fission. It is

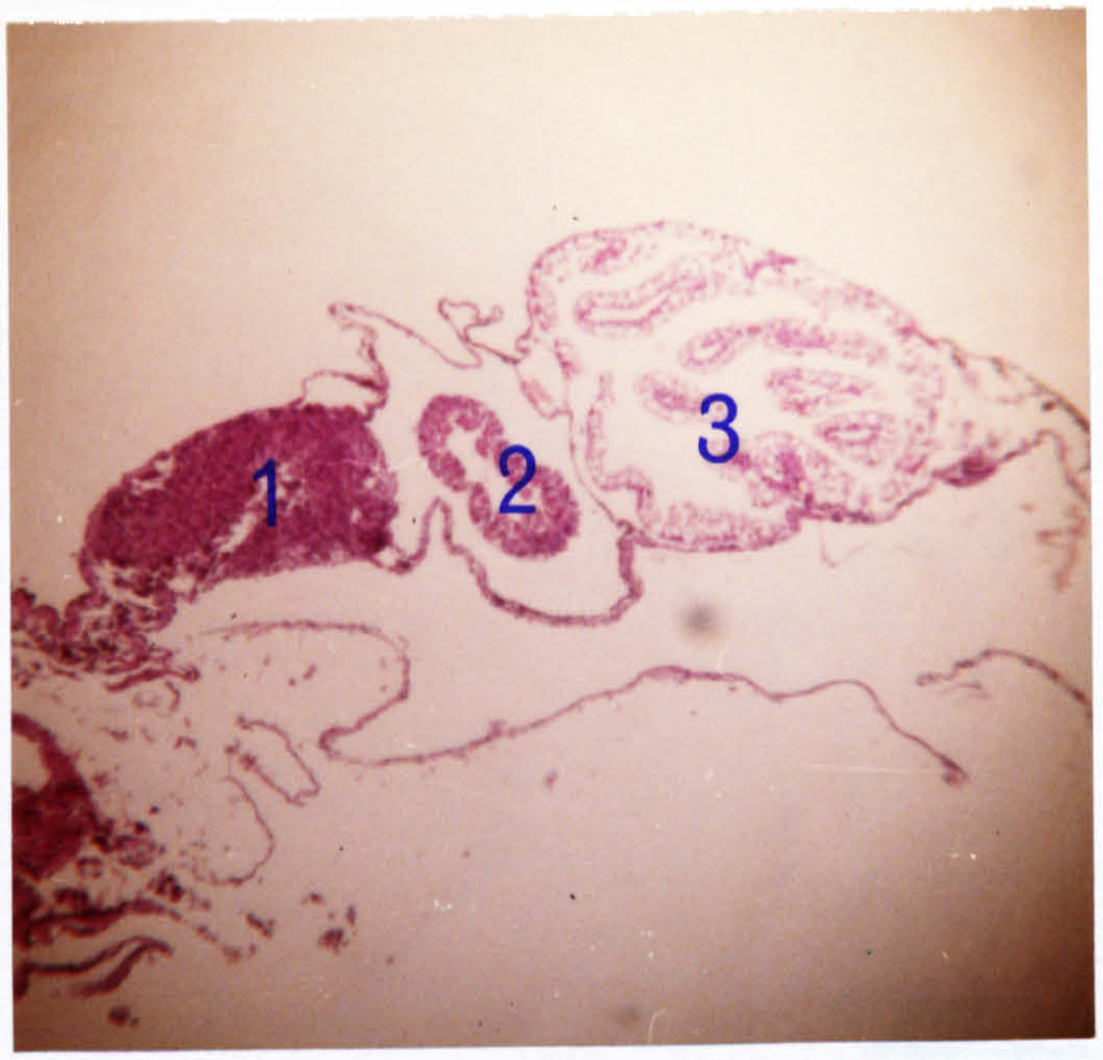


Fig. 10. Transverse section of mantle of Bulinus to show relation of lymphoid (1) to heart (2) and saccular part of kidney (3) (H/E)

^ tissue

Fig. 11. Section of lymphoid tissue to show the lightly basophilic stroma (s), and the central blood spaces (a), and peripheral blood spaces (b) with amoebocytes (am)

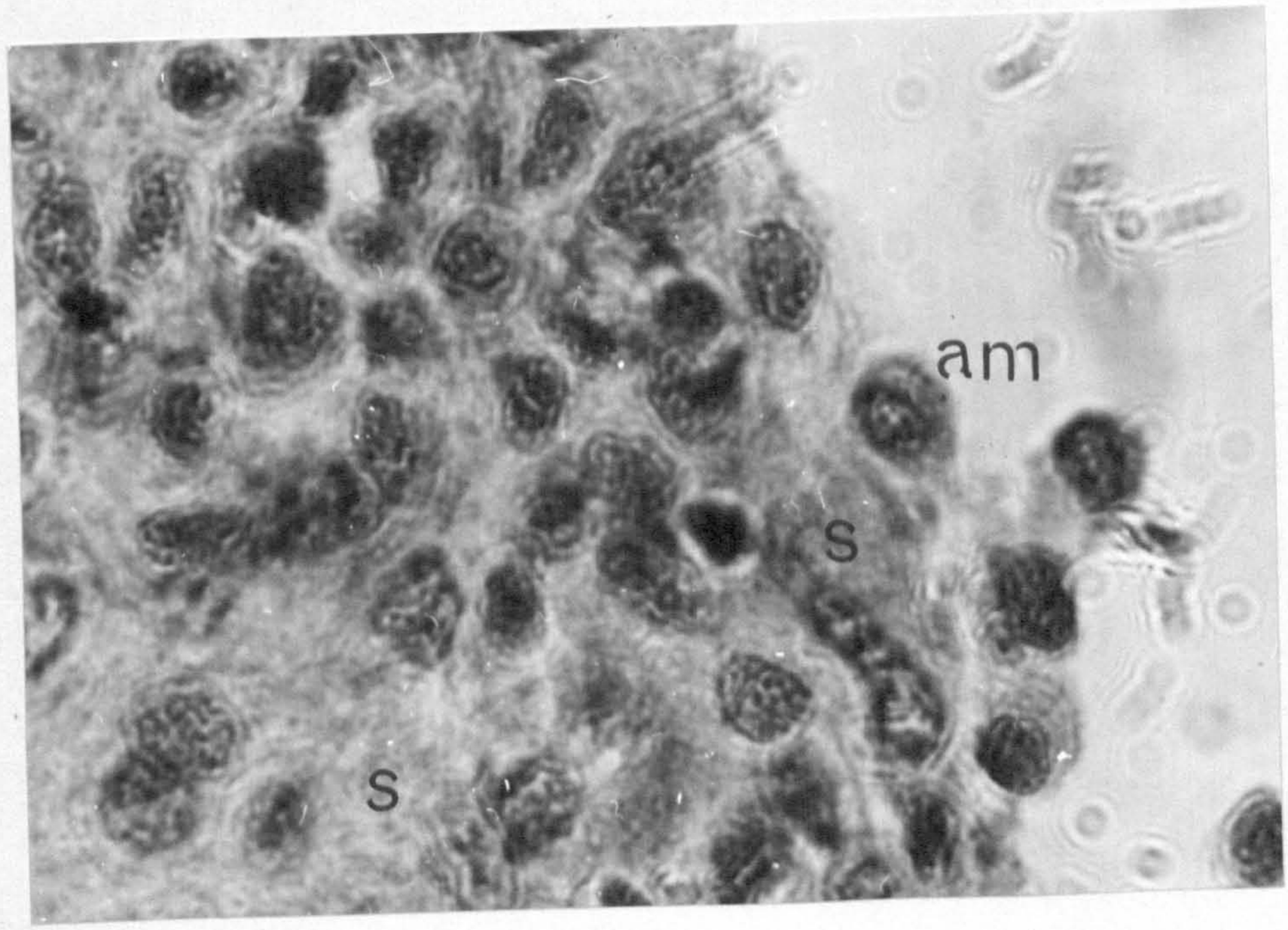


Fig. 12. Higher magnification (x1620) of an edge of lymphoid tissue to show the open nature of nuclei of the stroma(s) and amoebocytes (am) in the peripheral blood space.

therefore difficult to form a definite picture of what happens in the lymphoid tissue. A possible sequence of events is that fibroblasts enlarge, their cytoplasm becoming more basophilic, chromatin granules become disorganized and then re-organized to give a much larger nucleus, which then divides to give rise to two amoebocytes. The young amoebocytes then move into the blood spaces in the centre and especially in the periphery of the lymphoid nodule, whence they migrate to other parts of the body.

Although in the Mollusca amoebocytes are generally believed to come from the mantle (Baggo, 1955), a specialized tissue for their production has been reported in only one mollusc before. Pan (1958) found a lymphoid area in the mantle of A. glabratus, but in this snail amoebocytes are apparently produced in other parts of the snail especially on the walls of the kidney and of blood vessels as well. The origin of the blood cells of the Invertebrata has been very little studied and lymphoid tissues have only been reported in a few insects and other invertebrates (Haughton, 1934; Wigglesworth, 1959). In Bulinus no evidence was found to indicate that amoebocytes were produced in tissues other than the lymphoid area and it is probable therefore that the snail depends entirely on this tissue for its defences against parasitic and other infections.

(c)(ii)(f)

The Heart

This consists of a short, muscular ventricle and a long, tubular auricle. The walls of the ventricle consist of large muscle fibres which anastomose in all directions to form a three dimensional network. These muscle fibres have large nuclei and contain large deposits of glycogen. They appeared granular, not smooth or striated; this is apparently true of the cardiac muscles of most molluscs (Krijgsman and Bavaria, 1955).

The innervation of the heart of Bulinus was not studied with the special methods that are often necessary in work on nervous tissue, but in ordinary histological preparations no evidence of ganglia or any other nervous tissue was found in the complete serial sections of several hearts. In their review of work on the contractile and pacemaker mechanisms of the heart of molluscs, Krijgsman and Bavaria (1955) showed that there is considerable controversy over the nature of the pace-maker; they supported the view that the conduction of the heart beat is myogenic, not neurogenic. The observations on the heart of Bulinus lend some support to this view.

The chambers of the heart do not have an epithelial lining (endocardium); the ventricle has an epicardium of low cuboidal cells, but the auricle has not got one.

(c)(ii)(G)

The Renal Organ

The renal organ or kidney is a club-shaped tube which runs right across the mantle cavity, with the club end situated at the posterior border of the roof of the cavity. In other molluscs the club part of the kidney is generally called "saccular" and the longer and thinner part as "tubular" portion. The posterior end of the saccular part of the kidney lies on the left side of the pericardium from which it is separated by a thin membrane of connective tissue.

The kidney appears simply as a tube whose epithelial lining is thrown into numerous folds. The folds have a three-dimensional arrangement and completely fill the lumen of the tube, thus apparently forming an efficient filtration bed. Although the epithelial lining is continuous throughout the organ it is divided into two histologically distinct regions: the epitheliun of the saccular part is different from that of the tubular part of the kidney. The saccular epitheliun has large and cuboidal to low columnar cells, which stain very lightly with eosin. They have large nuclei and distinct nucleoli. Each of these cells has a single vacuole in the distal part of the cytoplasm. In the older snails the vacuoles were filled with large, spherical yellow

concretions which were refractile and did not take ordinary stains. Tests with an hexamine-silver mixture showed that the concretions were uric acid or some urate, probably sodium urate, the form in which uric acid usually occurs in animal tissues (Pearse, 1960). The deposits were found to be readily soluble in 1% lithium carbonate; this showed that they were a urate and not a phosphate or carbonate.

The cells of the epitholium of the tubular part of the kidney are much smaller; they are low cuboidal cells. The cytoplasm is basophilic and has a wavy appearance, the wavy linesrunning from the basal to the distal end of the cell. In most of the snails studied these cells did not contain the deposits found in the saccular epitholium, but in very old snails the deposits spread into the posterior part of the tubular epitholium.

It was found that some of the folds of the epitholium are modified so as to form a ciliated canal which runs down one side of the saccular part of the kidney. This fact, and the presence of deposits of uric acid, or its derivations, suggest that as fluid is passed down the tube by ciliary action, nitrogenous waste products are extracted from it by the epithelial cells of the saccular part. No studies on the excretory physiology of Bulinus or planorbids have been published; it is not possible to discuss the physiology of the bulinid kidney, therefore.

(c)(iii) Tunica propria

This is a very thin skin that covers the visceral hump, separating the viscera from the shell. In section it was found to consist of flat epithelial cells and a few strands of collagen fibres and the associated fibroblasts.

(d) The Digestive Gland

A section of the digestive gland of Bulinus superficially resembles that of the mammalian pancreas which consists of acini separated from each other by connective tissue. However, in the snail each functional unit, the tubule, consists of more than one type of cell. Three cell types were identified in the epithelium of each tubule: these are the digestive, secretory and goblet cells. Collagen fibres and fibroblasts constitute the intertubular connective tissue which is very loose and has numerous blood spaces in which amoebocytes occur. No muscle fibres or other cell types were found in the connective tissue.

The general arrangement of the cells of the tubule is shown in Figures 13 and 14. In haematoxylin and eosin preparations the most numerous cells, the digestive cells, stain very lightly with eosin. In transverse sections, the corners of the tubule are

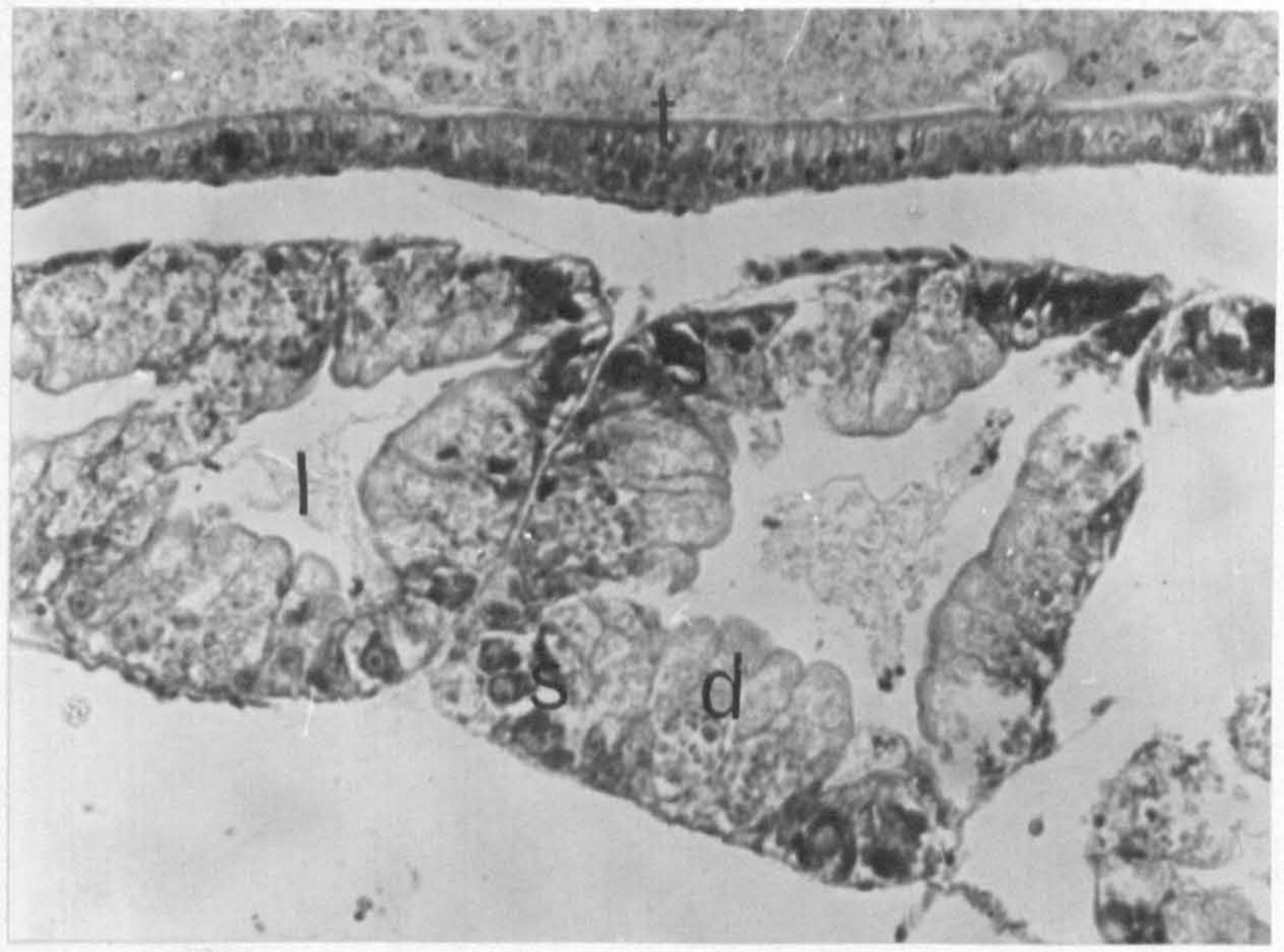


Fig. 13. Transverse section of 2 tubules of the digestive gland of Bulinus. Note secretory cells (s) at the corners. l = lumen; t = intestine. x 220

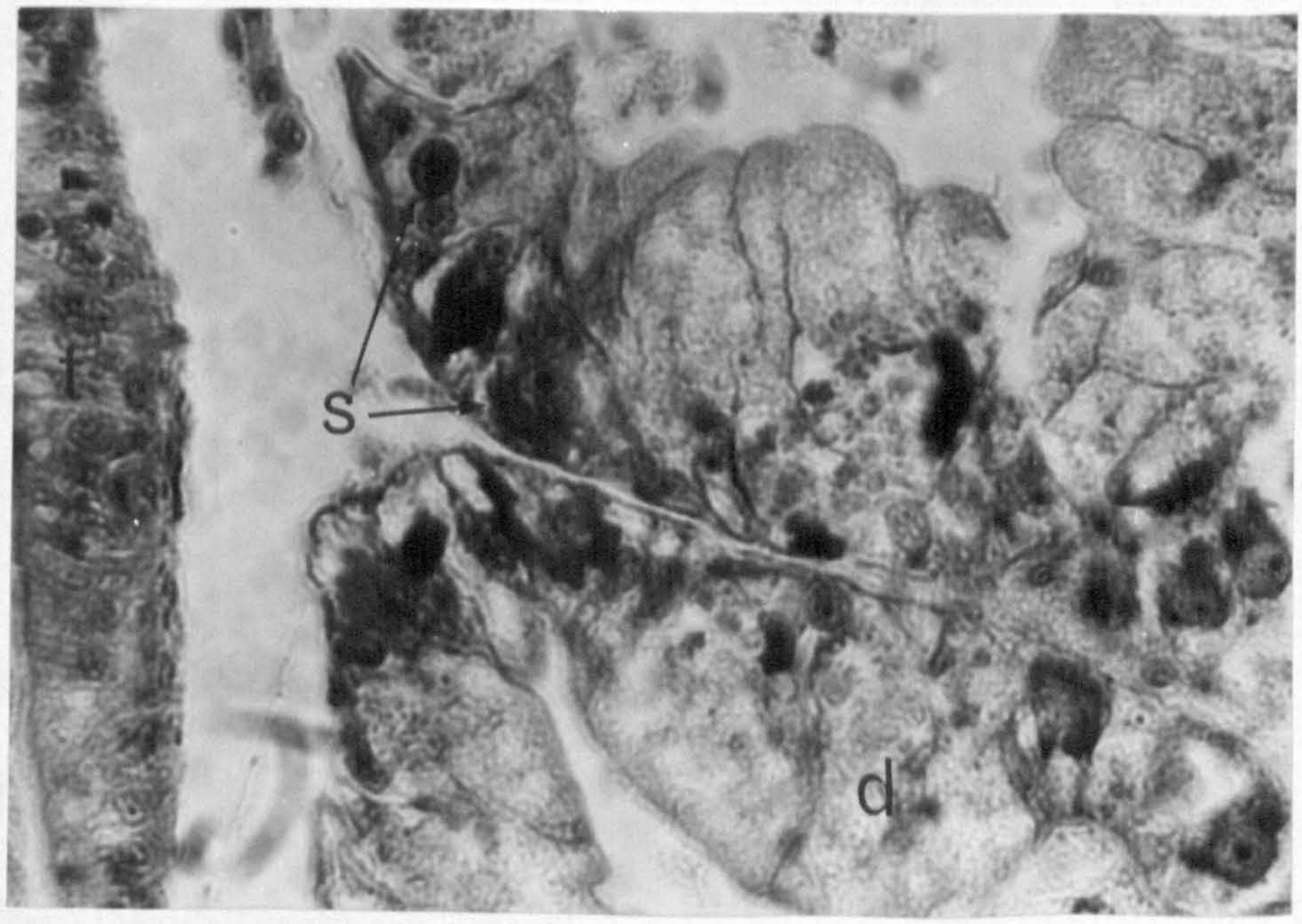


Fig. 14. Higher magnification (x 670) of part of the 2 tubules to show groups of secretory cells (s) and digestive cells (d)

occupied by groups of deeply basophilic cells, the secretory cells, but in sections cut in other planes the angular location of these cells is not obvious. The goblet cells have an irregular distribution in the tubules and it is difficult to distinguish them from the secretory cells because of their strong basophilia. The cytology of each of these cell types is described below. Results of histochemical tests made on the digestive gland are summarized in Table 1.

(d)(i) <u>The Digestive Cells</u>

These constitute most of the digestive gland. They are generally tall columnar cells which appear tightly packed, with the free distal end broader than the base. The nuclei are elongate (about 8 microns long and 3 microns broad) and basal. The nucleoli are quite distinct, but much smaller than those of the secretory cells.

A very distinctive feature of the digestive cells is the extensive vacuolation of their cytoplasm. The amount of vacuolation varies considerably from one specimen to another, and from one part of the same digestive gland to another. Cells with the least vacuolation are thin and tall, about 40 to 50 microns high

TABLE 1

Results of Histochemical Tests on the Digestive Gland of Bulinus

Test for	Test	Digestive cell	Secretory cell	Goblet cell
Calcium	Alizarin red S	-	-	-
Calcium	Nuclear Fast Red	-	-	-
Calcium	Ammoniacal haema-toxylin	-	-	-
Mucin	Alcian blue	-	-	++
Haemoglobin	Benzidine	-	-	-
RNA	Methylene blue-pyronin	+	+++	
Esterases	Alpha-naphthyl acetate	+	-	-
Esterases	Indoxyl acetate	+	-	-
Alkaline phosphatase	Cobalt (Gomori)	+	-	
Glycogen	Best's carmine	+	+++	

* Number of + signs indicates the relative intensity of a reaction; - means negative results; blank means the reaction was not studied.

and with a fairly uniform broadth of about 7 microns. Numerous small vacuoles which do not have any contents, occur in the central region of the cell. In those cells the elongate nuclei are situated in the basal third with their long axis parallel to that of the cell. These are probably young cells. In the next stage the vacuoles are larger and occupy most of the cell leaving a thin layer of cytoplasm in the distal end and all round the margins of the cell. The distal cytoplasm has small vacuoles (Fig. 15) which contain greenish yellow flakes while the central vacuoles contain yellowish, refractile granules and flakes of various sizes, but those are generally smaller than those in the apical cytoplasm. In a more advanced stage the central vacuoles coalesce to form one large vacuole which occupies the entire cell, except for a thin layer of cytoplasm at the distal and basal ends; the sides of the vacuole are lined by an extremely thin layer of cytoplasm. The central vacuole appears to press the nucleus to the bottom of the cell where it often becomes deformed, taking a crescent shape with the concave surface lining the lower limit of the vacuole. The vacuole contains masses of refractile granules and flakes of varying shapes and sizes. In the final stage of vacuolation the distal end of the cell breaks down and the contents of the vacuole pass into the lumen of the tubule.

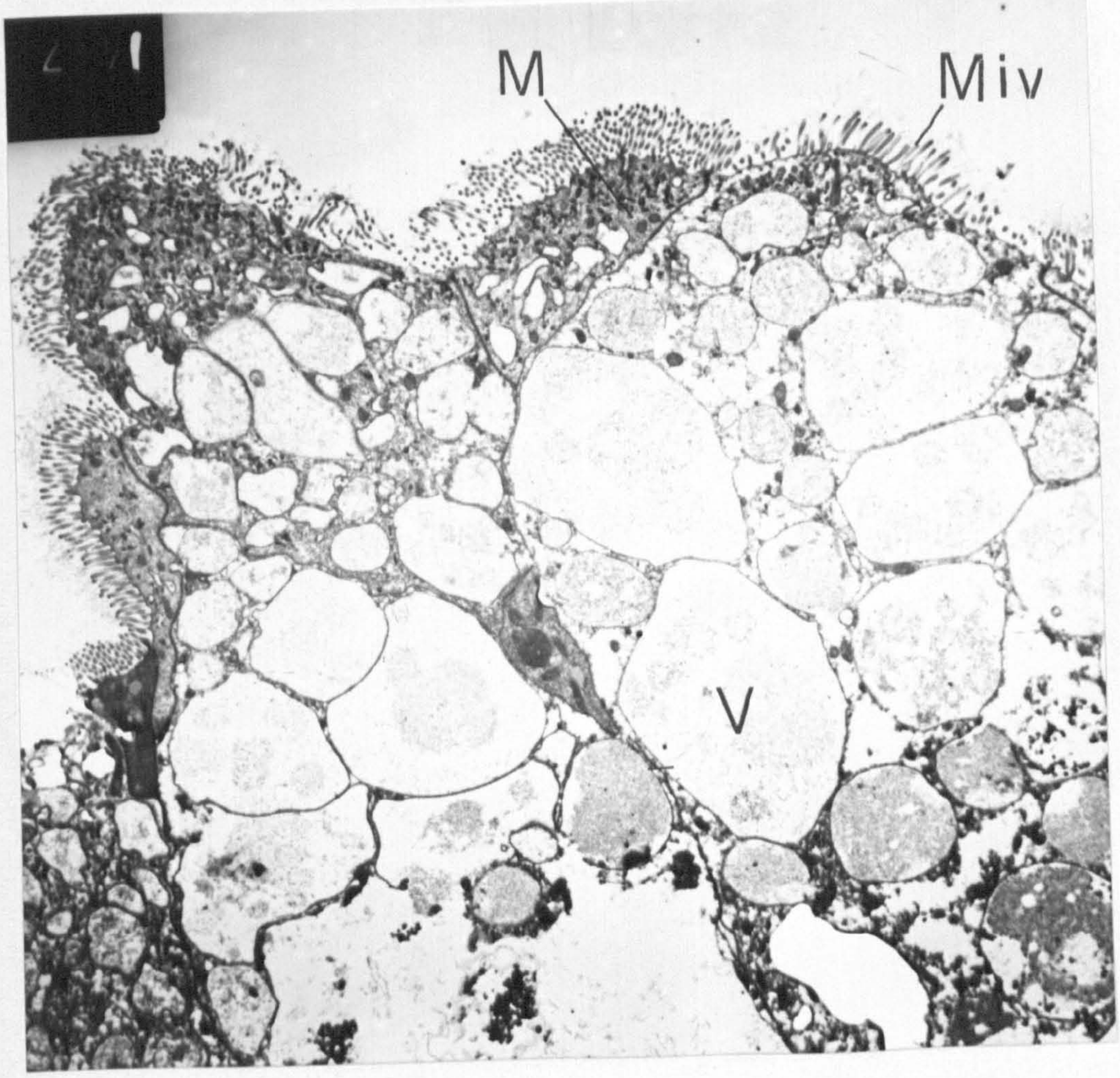


Fig. 15. Distal portion of digestive cells of R (P) africanus.
M = mitochondrion,
Miv = microvilli
V = vacuole

(x 6,000)

The cytoplasm of the digestive cells did not show any evidence of basophilia even in cells with plenty of cytoplasm. The methylene blue-pyronin method revealed very little ribonucleic acid (RNA) and this was confined to the apical layer of the cytoplasm (see Fig. 18). Preliminary observations with the electron microscope were not very successful because the extensive vacuolation made it difficult to get good sections of the digestive cell, but sections of the most compact part of the cell, namely, the distal layer of cytoplasm, did not show any evidence of endoplasmic reticulum or ribosomes (Fig. 15).

Tests for non-specific esterases showed their presence in the apical layer of the cytoplasm of the digestive cells. Both the alpha-naphthyl acetate and the beta-bromoindoxyl acetate methods localized the enzymes in the distal part of the cell. That the activity in this part of the cell was due to non-specific esterases was confirmed by the fact that it was inhibited by the prior treatment of sections of the digestive gland with aqueous 0.00001 M di-isopropyl fluorophosphonate, or aqueous 0.02 M silver nitrate, or 0.1 M sodium taurocholate in phosphate buffer. Sections of the convoluted tubules of a mouse kidney were used as positive controls and served to confirm further the presence of esterases in the apical cytoplasm of the digestive cells.

Gomori's cobalt method showed the presence of alkaline phosphatase all along the free surface of the digestive cells. No enzyme activity was found in the cytoplasm of the cells. Prior treatment of sections with Lugol's iodine or 10% acetic acid abolished the staining reaction of the free borderlaw of the digestive cells confirming that the activity in this area was due to alkaline phosphatase. Sections of the convoluted tubules of a mouse kidney served as positive controls. (See Chapter 5 for a fuller account of the enzyme tests). Electron microscope observations showed that the free border of the digestive cells is thrown into numerous microvilli (Fig. 15). In light microscopy the microvilli appear as a brush border which was seen in some of the cells, but not in others. Sumner (1965) working on the digestive gland of the land snail, Helix aspersa, found brush borders in digestive cells which had a flat surface, but not in those with a convex one: no such association between microvilli and the curvature of the free cell surface was found in Bulinus.

Best's carmine method showed that in comparison with the secretory cells, the digestive cells contained little glycogen. When present, the polysaccharide occurred in the apical and basal parts of the cell as small

amorphous masses or as granules (see Fig. 22). Digestion of the material taking up carmine with 1% malt diastase for filtered human saliva confirmed the presence of glycogen.

(d)(ii) The Secretory Cells

In their morphology (as seen in light microscopy) and location in the digestive gland these correspond to cells which in other molluscs have been described as "calcium" or "lime" cells. Evidence presented below shows that in Bulinus there is no calcium in the cells and that they are actively engaged in protein synthesis. Here they are therefore called secretory, not calcium cells.

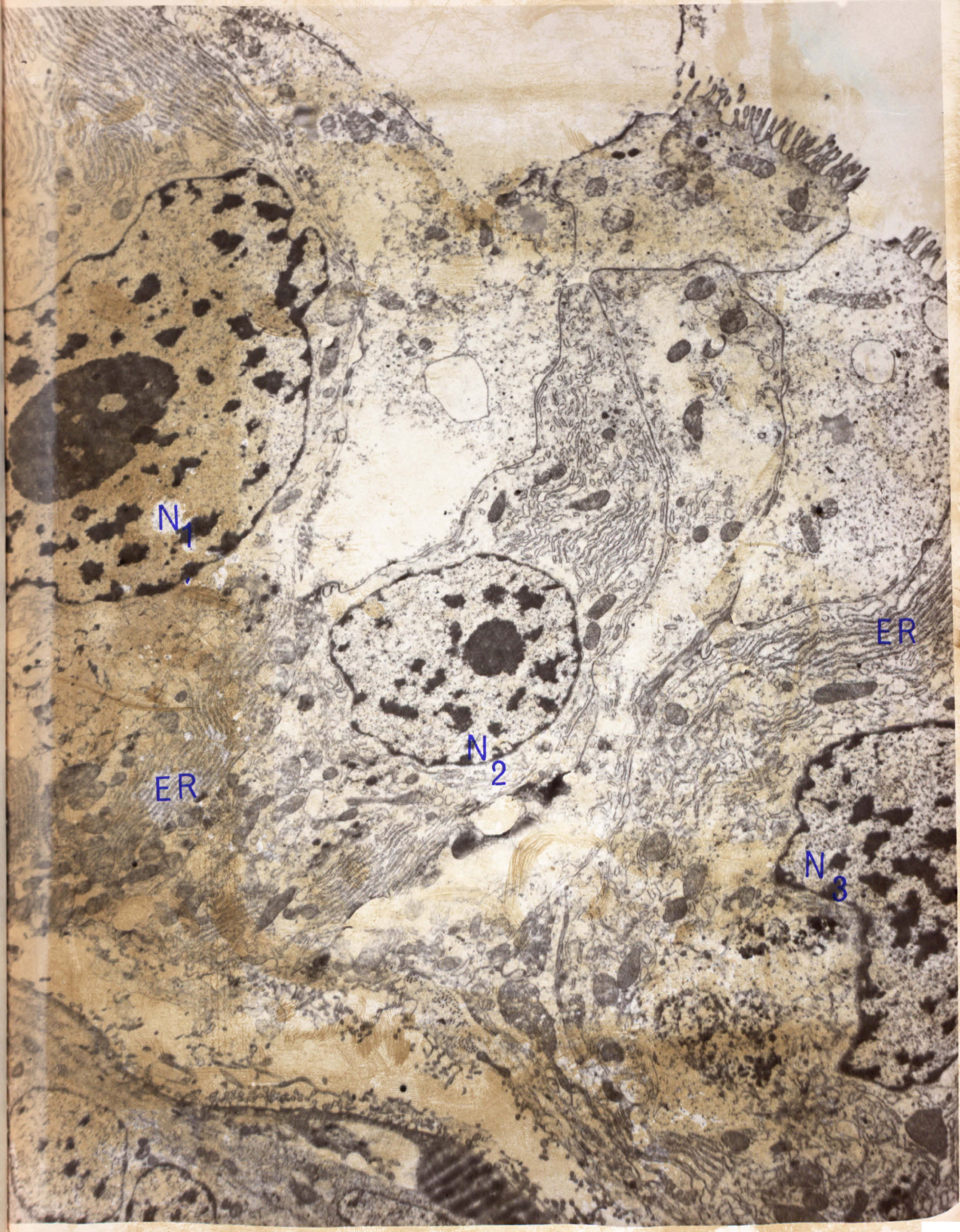
In transverse sections of the tubules these cells occur in groups in crypts of the digestive cells. In light microscopy the intense basophilia of the secretory cells often obscured the cell membranes and it was difficult to know whether what was observed was a group of cells or a single cell with two or more nuclei. Electron microscopy, however, showed that each cell has a single nucleus and that several secretory cells occur together (Fig. 16).

Fig. 16. A group of 3 secretory cells of B (P) africanus.

N_1- N_3 = nuclei of three secretory cells

ER = endoplasmic reticulum.

(x 10,000)


N1
N2
N3
ER
ER

In section the cells appear triangular, with the angles rounded. The height and the length of the base vary but they are, respectively, of the order of 15 to 20 microns and 12 to 18 microns. The cells have very large nuclei which are spherical to slightly eliptical, with the long diameter measuring about 8 to 10 microns and the short one about 5 to 8 microns. The nucleus usually occupies the basal third of the cell and it has a very prominent nucleolus which usually takes an eccentric position (Fig. 10). The nucleolus is nearly always spherical, varying very little from a diameter of approximately 3 microns.

In most of the large number of snails which were examined in the course of the work reported in this thesis, many secretory cells were found that had refractile yellow concretions. The development of these concretions appeared to start with the development of several small vacuoles in the apical cytoplasm of the cell. This was followed by the appearance of small yellow granules in the vacuoles and considerable loss of the basophilia of the cell. The vacuoles then coalesced to form one large vacuole which occupied most of the cell, forcing the nucleus to one of the basal corners where it was surrounded by a layer of cytoplasm. In such cells the concretions occurred mostly as large spheres with

diameters of up to 8 microns, but small granules were often seen in the same cells. Most secretory cells did not appear to be in direct contact with the tubule lumen as were the digestive cells, but all the cells which had the concretions abutted into the lumen (see Fig. 21). When finally the apical part of the cell broke down, the concretions passed into the lumen where they were readily seen. However, not all cells with a free surface contained the concretions: Figure 17 is an example of a cell with a free surface as is shown by the microvilli and yet it did not have the large vacuoles characteristic of cells with concretions.

Intense basophilia is a characteristic of cells which are rich in RNA (see discussion below). The methylene blue-pyronin test for RNA and DNA was therefore performed on the digestive glands of B (P) africanus. It was found that the cytoplasm of the secretory cells stained intensely with pyronin (Fig. 18) suggesting that the cells were rich in RNA. That the pyronin-positive material was RNA was confirmed by the fact that incubation of sections of the digestive gland in an aqueous solution of the enzyme ribonuclease (0.5 mg/ml.) for one hour at 37°C. completely abolished the affinity of the secretory cells for pyronin:

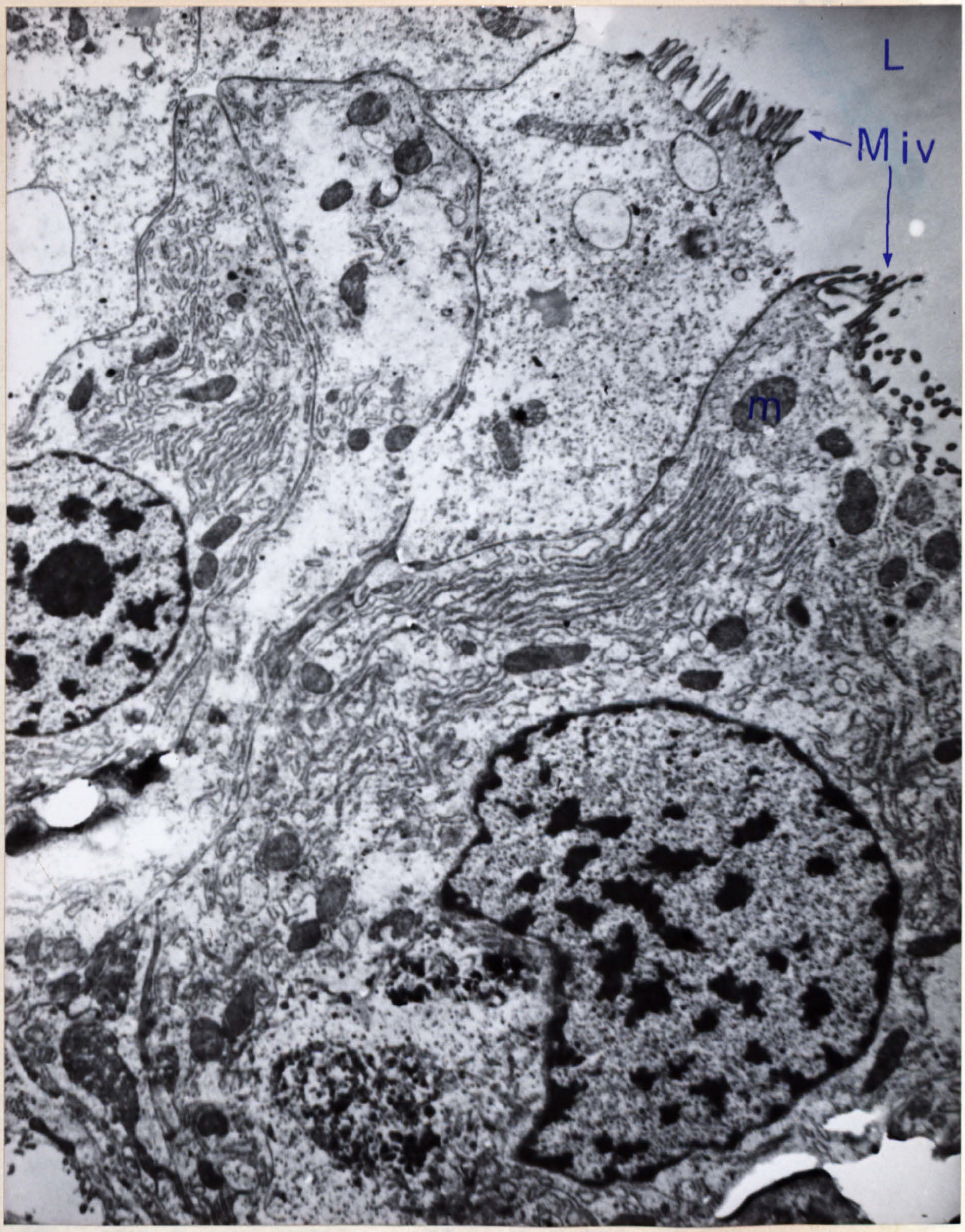


Fig. 17. Secretory cells of B (F) africanus to show their free surfaces.
Miv = microvilli; L = lumen of tubule;
M = mitochondrion. (x 10,000)

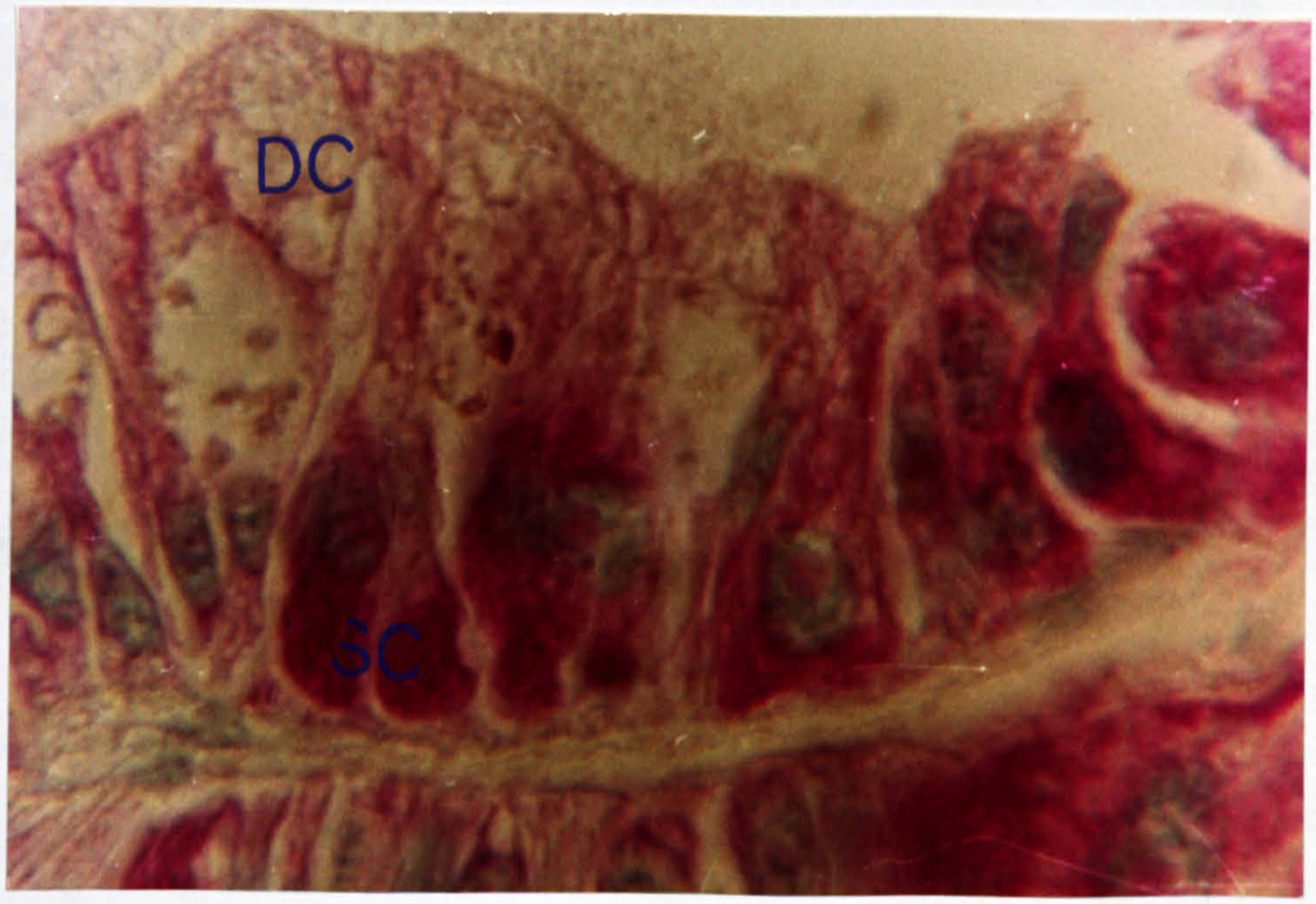


Fig. 18. RNA in secretory cells of B (P) africanus (Methylene blue-pyronin).
SC = secretory cell, DC = digestive cell.

incubation of sections in distilled water at the same temperature and for the same length of time had no effect on the staining properties of the cells.

Electron microscope observations on the secretory cells revealed an extensive endoplasmic reticulum (Fig. 19). In the region immediately above the nucleus the endoplasmic reticulum is tightly packed in a whorled pattern. The membranes of the endoplasmic reticulum were studded with dark granules which were undoubtedly ribosomes (Fig. 20). Large numbers of well developed mitochondria were found in the central and basal parts of the cells (Fig. 20). Fewer, but larger mitochondria occurred immediately below the free surface of the cells (Fig. 17).

The presence of large quantities of RNA and an extensive granular endoplasmic reticulum showed that the cells were actively engaged in protein synthesis (see discussion). The cells are so situated that the protein products of the synthesis could be passed either into the blood spaces in the intertubular connective tissue, or into the lumen of the tubule, or possibly into both. Only one protein, haemoglobin, is known to occur in the blood of both subgenera of Bulinus (see Part I under "Host Factors") and this fact provided an easy way of testing the direction in which the cells

Fig. 19. Whorled pattern of the endoplasmic reticulum of ~~the~~ secretory cell of B (P) africanus.

ER = endoplasmic reticulum
L = lumen of tubule

(x 10,000)

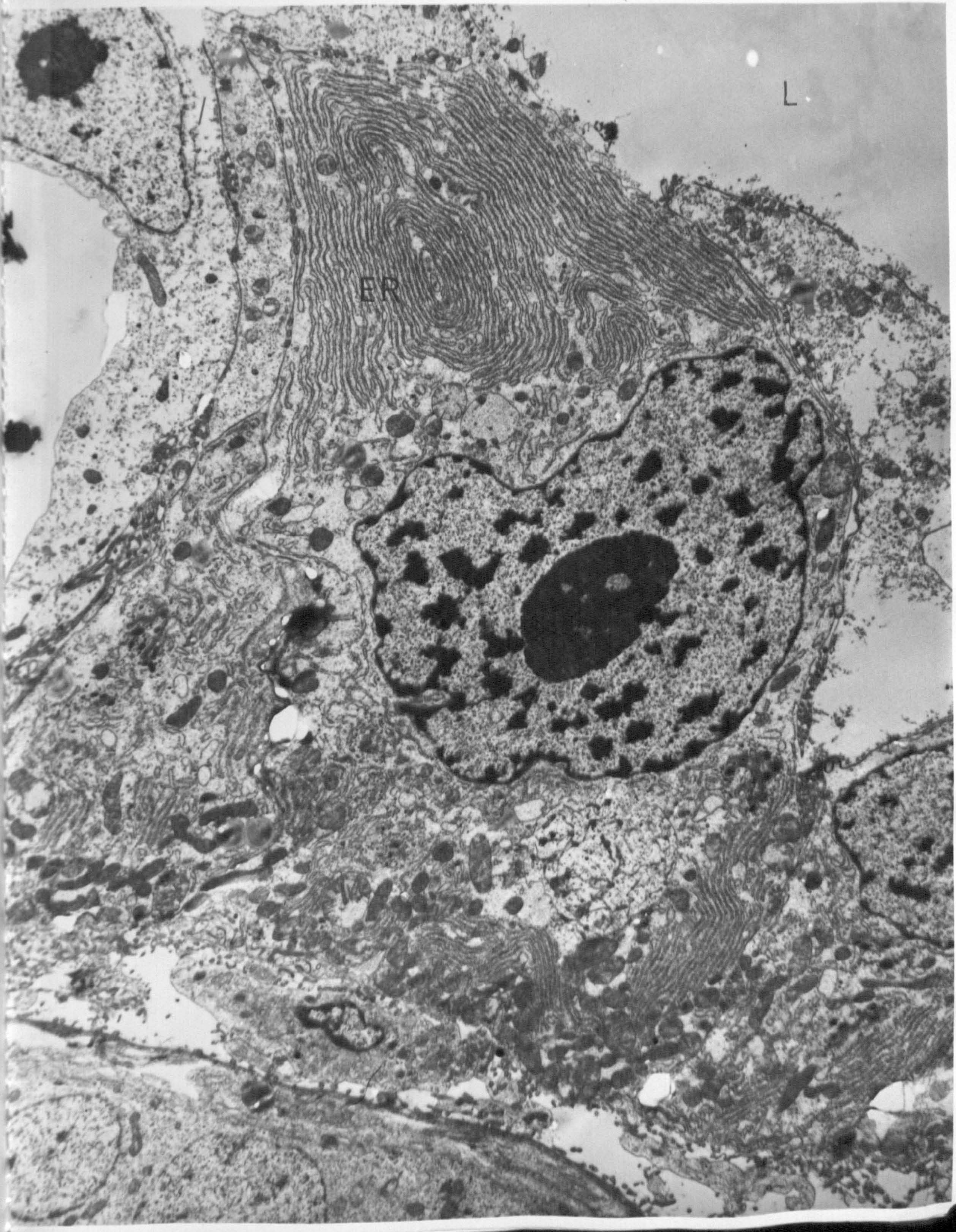
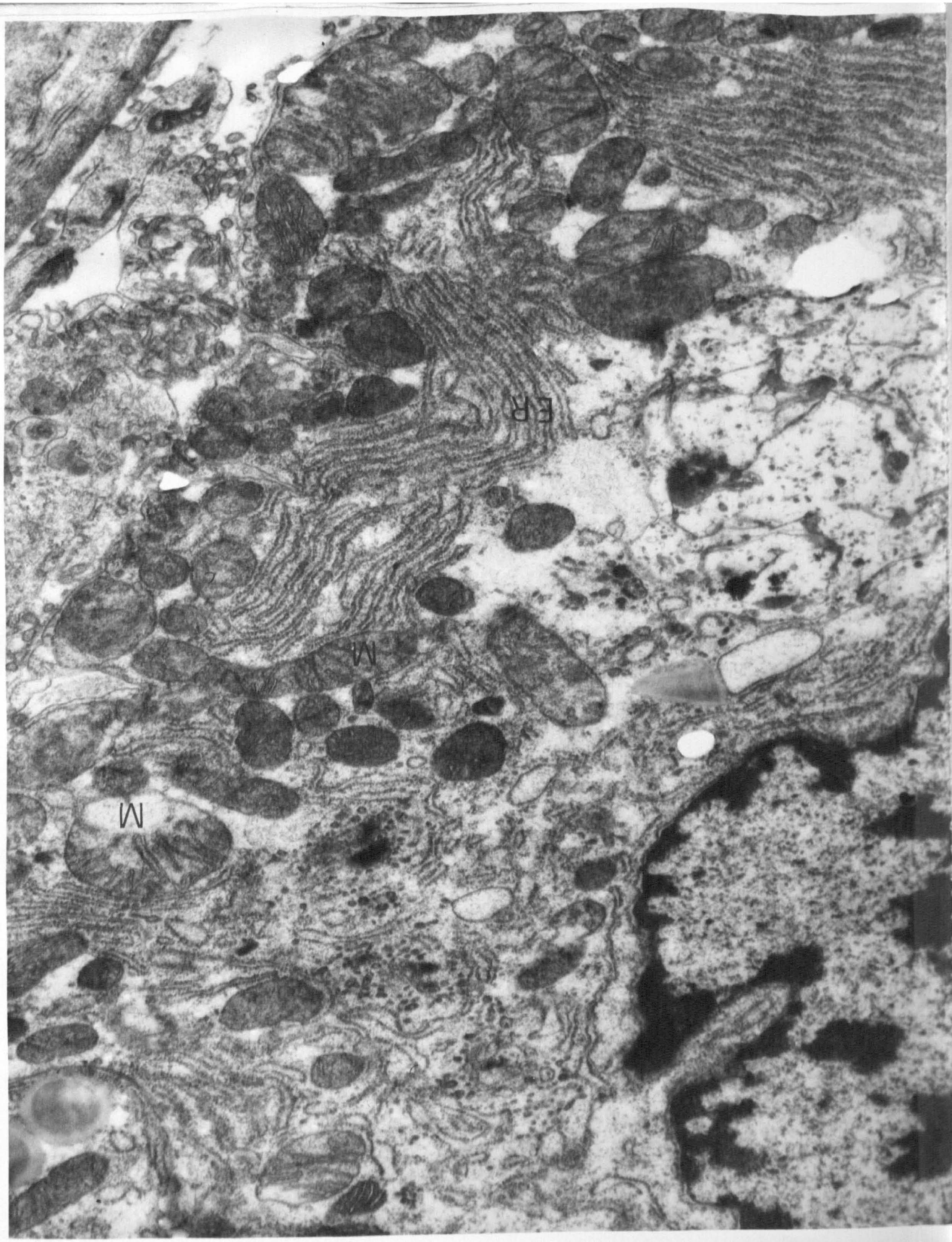

L
ER

Fig. 20. Numerous well-developed mitochondria (M) and endoplasmic reticulum (ER) with ribosomes in secretory cells of D (P) africanus

(x 30,000)


ER
M
M

secrete their products. If these cells synthesize haemoglobin, iron would be expected to accumulate in them. The benzidine method of Pickworth for haemoglobin was therefore applied to the digestive gland of B (P) africanus. (The principle of the method is first to unmask iron from its protein matrix and then after oxidising it from the ferrous to the ferric state, to stain it with nitroprusside). No reaction for haemoglobin was obtained in secretory or any of the other cells of the digestive gland, although positive results were readily obtained in the mouse red blood corpuscles which served as positive controls. It was concluded therefore that the secretory cells did not synthesize haemoglobin.

No deposits of calcium salts or any other inorganic salts were seen in the secretory cells which, as pointed out at the beginning of this section, correspond to cells in which calcium salts have been found by workers on other molluscs. In order, however, to check the possibility that calcium might occur in a form that could be obscured by the basophilia of the cells, histochemical tests were carried out. The three methods chosen were among those that McGhee-Russell (1958) recommended in his critical evaluation of all the available histochemical procedures for calcium. The methods were ammoniacal haematoxylin, Nuclear Fast Red and alizarin red S.

No reaction was obtained in any of the cells of the digestive gland with any of the three methods, although *in vitro* tests of the staining mixtures on calcium salts showed that they were working. It was concluded that the secretory cells do not contain calcium deposits.

Tests with the carmine method of Best showed that although glycogen occurred in all the tissues of *Bulinus* - including the connective tissue of the head-foot, mantle, digestive gland and gonad and the muscles - the largest deposits of the polysaccharide were in the secretory cells (Fig. 22). The amount of glycogen varied from one snail to another and some secretory cells contained little or no glycogen, but in general the heaviest concentrations were always found in these cells. It was found that at about 35°C. 1% malt diastase in 1% sodium chloride removed the heaviest deposits of the carmine-positive material from the secretory cells within 60 to 90 minutes. Filtered human saliva removed the deposits much more rapidly even at lower temperatures (e.g. 16°C.) These digestion experiments confirmed that the polysaccharide staining with Best's carmine was glycogen.

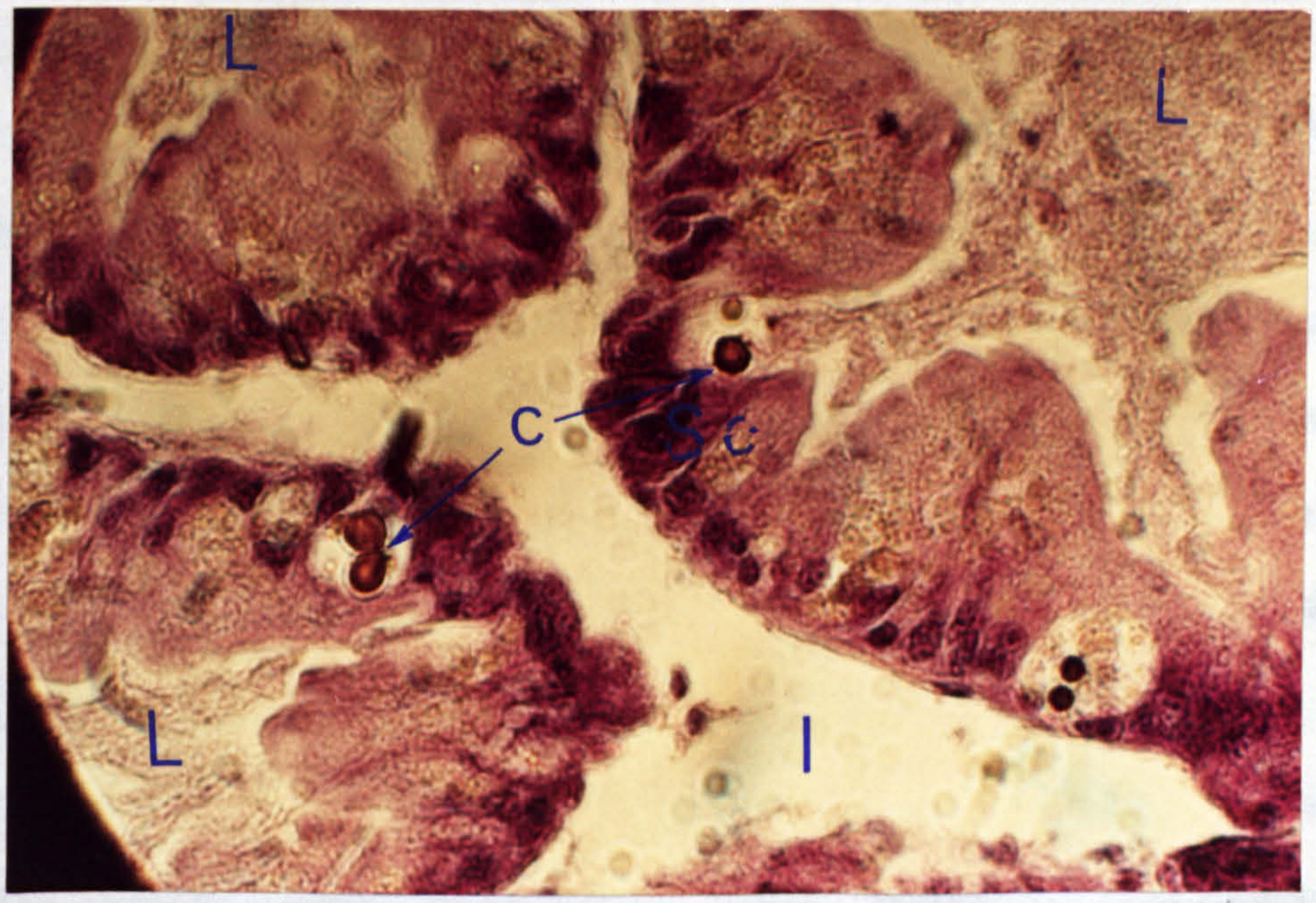


Fig. 21. Concretions in secretory cells of B. tropicus
C = concretions in groups of secretory cells (Sc)
L = lumen of digestive gland tubules
I = intertubular space
Note Colour of concretions is due to photographic processing (H/E).

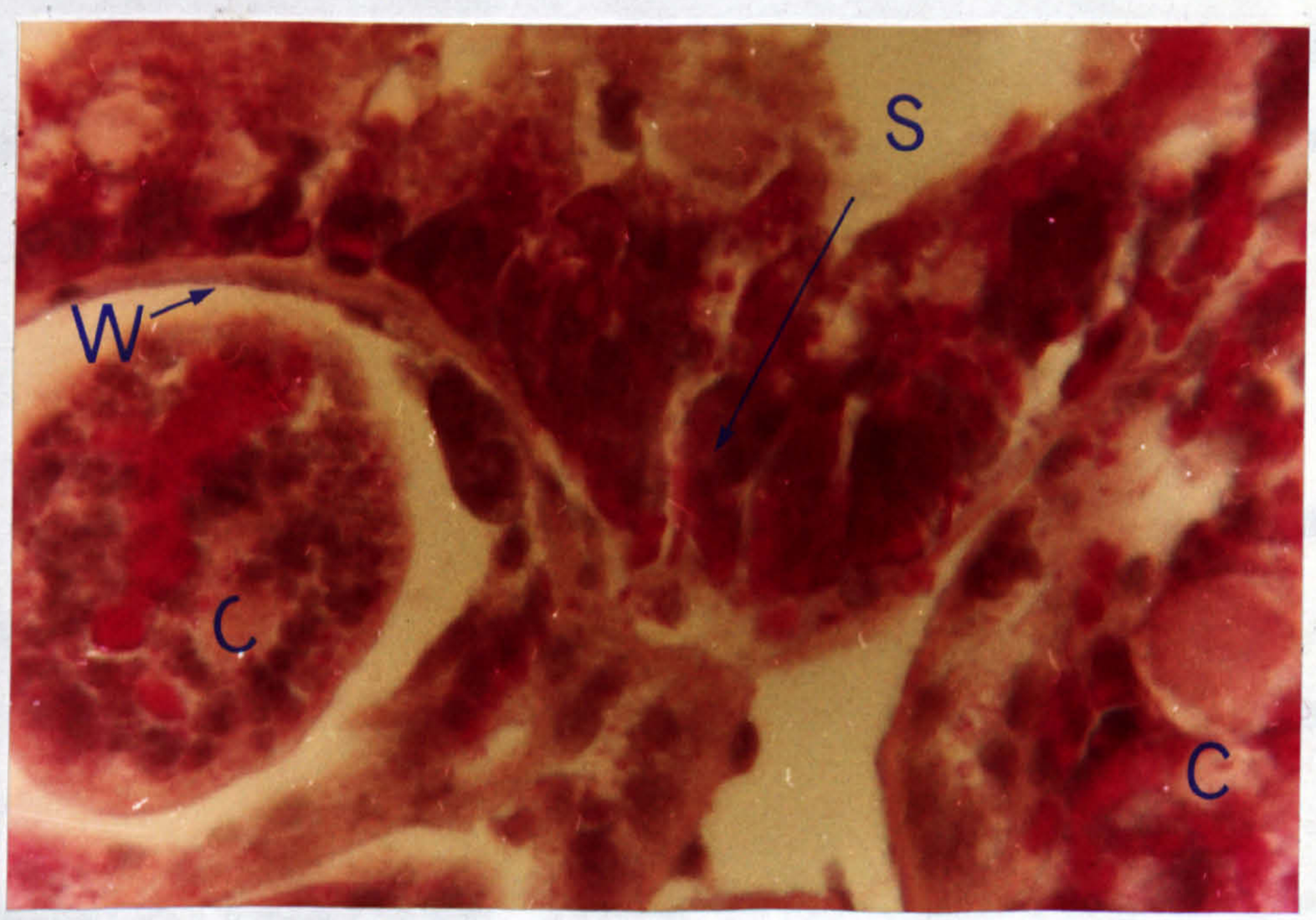


Fig. 22. Glycogen (red) in secretory cells (S) of S(P)africanus developing cercariae (C).
W = sporocyst body wall.

(d)(iii) The Goblet Cells

It has already been pointed out that in haematoxylin and eosin sections the goblet cells are very difficult to distinguish from the secretory cells because both types are intensely basophilic. They are, however, readily stained with alcian blue. They are proper goblet cells which occur in the epithelial lining of the tubules of the digestive gland. They are rather shorter and narrower than the digestive cells and open into the lumen by processes of varying lengths. They have an irregular distribution, occurring in considerable numbers in some parts of the digestive gland and being widely scattered in other parts.

The mucus secretions of the cells are discharged into the lumen of the tubules and have therefore no obvious relevance to the development of schistosomes.

(d)(iv) Discussion

Faust (1920), referring to the digestive gland of the Gastropoda, stated that "numerous students of histology and physiology have published their researches, which, on the whole, are decidedly conflicting and contradictory". Forty-five years later Faust's statement is essentially correct (see Fretter and Graham, 1962;

Sumner, 1965). This conflict of evidence on the functions of the organ is reflected in the names by which it is known: digestive gland (or diverticula), hepatopancreas, and liver. In general, two types of cell have been described in the epithelium tubules of the gastropod digestive gland, although many variants of one of the types have also been reported (Sumner, 1965). The two types are (1) digestive cells, which have also been called secretory, excretory, ferment, liver and absorptive cells (see e.g. Faust, 1920; Carriker, 1946) (2) calcium cells, which have also been referred to as lime, or secretory cells. In the present work only one type of digestive cells and one of secretory cells was recognized in both B (P) africanus and B. truncatus. In addition, goblet cells have been found in the two snails.

Secretory cells

Calcium or lime cells have been described in both fresh water and terrestrial pulmonata (Hurst, 1927; Carriker and Bilstad, 1946; Wagge, 1951; Sumner, 1965). The evidence presented here has shown the cells described as calcium cells in other molluscs do not contain deposits of calcium in Bulinus. It is now well established that intense basophilia is characteristic of cells whose cytoplasm is rich in RNA (De Robertis, Nowinski & Saez, 1965).

Most of the RNA of a cell is contained in particles called ribosomes in which protein synthesis takes place (Watson, 1963). The degree of development of the endoplasmic reticulum and the ribosomes with which it is often associated are indications of the level of the activity of a cell in protein synthesis (Sjöstrand, 1964; Watson, 1963). Observations on Bulinus have shown that the strongly basophilic cells of the digestive gland contain large amounts of RNA. Observations with the electron microscope have shown an extensive endoplasmic reticulum whose membranes carry large numbers of ribosomes. These observations show that the cells are actively engaged in protein synthesis. The large nucleoli of the cells and their plentiful nucleolar RNA (see Fig. 18) are also an indication of very active protein synthesis (Ingram, 1964). The large numbers of well-developed mitochondria indicate in general a high level of metabolic activity (see Bourne and Tewari, 1964 for review of mitochondria). This interpretation of the histochemical and electron microscope observations on the digestive gland of Bulinus is by analogy with findings with vertebrate tissues on which most of our knowledge of the cytology and fine structure of cells is based. However, recent cytological and electron microscope observations have shown that there is no essential difference between vertebrate and inverte-

brate cells. For example, cytological and electron microscope studies on the epithelial cells of the empty midgut of the females of the mosquito, Aedes aegypti, have shown that, as in the vertebrates, cytoplasmic basophilia is due to RNA and that numerous ribosomes are arranged on the membranes of an elaborate endoplasmic reticulum (Bertram and Bird, 1961; StHubli, Preyvogel & Suter, 1966). It is interesting to note that the organization of the endoplasmic reticulum and the distribution of mitochondria in the distal part of the cells are very similar in the mosquito midgut cells and the secretory cells of the digestive gland of Bulinus; in both types of cell and just above the nucleus the endoplasmic reticulum is in the form of tightly packed, whorled pattern which resembles a finger print.

In a review of the haemoglobins of parasitic animals, Lee and Smith (1965) have pointed out the lack of information about the sites where haemoglobins are synthesized in invertebrates. They suggested that in Ascaris certain intestinal cells might be responsible for the synthesis of the haemoglobin of this nematode. It was thought, therefore, that the secretory cells of Bulinus might be synthesizing haemoglobin, the only protein constituent of the blood of the bulinid snails

(Targett, 1961). However, tests did not reveal any haemoglobin in these cells. The origin of this protein which, as the work of Targett (1961) has indicated, might be an important source of the amino acids needed by developing schistosomes, is therefore still unknown. The absence of haemoglobin from the secretory cells suggests that the secretions of the cells are passed into the lumen of the tubule. Digestive enzymes are the most obvious proteins that might be passed into the lumen and whose synthesis could call for the high level of activity that is evident in the secretory cells. Although they were generally larger, the granules found in many of these cells resembled the zymogen granules of the mammalian pancreatic cells. Sumner (1965) was of the opinion that similar concretions present in the calcium cells of the land snail, Helix aspersa, were excretory products, but in Bulinus excretion is carried out by the kidney as evidenced by the large accumulations of uric acid (or its derivates) in the renal epithelium. I believe, therefore, that the refractile yellow granules present in the secretory cells are digestive enzymes, or more probably their precursors, which are synthesized in the cells and passed into the lumen of the tubule by holocrine secretion.

The presence of large quantities of glycogen in the secretory cells is undoubtedly related to the carbohydrate requirements of the high level of metabolic activity characteristic of these cells. These large stores of glycogen must be a very important source of carbohydrate for the developmental stages of schistosomes. (This is discussed more fully in Chapter 5).

Digestive Cells

In other molluscs these cells have been described as digestive, absorptive, storage, secretory and excretory (see, for example, Fan (1958) on Australorbis and Carriker & Dilstad (1946) on Lymnaea). In this work evidence was obtained which suggests that in Bulinus digestion and absorption are the only functions of the cells. The occurrence of non-specific esterases shows that hydrolysis of fats (mostly simple aliphatic esters) takes place in the apical cytoplasm of the digestive cells (see Burstone, 1964 for a review of the distribution and functions of esterases). Since Bulinus is a herbivore these esters are probably of plant origin. The various stages described in the formation of vacuoles and the accumulation of material and the eventual breakdown of the digestive cells also suggest that the cells play some part in digestion. It appeared that young cells take up very fine food parti-

cles from the lumen into their apical cytoplasm where some digestion (probably of simple fats mostly) takes place and the indigestible residues are passed into the central parts of the cell. This procedure is probably repeated many times until the cell is distended to capacity with the indigestible material, when the apical part breaks down and the contents pass into the lumen whence they are taken into the intestine and formed into strings of faeces. This would to a large extent agree with the scheme proposed by Krijgsman for the digestive activity of the digestive gland of Helix (Fretter and Graham, 1962). But the digestive activity in the two snails would differ in two respects. First, no evidence of the synchronization of the various phases - ingestion, digestion and elimination - was found in Bulinus. Cells in all stages were seen side by side in the snails examined. Secondly, in Bulinus only very fine food particles were found in the lumen of tubules and although some reduction in their size occurred inside the cells, relatively little digestion appears to take place here. It is suggested, therefore, that in Bulinus digestion is mostly extra-cellular and takes place in the alimentary canal and enzymes from the secretory cells are probably responsible for it. Hydrolysis of simple aliphatic esters is probably the only important intra-cellular digestive activity of these cells.

Absorption of substances from the tubule lumen may be a very important function of the digestive cells. This is shown by the fact that the free surface of these cells is thrown into numerous microvilli, a development that very greatly increases the effective absorption surfaces of cells (De Robertis, Nowinski & Saez, 1965). Probably the most important physiological function of the enzyme alkaline phosphate is the transport of metabolites across cell membranes (see Burstone, 1964 for review). The presence of alkaline phosphatase in the free surface of the digestive cells is further evidence of the absorptive function of these cells.

In comparison with the secretory cells, the digestive cells contained only a little glycogen and are therefore of little importance in the storage of the polysaccharide probably. The absence of a granular endoplasmic reticulum and the lack of basophilia even when plenty of cytoplasm was present shows that the digestive cells are not secretory in Bulinus. And, finally, no evidence was found to suggest that these cells have an excretory function.

In conclusion, the results of the present work suggest that absorption and some intracellular digestion of fats are the main functions of the digestive

cells and that the production of digestive enzymes and some absorption are the main functions of the secretory cells of Bulinus. Schistosomes developing in the snail, especially the stages which develop in the digestive gland, must be dependent on the activities of the gland in the digestion, absorption and storage of the metabolites that the parasites require for development.

4. SUMMARY AND CONCLUSIONS

In this chapter are recorded some histological, histochemical and electron microscope observations on the head-foot, mantle, tentacle and digestive gland of Bulinus (Physopsis) africanus and Bulinus (Bulinus) truncatus. These are organs that have a direct bearing on the development of schistosomes in these snail hosts.

It was found that in the head-foot and mantle epidermal epithelia enclose a matrix comprising collagen fibres and fibroblasts and in which large mucus-secreting cells, vesicular cells and muscle and nerve fibres are embedded. In both the head-foot and mantle the mucus cells discharge their copious secretions outside the ventral epithelium: these secretions may contain substances which attract schistosome miracidia to the organs. The connective tissue has numerous blood spaces, and it is very loose

over most of the mantle and immediately inside the ciliated epithelium of the sole of the foot. Amoebocytes occur in the blood spaces and are most numerous in the mantle. It is suggested that they are not transported in the circulating blood, but that they actively migrate through the snail tissues. The numbers of these cells increase in snails with bacterial infections and the amoebocytes probably play an important part in the defences of Bulinus against parasitic infections. Pigment cells which also occur in blood spaces, are probably amoebocytes that are enlarged for more efficient phagocytosis of invading bacteria and other foreign material. A lymphoid tissue is described (for the second time in a mollusc) in the mantle. Evidence is presented which suggests that the lymphoid tissue is responsible for the production of all the amoebocytes of Bulinus. In addition to the mucus-secreting cells, other secretory cells occur in the mantle, but their functions were not studied. They may be associated with the synthesis of the protein part of the shell. Uric acid, or a salt of it, occurs in the epithelial cells of the saccular part of the renal organ. This suggests that in Bulinus the kidney is the main excretory organ and that the digestive gland, apparently unlike that of some molluscs, plays very little part, if any, in excretion.

The tentacle appears as a cylinder with a thin skin enclosing a large blood space inside which there is a core of nerve trunks and longitudinal muscle fibres. Amoebocytes occur in the large blood spaces which, as the other loose tissues of the snail, probably provides a very suitable habitat for schistosome sporocysts.

The digestive gland consists of tubules which are separated from each other by very loose connective tissue. Relatively few amoebocytes normally occur in the blood spaces of this tissue. The tubule epitholium comprises three cell types: digestive, secretory and goblet cells. Histological observations and the occurrence of esterases in the highly vacuolated digestive cells suggest that a certain amount of intra-cellular digestion takes place in them. The enlargement of their free surfaces and the occurrence of alkaline phosphatase on the surfaces shows that the digestive cells are very actively absorptive.

The secretory cells are shorter and fewer than the digestive cells. They have large nuclei and nucleoli and the intense basophilia of their cytoplasm is due to ribonucleic acid (RNA). An elaborate granular endoplasmic reticulum and numerous well-developed mitochondria are other characteristics. These cytological and electron microscope observations show that these cells are very actively engaged in protein synthesis. Attempts to

identify haemoglobin, the only protein known to occur in the blood of adult bulinid snails in the cells, were not successful. It is suggested therefore that digestive enzymes, or probably their precursors, are the main and possibly the only proteins that are synthesized in the secretory cells. Although glycogen occurred in all the tissues of Bulinus the largest deposits of the polysaccharide were in the secretory cells. The digestive gland must therefore be of fundamental importance in the development of schistosome parasites, since it is responsible for the digestion and absorption of food substances and the storage of carbohydrate.

C H A P T E R 4

STUDIES ON THE RESISTANCE OF BULINUS (PHYSOPSIS) AFRICANUS AND BULINUS (BULINUS) TRUNCATUS TO INFECTION WITH SCHISTOSOMA MATTHEEI

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1. INTRODUCTION

Although there is good evidence to show that in refractory biomphalarid snails, infection with Schistosoma mansoni is actively resisted by a cellular defence mechanism, the mechanisms of resistance* to schistosome infection in bulinid and oncomelanid snails has been very little studied. (See "Resistance to Infection" under "Host Factors" in Part I). It is not known, therefore, how far the findings with biomphalarid hosts are applicable to the other mollusc-schistosome relationships. The purpose of the work recorded in this chapter was to investigate the mechanisms by which bulinid snails resist parasites of the S. haematobium group.

It was considered that the best approach to the problem would be a comparative one. The plan was to study the host-parasite relationships of a single strain of S. mattheei and a refractory, a susceptible and a highly susceptible strain of Bulinus. It was found that an Iranian strain of B. truncatus, a Tanzanian strain of

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* In this thesis the terms "resistance" and "immunity" are used interchangeably to include active defence reactions and natural or innate resistance such as that provided by the surfaces of animals. The terms used are with reference to invertebrate hosts and whenever reference is made to vertebrate immunology the appropriate, more specialized terminology is used.

3. RESULTS (cont'd)

(d) Penetration of miracidia into Bulinus

(i) The numbers of miracidia penetrating

(ii) The process of penetration

(e) The response of the host tissues to schistosome infections

(i) B (P) africanus

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4. DISCUSSION

5. SUMMARY AND CONCLUSIONS.

B (P) africanus and a South African strain of B (P) africanus, in that order, meet these requirements. Three aspects of the interaction between the snail hosts and the parasite were studied:-

(1) the attraction of the schistosome miracidia by the snail hosts;

(2) the penetration of the miracidia into the snails;

(3) the response of the tissues of the snail hosts to invasion by the parasite.

It was proposed to then repeat these observations with S. bovis, but difficulties in the maintenance of this schistosome did not allow more than a few preliminary observations.

2. MATERIAL AND METHODS

(a) Biological Material

(1) Schistosomes

(A) Schistosoma matthooi

The same strain of this schistosome was used throughout this study. It was derived from 5 heavily infected rats (Mastomys natalensis)which were kindly supplied by Dr. R. J. Pitchford from Nelspruit, South Africa and it was subsequently passaged in Mastomys natalensis and mice in London. Attempts to infect 2 English sheep were unsuccessful. The sheep were exposed by placing a heavy concentration of cercariae, totalling 1,000 - 2,000, into the groin pouch of the anaesthetized animal. Although the same batch of cercariae produced good infections in mice and Mastomys only a few male schistosomes were recovered from the sheep at necropsy.

(D) Schistosoma bovis

This parasite was isolated from specimens of B (P) africanus collected from streams near Kisumu, Kenya. Mice were exposed to cercariae from these snails and sent to London by air. The eggs from the first passage in mice had the typical spindle shape of S. bovis,

but in subsequent passages in mice, hamsters and Mastomys a wide range of egg shapes, most of which were abnormal, were obtained. Four sheep were each exposed to at least 750 cercariae of the first laboratory generation pooled from 15 snails which were shedding heavily. Only a few dead eggs were found in the liver, although a number of worms, mostly males, were recovered on perfusing one sheep.

The variable egg characteristics were accompanied by great fluctuations in the infectivity of the miracidia to the normal snail host. For example, with 4 - 6 miracidia per snail, first passage miracidia infected 80 to 100% of the snail host, but by the 4th passage only about 40% could be infected. This variability in the infectivity of the parasite miracidia ruled out the proposed comparison of the host-parasite relationships of S. bovis and S. mattheei in the snail hosts.

(a)(ii) Snails

In the work recorded here the same two races (Nelspruit and Mwanza) of B (P) africanus/and B. truncatus (Iran) whose histology was described in the preceding chapter were employed. In addition, an Egyptian strain of B. truncatus and a

Kisumu strain of B (P) africanus from which S. bovis was isolated were maintained in the laboratory. The same techniques were employed in the maintenance and breeding of all these strains of snail, but it was found that the Egyptian B. truncatus which had been in the aquarium longest was by far the easiest snail to keep.

The present work concerns the Nelspruit and Nwanza races of B (P) africanus and the Iranian strain of B. truncatus, but a few observations with the other two snails are mentioned.

(b) Miracidia, Exposure and Maintenance of Snails

Miracidia for infecting snails and for all other purposes were hatched from heavily infected livers of Mastomys, mice and sometimes hamsters. The livers were gently crushed between two glass plates, washed into urine jars in which the material was washed by sedimentation in 1% saline until the supernatant fluid was clear. The clean sediment was washed once in filtered tap water and then hatched in aquarium water under a 50 watt tungsten light.

When miracidia were for exposure of snails, material from two or more livers was mixed and sedimented together and then divided into portions convenient for hatching. The required number of miracidia was counted

under the dissecting microscope and pipetted into specimen tubes (3 in. x 1 in.) Enough aquarium water was added to the tubes to give a volume of 2 to 3 ml. and then young adult snails were introduced individually into each tube. Exposures were carried out under a 50 watt electric lamp at 27°C. to 28°C. and

After exposure snails were maintained at room temperature. For the determination of infection rates snails were exposed late in spring, during the summer and early autumn, when the temperature of the aquarium varied from 23 to 27°C. In some of the experiments on rates of infection, the temperature was maintained at 25 to 27°C. with the aid of electric heaters.

Examination of snails for infection began after 28 days and continued until either the snails died, or up to 50 to 60 days after exposure. The technique used was the usual one of leaving snails in water under a strong light and then examining the water for cercariae. At 25 to 27°C. the prepatent period was about 30 days in the Nelspruit race and about two days later in the Kwanza race of B (P) africanus.

(c) Experiments with Hydrocortisone acetate

The stability and insolubility of this compound provided a convenient way of feeding it to snails.

The principle of the method was to suspend the compound in a gel upon which the snails could feed. A modification of the calcium alginate gel developed by Standen (1951) was employed. Fresh lettuce leaves were washed thoroughly under the tape, scorched in boiling water, flattened between two large glass sheets and left to dry for two or three days in an incubator at 37°C. When completely dry and crisp the lettuce leaves were ground into a fine powder by means of a domestic mincer. The powder was slowly poured into a beaker of boiling water to give a 4% lettuce suspension. Commercial sodium alginate (B.D.H.) was very slowly added, with vigorous stirring until a concentration of 2% had been reached. When about half the required amount of the calcium alginate had been added and while the temperature of the mixture was about 50°C. the desired quantity of the injection suspension of hydrocortisone acetate (Boot's Pure Drug Co.) was added, by means of a hypodermic syringe, into the mixture. When the right concentration of calcium alginate had been added, the contents of the beaker were poured into an enamel tray which was then slowly tilted to give an even and thin layer of the colloidal mixture. The mixture was then gelled by adding 2% aqueous calcium chloride. When the gel was firm, calcium

chloride was thoroughly washed away under domestic water filter. The gel was then cut into thin strips so that the hydrocortisone content of each strip was known approximately. The strips were placed in clean plastic bags and stored in a refrigerator at 0 to 4°C. for up to 2 weeks, after which they were discarded and a fresh supply was prepared.

Small pieces of the alginate were fed to the experimental snails at a time. Control snails were fed on strips which had been prepared in the same way, except for the omission of hydrocortisone acetate. Other control snails were fed on lettuces. In most cases the snails ate all the alginate mixture before a fresh supply was introduced. Sometimes, however, pieces of old strips had to be removed from the tanks when they showed evidence of fungal growth. It was thus often possible to estimate the amount of hydrocortisone acetate that snails consumed.

Snails were exposed to miracidia, maintained and examined for cercarial shedding in the usual way (see (b) above).

(d) Attraction Experiments

Two methods were used in a comparison of the attraction of the snails for S. mattheei miracidia. These were (A) modifications of the apparatus of Etges

and Decker (see under "Attraction for Miracidia" in Part 1) and (B) a "dumb-bell method".

(A) The Etzen-Decker apparatus

Two modified forms of this apparatus were used. The apparatus simply consists of a central chamber and four side chambers with each of which it communicates by means of a side arm. In one form the modification involved the use of perspex material instead of the original cast-iron and a reduction in size to give an internal diameter of 6 cm. and a height of 5 cm. for the central chamber, an internal diameter of 3.5 cm. and a height of 5 cm. for each of the side chambers. A third attraction was that each of the side chambers carried a short side arm which was joined to a similar short arm of the central chamber by means of a short piece of polythene tubing. This arrangement permitted the introduction of a clip by means of which the chambers could be disconnected and the number of miracidia in each chamber could thus be conveniently counted.

The second form of this apparatus which was constructed for me by Mr. J. Turner, the School Glass Blower, was made of glass and the side arms were all one piece of glass. When desired the chambers were disconnected by stoppering with rubber bungs introduced from the central chamber.

Experiments with these modifications of the Etges-Decker apparatus were conducted as follows. Aquarium water was added into the apparatus so that the meniscus was just above the side arm. This meant adding about 150 ml. into the perspex and 200 ml. into the glass apparatus. Two or three specimens of the snail being studied were introduced into two of the side chambers and the apparatus was left to stand for one to two hours either in the aquarium or in a photographic dark room. To stop the penetration of miracidia, the snails were removed and 100 to 500 miracidia pipetted into the central chamber and the apparatus then left to stand undisturbed for another hour or two in total darkness or in the aquarium. At the end of this period the chambers were quickly disconnected by means of clips or plastic stoppers. The number of miracidia in each of the side chambers were counted and pipetted out under the dissecting microscope. Between experiments the apparatus was thoroughly washed under the tap and left to dry at room temperature. At the beginning this was the main purpose of using transparent material and reducing the apparatus to a size that could be observed under a dissecting microscope. When no more miracidia could be seen the contents of each of the chambers wavedrained into petri dishes and further searched for miracidia.

(B) The "Dumb-bell" Method

Preliminary observations showed that when a piece of paper which had been soaked in water in which the bulinid snails had been standing was introduced into petri dishes containing miracidia, the miracidia were attracted to it in the same way that they were attracted by the living snails. It was also noted that the length of time for which a piece of filter paper remained attractive depended on the degree of contamination of the water by snails; leaving four or five adult snails in about 10 c.c. of water gave a concentration of the attractants that was effective for at least 15 minutes when small (4 x 1 cm.) pieces of filter paper were used. These observations led to the development of a very simple test system.

The system consisted of two drops of liquid joined together by a short bridge (also of liquid). When miracidia were present in the system attraction of two liquids for miracidia could thus be easily compared. The most important requirements for this system is a completely flat and dry surface which is free of grease. These requirements were met by using petri dishes which had completely flat bottoms and which had been cleaned thoroughly and dried in the oven. A graduated Pasteur pipette was used to deliver drops of known volumes of the liquids.

The experiment was carried out as follows. Four or five snails were left to stand for 1 hour or more in about 10 ml. of water in small specimen tubes. A drop (0.1 ml.) of the contained water (referred to as "snail water") was placed in a petri dish on the dark stage of a dissecting microscope. A known number of miracidia was taken up in a drop of the same volume and placed about 10 mm. away from the first. The two drops were then joined by a narrow bridge (3 to 5 mm.) which was very gently drawn from the drop containing the miracidia. The numbers of miracidia in the "snail" side, the bridge and the clean water side were counted at convenient intervals of time, 3, 6, 10 and 15 minutes, after the drops had been joined. Use of a known number of miracidia (10 was found to be a convenient and large enough number) made possible a rapid and simultaneous counting of the number of miracidia in each part of the system, because the whole of one side and the whole bridge were in view under the microscope at the same time. Between counts the system was very gently arranged so that the bridge was in the centre of the field of view. Light was shone onto the stage, and in order to balance out any effects that it might have on the distribution of the miracidia in alternate experiments, the drops were arranged so that the "snail" side was away or near the observer.

In this method, only one point needs special care: water from one side, particularly the "snail" side, must not flow into the other side. To overcome this the drops must be of the same height; this is made possible by gently, but rapidly delivering drops of the same volume on a greaseless surface. The microscope stage and the bottom of the petri dish must also be smooth to permit a gentle and smooth movement of the dish when the system is being arranged for counting. It was found that after a little practice, the apparatus could be used with great ease to give clear-cut results.

(c) Histological Methods

Bouin's fluid was often a more convenient fixative than Zenker's. When convenient to do so, some snails were fixed in Zenker's fluid. After dehydration and embedding in paraffin wax, the snails were sectioned at 5 to 8 microns and the sections were stained with Erlich's haematoxylin and counterstained with eosin.

Complete serial sections were cut of all the snails studied within 32 days of exposure to miracidia. Every section of snails with infections younger than 8 days was carefully searched. With older infections at least every second or third section was examined. All this was done to ensure that no parasite was missed and

that in infected snails any changes in the head-foot, mantle, digestive gland and also the gonad were noted. The amount of labour involved in the preparation and examination of many thousands of sections put a limit to the number of snails that could be studied. However, it is considered that sufficient numbers were examined to give a clear picture of the nature of the resistance of the three strains of Bulinus to infection with S. mattheei. With infections older than 32 days at least half of each snail was sectioned and examined, but slides were also made and examined of material being employed in other experiments (e.g. for glycogen - see Chapter 5).

(f) Electron Microscopy

(i) Preparation of Miracidia for Fixation

Schistosoma mattheei miracidia were hatched, under an electric light, from the homogenate of the livers of Mastomys or albino mice. In order to avoid diluting the fixative by a factor greater than 1 in 20, it was often necessary to concentrate the miracidia further until a small drop of the suspension contained numerous miracidia. This was achieved by spinning large quantities (about 50 ml.) of the suspension two or three times for 3 to 5 minutes at 3,000 to 4,000 revolutions per second to remove all suspended debris and then filtering through paper. The

filtration apparatus consisted of vertical tubes (about 15 cm. high and with a diameter of 1 cm.) with a piece of a Whatman rapid filter paper fixed to the bottom by means of a resin/pitch mixture made by Mr. F. H. N. Foster. Usually all the miracidia present in about 25 ml. of water were concentrated into 0.5 ml. at the bottom of the filtration tube whence they were pipetted into 10 ml. of the fixative in a graduated centrifuge tube.

The first observations were carried out using S. mansoni miracidia hatched from the faeces of a heavily infected patient from St. Lucia (West Indies), but because of the original difficulties with the fixation of the material, the results given in this thesis are based on S. mattheei.

(ii) Fixation of Miracidia

Except for a few observations on the eyespots (see Isseroff, 1964), no studies on the fine structure of trematode miracidia have been published. Electron microscope techniques for the study of miracidia are therefore not available and these had to be worked out for this study. Although initial difficulties, particularly in the fixation of the material, have been to a large extent overcome, the techniques require studying further.

The first attempts were made using 3% gluteraldehyde in the buffer of Rhodin and Zetterquist for pre-fixing and 1% Osmium tetraoxide in the same buffer for post-fixing. Miracidia treated in this way became greatly distorted and, in electron microscope sections, appeared much vacuolated suggesting considerable loss of material from the tissues. Further attempts were made using both 3% gluteraldehyde and 1% osmium tetraoxide, either in tap water or in a phosphate buffer (pH 7.3) containing 10% sucrose. Yet in other experiments gluteraldehyde and Osmium tetraoxide were dissolved in the phosphate buffer with sucrose or in tap water and when miracidia were pre-fixed in gluteraldehyde and post-fixed in Osmium tetraoxide.

Heavy concentrations of the miracidia were pre-fixed for 30 to 40 minutes after which they were spun down to a pellet by means of a centrifuge. Gluteraldehyde was carefully pipetted out and the miracidia were re-suspended in the same medium as the fixative was dissolved in. After about 5 minutes the miracidia were spun down again and the medium was replaced by a fresh supply. This procedure was repeated three or four times until all the gluteraldehyde had been removed. The miracidia were then post-fixed in Osmium tetraoxide for 7 to 10 minutes and, after washing three or four times in the appropriate medium, they were dehydrated through 70%, 80% and 100% ethanol.

After staining in 1% phosphotungstic acid in absolute ethanol, the organisms were washed several times in absolute ethanol and then in toluene. Individual miracidia were embedded in Araldite and sectioned with glass knives. The sections were studied using a Zeiss EM9 and an AEI EM6.

3. RESULTS

(a) THE SUSCEPTIBILITY OF THE 3 STRAINS OF BULINUS TO SCHISTOSOMA MATTHEEI

In a preliminary experiment involving a small number of snails and using cercarial shedding as the criterion, it was found that the Iranian strain of Bulinus truncatus was very slightly (5%) susceptible to S. mattheei when the parasite first arrived from South Africa. All subsequent exposures, however, did not produce any infections in this snail, even when snails were exposed individually to up to 30 miracidia each. These observations suggested that miracidia penetrated, but failed to develop to the stage of producing cercariae. This strain, therefore, appeared to be very suitable for a study of the defences of bulinid snails against schistosome infection. In this work this strain was consequently studied instead of the Egyptian strain with which several exposures failed to produce any infections.

The Nelspruit strain of B (P) africanus was highly susceptible and the Kwanza one less susceptible. Although the rates of infection varied from one experiment to another the Nelspruit race was consistently the more susceptible of the two races of B (P) africanus. In several experiments the mortality of snails during the prepatent

period was very high and the results are not included in Table 2, which shows the relative susceptibility of the two races of B (P) africanus and the Iranian strain of B. truncatus. The consistency of the relative susceptibilities of the two races of B (P) africanus is well demonstrated in experiments 4 and 5. In each of these experiments the three strains of snail were exposed to the same number of miracidia hatched from the same pool of the homogenates of 2 or 3 Mastomys livers. The snails were exposed for the same length of time (4 hours) under the same conditions of temperature and lighting and then maintained in adjacent tanks. After exposure the snails of experiment number 4 were maintained at a temperature of 25 to 28°C. and those of experiment number 5 at 24 to 26°C. An interesting and unexpected result was that, even allowing for differences in mortalities in different experiments, the percentage of snails infected bore no relation to the number of miracidia used for exposure (see especially results with the Nelspruit race).

(b) ATTEMPTS TO ENHANCE THE SUSCEPTIBILITY OF B. TRUNCATUS TO S. MATTHEEI INFECTION

It is well known that certain adrenocortico-steroids can inhibit the defence mechanisms of mammalian hosts against helminthic and other parasitic infections

TABLE 2

THE SUSCEPTIBILITY OF 2 RACES OF B (P) AFRICANUS AND 1 STRAIN OF B. TRUNCATUS TO B. NATALENSIS

Snail	*Experiment number	Number snails exposed	Number miracidia per snail	Number of survivors	Number survivors infected	% survivors shedding cercariae
1. B(P)africanus (Nelspruit)	1	16	15	15	13	86.7
	2	40	12	40	27	67.5
	3	50	12	42	30	71.4
	4	50	10	36	33	91.7
	5	30	5	24	21	87.5
2. B(P)africanus (Barnes)	1	15	15	8	2	25.0
	4	46	10	28	20	71.2
	5	32	5	25	11	44.0
3. B. truncatus (Iran)	4	42	10	20	0	0
	5	46	5	36	0	0
	6	60	15	42	0	0
	7	30	15	19	0	0

* Experiments bearing the same number were performed on the same day using miracidia from one pool of the schistosome eggs.

(see for example, Ogilvie, 1965). There is good evidence that in mammals these steroids act by depressing the phagocytic activity of the reticulo-endothelial system. Although an identical mechanism is unlikely to occur in invertebrates it has been reported by Bhattacharya and Chowdhury (1964) that mosquitoes that are insusceptible to Wuchereria bancrofti can be made susceptible by feeding them on corticosteroids. Since all the evidence available suggested that refractory and poorly susceptible snails defend themselves against schistosomes by means of a cellular reaction (see Part I) ("Host Factors"), it was thought that the use of cortisone or its derivatives might throw some light on the nature of the defence mechanisms of Bulinus against S. mattheei and other members of the S. haematobium group.

Preliminary observations in collaboration with Dr. M. F. A. Saoud indicated that the susceptibility of Australorbis glabratus to S. mansoni could be enhanced by feeding them on prednisone. A series of experiments was therefore designed to test the effect of hydrocortisone acetate on the susceptibility to S. mattheei of the two refractory strains of B. truncatus. The results of five experiments are shown in Table 3.

TABLE 3

THE EFFECT OF HYDROCORTISONE ACETATE ON THE SUSCEPTIBILITY OF B. TRUNCATUS TO S. MATTHEEI

Strain of *Bulinus truncatus*	Experiment number	Number snails on Hydrocortisone	Number control snails (alginate-lettuce)	Number control snails (fresh lettuce)	Number snails shedding cercariae
Iran	1	25	20	20	Nil
	2	25	20	20	Nil
	3	30	-	-	Nil
Egypt	4	25	20	-	Nil
	5	25	20	-	Nil

Note (1) All the snails were individually exposed to 15 miracidia in 5 ml. of water.

(2) Snails of experiment numbers 1 and 2 were exposed to miracidia 3 days after beginning to feed on hydrocortisone acetate. Each snail had consumed about 0.16 mg. of hydrocortisone.

(3) Snails of experiment numbers 3, 4 and 5 were exposed on the 2nd day, when each had consumed about 0.08 mg. of hydrocortisone.

(4) Except for experiment number 3 where snails were put back to fresh lettuce after exposure, all the snails were maintained on the same food throughout the experiment.

within a few days. Examination of the snails started about 30 days and continued up to 60 days after exposure to miracidia. It was concluded therefore that hydrocortisone acetate has no effect on the susceptibility of B. truncatus to infection with S. mattheei. Later studies showed that only a negligible proportion of S. mattheei succeeded in penetrating the Iranian strain of this snail (see 4(d) below). It appeared unlikely therefore that adrenocorticosteroids would influence its susceptibility to infection with S. mattheei and so these studies were discontinued. Contribution of the salivary glands. The two main pairs

(c) ATTRACTION OF BULINUS FOR SCHISTOSOMA MATTHEEI MIRACIDIA

The quantitative studies of Etges and Decker (1963), MacInnis (1965) and of Klootzel (1958) have provided evidence which strongly suggests not only that snail hosts exercise some attraction for schistosome miracidia, but also that there might be some degree of specificity in the attraction. (see "Attraction for Miracidia" under "Host Factors" in Part I). Preliminary observations under the dissecting microscope showed that when the miracidia of S. mattheei came close to specimens of any of the three strains of Bulinus under study, they were stimulated to a striking rate of swimming, turning and twisting. These turning and twisting activities tended to keep the miracidia

within a few millimetres of the snail. When the snail was free to move about, many miracidia followed its path, often getting out of the path, but always returning to it. The behaviour of miracidia following a snail resembled that of a hunting dog tracking its prey. However, as has been shown in Part I, only an objective test can produce really satisfactory evidence both for the existence and the specificity of attraction. Attempts were therefore made to estimate quantitatively the effect of the substances that the snails evidently passed into the water on the distribution of S. mattheei miracidia. The two test systems described above were employed for this purpose.

(i) The Ettos-Docker Method

It was found that in each of 12 experiments carried out with B (P) africanus and S. mattheei miracidia using the perspex apparatus, only a small proportion (usually less than 50%) of the miracidia could be recovered. And of those only a tiny fraction (less than 15%) entered the side chambers. Similar results were obtained in 6 experiments using Australorbis glabratus and S. mansoni miracidia. Experiments on the longevity of the organisms in the aquarium water (pH 8.0 to 8.2) used in the attraction experiments, showed that at 26°C. more than one half of

the initial number of S. matthoei miracidia was still active after 8 hours. This apparatus was in use for more than a year and it appeared unlikely that a substance (s) so rapidly lethal to miracidia was diffusing from the inert perspex material into water so quickly and for such a long time. Results of observations on the longevity of miracidia in water which had been standing in the apparatus for four or five hours supported this view.

It appeared possible that miracidia were being left behind in small drops of water when the perspex apparatus was drained. A glass apparatus which could be cleaned of all grease was therefore made. The only material other than glass that was used with this apparatus was plastic bungs which were introduced only at the end of the experiments and which had been shown to have no lethal effects on S. matthoei miracidia. Before use, the apparatus was left to stand overnight in a chromic acid cleaning mixture and then rinsed under the tap for several hours and finally in distilled water several times. The results of 6 experiments carried out on different days showed no improvement in the percentage of the miracidia recovered, nor in the numbers entering the side chambers. It was concluded, therefore, that use of the apparatus of Etges and Decker was not a reliable method of studying the

attraction of host snails for schistosome miracidia. A very simple and yet more effective "dumb-bell" method was consequently used to test the attraction of the snails for S. matthoei miracidia.

(ii) The "Dumb-bell" Method

It was found that during the first one or two minutes, miracidia continued to swim randomly in the side in which they had been introduced and in the bridge. Soon, however, miracidia entering the bridge exhibited the rapid swimming and turning behaviour characteristic of the organisms when they were close to the snail hosts. All the miracidia entering the "snail" side of the system showed this type of behaviour: when they came to the edge of the drop some of the miracidia behaved as if they had made contact with a snail surface, probing it and wagging the posterior end of their bodies in a way which closely resembled their behaviour when they attached to the soft parts of the snail hosts. When miracidia swam back into the other side of the system they quickly resumed their original, relatively slow swimming behaviour.

After about two or three minutes there was constant movement of miracidia from one side to the other and back again. However, the miracidia strongly tended to accumulate in the "snail" side of the test system.

This tendency is demonstrated in Table 4a which shows the distribution of S. mattheei miracidia in the three parts of the test system over a period of 15 minutes. The table shows the total number of miracidia counted at 3, 6, 10 and 15 minutes in the "snail" side, the bridge, and the clean water (called "miracidial" in the table) side for each of 10 experiments with each of the Nelspruit strain of B (P) africanus and the Iranian strain of Bulinus truncatus. For comparison, experiments were carried out in which aquarium water, uncontaminated by snails, was used instead of "snail" water. The results of nine of these experiments are included in the table. Whereas miracidia distributed themselves more or less evenly between the two ends of the system using uncontaminated water, when contaminated water was used there were, on average, at least twice as many miracidia in the "snail" side as there were in the "miracidial" side.

This marked tendency of the miracidia to accumulate in the water in which snails had been standing was evident in each of the readings at 3, 6, 10 and 15 minutes. The results of the experiments recorded in Table 4a were analysed in a different way, and Table 4b shows the total number of miracidia counted at each reading for each of the 20 experiments with bulinid snails and 9 with clean

TABLE 4a

THE TOTAL NUMBERS OF S. MATTHEAI MIRACIDIA COUNTED OVER A PERIOD OF 15 MINUTES IN EACH OF THE "SNAIL" AND "MIRACIDIAL" SIDE AND IN THE BRIDGE IN THE "DUMB-BELL" SYSTEM

Experiment number	B (P) africanus (Nelspruit)			Clean Water (Control)			B. truncatus (Iran)		
	"Snail"	Bridge	"Miracidial"	Water	Bridge	"Miracidial"	"Snail"	Bridge	"Miracidial"
1	21	5	14	20	7	13	16	5	19
2	27	2	11	14	9	17	36	1	3
3	30	2	8	15	5	20	30	3	7
4	25	6	9	20	6	14	20	1	19
5	27	4	9	13	2	13	29	6	5
6	31	4	5	22	1	17	26	2	12
7	23	3	14	17	7	16	23	3	14
8	30	1	9	16	3	21	26	2	12
9	30	2	8	20	4	16	26	2	12
10	10	1	29	-	-	-	27	1	12
Total	254	30	116	157	44	147	259	26	115

aquarium water. The relatively small numbers of miracidia found in the bridge are omitted from the table for the sake of clarity.

TABLE 4b

THE TOTAL NUMBER OF S. MATTHEEI MIRACIDIA COUNTED AT 3, 6, 10 AND 15 MINUTES IN THE "DUMB-BELL" SYSTEM

		Side	Minutes 3	6	10	15
1.	B (P) africanus (Nelspruit)	"Snail"	65	70	65	54
		"Miracidial"	27	26	26	37
2.	Control	"Water"	40	42	40	35
		"Miracidial"	37	32	38	40
3.	B. truncatus	"Snail"	71	62	62	64
		"Miracidial"	24	29	30	30

The results recorded in Tables 4a and 4b showed clearly that S. mattheei miracidia were attracted by both B (P) africanus and B. truncatus. Therefore, attraction for miracidia could not be an important factor in the differential susceptibilities of the three bulinid strains to infection with S. mattheei.

(d) PENETRATION OF SCHISTOSOMA MATTHEEI MIRACIDIA INTO BULINUS

With the knowledge that attraction for S. mattheei miracidia could not account for the differences in the susceptibility of the Nelspruit and Lwanza races of B (P) africanus and the Iranian strain of B. truncatus, it was decided to use histological techniques to compare (i) the number of miracidia penetrating the three snails; and (ii) the process of penetration.

(i) Numbers of Miracidia Penetrating

Direct observations with a stereo-microscope showed that miracidia quickly attached themselves to the soft parts and attempted to penetrate all three bulinid strains. Once they were attached, the miracidia were very active, with repeated contractions and rotation of the body and wagging of the posterior part of the body of the organism, suggesting a determined effort to penetrate. But the majority of miracidia attached for only a short period and then swam away to some other part of the snail. This, and the fact that many miracidia settled under the snail, where they could not be seen, made direct observation an unreliable method of determining the numbers of miracidia that penetrated the snails, and because of the rapid disintegration of a proportion of the miracidia in

water, it was of little value to assess penetration from the numbers left behind. It became necessary therefore to use the extremely laborious method of exposing snails individually to known numbers of miracidia and counting the number of parasites in complete serial sections of the snails.

The observations on the numbers of miracidia which penetrated were combined with a study of the response of host tissues to the sporocysts during the first 48 hours. For this purpose, after exposure, snails were fixed at 7 to 8, 20 to 22, and 48 hours. The number of parasites recovered at these times in the three strains of Bulinus is shown in Table 5a. The results showed that the highest number of miracidia penetrated the Nelspruit race of B (P) africanus and the lowest into the Iranian snail. Slightly fewer miracidia penetrated the Mwanza race of B (P) africanus than the Nelspruit one. The difference between the number of parasites which penetrated B (P) africanus and B. truncatus is emphasized by the fact that whereas each snail of the former species had been exposed to 15 miracidia, those of the latter had been exposed to 30 miracidia each. The results represent a recovery rate of 26% in B (P) africanus (Nelspruit), 15% in B (P) africanus (Mwanza) and 1.6% in B. truncatus. It will also be noted from Table 5a that there was a marked difference between the

TABLE 5c.

THE NUMBER OF S. HAEMATOBI PARASITES RECOVERED IN THREE SERIES OF SNAILS*

Hours after exposure	B (P) africanus (Nelspruit)			B (P) africanus (Umtali)			B. truncatus (Iran)		
	Number snails examined	Number snails infected	Number sporocysts recovered	Number snails examined	Number snails infected	Number sporocysts recovered	Number snails examined	Number snails infected	Number sporocysts recovered
7 to 8	5	5	26	5	4	15	5	2	5
20 to 22	5	5	18	5	4	13	5	1	1
40	5	4	14	5	3	5	-	-	-
Total	15	14	58	15	11	34	10	3	6

* All the snails fixed at 7 to 8 and 20 to 22 hours had been exposed on a single occasion, and those at 40 hours on another occasion.

numbers of B (P) africanus and B. truncatus which miracidia succes fully penetrated. The time differences in the number of parasites recovered in each of the races of B (P)africanus are, for the most part, accounted for by an apparently spontaneous degeneration of miracidia inside snail tissues (see below under "Host Response"7.

In view of the small number of miracidia which penetrated the Iranian snail, another batch of 26 young and adult B. truncatus were exposed to 60 miracidia each and fixed at 8, 20 and 48 hours. The numbers of parasites recovered are shown in Table 5b.

TABLE 5b

THE NUMBER OF S. MATTHEEI SPOROCYSTS RECOVERED IN BULINUS TRUNCATUS (IRAN)

Hours after exposure	Number snails examined	Number snails infected	Number sporocysts recovered
8	8	2	5
20	8	2	2
48	10	5	5
Total	26	9	12

The results of this experiment confirmed those of the previous one. Only a small proportion (about 0.3%) of the miracidia offered to B. truncatus successfully penetrated, and only 9 out of 26 snails had parasites in them.

Similar results were obtained in a series of observations carried out on a small number of snails of the Kisumu strain of B (P) africanus and the Iranian B. truncatus using the Kisumu strain of S. bovis. It was found that when the parasite was first isolated, a larger proportion of the miracidia penetrated the highly susceptible B (P) africanus than the slightly susceptible B. truncatus. However, because of the subsequent changes in the infectivity of the miracidia and the variation in egg shape (see under "Material and Methods") these observations were not extended.

(d)(ii) The process of penetration

The results presented above clearly indicated that the main factor or factors responsible for the differences in the susceptibility between B (P) africanus and B. truncatus were associated with the surface of the snail. A first step towards an understanding of the mechanism operating at the snail surface would be the discovery of the stage at which, in B. truncatus, the process of penetration fails. Two obvious possibilities are that either when attached to B. truncatus most of the miracidia fail to discharge the

contents of their penetration and apical glands, or the contents are discharged but they are ineffective in digesting the tissues of the snail host. It was decided to study the contents of the glands of the miracidia, as a preliminary to an examination of the stage where the organisms failed to penetrate B. truncatus. For this purpose, two substances were chosen which, if present, would occur in relatively large quantities. These are mucin, which has been reported in S. mansoni miracidia (see Part I under "Parasite Factors"), and the proteolytic enzyme leucine aminopeptidase which might be expected to occur in histolytic secretions.

Observations were first carried out using S. mansoni because adequate supplies of the miracidia of this species were more readily available at the time. For mucin tests, heavy concentrations of the mirncidia were fixed in Bouin's fluid and, after dehydration, the larvae were incubated with alcian blue (see Chapter 5), either on slides or in watch glasses. In 3 experiments, which involved a large number of miracidia, no evidence of mucin was found in any part of the organisms. Similarly, several attempts with fresh miracidia failed to show any mucin in the penetration or apical glands or any other part of the miracidium.

The aminopeptidase tests were carried out on heavy concentrations of miracidia which had been fixed for 2 or 12

hours in acetone at 0 to 4°C. Miracidia were individually placed on glass slides and left for 1 to 6 hours at 37°C. in the incubation mixture, with L-leucyl-4-methoxy-beta-naphthylamide (Sigma Co.) as the substrate. After incubation the material was treated with copper sulphate solution and mounted in glycerine jelly. (Details of the method followed here are given fully in Barka and Anderson, 1963). Fresh frozen, cryostat sections of the proximal convoluted tubules of the mouse kidney served as positive controls. In 4 experiments, no aminopeptidase activity was found in the miracidium although it was readily demonstrated in the epithelium of the convoluted tubules. It was concluded therefore that none of the glands of the miracidia contained the enzyme leucine aminopeptidase. The miracidia of different species of Schistosoma are so similar in their other characteristics that it appeared unlikely that those of S. mansoni and S. matthei would differ in the occurrence of such major substances as mucin and aminopeptidase. These observations were therefore not extended to S. matthei.

Electron microscopy offered another approach to the problem of penetration. A knowledge of the fine structure of the miracidium might show, for example, the means by which the organism attaches to the snail surface and the factors of the miracidium that lead to the discharge

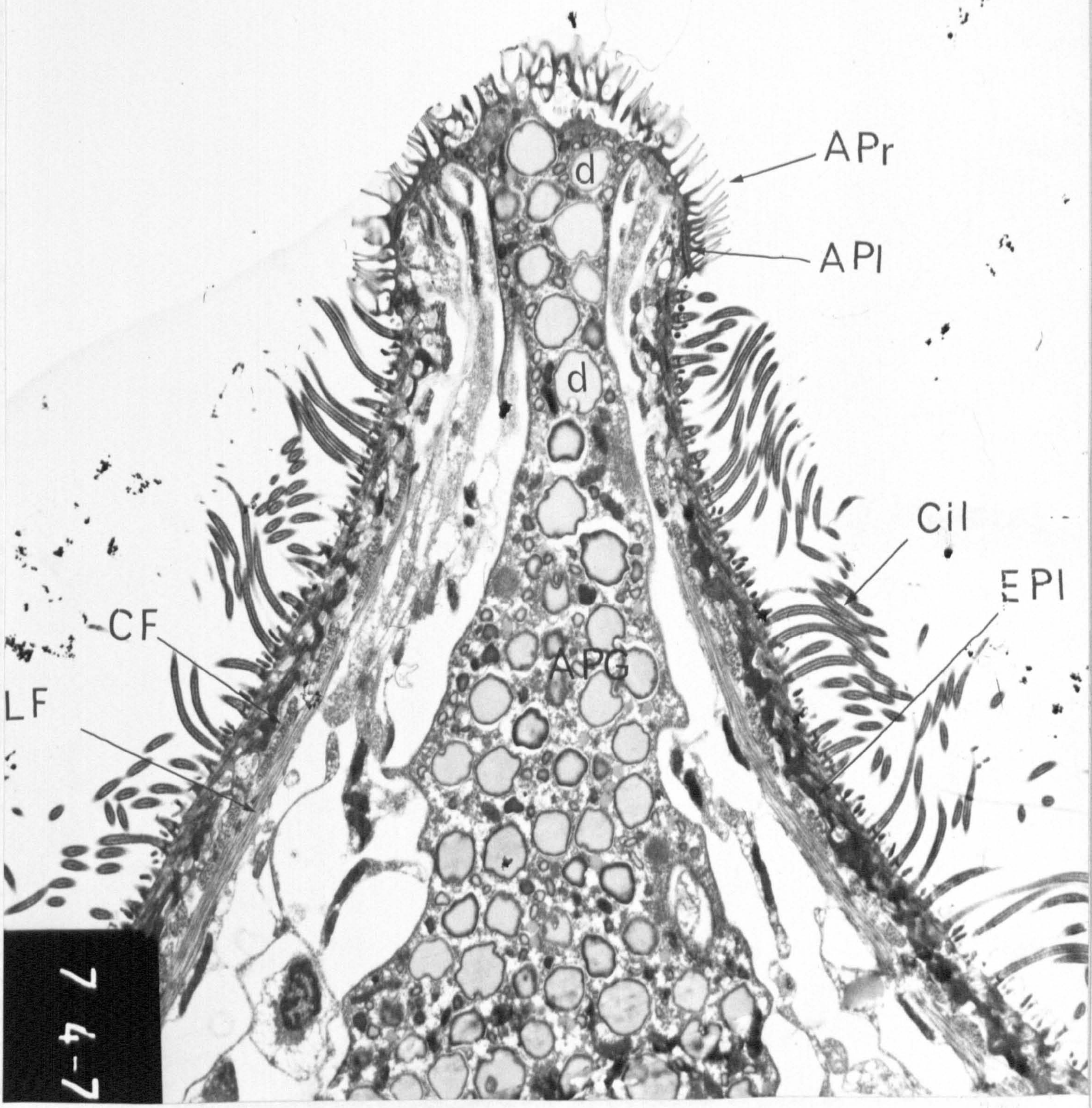
of the contents of the penetration and apical glands. This information would indicate the sort of specificity between the miracidium and the snail surface that is suggested by the results of the observations on the numbers of the larvae that penetrated the three strains of Bulinus. Observations on the fine structure of S. mattheei miracidium were therefore started.

Initial observations have revealed a number of interesting facts about the structure of the apical papilla and the so-called glands. In light microscopy with both fixed and fresh material, the surface of the papilla appeared completely smooth. The electron microscope, however, showed that the papilla is covered by an epidermal plate which bears an elaborate system of branching and anastomosing processes (Figs. 23, 24). The papilla itself has a more complicated organization than light microscopy reveals: for example, what appears like a sensory organ was found very close to the tip of the papilla (Fig. 24). Although difficulties with the fixation of the material have not yet been completely overcome, no differences have been found in the structure of the apical and penetration glands. Each of the glands is a single flask-shaped cell with a long neck whose tip rests against the inner surface of the apical epidermal plate (Figs. 25, 26, 23). The nucleus of

Fig. 23. Longitudinal section of anterior part of a miracidium of S. matthoei

Apl = apical epidermal plate
APv = apical processes
APG = apical "gland"
EPl = Epidermal plate
CF = Circular fibres
LF = Longitudinal fibres
d = droplets in the apical "gland"
Cil = cilium.

(x 10,500)



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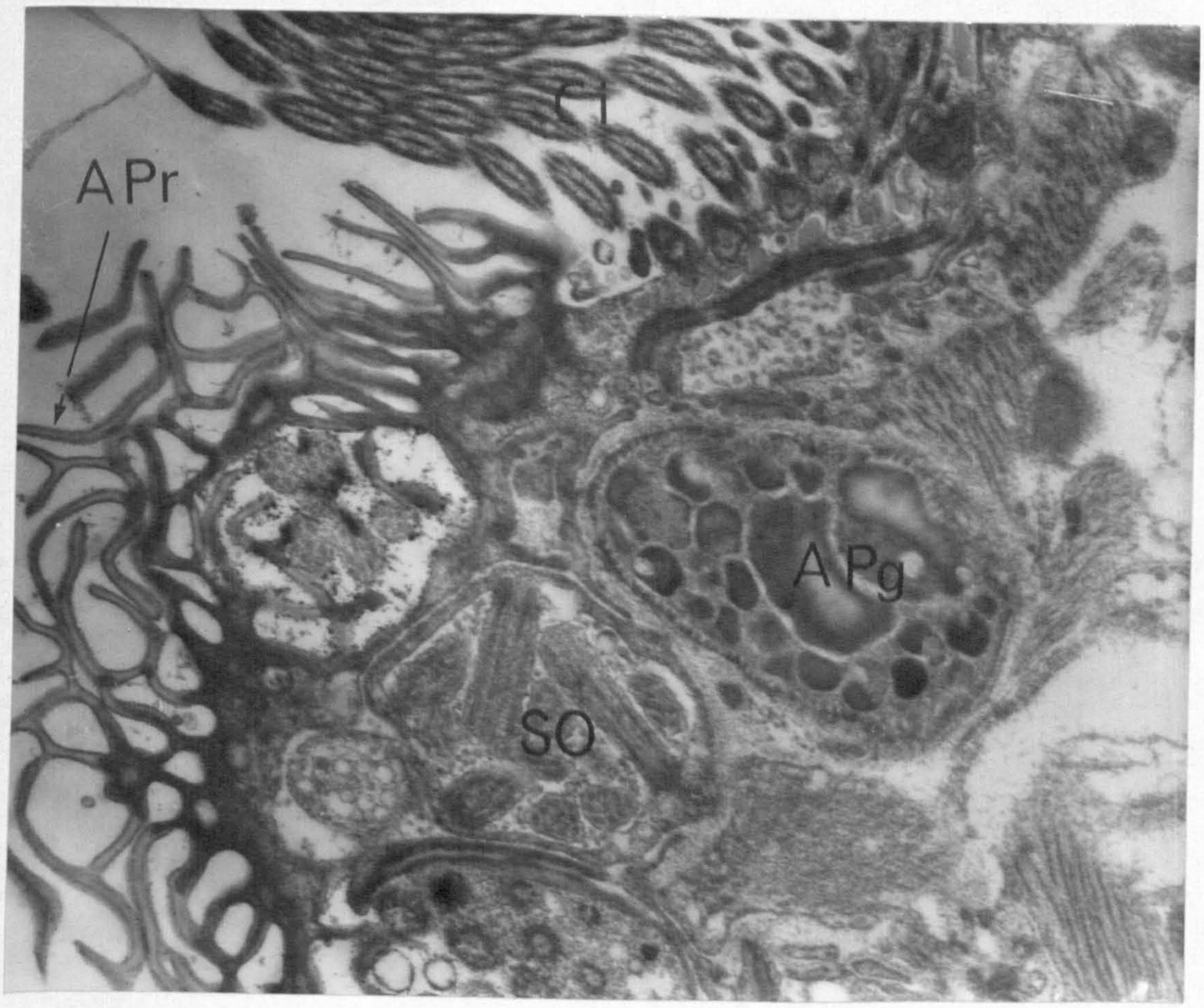

Fig. 24. Apical processes of S. mattheei miracidium seen under higher magnification (x 40,000)

APr = apical processes
SO = ? sense organ
APG = apical "gland"
Cil = cilia

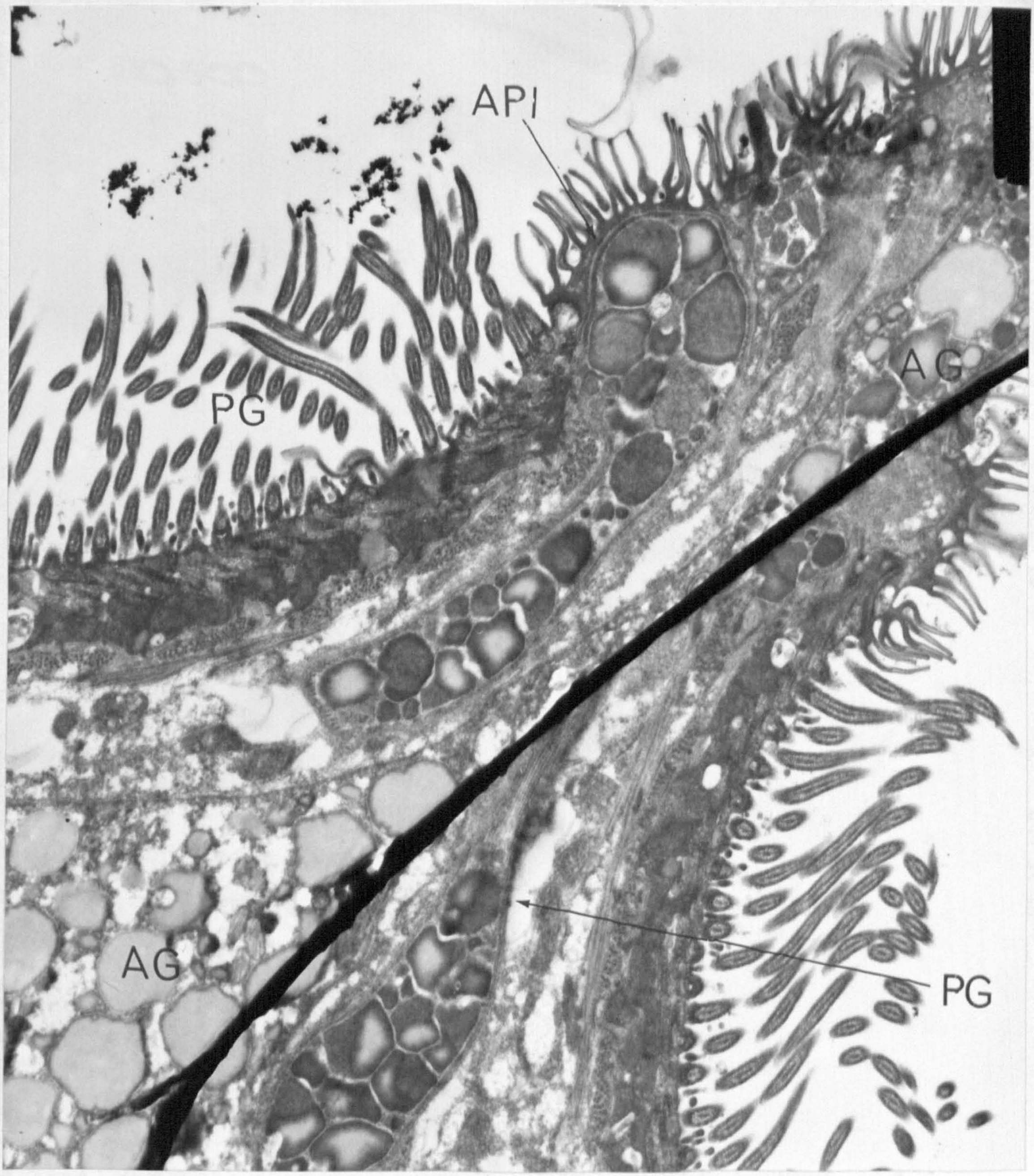

Fig.26. Longitudinal section of anterior part of S. mattheei miracidium. Note: (1) the "glands" end blindly below the apical epidermal plate (2) diagonal black line is due to a fold in the section.
PG = penetration "gland" API = apical plate
AG = apical "gland" (x 10,500)

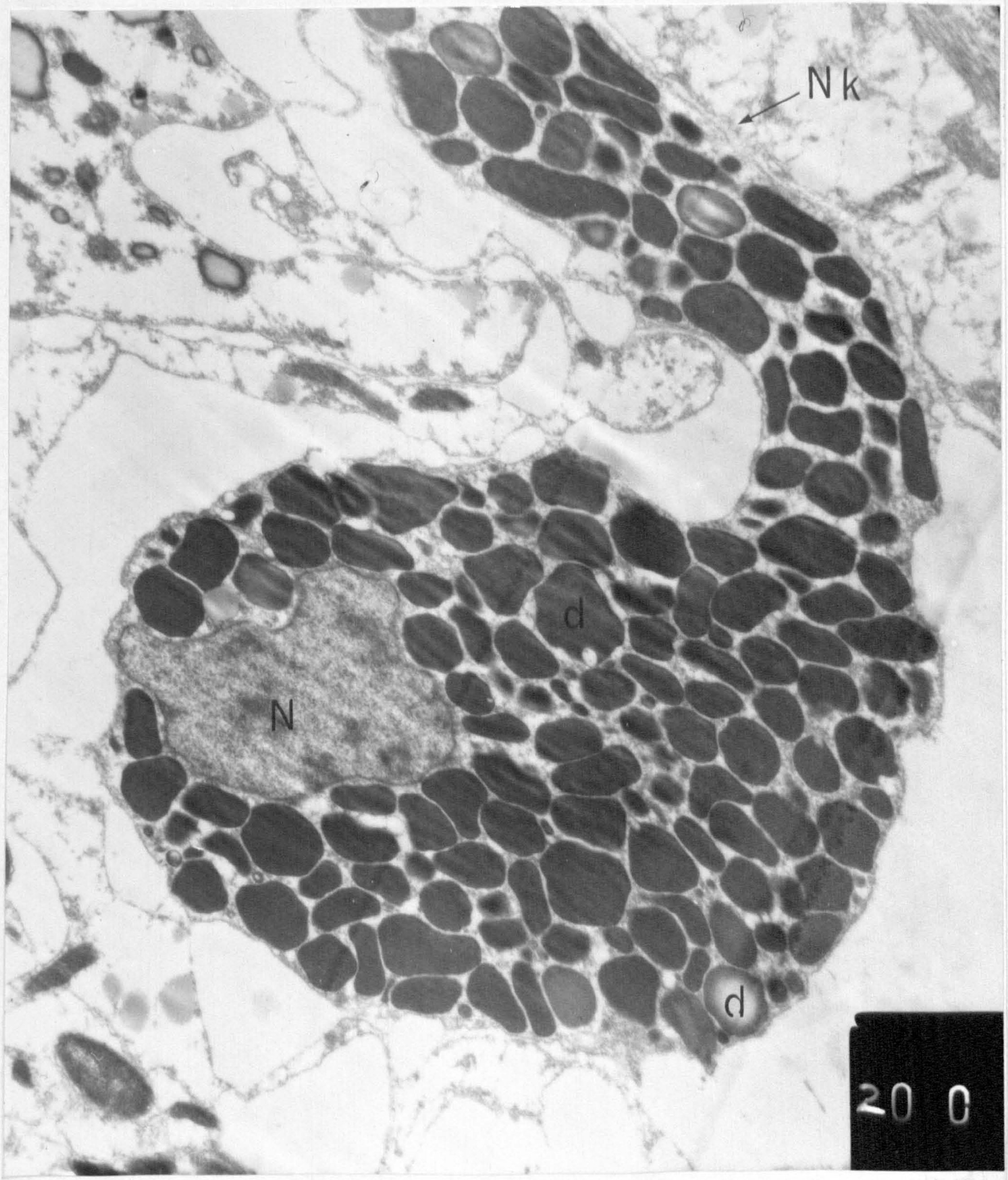

Fig. 25. Penetration "gland" of S. mattheoi miracidium.
N = nucleus
Nk = neck leading to apical plate
d = droplets.

the cell is basal (Fig. 25) and the cytoplasm which is delimited by a simple membrane, is full of droplets of different sizes. These droplets probably contain a hystolytic substance(s) of the miracidium. No evidence was found of an endoplasmic reticulum, either granular or agranular; nor were free ribosomes prominent in the cytoplasm. In the mature miracidium, these cells cannot therefore be actively glandular. They seem to be simple bags of the histolytic substances which must have been synthesized at an earlier stage in the life of the organism. No muscle or other fibres have been found in association with the cells and the cells are probably emptied by the contractions of the longitudinal and circular fibres (probably muscle) of the body wall of the miracidium.(Fig.23).

These observations suggest that further electron microscope studies may help towards an understanding of the parasite factors that determine the successful penetration of the miracidium into its snail hosts.

(c) THE RESPONSE OF HOST TISSUES TO SCHISTOSOME INFECTION

It was found that when a miracidium of S. mattheei had penetrated the tissues of the host snail, one of three things happened to it: (1) it rapidly degenerated apparently spontaneously or (2) it was soon encapsulated by a cellular

reaction, or (3) it proceeded to develop without any evidence of interference by the host. Although there is no evidence that spontaneous degeneration of the parasites was caused by a response of the host snail, it is convenient to consider it in this section.

(1) Spontaneous Degeneration

Using complete serial sections of snails which had been exposed to 15 miracidia each, it was found that a high proportion of the miracidia which penetrated B (P) africanus degenerated within 40 hours. In the early stages of degeneration, the miracidium shrank, the body lost its turgidity and became shrivelled, and the nuclei of the germinal cells appeared piknotic. In later stages the miracidium could only be identified by the neural mass whose small nuclei and eosinophilic fibres lasted longer than the other structures of the organism (see Fig. 27). The proportions of the numbers of degenerate to normal parasites that were recovered in each of the two races of B (P) africanus are shown in Table 6a. The results show that the number of degenerating parasites recovered had an inverse relation to the time after exposure when the host snails were killed. Consequently, in each race of B (P) africanus the total number of parasites was directly dependent on the age of the infection. It has already been

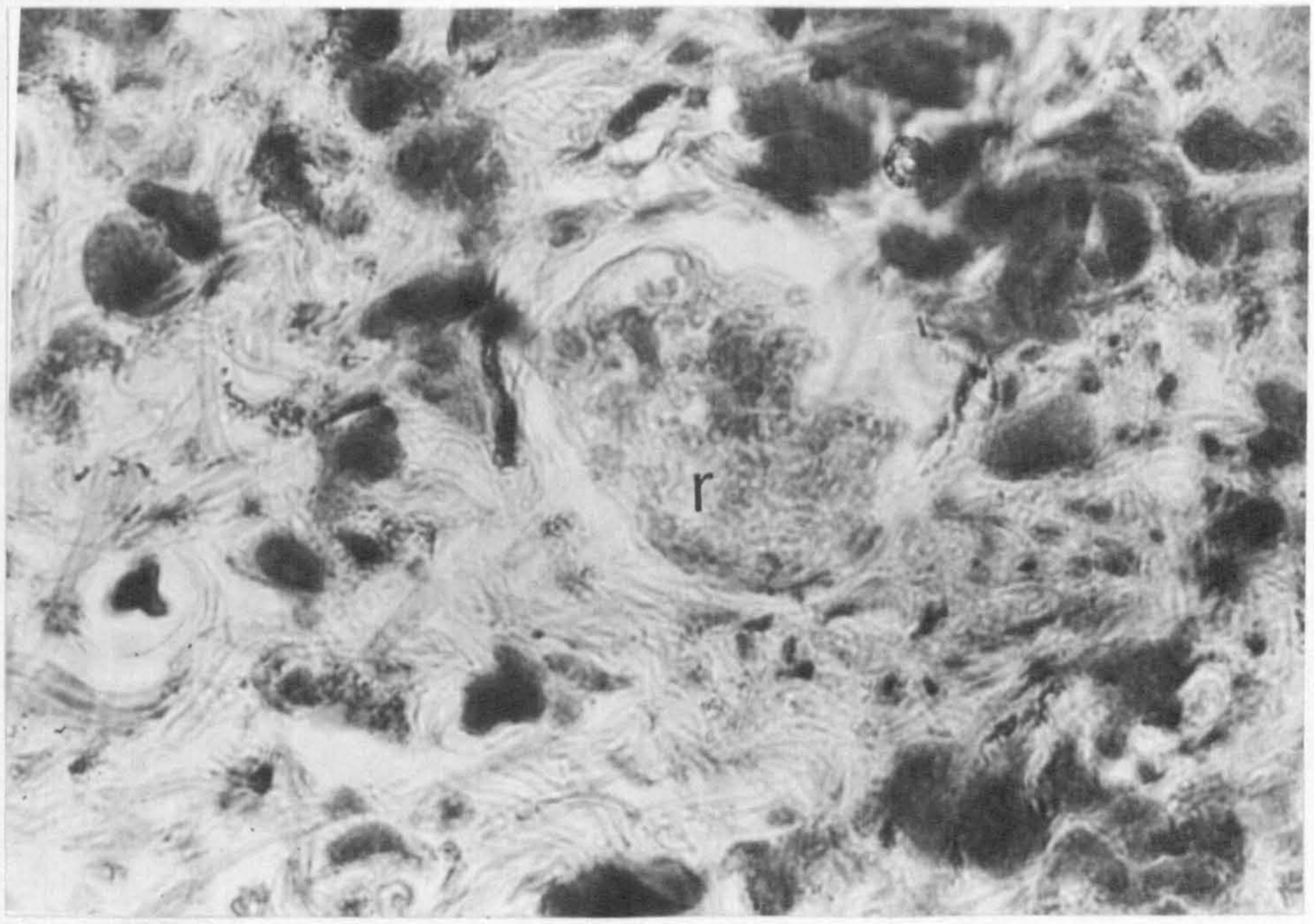

Fig. 27. Degenerate S. mattheei miracidium (8 hours) in dense tissue of B (P) africanus head-foot (cf. Fig. 27b) r = remains of miracidium. x 670.

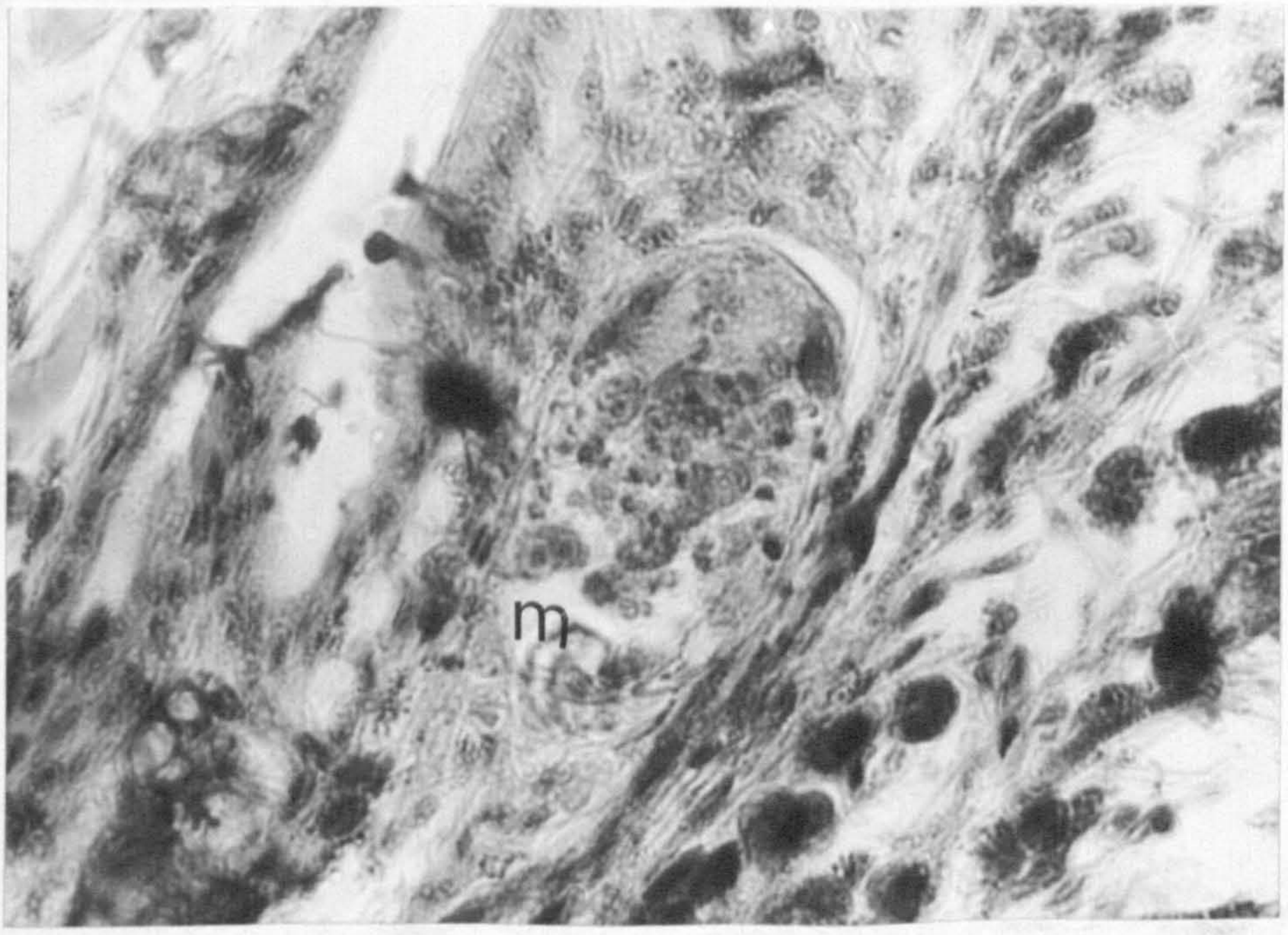

Fig. 27b. Normal S. mattheei miracidium (8 hours) in loose tissue of B (P) africanus head-foot.
m = miracidium x 670.

pointed out that very few miracidi penetrated B. truncatus. Therefore the results with this snail are not included in Table 6a, but it was noted that some of the parasites that penetrated B. truncatus degenerated in the same way as those in B (P) africanus.

TABLE 6a

THE NUMBER OF DEGENERATE AND NORMAL S. MATTHEEI PARASITES RECOVERED IN 2 RACES OF B (P) AFRICANUS

Race of snail	Hours after exposure	Number degenerate parasites	Number normal parasites	Number snails examined
1. Nelspruit	7	1	15	5
	20		13	5
	48		12	5
	Total	18	40	15
2. Lwanza	8	9	7	5
	20	4	9	5
	48	1	4	5
	Total	14	20	15

The density of the snail tissue in which the parasites settled appears to be the main factor which determined whether a miracidium would degenerate spontaneously. All the miracidia and mother sporocysts recovered in all the three strains of Bulinus occurred in the head-foot; none were found in the mantle or tentacle. The thickness of the tissues of the head-foot were classified into the loose, the dense, and the very dense. Loose tissue is characterized by large blood spaces and relatively few muscle and collagen fibres. It is found all round the head-foot immediately below the epidermal epitholium, but the largest expanses of it occur on the ventral side, that is, inside the ciliated epitholium of the sole of the foot. Towards its origin the columnolar muscle is the most dense tissue in the head-foot. Between these extremes lies dense tissue, which covers most of the organ. This classification is obviously arbitrary because the density of the tissues changes gradually not abruptly, but it provided a convenient working basis. It was found that whereas nearly all the parasites which settled in the loose tissue started to develop normally, the majority of those which settled in the dense tissue degenerated, as did all those in very dense tissue. The relationship between the condition of the parasites and the type of tissue in which they occurred is shown in Table 6b. The figures shown in this table

represent the total number of parasites recovered at 7 or 8, 20 and 48 hours for each of the two races of B (P) africanus. These results suggest that the density of tissue is very important in determining the fate of the parasite and it may be that some factor such as oxygen tension may be responsible for the degeneration of the parasites.

TABLE 6b

THE NUMBER AND CONDITION OF S. MATTHEEI PARASITES IN LOOSE, DENSE AND VERY DENSE TISSUE OF B (P) AFRICANUS

Race of snail	Condition of parasites	Loose tissue	Dense tissue	Very dense tissue	Number of snails sectioned
1. Nelspruit	Normal	33	7	0	15
	Degenerate	2	13	3	
2. Mwanza	Normal	20	0	0	15
	Degenerate	4	8	2	

(2) Cellular Response

It was found that B (P) africanus and B. truncatus differed in the stage of S. mattheei to which they reacted. This is indicated on Table 7 which shows the relationship between the age of the infection and the onset of cellular reactions in the three strains of Bulinus.

(c)(i) B (P) africanus

The observations recorded here were based on the snails shown in Table 7 as well as on a large number of snails which were examined with infections older than 70 days. It was found that in the Nelspruit race of B (P) africanus embryos of daughter sporocysts started forming by about the 8th day after exposure. Mature daughter sporocysts started migrating from the head-foot after another week and by the 16th day a few had arrived in the digestive gland. A week later, almost all the available space in the digestive gland had been taken up by the sporocysts which had grown very rapidly and already contained embryo cercariae. Cercariae started emerging from the snails by about the 30th day. Some daughter sporocysts settled in the gonad and the mantle, especially in the large blood spaces of the lymphoid tissue: in some snails a few daughter sporocysts were still present in the head-foot 70 days after exposure to miracidia. Apart from a slightly

TABLE 7

THE OCCURRENCE OF CELLULAR REACTIONS IN B (P) AFRICANUS AND B. TRUNCATUS

Snail	Time after exposure	Number snails examined	Number snails infected	Intensity of reaction
1. B (P) africanus (Nelspruit)	7 h.	5	5	Nil
	20 h.	5	5	Nil
	48 h.	5	4	Nil
	8 days	5	4	Nil
	24 "	4	4	Nil
	32 "	4	4	Severe
	70 "	4	4	Very severe
2. B (P) africanus (Mwanza)	7 h.	5	4	Nil
	20 h.	5	4	Nil
	48 h.	5	3	Nil
	8 days	5	4	Nil
	24 "	4	3	Nil
	32 "	4	4	Severe
3. * B. truncatus (Iran)	7 h.	15	4	Mild
	20 h.	15	3	Nil
	48 h.	10	5	Mild

* Most of the few parasites present in this snail underwent spontaneous degeneration (see preceding section).

(about 2 to 4 days) slower rate, the same pattern of development took place in the Nyanza as in the Nelspruit race of the snails and the two races behaved in the same way towards the parasite.

B (P) africanus did not show any evidence of cellular reaction to mother sporocysts at any stage of their development (Figs. 28a, 28b), not even when they degenerated after the migration of the daughters. Until the onset of cercarial shedding no reactions directed against daugh er sporocysts were found in the lymphoid tissue, digestive gland, or any other part of the snail (Figs. 29a, 29b). When cercariae started appearing in the tissues of the snail, the most noticeable change in the histology of the host was a marked increase in both the size and number of amoebocytes (Fig. 30). Considerable numbers of these enlarged cells occurred in the blood spaces of the mantle and in the few and small spaces that in
t e sporocysts left unoccupied/the digestive gland and gonad, especially in the space between the viscera and the tunica propria. As the infection progressed the number and evident migratory activity of the amoebocytes increased. There was also a great increase in the number and size of the pigment cells, especially in the mantle tissue. In the lymphoid tissue by the time the infection was 70

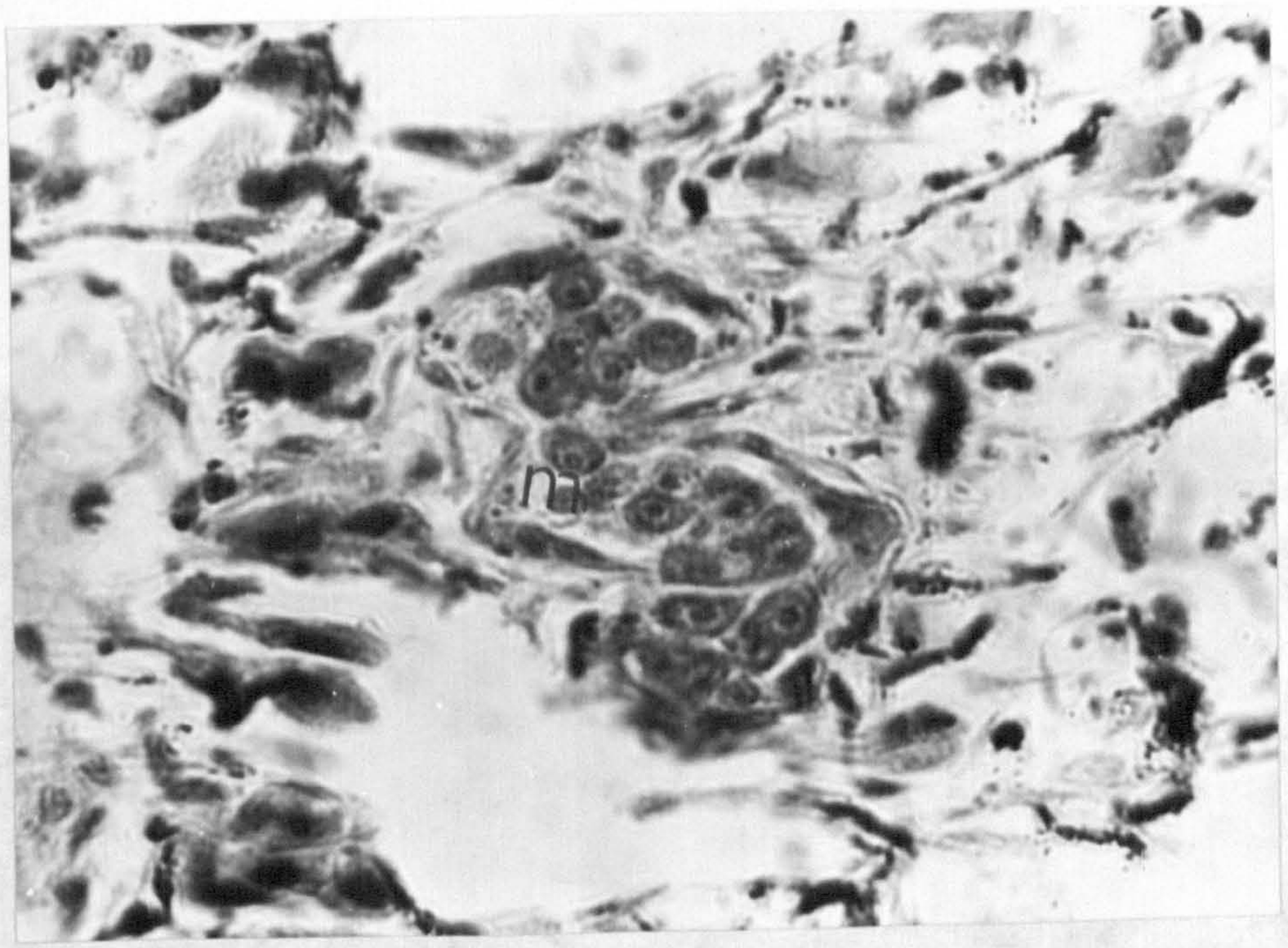

Fig. 28a. Mother sporocyst (48 hours old) of S. mattheei in the head-foot of B (P) africanus. x 670.

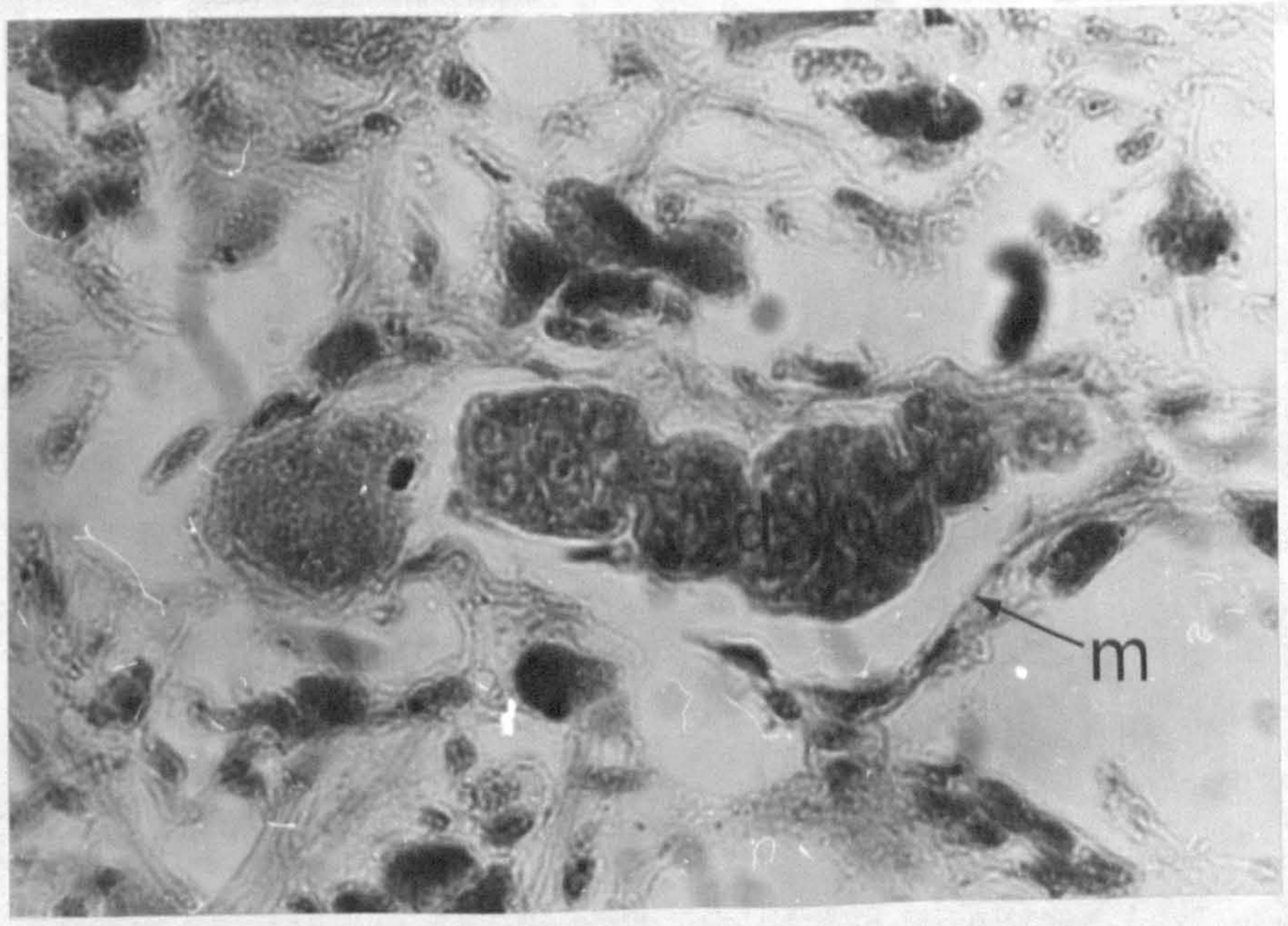

Fig. 28b. S. mattheei mother sporocyst (m) 16 days old, containing a daughter sporocyst (d). Head-foot of B (P) africanus. x 670.

Fig. 29a. Young S. mattheei daughter sporocysts (a) in blood spaces and stroma of the lymphoid tissue of B (P) africanus. x 670.

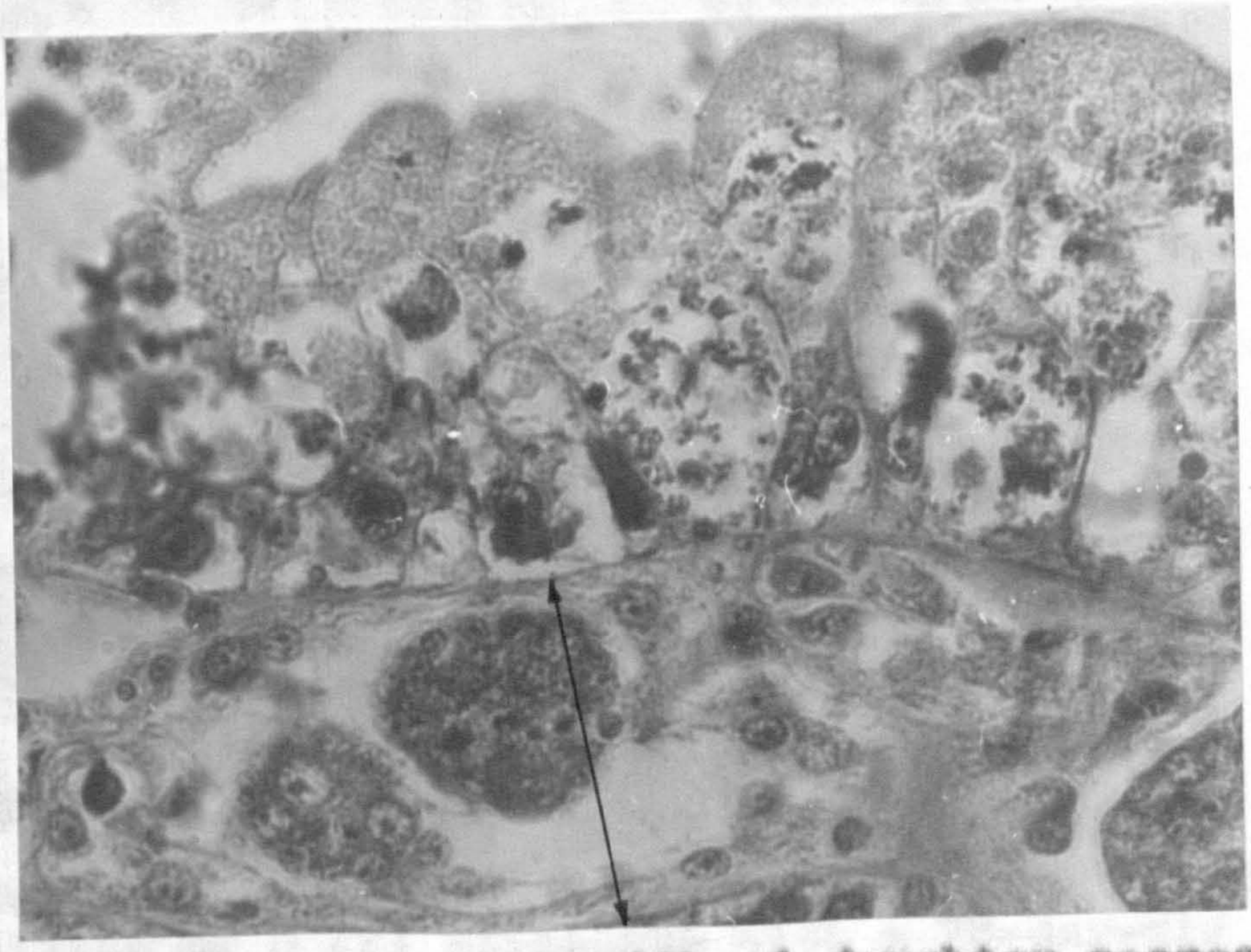

Fig. 29b. Part of a young S. mattheei daughter sporocyst (between arrows) in the digestive gland of B (P) africanus x 670.

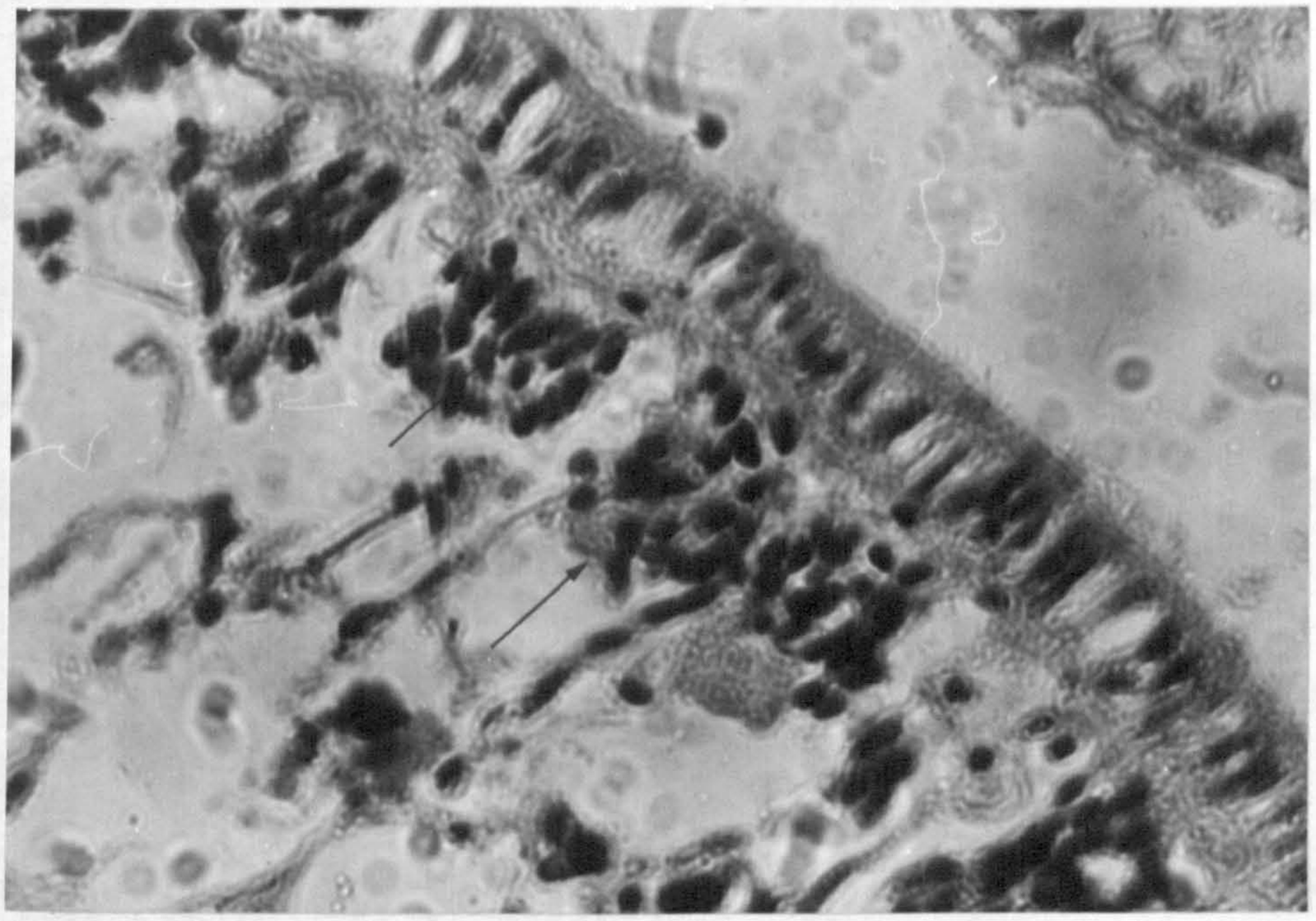

Fig. 30. Masses of amoebocytes enlarged (arrow) in blood spaces of head-foot B (P) africanus infected with S. mattheei. x 670

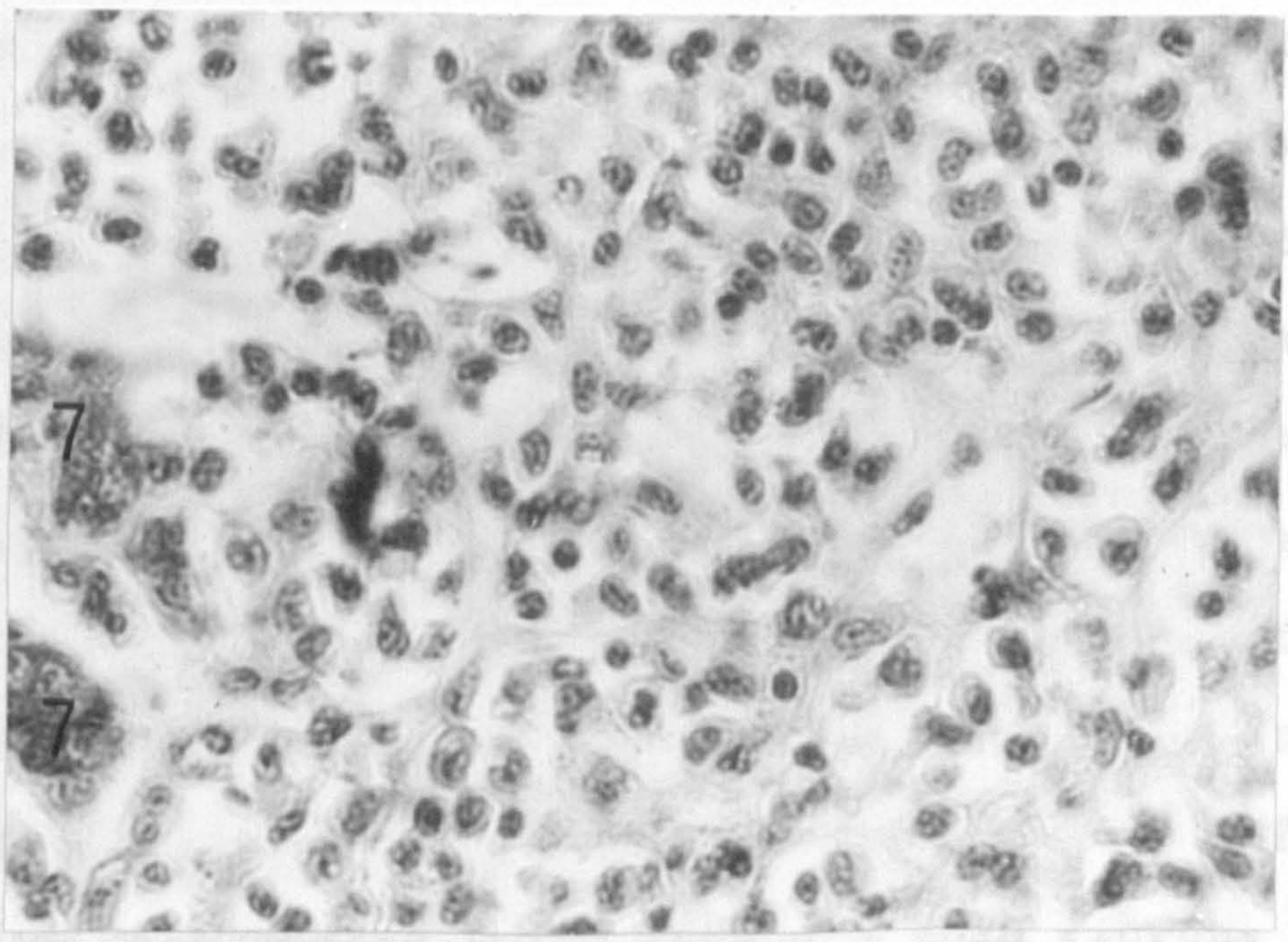

Fig. 31. Numerous amoebocytes in the lymphoid tissue of B (P) africanus with a 70-day-old infection of S. mattheei
(7 = sporocyst) x 670

days old almost all the cells of the stroma had changed into large amoebocytes (Fig. 31). No evidence of increased mitosis was found and in some snails there was evidence of considerable depletion of cells from this tissue (Fig. 32).

Reactions directed against individual cercariae were found in the digestive gland, gonad, mantle and head-foot. These reactions varied greatly in intensity but they consisted of the same cells, namely the amoebocytes. In intense reactions a large number of amoebocytes collected in concentric layers around a parasite (Fig. 33). Insome of these reactions the amoebocytes closest to the parasite retained their normal shape or became slightly elongate; outside this layer the amoebocytes stretched themselves considerably (appearing spindle-shaped and thin in section) and they formed a capsule which consisted of fibrous material and the nuclei of the cells. In other cases no free amoebocytes separated the parasite wall from the fibrous capsule. The intense reaction destroyed the parasite rapidly, after which the amoebocytes dispersed and the capsule disappeared. A less intense reaction against cercariae, consisted of relatively few amoebocytes which formed a thin fibrous capsule round the parasites (Fig. 34a). This type of reaction was commonest in the mantle and head-foot. The encapsulation apparently had the effect of sealing off the parasite from the tissues of the snail and such capsules

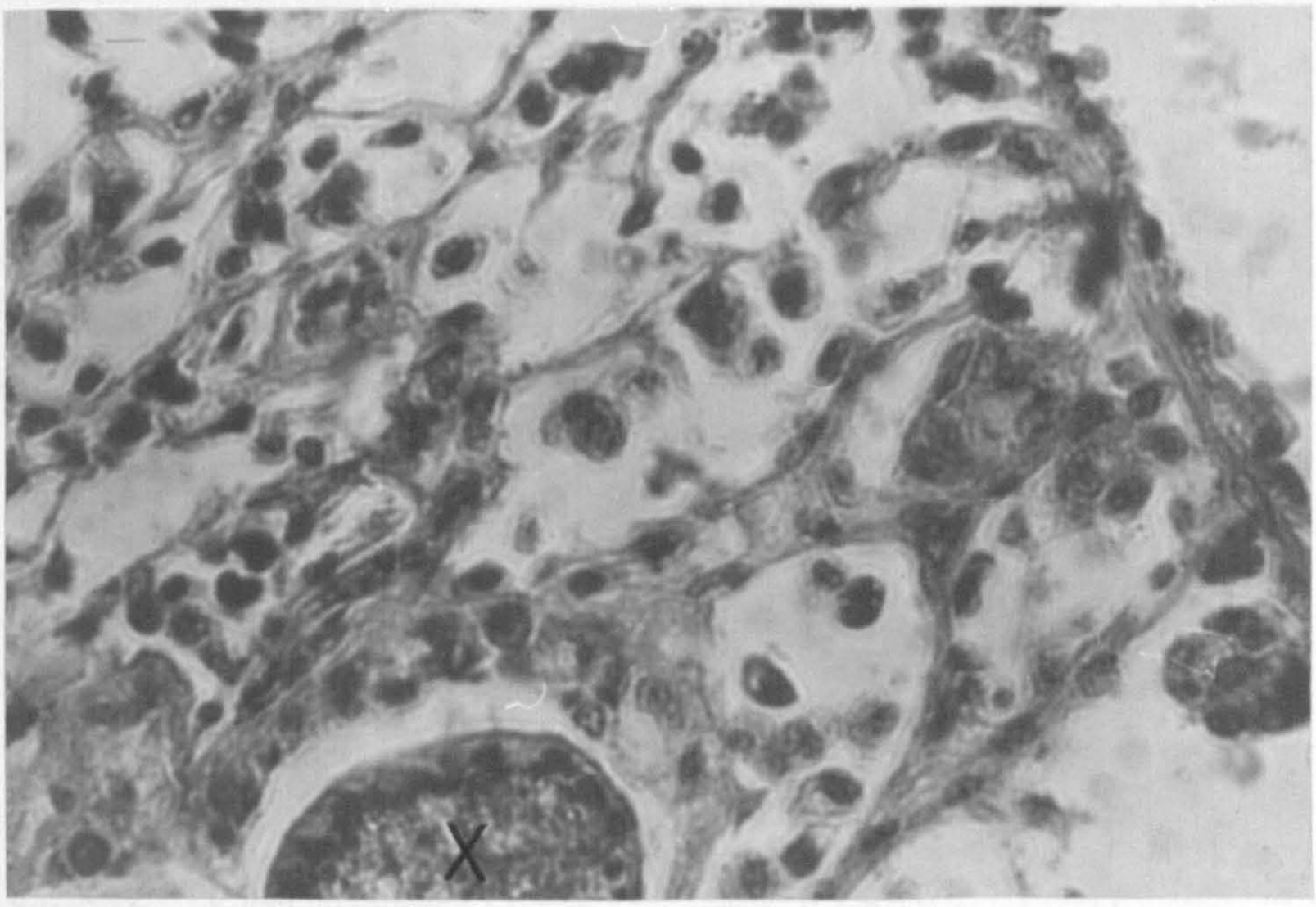

Fig. 32. Section of lymphoid tissue infected with S. mattheei to show reduction in number of amoebocytes (cf. Fig. 31). (X = section of cercaria) x 670.

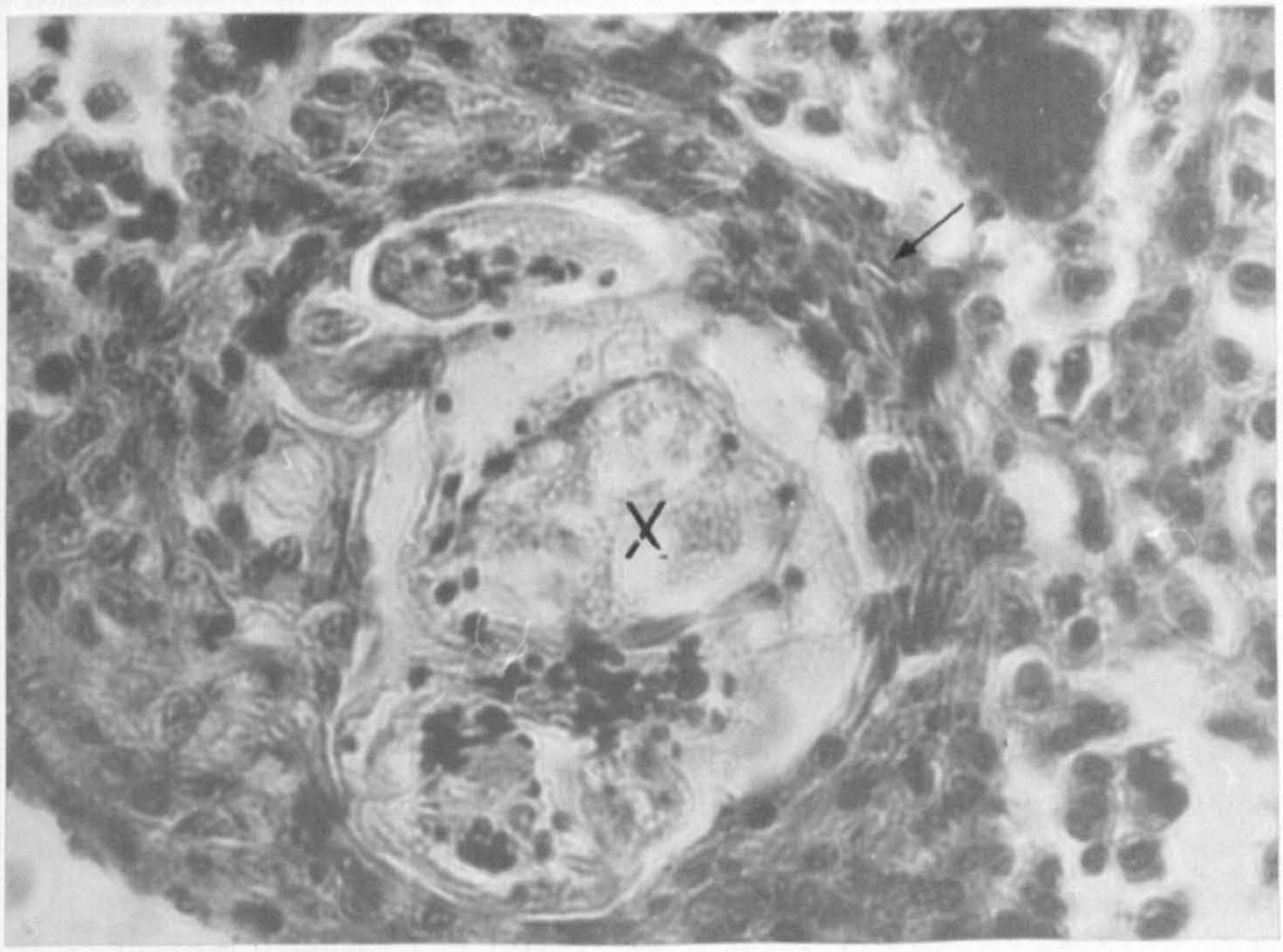

Fig. 33. Concentric layers of amoebocytes (arrow) round a cercaria (X) of S. mattheei in B (P) africanus. x 670.

remained in the body of the host for long periods of time. This is suggested by the fact that in infections which were 70 days old or more, the tissues of the parasites had disintegrated to a degree where only an amorphous substance remained and the contents of many capsules could be identified as cercariae only by their outline (Fig. 56b).

After the beginning of cercarial shedding, reactions directed against the parent daughter sporocysts or parts of the sporocyst became manifest. In the mantle, where this was (apparently) physically possible, capsules of amoebocytes formed round the sporocyst (Fig. 55). In the digestive gland where the sporocysts were, for the most part, pressed tightly against the epitheliun of the tubules, amoebocytes formed small plugs wherever there was room between the sporocyst wall and the host epithelium. These plugs consisted of amoebocytes and an eosinophilic material with a fine fibrous appearance. In some cases a few amoebocytes apparently squeezed themselves between a part of the sporocyst and the snail epithelium, forming a very thin fibrous layer which was no more than two or three cells thick. Although in comparison with the response against free cercariae these reactions were feeble, they nevertheless, gradually replaced sporocysts over quite extensive areas of the digestive gland. In old infections

Fig. 34. Remains of an S. mattheei cercaria (R) surrounded by a thin fibrous capsule (arrow) in B (P) africanus. x 670

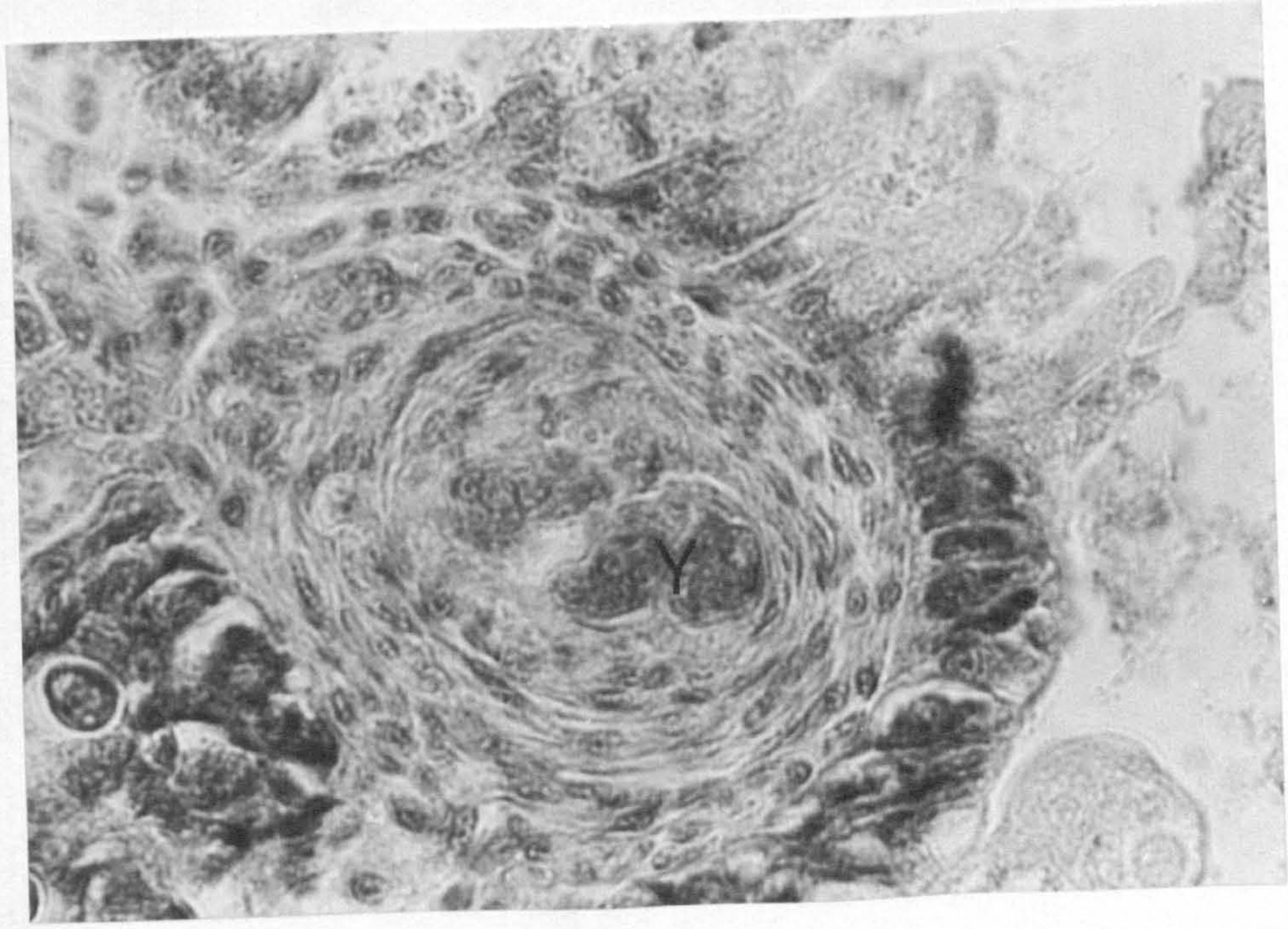

Fig. 35. Concentric layers of amoebocytes having replaced wall of S. mattheei sporocyst wall. Sporocyst situated in intertubular space of digestive gland of B (P) africanus
Y = germinal cell mass. x 670.

naked germinal cells were seen surrounded by the eosinophilic substance laid down by amoebocytes (Fig. 36). However, in none of the many infected snails that survived for two months or more after the beginning of cercarial discharge was there a complete destruction of the sporocysts. Amoebocytic reactions are therefore not totally effective in eliminating the parasites.

Although the main purpose of this study was to determine the effect of the host on the parasite it was impossible to dissociate this from the converse effect of the parasite on the host. These pathological changes are dealt with only briefly in this section; the subject has been reviewed recently by Wright (1966) with special reference to other schistosomes and other trematodes. With S. mattheei infections it was noted that in the head-foot both mother and daughter sporocysts caused very little damage to the tissues; this involved destruction of some collagen and muscle tissue. In the mantle the occurrence of large fibrotic areas, particularly in the large blood spaces of the lymphoid tissue, must cause some blockage of the blood circulation. The digestive gland and, to a lesser degree, the gonad, were the organs most seriously damaged. Although migrating cercariae must have caused some destruction, it was the pressure of the

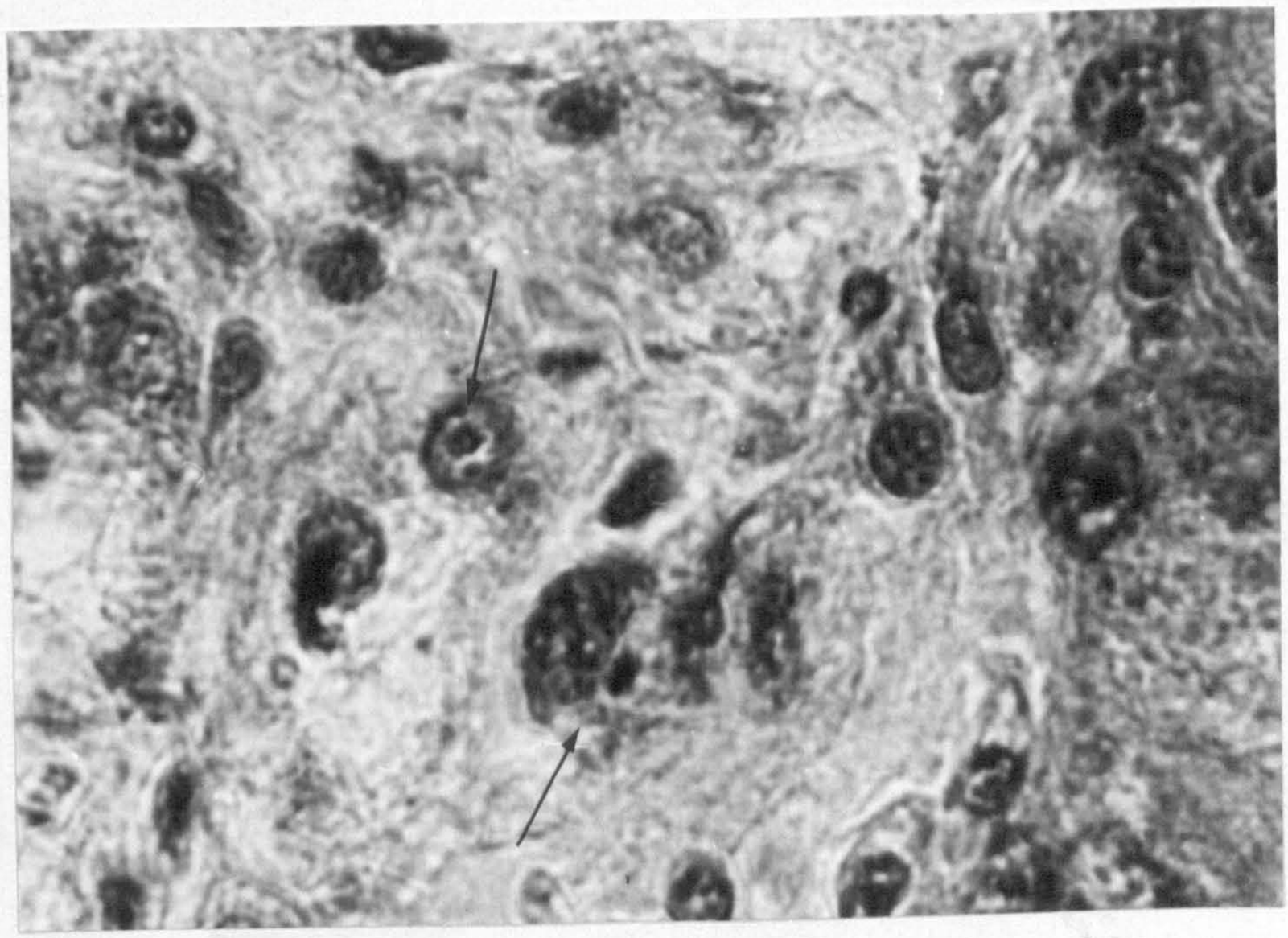

Fig. 36. Germinal cells (arrows) scattered in fibrous tissue and amoebocytes after destruction of the body walls of sporocysts of S. mattheei in the digestive gland of B(P)africanus (x 670)

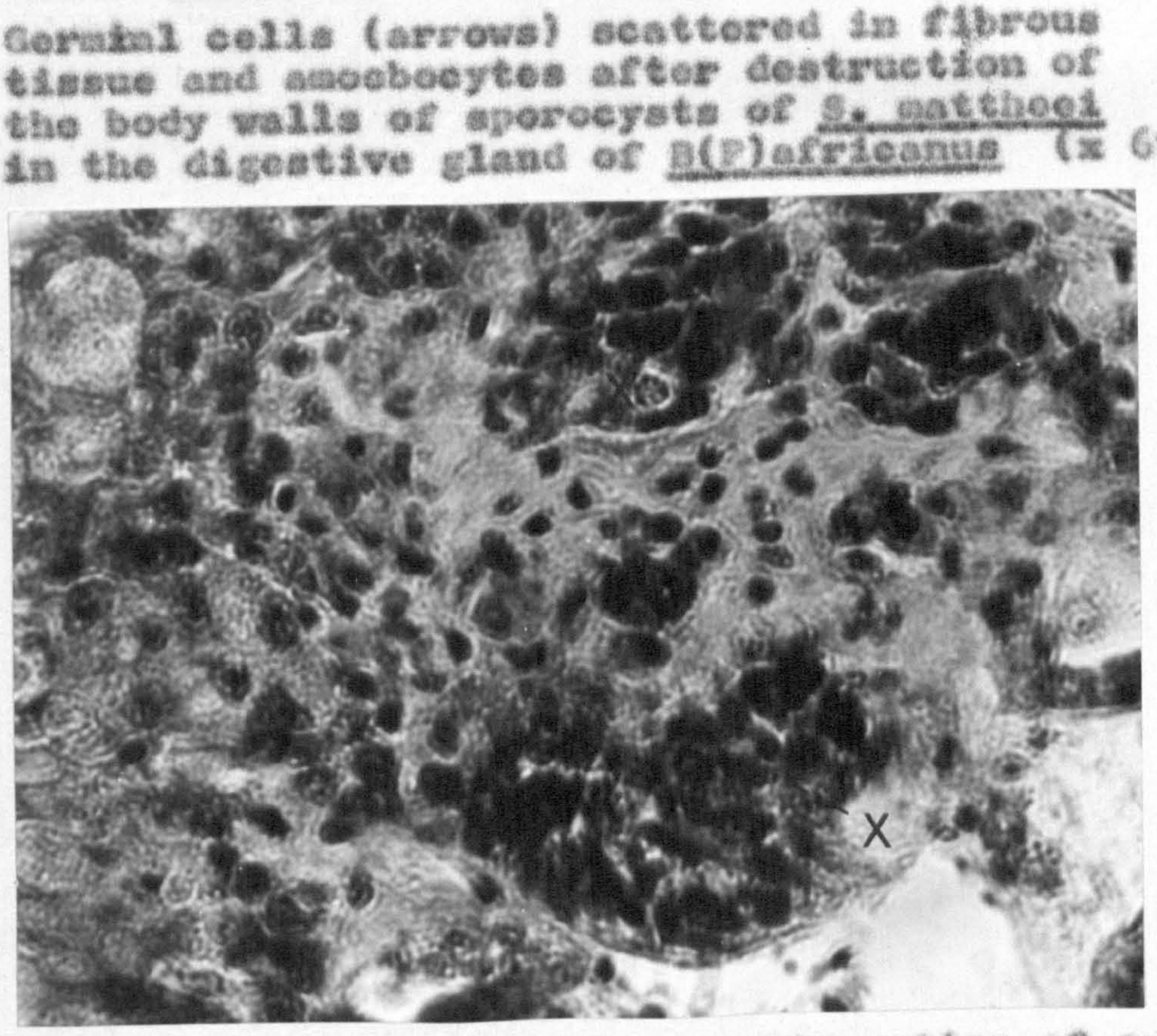

Fig. 37. Groups of nuclei (X) after degeneration of cytoplasm of epithelial cells of tubules of infected B(P)africanus; small nuclei are of amoebocytes. x 670.

rapidly expanding daughter sporocysts that caused the atrophy of many tubules of the digestive gland and of much of the germinal tissue of the gonad. Within about one week of their arrival in the digestive gland, the daughter sporocysts greatly distended the intertubular spaces, thus exerting considerable pressure on many of the tubules and causing the breakdown of the epithelial cells. The nuclei of these cells often persisted and appeared in a group, surrounded by parasite tissue or, in older infections, by amoebocytes and the granular eosinophilic substance that they produced (Fig. 37A). Sometimes, however, whole secretory cells escaped destruction and they appeared in groups (Fig. 37B), or, sometimes, scattered among the amoebocytes. But even when total destruction of the tubules did not take place the presence of the parasites had an important effect on the physiology of large parts of the digestive gland. This was evidenced by the loss of much of the basophilia of the secretory cells over parts of the digestive gland. Similar changes occurred in the gonad where, in old infections, only the remains of the germinal tissue, among amoebocytes, could be identified .

Observations on a limited number of snails, including a few that had been shedding cercariae for three

months showed that the Kisumu strain of B (P) africanus behaved towards S. bovis infection as did the Nelspruit and Mwanza races towards S. mattheei.

Most infected snails died within one month of starting to shed cercariae and this high mortality must have been mainly the result of the damage caused by the infection, particularly in the digestive gland.

(e)(ii) B. truncatus

The few miracidia that penetrated this snail and which did not degenerate spontaneously were surrounded by amoebocytes within 20 hours. The amoebocytic reaction was the same as that directed against cercariae in B (P) africanus: amoebocytes flattened themselves over the surface of the parasite and formed a complete capsule around it. The capsule consisted of a fibrous matrix formed by amoebocytes and layers of free amoebocytes on the outside (Figs. 39a, 39b). In every case these parasites must have been finally destroyed since, even after exposure to large numbers of miracidia (see (a) above), no cercariae were produced.

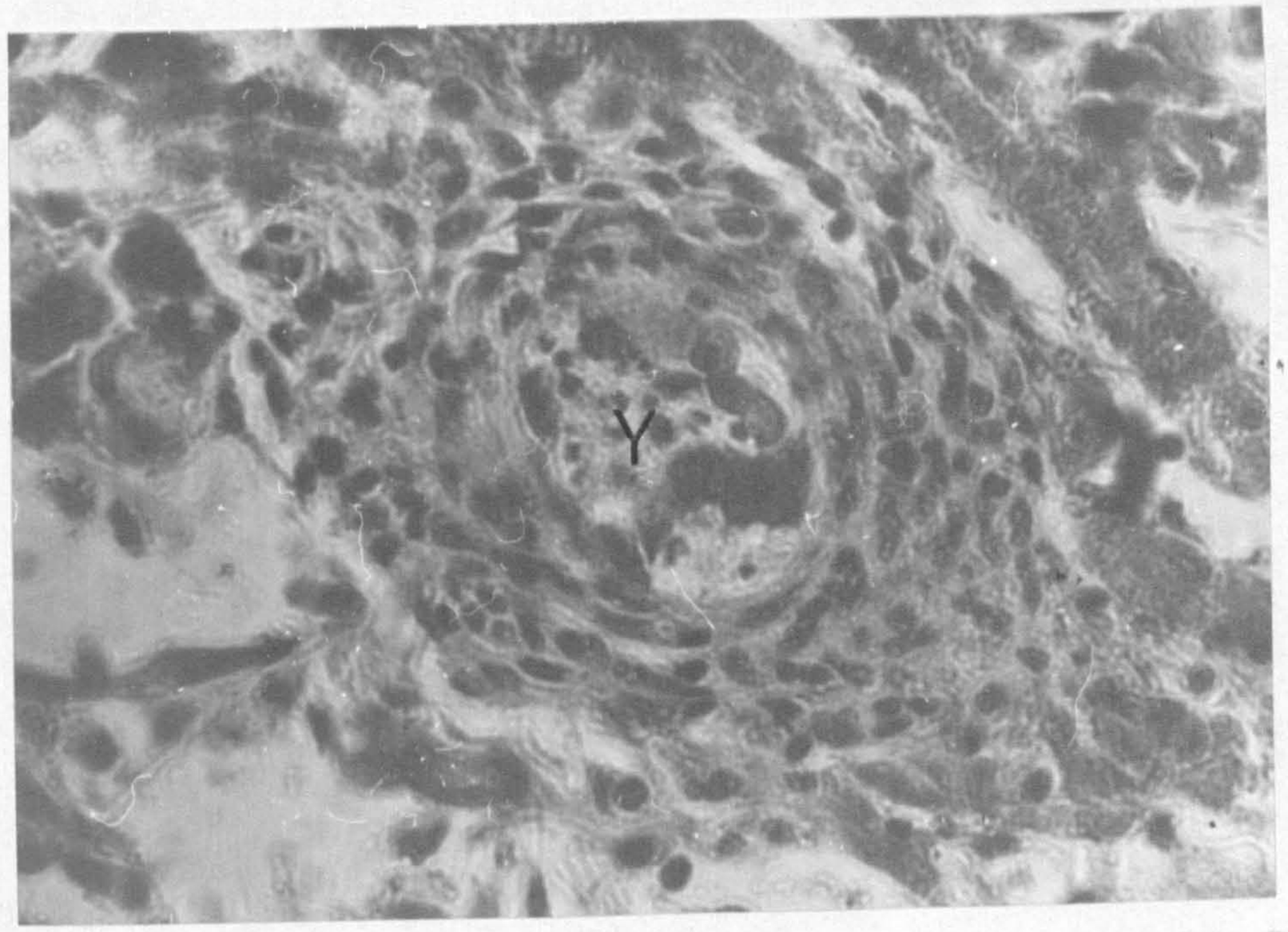

Fig. 39. Concentric layers of amoebocytes round a healthy S. mattheei miracidium (Y), (8 hrs. old) in head-foot of B. truncatus. x 670.

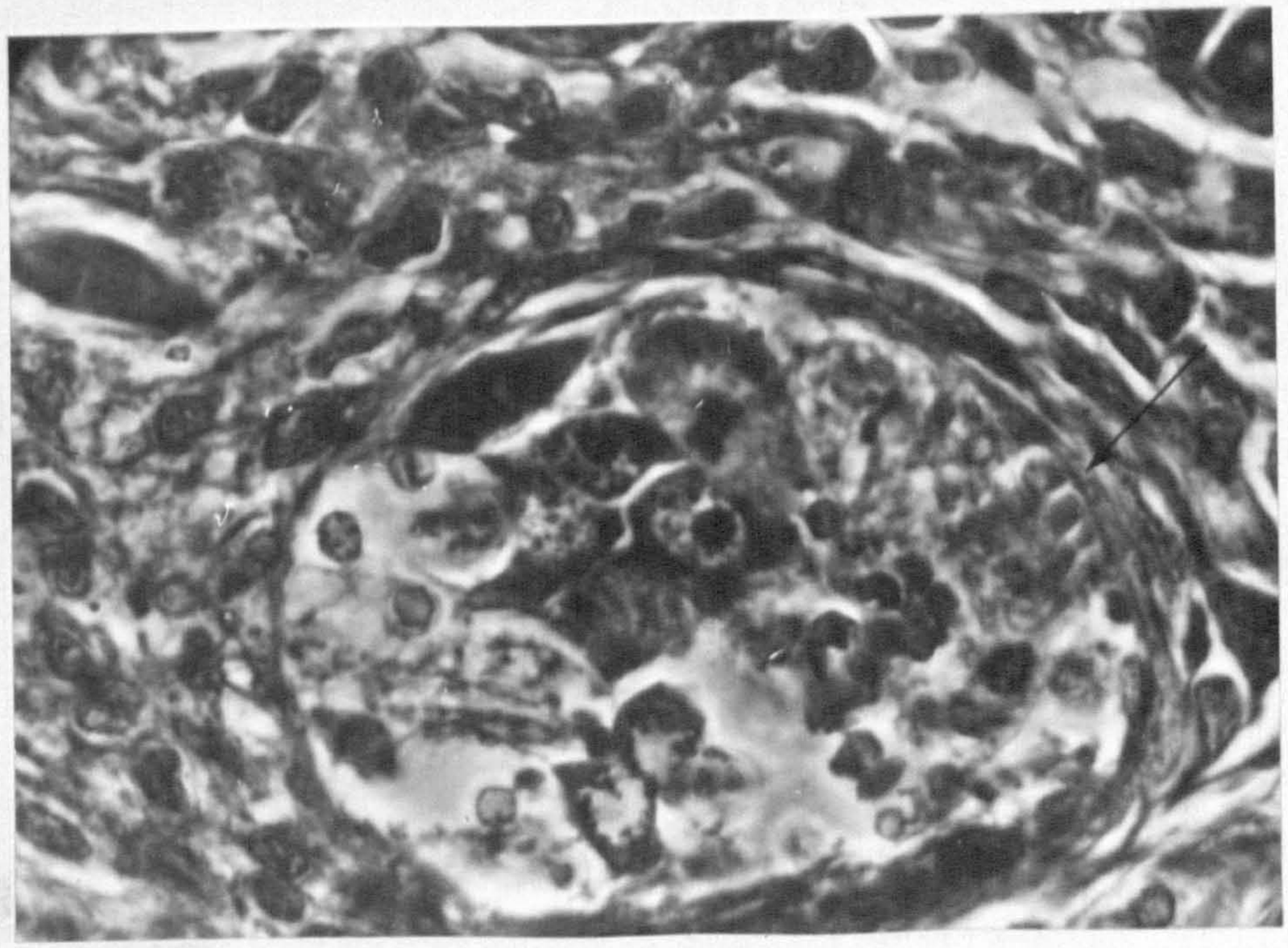

Fig. 39b. Higher magnification (x 1,620) of part of the capsule and miracidium shown in Fig. 39. Note large nucleoli of nuclei of the germinal cells of miracidium. Arrow indicates wall of miracidium.

4. DISCUSSION

Rates of Infection

The results of the susceptibility experiments (see Table 2) show that although within each strain of Bulinus the rate of infection varied from one exposure to another, yet the relative susceptibilities of all the three strains to S. mattheei infection remained constant throughout the 12 months of the study. These results therefore give further support to the argument that despite inter-populational differences, there are important cohesive forces that hold together a schistosome or snail species (see under "Race of Parasite" in Part I).

Attraction of Miracidia and Rates of Infection

It is clear from the results obtained with the "dumb-bell" test system that both B (P) africanus and B. truncatus attracted S. mattheei. Attraction for miracidia therefore was not an indication of the susceptibility of the snails to the schistosome infection. A quantitative assessment of the attractiveness of the three strains of Bulinus was not attempted, but the results on Table 4a suggest that both the highly susceptible and the totally refractory snails were equally attractive for the miracidia. Obser-

vations under the microscope showed that miracidia probed and attempted to penetrate all the three snails with apparent readiness. This indicates that whether the attractive substance(s) or separate "settling" factors cause the miracidia to attach and attempt to penetrate the snail skin, the same or very similar factors are operative in all the bulinid snails studied. These attractive and "settling" factors cannot therefore account for the differences in the rates of infection of the snails.

Penetration

Work on S. mansoni and its biomphalarid hosts has shown that equal or very similar numbers of the miracidia penetrate susceptible and refractory or slightly susceptible snails, and that whereas in the susceptible hosts the parasites develop without any host reaction, in the refractory and poorly susceptible ones the parasites are destroyed by cellular reactions (Brooks, 1953; Barbosa and Barreto, 1960). A similar picture was expected with S. matthesi and bulinid hosts. Though unexpected, it was not surprising to find that in Bulinus a surface phenomenon which prevented the penetration of miracidia was, for the most part, responsible for the refractoriness of B. truncatus and for the difference between the two races of B. (L.)

africanus in their susceptibility to infection with B. matthecei: it is well known that the surface of an animal is a very important line of defence against invasion by infective agents. In Africa the bulinid snails share their habitats with snail hosts of other trematodes, and if their tissues were readily accessible to any trematode parasite the snails would probably be rapidly wiped out, in view of the adverse pathological effects of trematode infections on molluscan hosts (see review by Wright, 1966).

The electron microscope observations on B. matthecei miracidia indicate that the failure of miracidia to penetrate B. truncatus may be due to purely physical factors. The branching and anastomosing processes of the apical epidermal plate appear to be well adapted for attachment to the snail surface: the arrangement, especially the anastomosis, of the processes probably also serves to provide a cushioning effect as the miracidium lands on the snail. There is little doubt that the miracidium is held on to the surface of the snail by the action of its own cilia which propel it forwards. The processes of the apical plate would therefore simply serve to give the miracidium a grip on the surface so that the organism keeps to the same position as it attempts to penetrate.

When the organism leaves a particular spot on the surface a reversal of the ciliary beat would easily effect a withdrawal. This would be a more plausible explanation of the mechanism of anchorage of the miracidium than the use of mucus as suggested by Lajdi (1963) for S. mansoni, or the dependence on a vortex produced by the host as suggested by Dawes (1960) for the miracidia of all digenetic trematodes. The effectiveness of the grip would depend not only on the digital arrangement of the apical processes of the miracidium, but also on the nature of the surface of the host. It appears possible therefore that the surface of the Nelspruit race of B (P) africanus was the most suitable for the attachment of S. mattheei miracidia and that of the Iranian B. truncatus, the least suitable, with the Swaziz race of B (P) africanus between the two extremes.

Electron microscope observations also indicate that the discharge of the penetration and apical glands may be dependent on physical factors. The miracidia of S. mattheei, as of many other trematodes, have a tendency to rotate on their long axis both when attached to snail surfaces and when swimming freely. The joint between the apical plate and next tier of epidermal plates appears weak. It is conceivable that, with the apical papilla firmly

anchored on the snail surface, the rotary motion of the rest of the body of the miracidium could result in the detachment of the apical plate from the rest of the organism. This would bare the extremely thin walls of the glands and these could be easily ruptured against the snail surface by the movements of the miracidium. Since no special structural arrangements for the emptying of the glands were found, the contractions produced by the longitudinal and circular fibres of the body wall of the organism would probably empty the glands. This would be a rather inefficient arrangement, but it would explain, for example, why in recently penetrated miracidia the same gland is sometimes intact and at other times empty (Maldonado and Acosta-Matienzo, 1947; Najdi, 1963).

Host Response

The histological observations show that although in B. truncatus the surface was a very effective barrier against infection with S. mattheei it was not the only defence mechanism: an amoebocytic reaction rapidly destroyed the few healthy miracidia that successfully penetrated the host were rapidly destroyed. The process of encapsulation involved the flattening of amoebocytes in concentric layers against the surface of the parasite and the production of fibrous

material. The amoebocytes of B (P) africanus behaved in the same ways towards cercariae as t ose of B. truncatus towards the miracidia of B. matthewi. Wigglesworth (1956) showed that the cytoplasm of the haemocytes of the insect Rhodnius prolixus contained a mucopolysaccharide with which the cells formed connective tissue and Salt (1963) has shown that it is the haemocytes of insects that produce a fibrous capsule round metazoan parasites. It would be expected therefore that in Bulinus, and other molluscs, amoebocytes would be capable of laying down fibrous material around parasites. It is unnecessary to postulate the migration of fibroblasts to the site of infection as suggested by Tripp (1961) who worked with Australorbis glabratus. If amoebocytes forming a fibrous capsule are regarded as fibroblasts then the present observations as well as those on lymphoid tissue (see Chapter 3) give support to the view that amoebocytes and fibroblasts have the ability to mutually transform into each other (see Wagge, 1955).

Immunologically, probably the most interesting thing to emerge from these observations is the fact that the susceptible snails only reacted to cercariae or to parts of the daughter sporocysts after the cercariae had started escaping from them: mother and daughter sporocysts, even when present in the lymphoid tissue itself, did not provoke

a host reaction. This suggests that since, strictly speaking, cercariae are parasitic in the sporocysts not in snail tissue, the successful establishment of an infection depends on the snail failing to react to the presence of the parasite. Salt (1960) has produced evidence which suggests that in insects it is the nature of the surface of a parasite which determines whether the host would react, but with Bulinus and S. mattheei this cannot be the explanation: the marked increase in the size, activity and number of amoebocytes with the onset of cercarial shedding suggests the cells "recognize" parasites at some distance. This argument is further supported by the fact that amoebocytes started attacking daughter sporocysts after the beginning of cercarial shedding, indicating that the cells were reacting to factors of the cercariae that leaked through the damaged sporocyst wall. Using the concept of "self" and "not-self" of Burnet (1959) it would appear that a successful infection will be established only when the snail fails to recognize the early stages of the schistosome as "not-self".

However, apart from the failure of the host to recognize the parasite as foreign material, other factors must affect its survival in the snail. It has, for example, been shown here that the nature of the tissue in which S. mattheei miracidia settle plays an important part in the survival of the organism.

5. SUMMARY AND CONCLUSIONS

It was found that, using cercarial shedding as the criterion, a South African race of B (P) africanus, a Tanzanian race of B (P) africanus and an Iranian strain of B. truncatus remained respectively, highly susceptible, susceptible and refractory to a South African strain of Schistosoma mattheei over a period of 12 months.

After the failure of many attempts using the apparatus of Yoges and Becker a "dumb-bell" test system was developed for a comparative study of the attraction of B (P) africanus and B. truncatus for the miracidia of S. mattheei. Results with this method showed that the differences in the susceptibility of the three bulinid snails were not due to differences in attraction because the refractory and susceptible snails were equally attractive for the miracidia.

A histological study of snails exposed to S. mattheei miracidia and killed at various intervals of time, showed that only a small fraction of the miracidia penetrated B. truncatus, and the miracidia penetrated only a few of the snails. With the South African and Tanzanian races of B (P) africanus much larger proportions of the miracidia penetrated larger numbers of the snails, figures for the

Tanzanian snail being a little lower than those for the South African one. It is concluded that the surface of B. truncatus forms a very effective barrier to the penetration of S. mattheei miracidia.

Electron microscope observations showed that the apical epidermal plate of the S. mattheei miracidium bears an elaborate system of branching and anastomosing processes. It is suggested that this arboreal arrangement serves to attach the miracidium to the surface of the snail. It is also suggested that the apical processes of this schistosome strain are well adapted for attachment to the surface of their normal host, B (P) africanus, but poorly adapted for that of B. truncatus.

Results of the electron microscope observations also suggest that the rotary movements of a firmly attached miracidium may result in the detachment of the apical epidermal plate from the rest of the organism, thus boring the extremely thin walls of the so-called penetration and apical glands which may then be ruptured mechanically. The glands would then be emptied of their contents by the contractions of the longitudinal and circular fibres of the body wall of the organism.

Histological studies have shown that the few *S. mattheei* miracidia that successfully penetrate *B. truncatus* are rapidly eliminated by amoebocytic reactions. In contrast, in *B. (P.) africanus* the host tissues do not actively respond to the newly arrived miracidia, mother sporocysts or daughter sporocysts. Severe reactions, which involved a marked increase in the number, size and (apparently) the migratory activity of the amoebocytes, as well as amoebocytic reactions directed against individual parasites, became evident only when cercariae appeared in the tissues of the snail hosts. It is suggested that a successful infection will only be established when the snail tissues fail to recognize the early stages of a schistosome as foreign material. However, active response is not the only factor affecting the fate of the parasites in the snail host. It has been shown that parasites penetrating dense connective tissue degenerate rapidly, but not those in loose tissue.

A brief account is given of the severe pathological effects that *S. mattheei* infection produces in the digestive gland and gonad of *B. (P.) africanus*.

C H A P T E R 5

STUDIES ON THE DAUGHTER SPOROCYST OF SCHISTOSOMA MATTHEEI AND SCHISTOSOMA BOVIS

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   (b) Histological methods

   (c) Electron Microscopical Methods

   (d) Histochemical Methods for

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3. RESULTS

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      (ii) Glycogen

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   (b) The Structure of the Sporocyst Body wall

   (c) Electron Microscope attempts to localize alkaline phosphatase in the sporocyst body wall

4. DISCUSSION

5. SUMMARY AND CONCLUSIONS.

1. INTRODUCTION

The main purpose of the work recorded in this chapter was to discover whether the passage of substances across the body wall of the daughter sporocysts of Schistosoma mattheei and S. bovis is active or passive. This is of special interest in the passage of food substances from the tissues of the host snail to the cercarine developing inside the daughter sporocyst.

It is now well established that high concentrations of the group of enzymes called alkaline phosphatases occur in regions of active molecular transfer such as the surface of the intestinal mucosa (Novikoff, 1953). Staining for alkaline phosphatase activity therefore provided a convenient test for active transport of substances, especially glucose (Burstone, 1964), across the sporocyst wall. Using the calcium-cobalt method of Gomori, high alkaline phosphatase activity was found in the sporocyst body wall. However, this was not enough evidence of active transport because with the light microscope the body wall of the schistosome sporocysts appeared to consist almost entirely of collagen fibres / which, in other animals, have a high level of alkaline phosphatase (Burstone, 1964) activity/ It was therefore decided to study the fine structure of the body wall and then attempt to localize the enzyme activity using the electron microscope.

Before undertaking electron microscope observations, attempts were made to estimate the importance of the sporocyst wall in the passage of glucose from the snail tissue into the developing cercariae. /no satisfactory Because it was decided to work with glycogen. histochemical method was available for glucose/ ~~However,~~ Since any glycogen present in the parasites must have come from host glucose, the accumulation of glycogen in the parasites is a measure of the amount of glucose passing across the sporocyst body wall. Efforts were made therefore to follow the depletion of glycogen from tissues of the snail hosts and its accumulation in the parasites developing inside the sporocysts.

There is evidence indicating that in adult strigeid trematodes the pseudosuckers and holdfast organs may secrete enzymes which digest host tissues (Lee, 1962). Preliminary tests were made therefore for the occurrence of esterases on the body wall of the schistosome sporocysts in an attempt to find out if the body wall could secrete digestive enzymes into the surrounding snail tissue. This would indicate whether the sporocyst does more to secure nutrients for the developing parasites than providing an active transport mechanism across its body wall.

The digestive gland is the main habitat of schistosome daughter sporocysts and all the observations recorded here were carried out using the daughter sporocysts of

S. matthaei or S. bovis in situ in the digestive gland of B (P) africanus. The first observations were made on S. bovis, but because of difficulties with the maintenance of this parasite (see Chapter 4) most of the work was repeated using S. matthaei.

2. MATERIAL AND METHODS

(a) Biological Material

The work recorded in this chapter is on S. matthaei in the Nelspruit strain of B (P) africanus or S. bovis in the Kisumu strain of the snail (see Chapter 4 for details of the strains of the schistosomes and their snail hosts).

(b) Histological Methods

For ordinary histology of the daughter sporocysts infected snails were fixed in the fluid of Zenker or that of Bouin. Paraffin sections, 4 to 6 microns thick, were stained in Heidenhain's haematoxylin alone, or in Erlich's haematoxylin and eosin.

Material for the study of the fibrous constituents of the sporocyst body was fixed in Bouin's fluid and the paraffin sections were stained in an orcein-aniline blue-orange G mixture (details of the method are given in Chapter 3).

(e) Electron Microscopical Methods

For the study of the fine structure of the daughter sporocysts infected digestive glands were plunged into cold (0°C.) 3% gluteraldehyde in the buffer of Rhodin and Zetterqvist and cut into small (about 1 mm.) cubes. The cubes were left to fix for 1 hour at this temperature the fixative of Rhodin and Zetterqvist modified to give and were post-fixed for 30 minutes in/1% Osmium tetroxide at 0°C. After dehydration in ethanol the blocks were stained in 1% phosphotungstic acid in ethanol and embedded in Araldite.

The description of the fine structure of the sporocyst body wall is based on S. bovis. The work was not extended to S. mattheei because observations during attempts to localise alkaline phosphatase activity in the body wall showed that the sporocysts of the two schistosomes had the same features of the fine structure that are relevant to the present study.

(d)(i) Histochemical Methods for Light Microscopy

(A) Alkaline Phosphatases

After removal from their shells, snails were plunged into ice-cold acetone (0° to 4°C.) and left to fix for 24 hours at this temperature, with one or two

changes of the fixative. The material was dehydrated, coated in 1% celloidin and then impregnated with paraffin wax under reduced pressure for 1 hour at about 54°C. with two changes of the wax. Paraffin blocks were stored for up to 2 months in a refrigerator at 0 to 4°C. without any noticeable loss of enzyme activity.

Paraffin sections were cut at 5 to 6 microns and incubated at 37°C. As controls alternate slides of the infected digestive glands were treated with inhibitors before incubation; other control sections were incubated in mixtures without the substrate. 10% acetic acid, freshly prepared Lugol's iodine or heat (boiling water) were the inhibitors used. Sections of the proximal convoluted tubules of mouse kidney, which had been treated in the same way as snail tissue, served as positive controls.

The calcium-cobalt method of Gomori for alkaline phosphatase, as given in Pearse (1960), was followed throughout. For the inhibition procedures, however, Barka and Anderson (1963) were followed.

(B) <u>Non-specific Esterases</u>

Snails were fixed in acetone at 0 to 4°C. for about 16 hours, with one or two changes of the fixative.

After rapid dehydration and coating with 1% celloidin the material was impregnated at 56°C. with paraffin wax under reduced pressure for 45 minutes.

Paraffin sections, 8 microns thick, were floated on warm, dilute albumin and if necessary the slides were stored in the refrigerator and studied within two or three days. Two methods for non-specific esterases were used. These are the alpha-naphthyl acetate and beta-indoxyl acetate methods. It was found that for the first method, the diazonemium salt, Fast Red B Salt (G. T. Gurr) gave clearer results than the Fast Blue B Salt whose dark blue deposits were difficult to distinguish from dark pigment which was already present in snail tissue. The incubation mixture was prepared by adding 1.0 ml. 1% of the substrate in acetone to 10 ml. of 0.1 M phosphate buffer (pH 7.4). After incubation at room temperature slides were counter-stained in Mayer's haemalum and mounted in glycerine jelly.

In the second method the substrate beta-bromoindoxyl acetate (Sigma Chemical Co.) was dissolved in 0.1 M tris (hydroxymethyl) aminomethane/HCl buffer at pH 7.7, or in sodium acetate buffer (pH 5.2). With this method sections were incubated for up to 16 hours. After incubation sections were counterstained with Kirkpatrick's haemalum which stained nuclei red.

With the naphthyl acetate method 0.00001 M di-isopropyl fluorophosphonate (DFP), 0.02 M silver nitrate or 0.5% sodium taurocholate were used as inhibitors. 0.02 M silver nitrate was the inhibitor used with the indoxyl acetate method. Sections of the convoluted tubules of mouse kidney served as positive controls with both methods. Except for the fixation of the material for which Burstone (1964) was followed, the techniques recommended by Pearse (1960) were adhered to here.

(C) Glycogen

Best's carmine method was used and details are given in Chapter 3.

(d)(ii) Electron Microscopy

Electron histochemistry is in its early stages of development so no standard methods for the localization of alkaline phosphatase are available. A number of methods reported in the literature, or modifications of them, were therefore tried on snail and schistosome tissues. These methods are described only briefly here.

(A) Lead methods

Following Molbert, Duspiva and von Deimling (1961), who worked on the tubules of the mouse kidney, infected digestive glands of D (P) africanus were plunged into 1% Osmium tetroxide in the buffer of Rhodin and Zetterqvist at 0°C., cut into small pieces (1 mm. cubes) and fixed for 3 minutes at the same temperature. The blocks were incubated in a medium containing potassium sodium tartrate, sodium beta-glycerophosphate, manganese chloride, magnesium chloride and lead nitrate, with a buffering system consisting of the tartrate and "tris" buffer (pH 7.6)

After incubation the blocks were post-fixed for 1 hour in the 1% Osmium tetroxide, dehydrated, rinsed in toluene and embedded in Araldite.

Examination of sections of several blocks did not show any deposition of lead on snail or parasite tissues. Fresh attempts were made using Clark's modification of the method of Molbert et al. (Clark, 1961). The main feature of the modification is the use of fixatives other than Osmium tetroxide which must destroy most of the enzyme activity. In the present study material was fixed in 3% gluteraldehyde in cacodylate buffer. With this technique very thin layers of lead were deposited on a few areas corresponding to the outer membrane of the cytoplasmic layer of the sporocyst body wall (see below

for structure of body wall.) But the results were not good enough to eliminate the possibility that the deposits of lead were an artefact.

It was thought that the unsatisfactory results were due to faults in the fixation of the material and so it was decided to use frozen, cryostat sections in order to get better preservation of the enzymes. For this purpose material was fixed for 30 to 40 minutes in 2.5% gluteraldehyde in a 0.2 M sodium cacodylate buffer (pH 7.2) at 0°C. After washing in 0.25 M sucrose in 0.1 M sodium cacodylate buffer (pH 7.2), they were left overnight in the buffer at 0°C. Frozen sections were cut at 40 to 60 microns and then incubated in the medium of Mölbert et al., but using 2.0 grams of sodium potassium tartrate instead of their 1.7 gm. After incubation the sections were washed in 0.25 M sucrose in 0.05 M "tris" buffer (pH 7.6) and post-fixed in 1% osmium tetroxide. The material was then treated in the usual way for electron microscopy. No deposits of lead were obtained with this method either, although very heavy precipitates of cobalt sulphide were found on the sporocyst wall when some of the frozen sections were treated according to the calcium-cobalt method of Gomori (see above), showing that considerable quantities of alkaline phosphatase had survived

the fixation in gluteraldehyde. This showed that the failure to deposit lead was due to some factor at the incubation stage and not to fixation.

(B) Calcium-Cobalt Method

The lead methods had been tried first because lead is more electron dense than cobalt, but after the failure of several attempts with these methods, it was decided to use the calcium-cobalt method of Gomori. Material for this purpose was fixed in gluteraldehyde, stored, sectioned, postfixed and embedded in the way outlined above for frozen sections, except that a veronal-acetate buffer (pH 7.3 to 7.4) containing 0.045 gm/ml sucrose was used for rinsing material after post-fixation instead of the R/Z buffer which was used in the lead methods. The sections were incubated and treated with cobalt nitrate and then with ammonium sulphide as for light microscopy.

Although the frozen sections were incubated for only 5 to 10 minutes, very heavy precipitates of cobalt sulphide were deposited on the sporocyst body wall. Although the deposits were too heavy and diffuse for their localization with the electron microscope, this is a very hopeful approach. However, there has not been sufficient time to complete these attempts to localize alkaline phosphatase activity with the electron microscope.

3. RESULTS

(a) Histochemistry

(1) Alkaline Phosphatase

Observations on uninfected digestive glands of B (P) africanus showed alkaline phosphatase activity on the basement membrane upon which the tubule epithelial cells stand. The contents of the tubule lumen and the free borders of the digestive cells also stained for alkaline phosphatase. This activity could be demonstrated after incubation periods of 30 to 45 minutes. After prolonged incubation periods (e.g. 16 hours) the black deposits representing alkaline phosphatase activity spread into the cytoplasm of the digestive and secretory cells. This was probably a result of the diffusion of the products of the reaction between the enzymes and the substrate. The nuclei of the epithelial cells also stained black, but the staining of nuclei for alkaline phosphatase is generally thought to be due to artefact (Pearse, 1960).

In snails infected with S. mattheei or S. bovis there was a dramatic increase in alkaline phosphatase activity in the intertubular regions of the digestive gland. Although some activity occurred in the collagen fibres which partition the cavity of the sporocysts into

several compartments (see below); most of the enzyme activity was in the walls of the sporocyst. The developing cercariae, germinal cells and germinal masses were enclosed by areas of strong enzyme reaction (Fig. 40). In sections that had been incubated for short periods (e.g. 30 minutes), or in those in which most of the enzyme activity had been destroyed by brief treatment with Lugol's iodine, almost all the staining reaction was confined to the sporocyst body wall (see Fig. 41). This showed that the increase in alkaline phosphatase activity was due to the sporocyst wall and not to the effect of the infection on the host tissues. Incubation periods of 5 to 6 hours resulted in a little diffusion of the cobalt sulphide into the cytoplasm of the tubule epithelial cells where it formed a thin layer. After prolonged incubation periods (16 hours or more) the black deposits occurred over most of the cells, especially the digestive cells.

The complete inhibition of the staining reaction by 10% acetic acid, Lugol's iodine, or boiling water (see Barka and Anderson, 1963) and the parallel behaviour of the reaction in the sporocyst body wall and on the surfaces of the epithelium of the mammalian concoluted tubules showed clearly that the activity in the body wall was due to alkaline phosphatase. However, as was pointed out

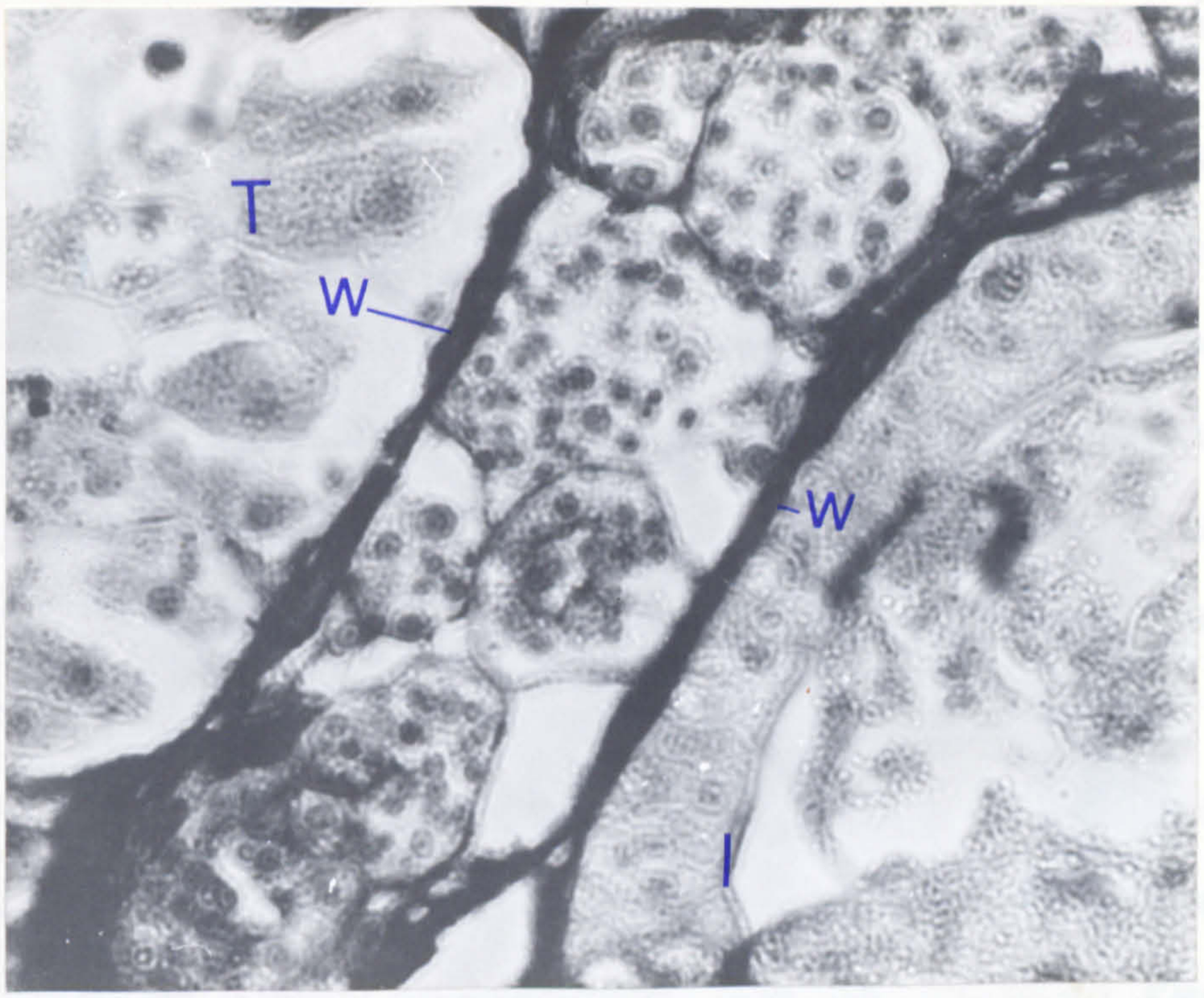


Fig. 40. Alkaline phosphatase activity in body wall (w) of S. mattheei sporocyst between a digestive gland tubule (T) and intestine (I) of B (P) africanus. x 670

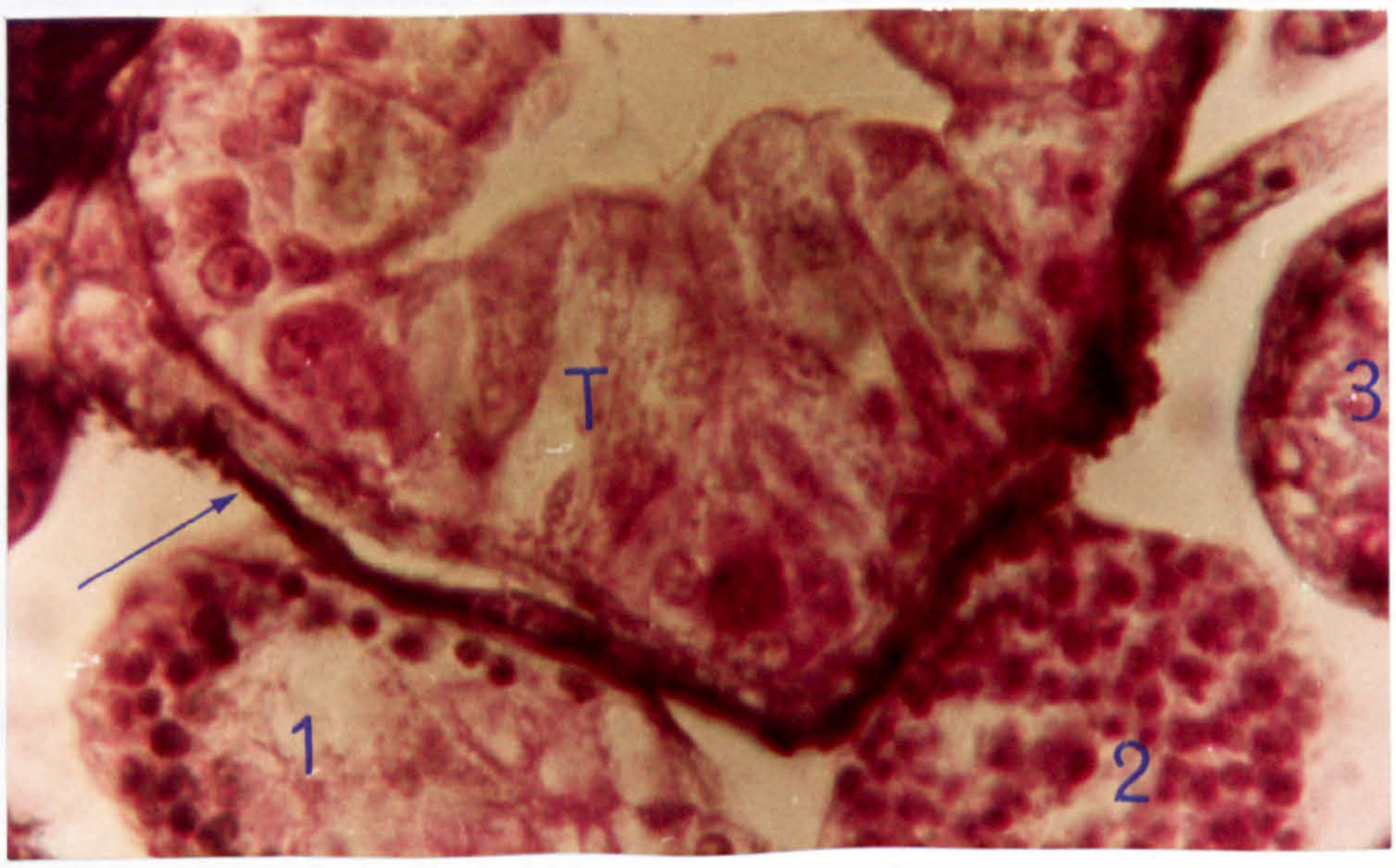


Fig. 41. Alkaline phosphatase activity on sporocyst wall (arrow) but not on basement membrane of tubule epithelium (T) 1 - 3 = developing cercaria.

in the introduction this high level of alkaline phosphatase could be due to the presence of collagen fibres in the sporocyst body wall and not to enzyme activity concerned with the active passage of substances from the host into the parasite. Electron microscope observations have shown that the outermost layer of the body of some adult trematodes consists of living protoplasm (Threadgold, 1963; Burton, 1964), and not of inmert material as the conventional name "cuticle" implies. These observations have indicated that the surface of adult trematodes may be adapted for the uptake of substances, mostly by pinocytosis, from the media in which they live. It appeared possible therefore that the surface of the schistosome sporocyst could have a similar organization which could not be seen with the light microscope and that at least some of the alkaline phosphatase might be associated with the surface. It was for this reason that observations were made on the fine structure of the sporocyst body wall. But before describing the results of the electron microscope study it is convenient to give the results of the other histochemical tests here.

(a)(ii) Glycogen

Relatively large deposits of glycogen were found in the secretory cells of the snail digestive gland and in the various stages of cercaria inside the sporocyst (Fig. 22). Smaller quantities were found in the sporocyst body wall, especially in the somatic cells (see below under structure).

Observations on uninfected snails and on snails with a 32-day old infection of S. matthaei shewed that in each group there was great variation in the amount of glycogen present in the digestive gland epithelium. Even with the same snail, some sections of the digestive gland had very little or no glycogen while in other sections heavy deposits of the polysaccharide occurred, especially in the secretory cells. It appeared likely therefore that the technique might be largely responsible for the variation in the amount of glycogen deposits present in both the parasites and the snail hosts. Best's carmine method is probably the best histochemical test for glycogen available (Pearse, 1960), but like other histochemical tests it appears not to be sufficiently reliable for quantitative work. The original intention of using the rate of depletion of glycogen deposits from the digestive gland as a rough measure of the absorption of glucose across the sporocyst wall was therefore abandoned.

(a)(iii) Non-specific Esterases

No esterase activity was found in the body wall of S. bovis with any of the two methods employed. These observations were not extended to S. mattheei because it appeared unlikely that the two sporocysts, which are very similar in other ways, would differ in the occurrence of such a large group of substances as the non-specific esterases.

The distribution of these enzymes in the digestive gland of B (P) africanus is described in Chapter 3. In this snail the enzymes have the same distribution as in Australorbis glabratus (Muller, 1965).

(b) The Structure of the Sporocyst Body Wall

With the light microscope, the mature sporocysts of S. mattheei and S. bovis as other trematodes (Cort, Ameel and van der Woude, 1954) appeared as sacs full of germinal cells, germinal cell masses and cercariae in various stages of development. It was found that with the schistosomes the cavity of the "sac" was criss-crossed by partitions and that the resulting compartments were occupied by germinal cells, germinal cell masses and cercariae (Fig. 12). The wall of the "sac" appeared to consist of lightly eosinophilic fibres mostly (Figs. 12, 13.)

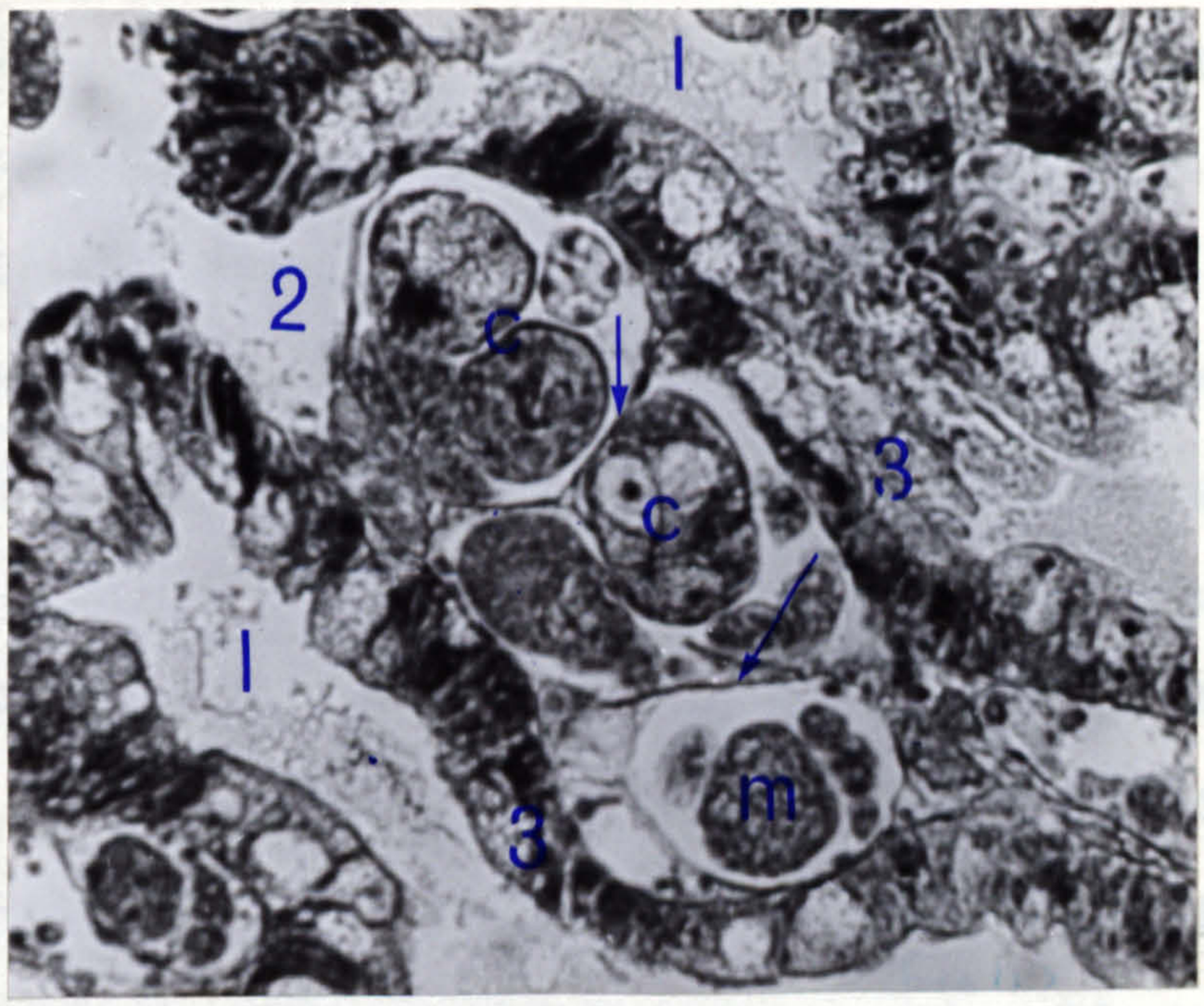


Fig. 42. Section of S. mattheei sporocyst lying in intertubular spaces of digestive gland of B (P) africanus.
1 = lumen of tubule, 2 = intertubular space 3 = tubule epithelium (c) = cercaria (m) = mass of germinal cells. Note: Fibres (arrow) dividing the sporocyst into compartments x 220 (Heidenhain).

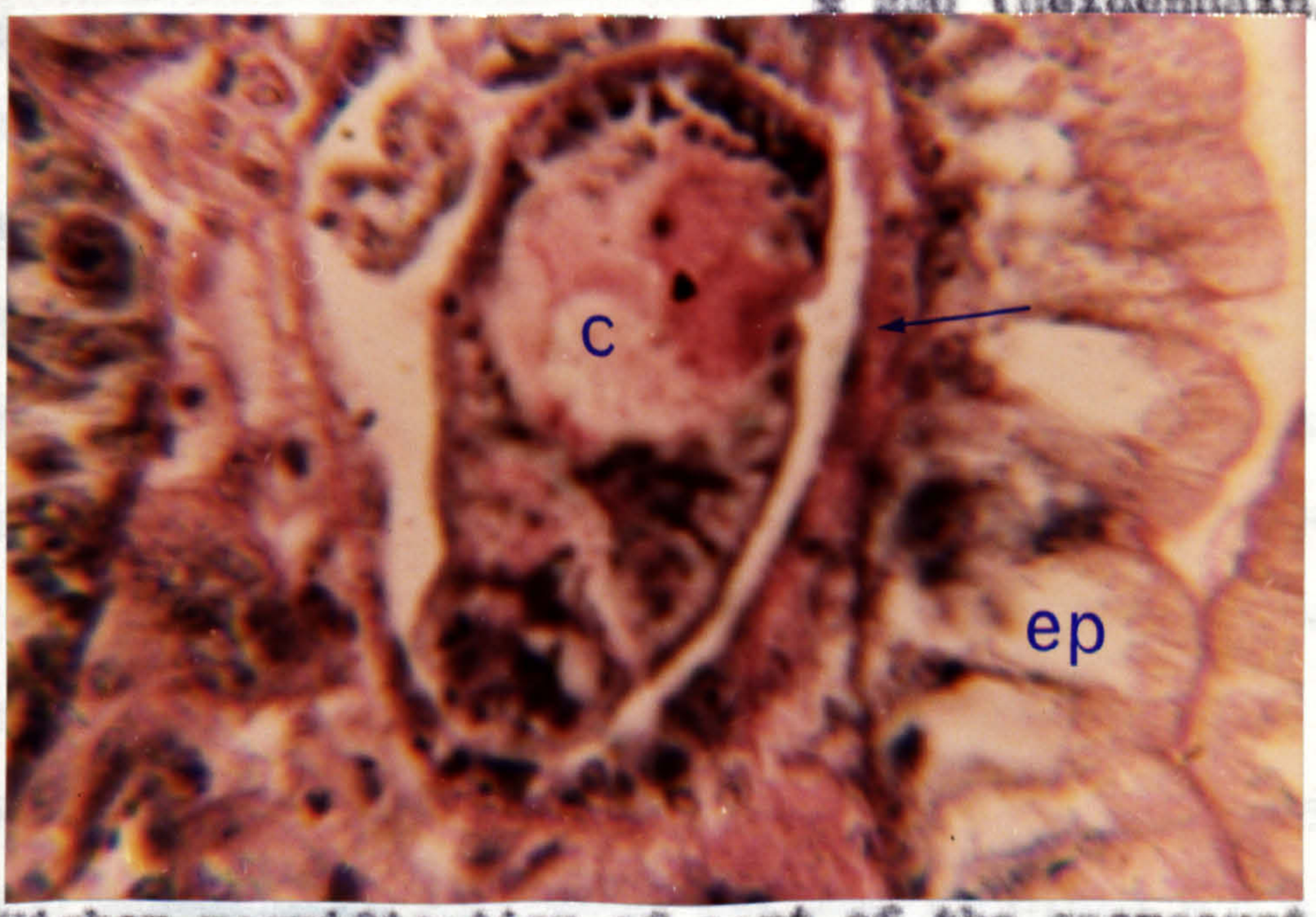


Fig. 43. Higher magnification of part of the sporocyst to show the wall (arrow) pressed against basement membrane of the snail epithelium (ep).
c = cercaria. (Heidenhain).

Elongate cells with large nuclei and nucleoli as well as germinal cells were commonly seen attached to the inner side of the wall. The elongate cells appeared to correspond to those described as "somatic" cells by Cort et al. (1954), and they are therefore referred to by this name in this thesis. The partitions, which are continuous with the wall, also consist of lightly eosinophilic fibres and fibroblasts; where they are wide enough, somatic cells occur among the fibres. Tests with the orcein-aniline blue-orange G stain showed the presence of collagen fibres only in both the wall and the partitions. None of the fibres stained like muscle tissue with this mixture or with Heidenhain's haematoxylin with which the muscle fibres of the snail host stained readily.

The main interest of this chapter is in the wall of the "sac", which is called the sporocyst "body wall" in this thesis. Electron microscope observations showed that the body wall has a much more elaborate organization (Fig. 44) than was apparent with the light microscope. On the outside the wall is bounded by a continuous layer of cytoplasm (Fig. 45) and on the inner side by a thick membrane (Figs. 44, 46). Immediately below the cytoplasmic layer bundles of circular and longitudinal collagen fibres occur. The somatic cells lie close to the membrane lining the cavity of the sporocyst (Fig. 44). That the

outermost layer of the body wall consists of living cytoplasm is shown by the occurrence of mitochondria in it (Fig. 45, also Fig. 46) and the fact that the cytoplasm is thrown into numerous microvilli (Figs. 45, 46). The microvilli form an impressive layer between the parasite and the snail tissue (Fig. 46). No other intracellular structures were found in the cytoplasmic layer. The cytoplasm of the somatic cell contains numerous inclusions which appear darkly stained with both the light and the electron microscope. With the electron microscope the Golgi apparatus appeared to be the most prominent feature of the cytoplasm (Fig. 47). However, the very prominent nucleolus (Fig. 44) suggests that the cell may be an important site of protein synthesis. There is evidence to show that at least some secretory cells go through cycles of formation and dispersal of the endoplasmic reticulum (Bertram and Bird, 1961). It is possible therefore that the present observations could have caught the cells in a "dispersal phase". In places the cytoplasm of the somatic cell lies very close to the cytoplasmic layer which covers the surface of the body wall and the two might be continuous (Fig. 44, 47).

Fig. 44. Part of S. bovis sporocyst in B (P) africanus

1 = snail tissue
2 = sporocyst body wall
3 = somatic cell of the sporocyst
4 = ?fibroblast
5 = developing cercariae.

x 7,000

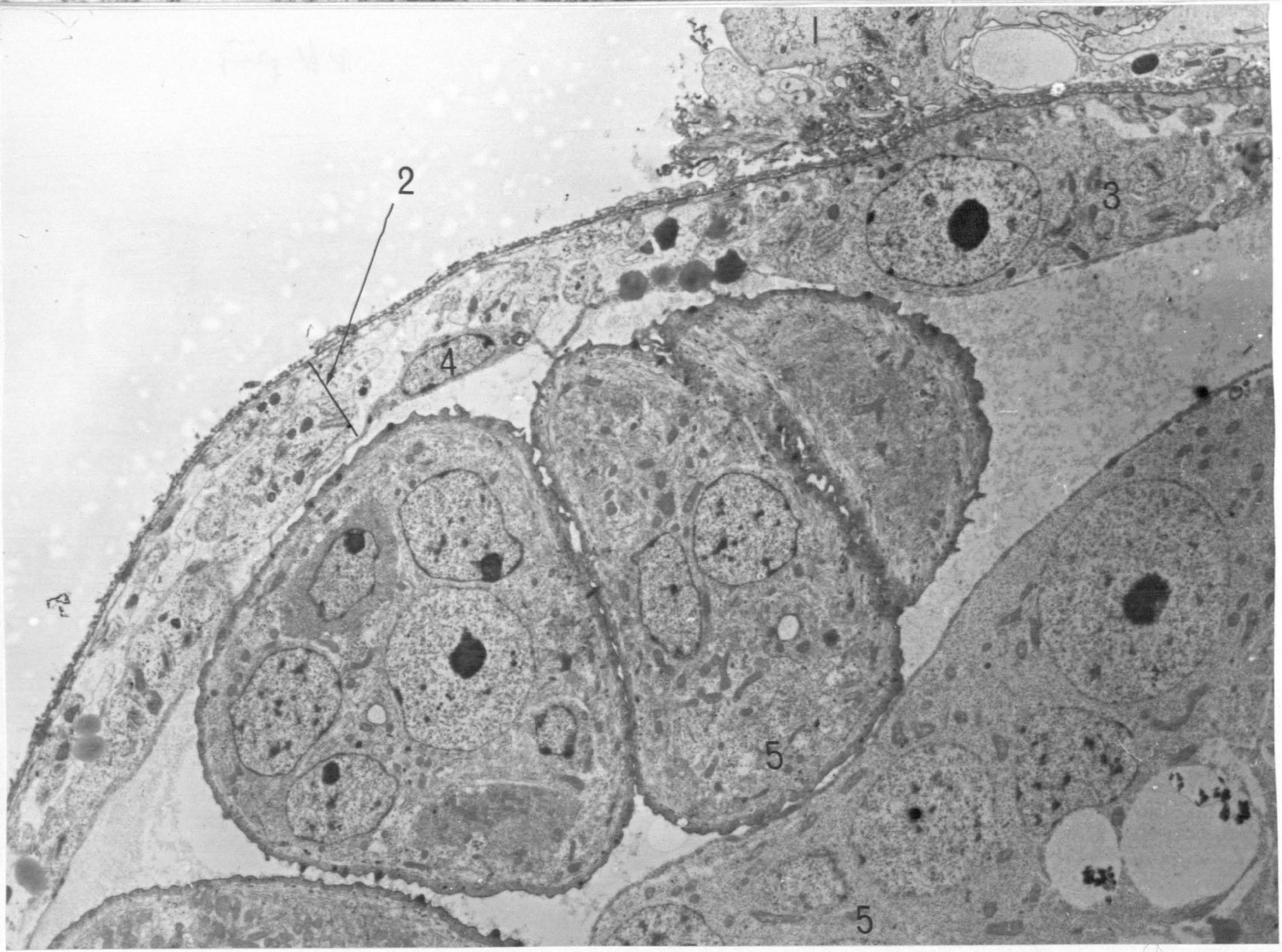
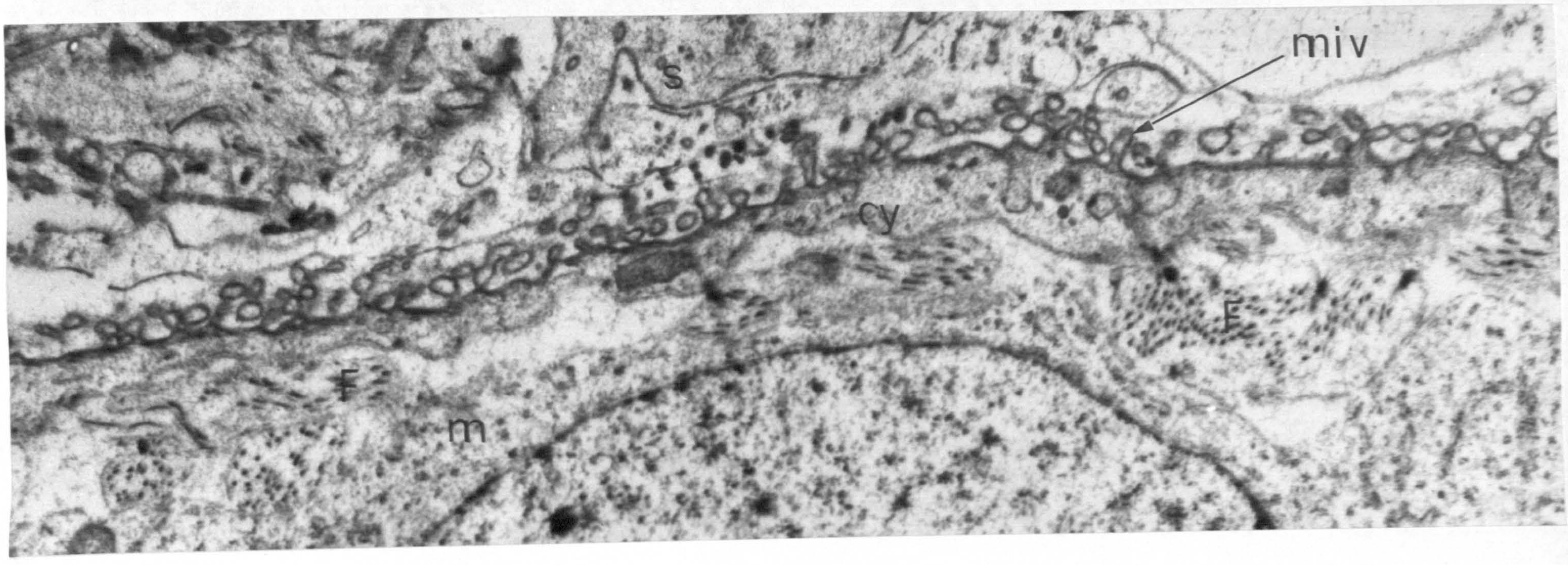

1
2
3
4
5
5

Fig. 45. Higher magnification of part of S. bovis sporocyst wall to show layer of cytoplasm (cy) with mitochondrion (m) note microvilli (miv)
S = basal parts of snail epithelial cells
F = bundles of fibres of body wall
N = nucleus of somatic cell.

x 25,000


miv
cy
F
F
m

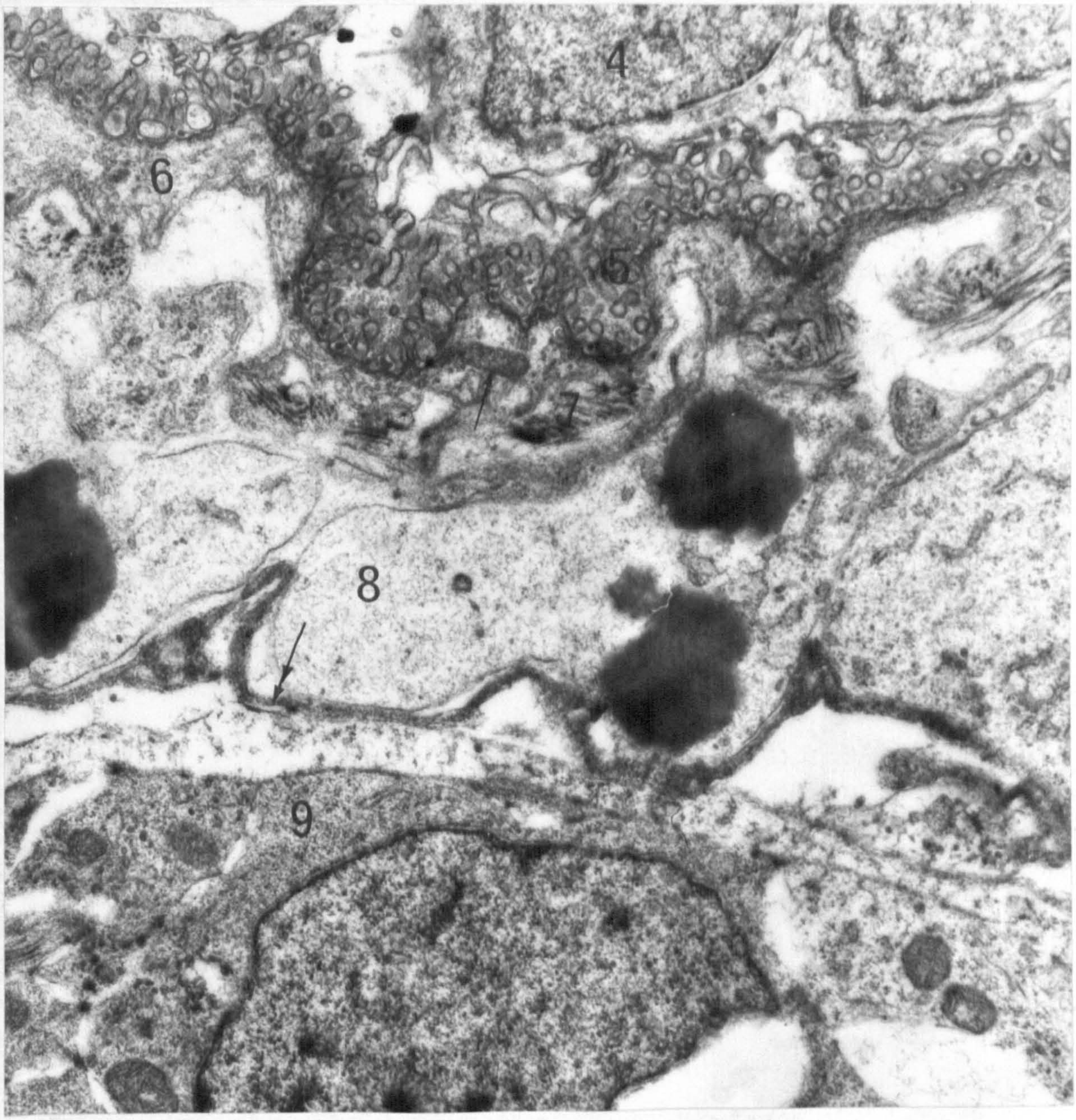


Fig. 46. Part of S. bovis sporocyst to show layer of numerous microvilli of the body wall lying below snail epithelial cells.

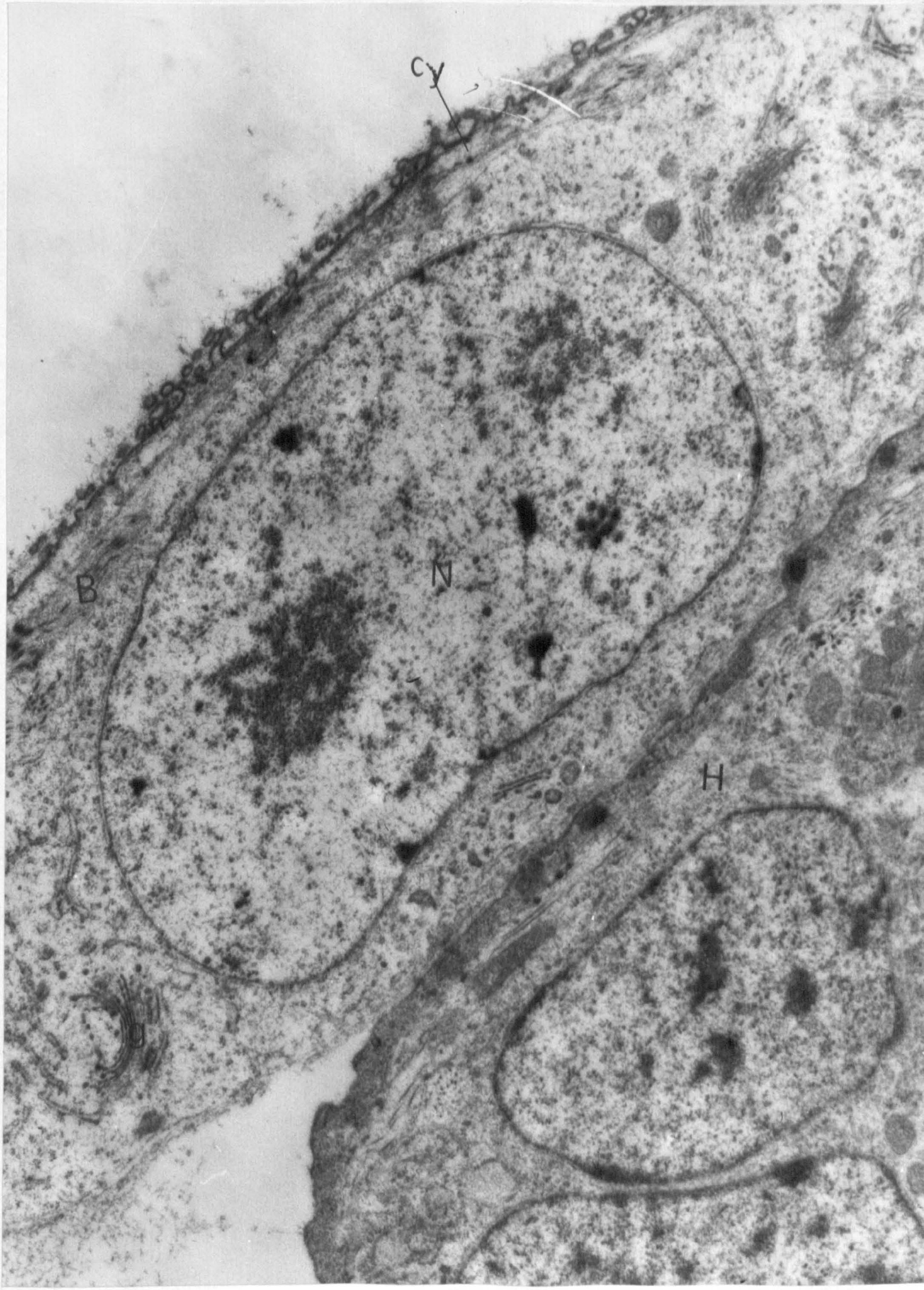
4 = basal part of snail epithelial cells
5 = layer of microvilli
6 = laypr of cytoplasm with a mitochondrion (arrow)
7 = collagen fibres
8 = parts of somatic cell
9 = developing cercaria.

Note membrane (two arrows) forming inner limit of body wall.

x 24,000

Fig. 47. To show part of somatic cell of S. bovis sporocyst lying very close to the cytoplasmic layer (cy)
N = nucleus of the cell
G = Golgi apparatus of the cell
B = collagen fibres
D = developing cercaria.

x 24,000


Cy
B
N
H

(e) **Electron Microscope attempts to localize Alkaline Phosphatases in Sporocyst Body wall**

It has already been pointed out that these attempts were unsuccessful. The use of lead salts is not a very satisfactory method for alkaline phosphatases (Pearse, 1960), and attempts with lead were only made because of the need for a metal that was adequately electron dense. Light microscope observations had revealed a high level of alkaline phosphatase activity in the sporocyst body wall and the failure to localize it with the electron microscope was clearly therefore due to the technique and not to the absence of enzymes.

4. DISCUSSION

Little is known about the physiology of the intramolluscan stage of digenetic trematodes in general and of schistosomes in particular (see Smyth, 1966). The only study of the physiology of schistosome daughter sporocysts appears to be that of Dusanic (1959), who reported alkaline phosphatases in the sporocysts of Schistosoma mansoni in the digestive gland of Australorbis glabratus. However, the tests for enzymes do not appear to have been adequately controlled against non-specific staining and the

significance of the results in relation to the feeding mechanisms of the schistosome sporocyst is therefore not clear. Observations on other trematode larval stages have not thrown much light on the nutritional physiology. For example, Cheng (1964) reported a marked increase in alkaline and acid phosphatases in the cells of the digestive gland of Ilyliosoma infected with Echinostoryphium, but the importance to the parasite of such a distribution of alkaline phosphatases is not clear because it is now well established that the activity of these enzymes is mostly extra-cellular occurring on cell surfaces with active molecular transport (Novikoff, 1955).

In the present work, tests have shown the occurrence of a high level of alkaline phosphatase activity on the body wall of the daughter sporocysts of S. mattheei and S. bovis. The electron microscope observations have shown that the surface of the sporocyst body wall consists of a layer of living cytoplasm. Alkaline phosphatase would therefore be expected to occur on the outer membrane of this layer. The formation of numerous microvilli from the cytoplasmic layer is evidence of its importance as an absorptive surface: microvilli, microthrices and other modifications of cell and parasite surfaces are believed to be devices for increasing absorptive areas

(see Vickerman, 1963 for review). It appears therefore from the combined results of alkaline phosphatase tests and electron microscope observations that the surfaces of the daughter sporocysts of S. mattheei and S. bovis are well adapted for a high level of absorptive activity. As with other surfaces where high concentrations of alkaline phosphatases occur (Burstone, 1964), it is likely that glucose is the main substance being transported actively across the surface of the sporocyst body wall. In both light and electron microscopy the body wall is pressed against the basement membrane of the tubule epithelial cells (see Figs 22, 45). Glucose, therefore, probably passes directly from the epithelial cells into the microvilli and thence into the cercariae developing in the cavity of the sporocyst. The storage of glucose as glycogen in the cercariae would create a steady glucose gradient across the sporocyst body wall. Since a lowering of the glucose content of the cytoplasm of host cells would probably lead to the release of more of the sugar from the glycogen stores present in the host cells, it is not necessary to postulate, as Cheng and Synder (1962) suggested for sporocysts of Glyptheimius that the sporocyst secretes digestive enzymes into the host cells.

There is evidence that the surface of the body wall of adult S. mansoni also consists of living cytoplasm and that alkaline phosphatases occur in the wall (Senft, Philpott & Pelofsky, 1961; Robinson, 1961). However, the presence of a well-developed gut indicates that the adult schistosomes are not dependent on the body surface for the supply of food substances to the same extent that the sporocysts must be.

In addition to serving a vital function in the supply of carbohydrates to the developing cercariae, the living cytoplasmic layer of the sporocyst body wall is probably of great importance in the protection of the cercariae from the defence reactions of the host. Observations on the reaction of the tissues of B (P) africanus to its normal parasite S. mattheei (see Chapter 4) suggested the sporocyst body wall might effectively stop the passage of cercarial substances into the host tissues until mature cercariae started emerging when they probably damaged parts of the sporocyst body wall, thus allowing the leakage of the cercarial substances into snail tissues where they provoked a strong amoebocytic reaction. The results of the work reported here support this view since active molecular transport at the surface of the sporocyst must mean that passage across the body wall is selective. The sporocyst wall would therefore appear to be analogous to the mammalian placenta in some ways.

5. SUMMARY AND CONCLUSIONS

Using the calcium-cobalt method of Gomori, a high level of alkaline phosphatase activity has been found in the body wall of the daughter sporocysts of Schistosoma mattheui and S. bovis in the intertubular spaces of the digestive gland of their host snail, B (P) africanus.

Electron microscope observations have shown that the surface of the body wall consists of a layer of living cytoplasm in which mitochondria occur. It was found that the surface of the cytoplasmic layer is thrown into numerous microvilli which lie very close to the basement membrane of the host cells and which showed that the parasite surface was a very important absorptive area. Attempts to localize alkaline phosphatase with the electron microscope using lead were unsuccessful owing to technical difficulties, but there can be little doubt that much of the enzyme activity seen in light microscopy was on the surface membrane of the cytoplasmic layer.

It is therefore suggested that the body wall of the sporocyst plays a fundamental role in the active transport of glucose from the cells of the host where large stores of glycogen were found, into the cercariae developing inside

the parasite. It is also suggested that the sporocyst body wall may be responsible for protecting the developing cercariae from the defence reactions of the host snail.

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A NOTE ON THE SUSCEPTIBILITY OF SOME GASTROPOD MOLLUSCS TO *SCHISTOSOMA BOVIS* AND *S. MATTHEEI*

BY
GEORGE KINOTI

A NOTE ON THE SUSCEPTIBILITY OF SOME GASTROPOD MOLLUSCS TO *SCHISTOSOMA BOVIS* AND *S. MATTHEEI*

BY

GEORGE KINOTI*

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Schistosoma bovis is found chiefly in the more northerly parts of Africa, whereas *S. mattheei* is found in the south. East Africa appears to be a transitional zone in which both species are present (Dr. J. A. Dinnik, *personal communication*). In view of the danger of bovine schistosomes infecting man (Pitchford, 1959), laboratory experiments were carried out to assess the susceptibility of some East African bulinid snails to these two schistosomes. The local (Sukumaland) strain of *S. bovis* and a South African strain of *S. mattheei* were used in the experiments.

Five local species of snails were exposed to *S. bovis* miracidia: *Bulinus* (*Physopsis*) *nasutus productus* (Mandahl-Barth), in the Mwanza area the commonest bulinid snail, occurring in pools in the rainy season ; *B.* (*Bulinus*) *forskalii* (Ehrenberg), also common, with a widespread but sporadic distribution in streams, ponds and dams ; a snail from dams described by Dr. Mandahl-Barth as being intermediate between *B.* (*B.*) *tropicus* (Krauss) and *B.* (*B.*) *coulboisi* (Bourguignat) ; *B.* (*P.*) *globosus* (Morelet), from quiet pools and channels connected to Lake Victoria ; and *B.* (*P.*) *africanus ovoideus* (Bourguignat), found in streams and dams. Two further species of snails were exposed to *S. bovis* miracidia : *B.* (*B.*) *tropicus alluaudi* (Dautzenberg), found by Teesdale (1962) naturally infected with *S. bovis* in Kenya ; and *B.* (*P.*) *africanus africanus* (Krauss), from Iringa in the southern highland region of Tanganyika.

B. (*P.*) *africanus ovoideus*, *B.* (*P.*) *globosus* and *B.* (*P.*) *nasutus productus* were used in experiments with the South African strain of *S. mattheei*.

MATERIALS AND METHODS

Snails. All the snails used were laboratory-bred. Stock cultures were maintained in glass tanks or large enamel bowls with a layer of sand at the bottom supporting some *Vallisneria* sp. Snails for experimental work were raised by a technique successfully used by Mr. W. F. J. McClelland (*personal communication*) for rearing *B.* (*P.*) *nasutus productus* in the laboratory. Stock snails were left in surgical enamel trays, the sides and bottom of which supported a thin growth of a green unicellular alga. Newly hatched snails fed on this growth until they could take boiled lettuce, on which older snails were fed. When an appreciable number of egg-masses had been laid on the sides of the trays or on strips of polythene (which were introduced into the trays at the same time as the snails), the snails were removed and returned to their tanks or bowls. Boiled lettuce was introduced into the bowls when the snails were about a week or so old. This technique has two advantages :

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(*i*) the age of the snails used in experimental work is accurately known, and (*ii*) large numbers of snails can be reared, as plenty of food is available for the hatchings.

Miracidia and snail exposure. Ova were obtained by homogenizing, in saline in a blender, the liver and sometime pieces of gut of experimentally infected hamsters. The homogenate was repeatedly sedimented in measuring-cylinders or urine-glasses until the supernatant fluid was clear. Miracidia were obtained within 30 minutes of diluting the final sediment with chemically untreated water and placing it under an electric lamp. With a Point-o-lite light, known numbers of miracidia were pipetted into 2-3 ml. of water in 3-inch × 1-inch tubes. Snails were then introduced individually into the tubes. Miracidia older than two hours were not used and exposures lasted for 2-4 hours. After exposure, the snails were placed in glass tanks or enamel bowls containing sand and *Vallisneria*. They were maintained at room temperature (22-24° C.) and were fed on lightly boiled lettuce.

The snails were examined for cercarial shedding after about 25 days and at intervals of two or three days thereafter. They were placed individually in one inch of water in 3-inch × 1-inch tubes and were left for three or four hours under a strong electric light. Only snails that shed cercariae were considered infected.

Maintenance of the schistosomes. The *S. bovis* used in the experiments was originally obtained from *B.* (*P.*) *africanus ovoideus* collected from two streams at Bariadi in the Maswa district of Tanganyika. It was maintained in the laboratory in *B.* (*P.*) *africanus ovoideus* and the golden hamster. *S. mattheei* was maintained in *B.* (*P.*) *africanus africanus* from Nelspruit and in the local *B.* (*P.*) *africanus ovoideus*, with the golden hamster as the definitive host.

RESULTS

Table I shows the results of exposing the various species to miracidia of *S. bovis*. No *B.* (*P.*) *nasutus productus* were infected in any of the four experiments, which involved from 4-5 to about 100 miracidia per snail. In two trials involving 58 *B.* (*B.*) *tropicus alluaudi* and 10 and 30 miracidia, the snail proved refractory, as also did the *B.* (*B.*) *tropicus-coulboisi* form. *B.* (*P.*) *globosus* was only slightly susceptible, but *B.* (*B.*) *forskalii* showed a high susceptibility. When exposed to six miracidia per snail, an infection rate of 95 per cent. was obtained with *B.* (*P.*) *africanus ovoideus* ; an increase to 8-9 miracidia per snail yielded infection in all exposed snails. When *B.* (*P.*) *africanus africanus* (Iringa form) were exposed to 10-12 miracidia per snail, all 23 snails which survived were infected.

The results of the exposure of *B.* (*P.*) *nasutus productus*, *B.* (*P.*) *globosus* and *B.* (*P.*) *africanus ovoideus* to the miracidia of *S. mattheei* are shown in Table II. Here again *B.* (*P.*) *nasutus productus* was refractory, *B.* (*P.*) *globosus* was only slightly susceptible, and *B.* (*P.*) *africanus ovoideus* was highly susceptible.

In one experiment (not shown in the tables) 25 *B.* (*P.*) *nasutus productus* 2-3 weeks old were exposed individually to a mixture of 10-12 *S. bovis* and 10-12 *S. haematobium*. The *S. haematobium* miracidia had been hatched from ova in the urine of a human donor. Thirty-six days after exposure five of the surviving 22 snails shed cercariae, to which two hamsters were exposed. So few cercariae were shed that, to be sure of infecting the animals, the hamsters were exposed to all the cercariae shed on two further days. The hamsters

TABLE I

Showing the results obtained by exposing snails to the miracidia of *S. bovis*

Snail	No. of snails exposed	Age of snails*	No. of miracidia per snail	No. of snails surviving	at Days after exposure	No. of survivors infected	Percentage of survivors infected	Notes
B. (P.) nasutus productus	55	Young	4–5	6	30	0	0	
	44	Young	10–12	29	36	0	0	
	30	4–5 weeks	15	28	47	0	0	
	20	Adult	About 100	17	42	0	0	
B. (B.) tropicus alluaudi	28	Adult	30	24	34	0	0	18 snails survived up to 54 days but did not shed cercariae
	30	1–2 weeks	10	30	39	0	0	
B. (B.) tropicus-coulboisi	50	Young and adult	4–5	40	31	0	0	15 snails survived up to 53 days; no cercariae shed
B. (P.) globosus	25	10 weeks	10–12	24	36	1	4·1	
B. (B.) forskalii	31	Young	4–5	19	35	12	63·1	
	24	Young	5	17	36	11	64·7	
B. (P.) africanus ovoideus	40	5–7 weeks	4–6	35	42	23	65·7	
	20	6–7 weeks	6	20	34	19	95·0	
	40	6–7 weeks	8–9	20	34	20	100·0	
B. (P.) africanus africanus	25	Adult	10–12	23	48	23	100·0	

* Young = Snails which have not started breeding. Adult = Snails which have started breeding.

were autopsied four months later and their livers were homogenized in saline in a blender. The homogenate was sedimented as described above, and the whole of the final sediment was examined microscopically. Only *S. haematobium* ova were seen.

The original object of these experiments was to obtain information on the likely intermediate hosts of *S. bovis* among the more common bulinid snails in the Lake region of Tanganyika. The results suggest that *B.* (*P.*) *nasutus productus* is not a host, a suggestion strongly supported by the finding that the snail selectively took in *S. haematobium* when it stood an equal chance of being infected with *S. bovis* or *S. haematobium* or both. The low susceptibility of *B.* (*P.*) *globosus* and its limited distribution tend to make it relatively unimportant in the epidemiology of *S. bovis* in the area. Although *B.* (*B.*) *forskalii* is a good host experimentally, its very sporadic, though widespread, distribution would probably greatly limit its role in the transmission of the parasite. *B.* (*P.*) *africanus ovoideus* is the only known host in the field ; its high degree of susceptibility is in marked contrast to the resistance of *B.* (*P.*) *nasutus productus*.

It is interesting to note that the South African *S. mattheei* behaved in the same way as *S. bovis* in the three snail species tested : *B.* (*P.*) *nasutus productus* was refractory, *B.* (*P.*) *globosus* was only slightly susceptible, and *B.* (*P.*) *africanus ovoideus* was highly susceptible. The limited nature of the observations reported here does not warrant the drawing of any definite conclusions, but it is interesting that the two ' animal ' parasites showed no physiological differences in relation to the molluscan hosts. Our understanding of the evolution and relationships of the various African schistosomes—especially of the terminally spined species—is very meagre. It is not possible, for instance, to predict with any degree of certainty the behaviour of a strain of a schistosome to a snail. Although, for example, *B.* (*P.*) *africanus ovoideus* is a natural host of *S. haematobium* in parts of Kenya (Teesdale, 1962), Uganda (Schwetz, 1951) and Tanganyika (Kinoti, *in the press*), Cridland (1955) was unable to infect a strain of the species with *S. haematobium* from the Kisumu area of Kenya. In the observations reported in the present paper a South African strain of *S. mattheei* and a Tanganyikan strain of *S. bovis* behaved in the same way to three bulinid snails. McCullough (1959) and Wright (1962) have already appealed for greater efforts towards an understanding of the host-parasite relationships of African schistosomes and their molluscan hosts. Such an understanding would be of more than academic value, for the control of schistosomiasis, as of any other parasitic disease, calls for a thorough knowledge of host-parasite relationships on the one hand, and of the parasites and hosts as animals in their own right on the other.

SUMMARY

1. The susceptibility is reported of different species of bulinid snails to a Tanganyikan strain of *Schistosoma bovis* and to a South African strain of *S. mattheei*.

2. Both schistosomes were found to develop well in *Bulinus* (*Physopsis*) *africanus ovoideus*, but *B.* (*P.*) *globosus* was only slightly susceptible to either. *S. bovis* developed also in *B.* (*P.*) *africanus africanus* and in *B.* (*Bulinus*) *forskalii*, but *S. mattheei* failed to develop in *B.* (*P.*) *nasutus productus*, in a form intermediate between *B.* (*B.*) *tropicus* and *B.* (*B.*) *coulboisi* or in *B.* (*B.*) *tropicus alluaudi*.

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Observations on the Transmission of *Schistosoma haematobium* and *Schistosoma bovis* in the Lake Region of Tanganyika

GEORGE KINOTI [1]

Previous investigations have shown that in the Lake Region of Sukumaland, Tanganyika, where Schistosoma haematobium *is highly endemic,* Bulinus (Physopsis) nasutus *is responsible for the transmission of that schistosome in small, temporary rain pools. This area is one of low rainfall, and large artificial reservoirs are the chief source of water in the dry season. The role of these reservoirs in* S. haematobium *transmission was studied over a period of about a year.*

Previous work in South Africa had indicated the potential danger of bovine schistosomes to man. S. bovis *is a very common parasite in cattle in the Lake Region, and a search for its intermediate host or hosts, previously unidentified, was therefore also made.*

The results of this double investigation suggest that large bodies of water are relatively unimportant in the transmission of both S. haematobium *and* S. bovis. Bulinus (Physopsis) africanus *is shown to be a second intermediate of* S. haematobium *and a vector of* S. bovis *as well. Transmission of these parasites by this snail takes place principally in streams.*

Schistosoma haematobium is endemic in the Lake Region of Sukumaland, at the southern end of Lake Victoria (Jordan, 1961). Webbe (1962) studied in detail the bionomics of *Bulinus (Physopsis) nasutus productus* Mandahl-Barth and its infection rate with *S. haematobium* in a 6-square-mile (15 km²) area in the region and demonstrated the seasonal nature of transmission corresponding to the temporary existence of the habitats—small rain pools—of the molluscan intermediate host.

Rainfall is low in the area, about 30 inches (75 cm) a year, and there is an acute shortage of water during the long dry season from July to November. This has led to the impounding of water by different methods. The undulating nature of the country permits the building of dams across valleys and seepage areas while on the more level ground, " tanks " are excavated in the ground. The capacity of these reservoirs varies considerably, from several hundred million gallons (e.g., Sola dam) to a few hundred thousand.

The work reported in this paper was undertaken by the author, with the help of a technician, as part of the research programme of the East African Institute for Medical Research. It was particularly intended to supplement and extend the observations of Webbe (1962) into permanent waters and to cover a wider area of Sukumaland (see map). The present observations were made from July 1962 to August 1963, thus covering both the dry and the rainy seasons.

In addition to *S. haematobium* being endemic in the area, bovine bilharziasis occurs and 58% (37/64) of the cattle examined over a period of six months at the Mwanza abattoir were infected. All the eight cattle examined on three occasions at Nzega, and all the six examined on three occasions at Bariadi were infected. Both *Schistosoma bovis*, a more northerly, and *Schistosoma mattheei*, a more southerly species, occur in this region, East Africa being a transitional zone (Dinnik, personal communication). In spite of the high prevalence of bovine bilharziasis, however, no infected snails were found among the many thousands that were examined, over a number of years, during studies on *S. haematobium* (Webbe, 1962). It

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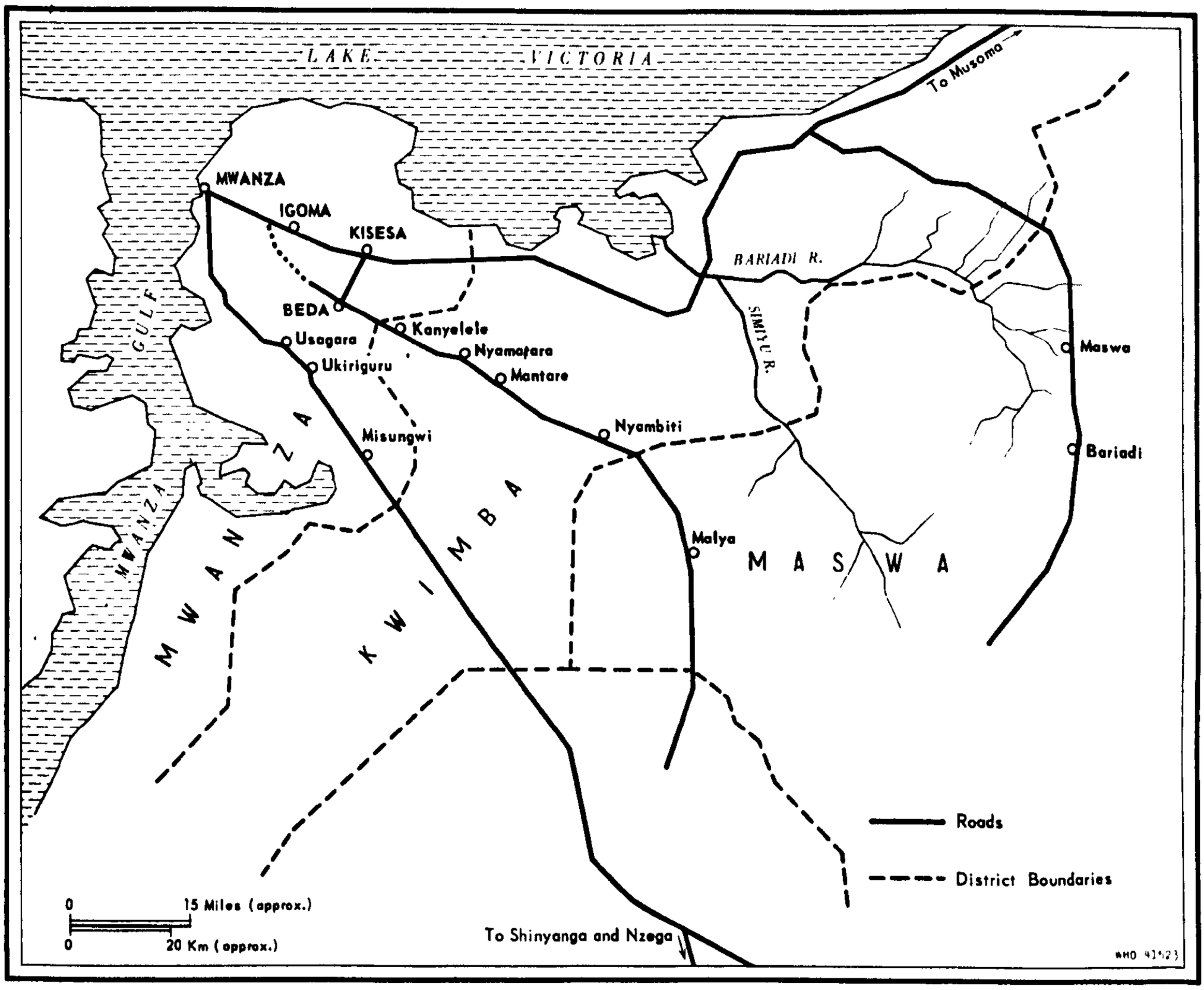
THE LAKE REGION OF SUKUMALAND, TANGANYIKA



was hoped, therefore, that observations on the reservoirs which supply most of the water for livestock, bathing and domestic consumption in the dry season would reveal the intermediate host or hosts of the bovine schistosomes. An intermediate host of *S. bovis* was later found in a stream and observations were extended to this type of habitat.

THE ROLE OF PERMANENT WATER RESERVOIRS

For the purpose of the present study water reservoirs are divided into (*a*) large dams, and (*b*) " hafirs ". Technically the distinction between a dam and a " hafir " is based on the gradient across which a reservoir is built, dams being across the steeper slopes and " hafirs " across the more gentle ones. Here "hafir " is used to mean a dam with a water surface of about 0.25-0.5 acre (about 1000-2000 m^2) and a capacity of about 1 million UK gallons (or about 4500 m^3). " Dams " are much larger bodies occupying 5 acres (2 ha) or more and containing several thousand million gallons of water.

The habitats

A general characteristic of " hafirs " is the open water surface owing to the removal of vegetation, especially at the onset of the dry season, when they come in use by man and his livestock. When present, vegetation comprises largely marginal grasses and the emergent water lettuce, *Pistia* sp., which also tends to grow round the margins. Large dams, on the other hand, may have much more vegetation, including water lettuce, water lilies, sedges and grasses. Eleven " hafirs " and three large dams in

Mwanza district were chosen for regular observations. In Kwimba district four " hafirs " were visited periodically as was one large dam in Maswa district. In addition a number of other dams were examined. Habitats under regular or periodic observations are numbered 1-19 to facilitate the recording of results.

Habitat No. 1. A " hafir " situated at Ukiriguru. Considerable human and cattle contact in the dry season. *B. (Ph.) nasutus productus* and *Bulinus (Bulinus) forskalii* (Ehrenburg) were the only bulinid snails present.

Habitats No. 2-4. " Hafirs " at Nyamatala. No. 2 had *B.(B.) forskalii* and *B.(Ph.) nasutus productus.* No. 3 *B.(B.) forskalii,* and No. 4 *B.(B.) forskalii* and *B.(Ph.) nasutus productus.* No. 4 may dry out in prolonged drought. There was considerable human and cattle activity around all four.

Habitats No. 5-10. " Hafirs " situated at Kitumba village near Kisesa. *B. (Ph.) nasutus productus* was present in No. 6, 7 and 8; *Bulinus (Physopsis) africanus ovoideus* (Bourguignat) in No. 9 and 10; *B.(B.) forskalii* sporadically in No. 5, 7 and 9. A bulinid intermediate between *Bulinus (Bulinus) coulboisi* (Bourguignat) and *Bulinus (Bulinus) tropicus* (Krauss) was present in No. 5.

Habitat No. 11. A " hafir " at Igoma cattle dip. Considerable bathing and cattle contact. *B.(Ph.) nasutus productus* and, sporadically, *B.(B.) forskalii* were present.

Habitat No. 12. A pair of very similar " hafirs " on the Nyambiti-Malya road, Kwimba district. In drought only very muddy water remained at the bottom. *B. (Ph.) nasutus productus* and *B. (B.) forskalii* were present.

Habitat No. 13. Three similar " hafirs " close together, situated about 10 miles (16 km) along the Mantare-Nyambiti road. Water volume subject to great fluctuations. *B. (Ph.) nasutus productus* and, sporadically, *B. (B.) forskalii* were present.

Habitat No. 14. A " hafir " about half a mile (800 m) below No. 13 on the same slope. Fluctuations of smaller volume than in No. 13. *B. (Ph.) nasutus productus* was present. There was considerable human and cattle contact.

Habitat No. 15. A " hafir " close to a large dam situated about 6 miles (9.5 km) from Mantare on the Nyambiti road, near a cattle dip. There was much human and cattle contact. *B. (Ph.) nasutus productus* was present.

Habitat No. 16. A large dam at Misungwi. Covers approximately 21 acres (8.5 ha) with a capacity of about 30 million UK gallons (or about 13 600 m^3). There was little direct human or cattle contact. *B. (Ph.) africanus ovoideus* was present.

Habitat No. 17. A large dam at Kisesa, slightly smaller than No. 16. There was much human and cattle contact. No bulinid snails were found.

Habitat No. 18. A large dam at Kanyelele, with little human and cattle contact. " *B. (B.) coulboisi-tropicus* " intermediate was present in the body of the dam, and *B. (Ph.) nasutus productus* and *B. (B.) forskalii* in an inlet and a spillway during the rains when flooding occurred.

Habitat No. 19. A large dam at Sola near Maswa District Headquarters with a capacity of about 100 million UK gallons or 360 acre-feet (about 454 600 m^3) and area of about 85 acres (34.5 ha). There was much human and cattle contact. *B. (Ph.) africanus ovoideus, B. (B.) forskalii and the* " *B. (B.) coulboisi-tropicus* " intermediate were present.

Methods

Habitats under regular observation were visited about once a fortnight in the case of " hafirs " and once a month in the case of large dams. In an attempt to detect any seasonal fluctuations in bulinid snail population densities in these habitats, as well as changes in infection rates with mammalian schistosomes, snails were collected in as uniform a manner as possible. Using a hand net, the same man collected steadily for 20 minutes in each " hafir " and for two hours in a dam. Snails were brought into the laboratory and examined individually for infection in about 2.5 cm of water in tubes 3×1 inches (7.5×2.5 cm) which were placed under a strong electric light. Golden hamsters and mice were exposed to any mammalian schistosome cercariae shed and dissected about three months later for the identification of the infection. Snails were returned to their habitats within 24 hours in the case of " hafirs " in Mwanza (i.e., No. 1-11), but the very small numbers of bulinids collected from the large dams were discarded as it was considered these made little difference to the population density of snails in these habitats. Snails from outside Mwanza district were also not returned to their habitats, because of the distances involved.

Results

The densities of bulinid populations were very low in both " hafirs " and dams, being lower in dams than " hafirs ". Table 1 shows the total numbers of

TABLE 1

TOTAL MONTHLY NUMBERS OF *PHYSOPSIS* FROM ALL HABITATS AND NUMBERS INFECTED

Snail	No. of snails	1962						1963								
		July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April	May	June	July	Aug.	Sept.
B. (Ph.) nasutus productus	Collected	846	31	231	98	244	75	3	408	722	469	87	106	93	448	
	Infected	1	0	5	1	5	0	0	5	1	4	0	5	3	5	
B. (Ph.) africanus ovoideus	Collected	3	0	19	16	3	0	10	0	51	0	2	66	243	257	269
	Infected	0	0	2	0	0	0	0	0	0	0	0	1	0	0	0

Physopsis spp. collected monthly and their rates of infection with mammalian schistosomes. The differences between monthly figures do not indicate fluctuation in the snail population as, for different reasons, not all habitats could be visited each month. Table 2 shows the contribution of each habitat to the total number of snails collected and the proportion infected with mammalian schistosomes. The number of collections made at each habitat includes those occasions when no snails were found. This indicates the relative densities of the snails in the various habitats.

TABLE 2

TOTAL NUMBERS OF *PHYSOPSIS* TAKEN FROM EACH HABITAT AND NUMBERS INFECTED

Snail	Habitat No.	No. of snails collected	No. of snails infected	Total collections made
B. (Ph.) nasutus productus	1	319	3	16
	2	462	11	18
	4	56	1	16
	6	13	0	13
	7	42	0	13
	8	511	1	13
	11	34	0	13
	12	1 231	6	7
	13	439	0	8
	14	507	11	4
	15	247	2	5
	Total	3 861	35	
B. (Ph.) africanus ovoideus	9	7	1	13
	10	808	1	7
	16	24	0	10
	19	100	1	5
	Total	939	3	

Identification of the infections was made difficult by the small numbers of infected snails, since hamsters or mice exposed to cercariae pooled from fewer than four snails almost always returned unpaired male schistosomes and there is no reliable character for distinguishing males of *S. haematobium* from those of *S. bovis*. However, *B. (Ph.) nasutus productus* was experimentally found refractory to *S. bovis* (Kinoti, 1964) and unpaired males were therefore identified as *S. haematobium*. Table 3 shows the schistosomes recovered from various habitats.

B. (B.) forskalii has a widespread but very sporadic distribution in the habitats and is probably of little epidemiological significance. Its susceptibility to the local *S. haematobium* has not been tested but Cridland (1955) was unable to infect it in Uganda. None of the 5000 specimens examined by Webbe (1962) was found infected. It is experimentally susceptible to *S. bovis* (Kinoti, 1964), but none of the several hundred wild specimens examined during the present study was found shedding mammalian schistosome cercariae.

A bulinid described by Dr Mandahl-Barth (personal communication) as being intermediate between *B. (B.) tropicus* and *B. (B.) coulboisi* had a more limited distribution than the other snails. It was present in habitats No. 5 and 18. In both habitats it had a low density during most of the period of study. In habitat No. 5, however, its density built up from three in March 1963 to about 200 snails per minutes' search in September. It is not known to be susceptible to *S. haematobium*, *S. bovis* or *S. mattheei*. In one exposure of 50 laboratory-bred progeny of this snail, originally from Shinyanga, to the local *S. bovis*, no infections were obtained.

TABLE 3
SCHISTOSOMES RECOVERED FROM VARIOUS HABITATS

Habitat No.	Date	Snail host	Schistosomes recovered	Remarks
1	Sept. 1962	*B. (Ph.) nasutus*	Males only	*S. haematobium* [a]
2	Sept. 1962	*B. (Ph.) nasutus*	Males only	*S. haematobium* [a]
2	May 1963	*B. (Ph.) nasutus*	Males only	*S. haematobium* [a]
4	Oct. 1962	*B. (Ph.) nasutus*	Unidentified	Too few cercariae for inoculation of hamsters
8	April 1963	*B. (Ph.) nasutus*	Males only	
12	July 1962	*B. (Ph.) nasutus*	Unidentified	Mouse died prematurely
12	Feb. 1963	*B. (Ph.) nasutus*	*S. haematobium*	Pairs in hamsters
12	March 1963	*B. (Ph.) nasutus*	Unidentified	
12	April 1963	*B. (Ph.) nasutus*	Males only	*S. haematobium*
14	Nov. 1962	*B. (Ph.) nasutus*	*S. haematobium*	Pairs in mouse and hamsters
14	Feb. 1963	*B. (Ph.) nasutus*	Males only	*S. haematobium*
14	Aug. 1963	*B. (Ph.) nasutus*		
15	Sept. 1962	*B. (Ph.) nasutus*	Males only	*S. haematobium* [a]
15	Nov. 1962	*B. (Ph.) nasutus*	Males only	*S. haematobium* [a]
10	June 1963	*B. (Ph.) africanus*	Unidentified	
19	Sept. 1962	*B. (Ph.) africanus*	Males only	*S. haematobium* or *S. bovis*

[a] So attributed because *B. (Ph.) nasutus productus* has been found refractory to *S. bovis* (Kinoti, 1964).

THE ROLE OF STREAMS

The majority of rivers and streams of Sukumaland are markedly seasonal. In the rains they are flooded in their lower courses but in the dry season their volume is greatly reduced, with flow ceasing altogether in the smaller rivers, leaving extensive sand beds. Before drying up completely some of these streams and rivers are reduced to a series of pools, the duration of which depends largely on their situation. In the upper courses particularly, where little or no silting occurs, these pools may last throughout the dry season. In some localities these form the main water supply for man and livestock.

Occurrence of B. (Ph.) africanus ovoideus *in streams and its infection with* S. haematobium *and* S. bovis

In November 1962, *B. (Ph.) africanus ovoideus* were collected from two streams at Bariadi in North Maswa. These streams feed the Bariadi river which in turn flows into the Simiyu river. Snails were collected from both streams; 33 of 214 (15.4%) specimens from one and 11 of 1158 (0.9%) from the other emitted large numbers of mammalian schistosome cercariae. Several hamsters and mice were exposed to cercariae pooled from all the snails. Paired *S. bovis* adults were recovered from the mesentery and liver, and typical *S. bovis* ova from the liver, of a hamster dissected 52 days after exposure. Ova and adults of *S. haematobium* were found in another hamster dissected about three months after exposure. The other hamsters and the mice returned *S. bovis* and *S. haematobium*.

Following the observation that *B. (Ph.) africanus ovoideus* transmitted both *S. haematobium* and *S. bovis* at Bariadi, a number of other streams were examined for this snail. In Mwanza District the snail was found in:

(*a*) Misungwi stream, where *S. haematobium* infection rates of up to 10% have been recorded.

(*b*) Beda stream near Fela railway station, where on two occasions snails have been found shedding mammalian schistosome cercariae.

(*c*) A stream near Misungwi. No snails were infected.

In Kwimba it has been recorded from:

(*a*) The Korimije tributary of Ngongwa river. No snails were infected.

(*b*) The first tributary of the Nyambehu river on the Mantare-Nyambiti road. Here one specimen was found shedding schistosome cercariae in May. Male schistomes were recovered from a hamster exposed to these cercariae.

In Maswa only streams in the Bariadi area were examined. Here *B. (Ph.) africanus ovoideus* is the major molluscan host of *S. bovis* and *S. haematobium.*

It should be noted that the surveys of these streams, with the exception of Bariadi and Misungwi, were carried out during or shortly after the rainy season, when snail populations are generally at their lowest level.

The transmission pattern of schistosomes in streams

It was not possible in the present study to make detailed quantitative observations on the snail populations or cercarial infection rates in any stream. Periodic visits to Misungwi and the Bariadi streams have, however, shown a transmission picture correlated to fluctuations in snail populations.

Bariadi streams. These streams were first examined in November 1962, which is the end of the dry season. The two streams examined consisted of large pools connected by a very gentle flow of water. Many of the pools contained large numbers of *B.(Ph.) africanus ovoideus*, with a schistosome infection rate of up to 15% in one stream.

The streams were again visited towards the end of January 1963, after about two months of rain. A third stream was also surveyed. They were flowing quite fast and the pools had been obscured by the flow. Snails were much more difficult to find than previously, and only two of the 156 specimens of *B. (Ph.) africanus ovoideus* recovered from the original two streams shed mammalian schistosome cercariae. During this visit a number of temporary pools were examined for *B. (Ph.) nasutus productus* in the area. This snail was found in two large pools. Most of the pools appeared to be too temporary for the establishment of snails in them. Streams are the main source of water, even in the rainy season, in the area covered by these observations. None of the 90 *B. (Ph.) nasutus productus* collected from the two pools shed mammalian schistosome cercariae.

When the streams were finally visited in June, their rate of flow had slowed down considerably, and pools were becoming more clearly marked along the original two streams. Large numbers of young snails and egg masses were present in them. No snails or egg masses were found in the third stream, which was flowing much more swiftly than the others. Four of the 80 adult snails collected were infected.

Misungwi stream. The large dam (Habitat No. 16) is built across this stream but in the rains two spillways connect the body of the dam and the stream below the dam wall. The stream, especially just below the dam, is used intensively for bathing and washing clothes by the inhabitants of Misungwi village throughout the year. Cattle are watered further down the stream. In the dry season it is reduced to a series of pools, which contain large numbers of *B. (Ph.) africanus ovoideus.*

It was first examined in the dry season (October 1962), when three of 30 *B. (Ph.) africanus ovoideus* collected from a pool were found infected with *S. haematobium.* Examinations of the upper mile in the middle of November and in January (rainy season) showed the presence of snails on marginal vegetation. The stream was flowing quite fast and in many places pools had been obliterated. In May and July flow was more gentle and there was a clear increase in the numbers of snails, especially of the young ones. Only one of the 130 large snails recovered during those visits was infected.

In September pools along most of the stream had formed and contained large numbers of snails. Infection rates had also increased—seven out of a total of 129 adult snails taken were infected. When these observations were terminated in August 1963 it was too early to identify the infection by dissection of hamsters exposed to the cercariae, but it is mostly likely to be *S. haematobium* as this part of the stream is intensively used by man and has little contact with cattle.

Teesdale (1962) found that in Kitui, Kenya, flood conditions greatly reduced the numbers of *B.(Ph.) africanus ovoideus* and other snails in streams and that as the dry season progressed snail populations built up and cercarial infection rates increased. The same general picture would seem to prevail in the Misungwi and Bariadi streams, and is probably applicable to many other streams in this region. The transmission of the liver fluke, *Fasciola gigantica*, in the Bariadi area is reported by the veterinary station there to be markedly seasonal, with a peak around September-November, at the end of the dry season.

Aestivation of B. (Ph.) africanus ovoideus

In April 1963, a pool in an old gravel quarry, supporting a large number of *B. (Ph.) africanus ovoideus*, *Biomphalaria pfeifferi* and two operculates (*Pila ovata* and *Bellamya* sp.) was discovered in the higher of the two spillways of the Misungwi dam. The pool had been left behind when outflow from the dam had stopped and, having a gravel bottom and sides, dried up rapidly. In June, when the pool had been dry for about a month, four aestivating *B. (Ph.) africanus ovoideus* and three *P. ovata* were found under stones and dry leaves. On being placed in water the snails extended within two hours. The *Physopsis* were kept in the laboratory and started laying egg masses after about a week. Collections were again made in July and September, i.e., two and four months after the drying up of the habitat. On the first occasion four *B. (Ph.) africanus ovoideus* were collected and one was alive, and on the second occasion 10 were collected and one was alive. Operculates collected on both occasions were alive. In the September collection a foam containing numerous young operculates which had not yet hatched at the time the habitat had dried up was taken. The young operculates emerged when the foam was left in water overnight and started feeding on boiled lettuce a day later.

Thus *B. (Ph.) africanus ovoideus* is able to aestivate for at least four months. The ability to aestivate is by no means a rare occurrence among pulmonate gastropods (Morton, 1963), and, although these observations were not extended beyond September, there can be little doubt that aestivating snails would have survived for another two months or so, when rain was expected.

DISCUSSION

The results of work on " hafirs " (Tables 1 and 2) show that some transmission of *S. haematobium* takes place in these habitats. The amount of transmission occurring in these sites, however, is relatively unimportant in comparison with the situation in smaller habitats—rain pools (Webbe, 1962) or pools in streams such as Misungwi. Considering " hafirs " as a group no pattern of seasonal transmission or fluctuation of snail populations can be discerned. Several factors are responsible for obscuring such a pattern. In the first place, snail populations in many of the " hafirs " were too small to allow an accurate assessment of fluctuations in their densities. Secondly, the effects of human and cattle activities vary from one habitat to another. The intensity, duration and timing of those activities depend on the availability of other water, which in turn depends on such factors as the timing, duration and amount of rainfall and on the situation of the " hafir ". Thus, for instance, whereas the disturbance by removal of vegetation of habitat No. 2 reduced the catch from 112 snails in April to 55 in June, habitat No. 1 remained relatively undisturbed during this period owing to the presence of water in pools and rice-fields in the surrounding ground and experienced disturbance in August when these pools and fields had dried out.

A consideration of individual " hafirs ", however, suggests that snail populations build up in the rainy season when disturbance by man and cattle is minimal. As drier conditions set in and increased use is made of " hafirs " there is an increase in cercarial infection rates until human and cattle activity seriously interfere with the snail population, when both infection rates and snail populations decline. As has been pointed out above, happenings in the various habitats are so staggered in time as to obscure a seasonal picture when " hafirs " are considered as a group.

In the large dams bulinid snail population densities are too low to allow detection of population changes by the sampling technique employed. *B. (Ph.) africanus ovoideus* was found in Sola (No. 19) and Misungwi (No. 16) dams but was absent from Kisesa (No. 17) and Kanyelele (No. 18). The "*B. coulboisi-tropicus* " intermediate form was the only bulinid recovered from four other dams examined—Nyambiti (Kwimba District), Sepewa and Mkulani (Maswa District) and one near Kolandoto (Shinyanga District). But even when present, the *B. (Ph.) africanus ovideus* population density is so low that, coupled with the dilution of any schistosome miracidia in these large water reservoirs, little, if any transmission, takes place in large dams.

It is interesting to note that *B. (Ph.) nasutus productus* has never been recorded from the body of large dams although during floods it may be found in spillways and channels leading into the dams. It is not known what factors limit its establishment in dams. *B. (Ph.) africanus ovoideus*, however, is found in both dams and " hafirs ". It appears to be essentially a riverine species and its presence in or absence from dams or " hafirs " seems to depend on the situation of these. The snail is found chiefly in the upper reaches of streams, where pools shaded by vegetation, particularly sedges and grasses such as *Typha* sp., form suitable habitats. Dams or " hafirs "

built across such streams or valleys are likely to contain the snail.

The evidence so far available suggests that *B. (Ph.) africanus ovoideus* is entirely responsible for the transmission of *S. bovis* in this region. No infected *B. (Ph.) nasutus productus* has ever been recorded from field studies. Experimentally it has proved refractory to the local *S. bovis* (Kinoti, 1964). Streams are the main habitats of *B. (Ph.) africanus ovoideus*, and it would therefore be expected that the transmission of *S. bovis* mostly takes place there. The observations on streams recorded in this paper do not cover a long enough period of time to reveal a definite transmission picture of this parasite, but they suggest that in pools snail populations build up rapidly in the dry season and with increased cattle watering cercarial infection rates go up. In the rainy season floods greatly reduce snail populations. The transmission of *S. haematobium* by this snail follows the same pattern as that for *S. bovis*.

The contribution of *B. (Ph.) africanus ovoideus* to the over-all *S. haematobium* transmission in this region cannot at present be accurately assessed. Two types of evidence, however, point to its being at least significant. Firstly, the snail has been found naturally infected with *S. haematobium* in two streams and is easily infected experimentally. In addition, it appears to have quite a wide distribution. Although transmission may take place in some "hafirs" (three of 16 *B. (Ph.) africanus ovoideus* from a "hafir" in Maswa town were found infected with *S. haematobium* in September 1962), streams are the most important sites. Secondly, since evidence strongly points to *B. (Ph.) africanus ovoideus* being entirely responsible for an average *S. bovis* prevalence in cattle of about 60% of this region, it appears reasonable to suppose that this snail makes a significant contribution to *S. haematobium* transmission, particularly as the same sites are often shared by man and cattle. The role of *B. (Ph.) nasutus productus* and *B. (Ph.) africanus ovoideus* in the transmission of urinary bilharziasis probably varies from one locality to another. In the area studied by Webbe (1962), for instance, *B. (Ph.) nasutus productus* is the principal intermediate host, whereas *B. (Ph.) africanus ovoideus* appears to be the more important intermediate host at Bariadi. But, considering the lake region of Tanganyika as a whole, our knowledge of the biology of schistosomes of importance to man can be regarded as only qualitative until the relative importance of the various snail species is determined more precisely.

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RÉSUMÉ

Dans la Province des Lacs, au Tanganyika, de vastes réservoirs artificiels, certains de très grandes dimensions, d'autres plus modestes nommés « hafirs », ont été aménagés pour pallier la pénurie d'eau en saison sèche. La forte endémicité locale de l'infection à *Schistosoma haematobium* a incité l'auteur à étudier l'influence de ces installations sur la transmission de ce parasite et sur la transmission de *Schistosoma bovis*, parasite du bétail très commun dans la région. L'enquête a duré un an environ.

On a pu démontrer que, dans la zone étudiée, *Bulinus (Physopsis) africanus*, un des vecteurs de *S. haematobium*, est également l'hôte intermédiaire de *S. bovis*. En revanche, *B. (Ph.) nasutus* ne semble pas intervenir dans la transmission de ce dernier parasite. Dans les grands réservoirs, on a relevé la présence de *B. (Ph.) africanus* mais en petit nombre. Ce fait, et la très forte dilution des éventuels miracidies dans ces énormes masses d'eau font penser que la transmission des deux types d'infection y est très faible. Dans les « hafirs », une certaine transmission de *S. haematobium* s'effectue en saison sèche, par l'intermédiaire de *B. (Ph.) nasutus* et de *B. (Ph.) africanus*, par suite de la fréquentation des réservoirs par un plus grand nombre de personnes. Il apparaît que réservoirs et « hafirs » ne jouent qu'un rôle secondaire dans la

propagation des deux types d'infection et que la transmission des parasites a lieu essentiellement dans les rivières de la région.

On a par ailleurs observé que *B. (Ph.) africanus* est capable d'estiver pendant plus de 4 mois et il est vraisemblable qu'il peut survivre pendant toute la saison sèche. Ce vecteur est très répandu et on peut supposer qu'il intervient pour une grande partie dans la transmission de *S. haematobium* et de *S. bovis* aux endroits fréquentés à la fois par les humains et le bétail.

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