STUDIES ON SURFACE MEMBRANE PROTEINS OF *SCHISTOSOMA MANSONI*

A thesis presented for the degree

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DOCTOR OF PHILOSOPHY

by

BATOOL HASHIM EL-ADHAMI

Department of Biochemistry

University of Glasgow

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Abbreviations

Abbreviations used in this thesis are recommended in the Biochemical Journal Instructions to Authors (revised, 1978), with the following additions:

GENERAL

BSA	Bovine Serum Albumin
BP	Basic Protein
$C_1(14)$	Coding System Utilized
$C_5(17)$	to Designate Individual
$C_6(12)$	Clones of *S. mansoni*
$C_2(4)$	
CIE	Cross-Over Immunoelectrophoresis
EA	Egg Antigen
E/Lac	Eagle's Medium + 0.5% Lactalbumin Hydrolysate
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
FP	Fast Pellet
FTS	Frozen-Thawed Supernatant
IEF	Isoelectric Focusing
IEP	Immunoelectrophoresis
m.p.	Mixed Population
MP	Membrane Pellet
PBS	Phosphate Buffered Saline
SDS	Sodium dodecyl sulfate
WHP	Worm Homogenate Pellet
WHS	Worm Homogenate Supernatant
SF	Soluble Fraction

CHEMICAL

DMSO	Dimethyl Sulfoxide
PEG	Polyethylene Glycole 6000
PPO	2,5-diphenyloxazole
TCA	Trichloroacetic Acid
TEMED	N,N,N',N Tetramethylethylenediamine

SEROLOGICAL

FCS	Foetal Calf Serum
Ig	Immunoglobulins
NMS	Normal Mouse Serum
NRS	Normal Rabbit Serum
RABP	Rabbit Anti-Basic Protein
RAIgA	Rabbit Anti-Mouse IgGA
$RAIgG_{2a}$	Rabbit Anti-Mouse IgG_{2a}
$RAIgG_{2b}$	Rabbit Anti-Mouse IgG_{2b}
RAIgM	Rabbit Anti-Mouse IgM
RAMIg	Rabbit Anti-Mouse Immunoglobulins
RAFT	Rabbit Anti-Frozen-Thawed Supernatant
RAM	Rabbit Anti-Membrane Fraction

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SUMMARY

SUMMARY

The protein composition of schistosome fractions extracted from adult worms of S. mansoni was studied by electrophoresis in thin-layer polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-gels). The SDS pattern showed a complex mixture of protein components with molecular weight range of approximately 28,000-112,000.

Isoelectric focusing in thin-layer polyacrylamide gel (IEF) of radiolabelled fractions from adult worms was carried out. Patterns of the radiolabelled membrane fractions (membrane pellet and frozen-thawed supernatant) showed the presence of protein bands at pH between 7.2-8.2. These bands were absent in fractions prepared from the whole worm without membrane. The stained IEF gels of the same fractions had confirmed the results obtained with the radiolabelled fractions. It was concluded that there is a specific component(s) in the membrane fractions with isoelectric points at pH between 7.2-8.2. This component(s) was termed the basic protein (BP) and found to be present in radiolabelled fractions extracted from the schistosomular stage of the parasite.

BP appeared to be synthesized by the parasite as shown by internal radiolabelling of adult worms and schistosomula. However, it has been found that the basic region of the IEF gel (pH 7.2-8.2) does contain mouse immunoglobulins. But, on the specific removal of these immunoglobulins, the parasite antigens were still detectable by the coomassie blue staining. In addition, antibodies in rabbit antiserum raised specifically against partially purified BP showed no binding to the schistosomular surface membrane. Thus, BP might not be expressed on the outer surface of the parasite.

The function of BP present in the surface membrane of the parasite is not clear. Yet, it might contribute to the integrity of

the electronegatively charged schistosome tegument, by associating with the acidic phospholipids shown to be present in the tegument of adult worms.

The frozen-thawed supernatant fraction (FTS) was used to study some characteristics of surface membrane proteins of adult schistosomes. It appeared to contain a complex mixture of proteins and glycoproteins. Antibodies in immune mouse serum collected at late stages of infection with S. mansoni recognized at least 3 antigens in the FTS fraction. Attempts to assess the amounts of radioactively labelled antigens recognized by mouse antibodies had met with limited success. This fraction contained some immunoglobulin classes and subclasses, mainly, IgG_{2a}, IgG_{2b} and IgA, but not egg antigen.

Isoelectric focusing of the FTS fractions extracted from individual clones of S. mansoni showed the presence of BP in all clones examined. But, BP was less labelled in some clones than in the others. It was concluded that BP is synthesized by all clones yet at different rates. Differences in the rate of incorporation of ^{35}(S)-methionine into the surface membranes of schistosomula and adult worms derived from individual clones were reported. In addition, a direct correlation between the percentage of recovery of adult worms from mice infected with individual clones of S. mansoni and the rate of incorporation of ^{35}(S)-methionine into schistosomula of these particular clones was noticed. It was suggested that the high rate of metabolism shown by an individual clone may account for the enhanced survival of the cercariae derived from that clone during penetration of the skin and migration through the vertebrate host.

CHAPTER I

INTRODUCTION

I. INTRODUCTION

I.1. General Introduction

Schistosomiasis is one of the very important human diseases caused by metazoan parasites. It is widely distributed in the tropical and subtropical regions of the world. The parasites are blood flukes belonging to the class Trematoda. Three major species may infect man: Schistosoma haematobium, S. mansoni, and S. japonicum. The first affects primarily the urinary bladder, the other two the intestine. S. haematobium and S. mansoni occur in many parts of Africa and South-West Asia, but South and Central America have S. mansoni only. S. japonicum is an oriental species. The most recent estimate of the prevalence of schistosomiasis has been 200 million cases (WHO, 1976). The distribution and intensity of the disease are increasing as a result of the extension of irrigation schemes and concentration of human populations particularly in the developing countries (WHO, 1980). Schistosomiasis does not only constitute a public health problem, but it also has long-term consequences for social and economic development. The economic importance of the disease may be assessed by the loss of potential wealth due to working disability, the costs of medical care and the loss due to failure in taking advantage of chances for economic development especially for agriculture and animal production (Macdonald and Farook, 1973). During the last decade, two main approaches have been applied as control measures of schistosomiasis: the use of chemotherapeutic drugs for treatment of infected people and the application of chemicals and biological control agents for the elimination of the snails. Some advances have been achieved in the control of this disease in limited areas of the world as in Japan and Puerto

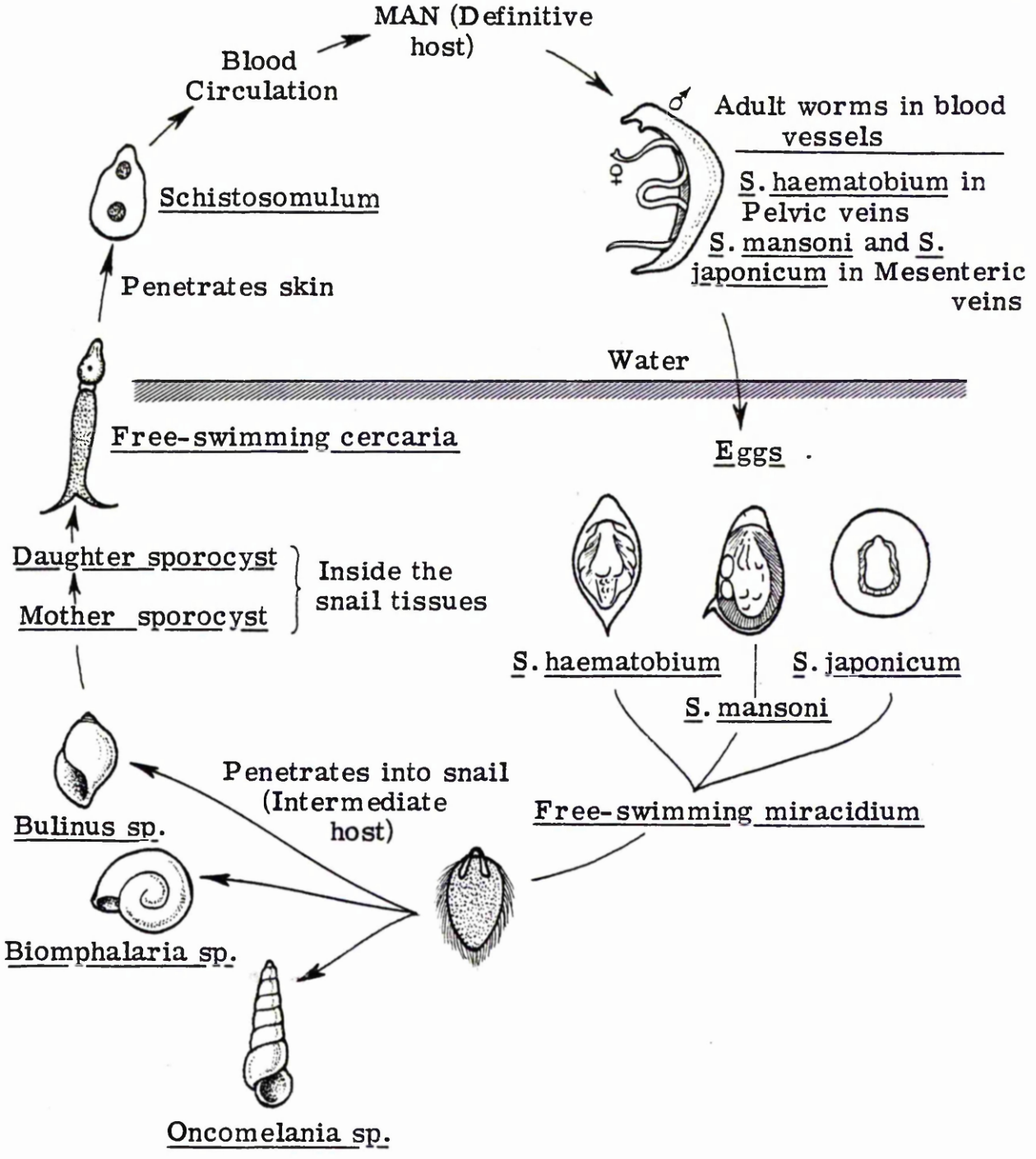
Rico (WHO, 1980). In recent years, there has been a marked movement towards the application of modern immunological methods to the problem. Improved understanding of the immunological aspects of the disease should help in the development of a vaccine which could be extremely beneficial.

I.2. Life Cycle

Schistosomes developed a complicated life-cycle including a sexual generation parasitic in vertebrates (the definitive host) and an asexual generation parasitic in molluscs (the intermediate host). Relatively short-lived, free-swimming larval stages infect each of these hosts. The vertebrate infecting stage is termed the cercaria and that infecting the molluscs is termed the miracidium. It is important to note that host specificity is more clearly defined in the intermediate molluscan host than in the definitive vertebrate host. The miracidium is usually host specific and can only survive in one or a limited number of species of snails, whereas adult worms can develop in a number of experimental animals. Although the three species of schistosomes which infect man have a basically similar life-cycle, they show some differences in the morphology of the adults, the larval stages, and the eggs. They also differ in their specific infectivity to each of the definitive and intermediate hosts (Fig. I.1.). Since S. mansoni represents the species which has been most studied and is also the species used in this study, I shall only describe its life-cycle in detail. Adult worms of S. mansoni live in the small branches of the mesenteric veins. The male is shorter and broader than the cylindrical female. The lateral borders of the male's body are folded together to form a groove (the gynaecophoric canal) within which the female is held. Both sexes have oral and ventral suckers which might be used to

Figure I.1. Schematic Life-Cycle of Human Schistosomes; S. haematobium, S. mansoni, and S. japonicum

Adult worms live in the pelvic veins (S. haematobium) or the mesenteric veins (S. mansoni and S. japonicum) where they produce eggs. Eggs voided with urine or faeces hatch into miracidia which infect the appropriate species of snails. Inside the snail tissues, the miracidium undergoes asexual reproduction involving two generations of sporocysts which produce cercariae. Cercariae represent the infective stage of the parasite which penetrates human skin. In the dermis, the heads of the cercariae become the schistosomula which thereafter migrate, probably via the circulatory system to the lungs then within 2 weeks they reach the hepatic portal system. Here, males mate with females and carry them into the pelvic veins (S. haematobium) or mesenteric veins (S. mansoni and S. japonicum), where full development of the females and egg production occur.


MAN (Definitive host)
Blood Circulation
Adult worms in blood vessels
S. haematobium in Pelvic veins
S. mansoni and S. japonicum in Mesenteric veins
Schistosomulum
Penetrates skin
Water
Free-swimming cercaria
Eggs
Daughter sporocyst
Mother sporocyst
Inside the snail tissues
S. haematobium
S. japonicum
S. mansoni
Penetrates into snail (Intermediate host)
Free-swimming miracidium
Bulinus sp.
Biomphalaria sp.
Oncomelania sp.

fix the worms into the walls of the blood vessels. Eggs laid by the female are usually immature and bear a large lateral spine by which the eggs may retain their position in the blood vessels and which may also help to penetrate through the vessel's walls. Eggs gradually work their way out of the blood vessel into the tissues of the intestinal wall and finally into the lumen, during which time the embryos mature inside the eggs into fully developed miracidia. Some eggs escape with the faeces, others become trapped in various tissues especially in the intestine and the liver where they cause inflammation leading eventually to granuloma formation. The eggs are important pathogenic agents. The pathology of schistosomiasis is essentially a series of chronic inflammatory lesions produced by the host's response against the eggs or their products (Warren, 1974). Under suitable environmental conditions and dilution of faeces with fresh water, the escaped eggs hatch into a ciliated larval stage called the miracidium. The free-swimming miracidium finds and penetrates a suitable species of fresh water snail, usually Biomphalaria glabrata. The mechanism by which the miracidium becomes attracted to a particular species of snails is thought to be dependent on chemicals produced by the potential snail host (MacInnis, 1965; MacInnis, Bethel and Cornford, 1974). After penetration, the ciliated outer coat disappears and the miracidium transforms into a mother sporocyst. Within the snail tissue, the mother sporocyst enlarges in size and begins to produce the daughter sporocysts which eventually break out of the mother sporocyst. The germinal cells of the daughter sporocyst develop into fork-tailed cercariae, which escape from the snail and become free-swimming in water. The cycle within the snail between the penetration of the miracidium and the production of the cercariae lasts about 4-5 weeks.

The cercaria is the infective larval stage which penetrates the skin of man. The penetration process of the cercariae involves the secretion of lytic enzymes from their penetration glands which may help to disrupt the ground substance between the skin cells and thus facilitate penetration (Stirewalt, 1966). Once the cercaria successfully enters the human host it is termed a schistosomulum. The changes which take place upon skin penetration consist of (a) loss of tail, (b) loss of precise shape and exhibition of elongated, worm-like appearance, (c) shedding of the cercarial coat (the glycocalyx), (d) secretion of the contents of penetration glands, (e) development of sensitivity to fresh water so that the schistosomulum becomes unable to live in fresh water even for a short period of time, and (f) replacement of the cercarial trilaminate surface membrane with a heptalaminate membrane (Stirewalt, 1974). Schistosomula can be found in the skin for about 18 hours after penetration. They eventually find their way into the blood circulation and are carried to the lungs via the heart. Within two weeks, young worms appear in the liver where they feed on portal blood and undergo rapid growth. Thereafter, males mate with females and carry them into the mesenteric veins. Egg production generally occurs by the 40th day after infection. The cycle within the definitive host, between the cercarial penetration and the appearance of the eggs in the stool, takes about 5-6 weeks after infection.

I.3. Resistance of Vertebrate Host to Schistosomes

I.3.1. Innate Resistance

Innate resistance to infection is generally defined as the resistance displayed by an animal which has never experienced the

particular pathogenic organism either as a pathogen or as a related non-pathogenic variant (Humphrey and White, 1970). Here, I shall describe the innate mechanisms believed to be of importance in determining the success of schistosomes in establishing infection in the vertebrate host. Variations in host-specificity shown by the different species of the parasite, mechanisms of attachment to host's skin and the presence of fatty acids and lipids in the skin surface to act as stimulus to penetration (Clegg, 1969) are examples of innate resistance.

Despite the fact that host-specificity is well-defined in the three species of schistosomes which infect man, different ranges of natural and experimental infections of various other animals with these species have been reported. Natural infections of monkeys and rodents with S. mansoni are known. Similarly, monkeys can be naturally infected with S. haematobium and various rodents can be experimentally infected with this parasite. S. japonicum is the least host-specific among the three human species of schistosomes. It can be found naturally in a wide range of domestic animals as well as in various wild rodents (Smithers and Terry, 1969a). There is no explanation for the great variations shown by different hosts with regard to their susceptibility to infection with S. mansoni. But, it is likely that many factors are concerned in controlling host-specificity and susceptibility to schistosome infection. Genetic variations of both parasites and hosts from geographically different areas may be one of the major factors. There are at least two strains of S. japonicum endemic to Formosa (Taiwan) which are non-infectious to man. In nature, rodents are the definitive hosts for these two strains, but man is innately resistant to infection (Hsü and Hsü, 1968). Another explanation for variations in host-specificity is the death of cercariae in the skin. Schistosome cercariae can penetrate the skin and establish an adult infection in a

normally susceptible host. Numerous investigations on the invasion of the skin by schistosome cercariae have shown that a relatively large number of cercariae die or show severe damage within a short time after penetrating the skin of a susceptible host. Clegg and Smithers (1968) studied the recovery of schistosomula of S. mansoni from the skin of mice, rats and hamsters. In hamsters, 10% of the cercariae which enter the abdominal skin die within 15 minutes after penetration, in mice about 30% and in rats 50%. Comparison of the proportion of cercariae which died in the skin of hamsters and mice with the proportion recovered as adult worms showed that fewer cercariae died in the skin of hamsters (10%) than mice (30%) and correspondingly a larger proportion survived to become adults (66% in hamsters and 45% in mice). On comparing mice with rats, the percentage of dead schistosomula in the skin was higher in rats (50%) than in mice (30%). But, the percentage of adult worms recovered from rats (19%) was less than that predicted from the numbers lost in the skin. This might be due to the death of the schistosomula at some later stage of migration beyond the skin in rats. It is clear that the skin of some hosts is an important factor in innate resistance against invading cercariae. Ghandour and Webbe (1973) demonstrated that 2 hour old cercariae exhibit mortality of about 30% in mouse skin. This result is closely comparable to that observed by Clegg and Smithers (1968). Further study by Smithers and Gammage (1980) indicated that approximately 65% of cercariae which penetrate mouse skin failed to be recovered from the skin, lung and liver of infected animals. This proportion is higher than that obtained in the previous studies (Clegg and Smithers, 1968; Ghandour and Webbe, 1973). However, comparison between these results is difficult since different strains of mice and different batches of cercariae were used in each of these studies. The causes of death of some cercariae in the vertebrate host's skin

have not been determined yet. Several factors seem to be involved in affecting the survival of cercariae during penetration of the skin and migration through the host. These include: (i) the age and sex of the host, (ii) the physiological conditions of cercariae at the time of contact with host's skin, and (iii) the genetic variations of penetrating cercariae.

(i) The effect of age and sex of the host

Lewert (1958) suggested that skin penetration may depend largely on glandular secretions of the cercariae, which contain enzymes capable of liquifying glycoproteins of the basement membranes and ground substance of the skin. This suggestion was extended by Lewert and Mandlowitz (1963) who found that the density of the basement membrane and ground substance of mouse skin increases with age. The increase in skin density was considered to be of major importance in innate resistance of old mice to cercarial invasion of S. mansoni. They supported this view by using LAF strain of mice whose aged individuals retain the activities and characteristics of the skin of young mice of other strains. They demonstrated that there is no significant difference to infection with S. mansoni between old LAF mice (2 years old) and young CF mice (less than 1 month old). But, both showed significant difference from old CF mice (2 years old). It has been shown that the schistosomula die within the epidermis of mouse skin (Clegg and Smithers, 1968). Thus, the density of the basement membrane may be an important factor in skin penetration, but the density of the ground substance is unlikely to play a part causing the death of schistosomula. Purnell (1966) showed that the yields of adult worm recovery in both mice and hamsters infected with S. mansoni decrease rapidly with increase in age of the animals. Ghandour and Webbe (1973)

gave evidence that the age of the mouse host affects directly the proportions of cercariae dying during penetration of the skin and also the recovery of adult worms. In 2 day old mice, the level of mortality in the skin was found to be less than one-third the level in adult mouse skin (about 1 month old). Losses of cercariae in the skin increase substantially with the age of the host up to about 28-35 days. The high worm recovery in 2 day old mice was correlated with the low level of cercarial mortality in the skin (Ghandour and Webbe, 1973). Therefore, it seems likely that the age of the vertebrate host is an important factor which might affect the survival of the parasite upon skin penetration. Other less understood factors are reported. For instance, the sex of the host may influence the cercarial penetration of the skin. Purnell (1966) examined the effect of the sex of the host on the development of the parasite in mice and hamsters infected with S. mansoni and S. haematobium. He found that in both hosts the male animals gave significantly higher average of worm recovery than the females. Further evidence to support this study is still not available.

(ii) The effect of the physiological conditions of cercariae at the time of contact with host's skin

Ghandour and Webbe (1973) studied the effect of the age of cercariae on their mortality during penetration of host's skin. The number of cercariae of S. mansoni which died during penetration of mouse skin increased steadily with the ageing of cercariae following their emergence from the snail. The mortality was approximately 30% for 2 hour old cercariae and rises to 50% at 8 hours and 85% at 24 hours. The increase in the mortality level was found to be correlated with the decrease in infectivity which was estimated as percentage recovery of adult worms. Bruce, Weiss, Stirewalt and Lincicome (1969) investigated

the metabolism and glycogen content of the cercariae of S. mansoni by exposing infected snails to ^{14}(C) glucose and thereafter detecting the distribution of radioactivity in the emerging cercariae. Newly emerged cercariae showed a relatively high level of radioactivity in the glucose fraction. But this level of radioactivity was reduced to about one-fourth of the initial value when labelled cercariae were maintained free-swimming for 18 hours after emergence from the snail. Rai and Clegg (1968) demonstrated a reduced level of glycogen in the cercariae of Austrobilharzia terrigalensis which died in bird skin compared with the level in living ones. There is evidence that glucose or other products of snail metabolism are rapidly metabolized by the cercariae for the synthesis of proteins, nucleic acids and lipids (Bruce, Weiss, Stirewalt and Lincicome, 1969). Therefore, the size of the glucose reserve may determine the longevity of the cercariae after emergence from the snail. Penetration through host's skin and transformation into schistosomula may require the energy reserves of the cercariae. Thus, it is conceivable that some cercariae might die in the epidermis as a result of decline in their energy reserves followed by subsequent reduction in their metabolic activities before reaching the dermis where they can be in contact with host's nutrients. This suggestion could be confirmed in the finding of Ghandour who was able to show that the mortality of cercariae shed from snails which have been incubated in 2% solution of glucose is about half of those cercariae shed from normal snails (Smithers, 1976).

(iii) The effect of the genetic variations of penetrating cercariae

Smith and Clegg (1979) have established the existence of heterogeneity in S. mansoni. They suggested that variations in the level of acquired resistance to S. mansoni is related to variations

between different pools of cercariae. They strengthened their hypothesis by the demonstration that individual clones of S. mansoni, in which all the cercariae are genetically identical, showed very different levels of susceptibility to the immune response induced by a single pool. Such genetic variations among the cercariae could be reflected in their metabolic activities, i.e. some clones may have a higher rate of metabolism than the others. Since the rate of metabolism appears to be an important factor in skin penetration (Rai and Clegg, 1968; Bruce, Weiss, Stirewalt and Lincicome, 1969; Smithers, 1976), it is conceivable that clones with high rate of metabolism can penetrate host's skin more successfully than clones with lower rate of metabolism.

In summary, innate resistance of the host to schistosome infection may vary with age, genetic strain or the physiological conditions of both the host and the parasite. The general knowledge and understanding of the precise mechanisms utilized by schistosomes in penetration of the tissues of the host is still relatively incomplete. Similarly, the knowledge of natural defences of the host as related to these mechanisms is still deficient. Thus, further studies are essential in the field to amplify our knowledge of the innate mechanisms of the host, and should also result in better understanding of the concepts of acquired resistance and reasons for their variability in different host species.

I.3.2. Acquired Resistance

I.3.2.1. General Considerations

It is believed that acquired resistance to schistosomiasis is different from that type known to be acquired in bacterial or

viral infections in the following respects: (a) it develops gradually taking years to become pronounced and even then may be only partial, (b) the parasite persists after the primary infection, and (c) it establishes a state in some animals and probably man which enables the host to become resistant to re-infection, but at the same time unable to destroy the adult worms from the primary infection. This state has been termed as "concomitant immunity" (Smithers and Terry, 1969b). This term implies components from the immune system of the host. It is usually used in the context of tumour-bearing animals, in which the secondary transplant of the primary tumour is rejected while the primary tumour grows steadily and kills the animal.

Although it is generally agreed that acquired resistance to infection by schistosomes exists in man there has been no direct demonstration of this phenomenon yet. Evidence for acquired resistance in man has come from epidemiological studies. Many epidemiological studies have indicated that man develops increased resistance to re-infection and can tolerate infection of much higher levels on continuous exposure to the parasite. An example has been described by Gelfand (1973) comparing the infection with schistosomes in native Africans and immigrant Europeans in Rhodesia. Some other examples have been reviewed by Phillips and Colley (1978). The epidemiological evidence for development of resistance in man is based mainly on the decrease in the prevalence and intensity of the disease of selected individuals measured by reduction in egg output. It has been suggested that the intensity of infection (as measured by the egg output) decreases with ageing. On examining the egg output of individuals in a community where S. haematobium is endemic, a relatively stable fall in the mean egg output after the age of 14 was noted (Bradley and McCullough, 1973). In a further study, McCullough and Bradley (1973) presented data on

urinary egg output patterns over 3 years in the same community. It showed marked stability of the egg output among the children examined over the 3 year study period. The authors considered this result as demonstration of the concept of concomitant immunity of Smithers and Terry (1969b) in man. However, evaluation of evidence based on epidemiological studies is extremely difficult as it is confused by changes in human behaviour involving water contact (Jordan, Cook and Davis, 1974). Furthermore, many other biological, sociological and ecological factors may play important roles in explaining these observations (Warren, 1973). Clearly more reliable standards are needed to establish criteria for the evaluation of infection and the mechanisms involved in acquired resistance in man. To establish such criteria, Capron and collaborators published a series of immunological studies in human schistosomiasis. Capron, Camus, Carlier, Figueiredo and Capron (1977) demonstrated the presence of complement-dependent cytotoxic antibodies of the IgG class in serum obtained from schistosome infected patients in a community where S. mansoni is endemic. The sera from infected patients gave a higher percentage of cytotoxicity (63%) than did the sera from normal subjects (8.7%). The cytotoxic activity of lethal antibodies appeared to have no correlation with the mean egg output in the examined patients. Camus, Carlier, Capron, Bina, Figueiredo, Prata and Capron (1977) examined the correlation between the clinical aspects of the disease and some immunological parameters including serum immunoglobulin level, specific antibodies titre and delayed hypersensitivity response to whole worm extract. Direct relationship was reported between the IgG level and the hepatosplenomegaly. Delayed hypersensitivity response was found more frequently in hepatosplenic patients and was also directly related to the faecal egg output. Sher, Butterworth, Colley, Cook, Freeman and Jordan (1977) studied the correlation between the occurrence

of eosinophil-dependent cytotoxic antibodies and the intensity of infection (as judged by faecal egg count) in schistosome infected patients. It was found that groups of infected patients with low egg counts (60 eggs/ml faeces or less) showed a significantly lower level of eosinophil-dependent cytotoxic antibodies than did the patients with high egg output. It appeared that the cytotoxic activity of these antibodies is proportional to the intensity of the disease. However, no significant relationship was detected between the antibodies activity and the duration of infection. This assay can serve as an index for the intensity of human infection in chemotherapy programmes. Further studies on a wider scale (for example in a community) would be required to confirm this diagnostic application.

Numerous studies on acquired resistance to schistosome infection have been carried out in a wide range of experimental animals. The occurrence of some degree of resistance has been clearly demonstrated in most experimental hosts, but it is variable and sometimes absent (Stirewalt, 1963). Hence, two points should be stressed; (a) resistance to re-infection by cercariae has been considered as the criterion for the acquired resistance to schistosomiasis, and (b) resistance to re-infection in laboratory animals can be measured by several methods including a rapid assay based on the recovery of the schistosomula of a challenge from the lungs (Perez, Clegg and Smithers, 1974; Sher, Mackenzie and Smithers, 1974). It can also be measured by assaying the number of adult worms recovered by perfusion from the hepatic portal system of the host several weeks after infection (Smithers and Terry, 1965a). Information gained from animal experiments showed that host species vary widely in their ability to acquire resistance to schistosomes. Among primates, the rhesus monkey (Macaca mulatta) develops solid resistance to S. mansoni 16 weeks after exposure to normal or

irradiated cercariae (Smithers and Terry, 1967); the chimpanzee (Pan satyrus) does not show acquired resistance with the time or with repeated infection (Sadun, Lichtenberg, Cheever and Erikson, 1970). Studies on baboons (Papio cynocephalus) have indicated that these animals can acquire partial resistance to re-infection which develops slowly over a period of several months (Damian, Greeneand Fitzgerald, 1974).

A variety of rodents have been utilized for experimental studies with the schistosome species that attack man. Most experimentation has been performed with S. mansoni. With this species most laboratory animals show marked variations in the level of resistance that develops and the speed with which it is acquired. The rat is extremely resistant and shows a spontaneous decrease in the worm burden 4 or 5 weeks after initial infection (Smithers and Terry, 1965b; Perez, Clegg and Smithers, 1974). Whereas, both the mouse (Sher, 1977) and the hamster (Smith, 1975; Smith and Clegg, 1976) show full susceptibility to schistosomiasis. Finally, although information obtained from experimental studies in different animal species has helped to establish whether there is resistance to re-infection with schistosomes, the relevance of innumerable experiments performed in animals in assessing the development of resistance in man remains equivocal.

I.3.2.2. Mechanisms of Acquired Resistance

(i) In vitro mechanisms

The mechanisms responsible for initiating resistance and for maintaining this resistance to schistosome infection have not been elucidated yet. However, the establishment of many in vitro systems involving various components of the immune system is important in a way that it may help to identify the possible effector mechanisms whose relevance to immunity can be tested by appropriate experiments in vivo.

There is *in vivo* evidence indicating that the young developing schistosomulum is the vulnerable stage to the host's immune attack, as the subsequent stages become protected from the effects of the immune response (Sher, Mackenzie and Smithers, 1974; Perez, Clegg and Smithers, 1974). For this reason, the young schistosomulum has been chosen by most workers for experiments *in vitro*. Schistosomula can be prepared in several ways; (a) skin transformation by allowing the cercariae to penetrate through an isolated piece of rat or mouse skin (Clegg and Smithers, 1972), (b) artificial transformation by mechanical disruption of the cercariae (Ramalho-Pinto, Gazzinelli, Howells, Mota-Santos, Figueiredo and Pellegrino, 1974), and (c) serum transformation by incubating cercariae in a medium containing 50% fresh serum for about 3 hours (Eveland and Morse, 1975). Schistosomula can be cultured *in vitro*, where they grow and reach partial maturity including sexual differentiation and mating but with no egg production (Clegg, 1965). Although schistosomula prepared *in vitro* are similar in many aspects to those found *in vivo*, they are not necessarily identical. For instance, a freeze-fracture study has shown that schistosomula prepared by skin penetration and cultured *in vitro* have much lower proportions of the intramembraneous particles on the outermost leaflet of the tegument than the *in vivo* specimens (McLaren, Hockley, Goldring and Hammond, 1978). In studies on schistosomiasis, several *in vitro* systems involving antibodies or cooperation of antibodies and cells and causing damage to schistosomula have been described. Direct lethal activity of immune rhesus monkey serum against young schistosomula has been demonstrated *in vitro* (Clegg and Smithers, 1972). The lethal activity was found to be associated with an IgG fraction of the immune serum, and was dependent on a heat-labile material, presumably complement, present in fresh normal serum. Schistosomula incubated for 4-5 days

in culture medium containing red blood cells and serum were protected from the lethal effects of the immune serum. This might imply that the worm acquired materials during cultivation in vitro which are related to the protection they developed against the effect of the immune serum (Clegg and Smithers, 1972). Similar complement-dependent lethal antibodies have been detected in sera from: infected baboon and human patients (Smith and Webbe, 1974); rats, rabbits, and mice (Murrell and Clay, 1972; Dean, Wistar and Murrell, 1974), and guinea pigs (Dean, Wistar and Chen, 1975). It was believed that lethal antibodies may participate in inducing resistance to re-infection in vivo. But, the finding reported by Sher, Kusel, Perez and Clegg (1974) indicated that there is no correlation between lethal antibody production and immunity in artificially immunized rats. A partially purified membrane antigen isolated from adult worms of S. mansoni can absorb the lethal antibody activity from immune rat and rhesus monkey sera. Immunization of rats with this antigen produced high level of lethal antibody, but failed to induce resistance to infection (Sher, Kusel, Perez and Clegg, 1974). Furthermore, passive transfer of immune rabbit serum which showed strong in vitro lethal activity failed to protect mice against challenge infection (Murrell and Clay, 1972). Mice immunized either with live or attenuated cercariae developed low levels of lethal antibodies, but at the same time showed high resistance to challenge infection (Murrell, Dean and Stafford, 1975). From all these studies it was concluded that lethal antibody was not able to confer immunity on its own. It must act in collaboration with another antibody or cells in vivo. In this regard, several studies were carried out to examine the combined effects of antibody and cells as effector mechanism in acting against schistosomula in vitro. Schistosomula were found to be rapidly damaged by complement-dependent IgG-antibodies from schistosome infected

rat (Dean, Wistar and Murrell, 1974) and guinea pigs (Dean, Wistar and Chen, 1975), in the presence of neutrophils from normal rats. It was suggested that the effect of neutrophils was to enhance the damage initiated by antibody and complement. A further mechanism involving the cytotoxic effect of complement-dependent antibodies and macrophages on young schistosomula has been described (Perez and Smithers, 1977). Peritoneal cells from normal rats when sensitized with heat-inactivated immune serum caused damage to schistosomula over 20 hours incubation in vitro. The time course of development and decline of the antibody activity in rats was parallel to that of resistance to re-infection. It was suggested that this serum-dependent macrophage adherence phenomenon may reflect the effector mechanism of protection in vivo. No further study was reported to follow up this suggestion. Another system involving rat macrophages and serum was described by Capron, Dessaint, Capron and Bazin (1975) through the use of macrophages and rat IgE. Normal macrophages incubated with heat-inactivated immune serum, then added to schistosomula without further washing adhered to schistosomula and damaged the parasite. Adherence was completely inhibited by treating serum with mercaptoethanol. The serum activity was heat-labile and can be separated by passage through IgE-specific immunoadsorbent column. In later work, Capron, Dessaint, Joseph, Rousseaux, Capron and Bazin (1977) indicated that the cytotoxic characteristic of normal rat macrophages against schistosomula was in fact induced by a complex of IgE-antibody and circulating schistosome antigens present in immune rat serum. Butterworth, Sturrock, Houba, Mahmoud, Sher and Rees (1975) implicated the involvement of another antibody-dependent cell-mediated cytotoxicity system against schistosomula in vitro. The cell involved is the eosinophil and the system is complement-independent. Serum from schistosome infected patients can damage schistosomula in the

presence of normal human eosinophils as indicated by a ^{51}(Cr) release assay. This damage was confirmed by both light and electron microscopy using eosinophils from normal rats (Mackenzie, Ramalho-Pinto, McLaren and Smithers, 1977; McLaren, Mackenzie and Ramalho-Pinto, 1977). Eosinophils adhere to IgG-coated schistosomula by Fc receptors (Butterworth, David, Franks, Mahmoud, David, Sturrock and Houba, 1977; Mackenzie, Ramalho-Pinto, McLaren and Smithers, 1977) and peroxidase is secreted from the matrix of the eosinophil granules onto the surface of schistosomula (McLaren, Mackenzie and Ramalho-Pinto, 1977). Ramalho-Pinto, McLaren and Smithers (1978) and Anwar, Smithers and Kay (1979) described an alternative mechanism by which eosinophils adhere to and kill schistosomula *in vitro*. It depends on the activation of complement by the alternative pathway at the schistosomular surface, followed by subsequent adherence of eosinophils through their complement receptor (C3). This mechanism can occur in the absence of specific antibodies. Mahmoud, Warren and Peters (1975) presented evidence that the antibody-dependent eosinophil system is involved in the immune mechanism operating in mice *in vivo*. Treatment of mice with anti-eosinophil antisera abolished their immunity to challenge infection. In addition, passive transfer of resistance with immune serum was prevented when mice were pre-treated with anti-eosinophil antiserum. Therefore, several effector mechanisms are capable of killing schistosomula *in vitro*. Experiments *in vivo* have implicated eosinophil-mediated damage as an important aspect in immunity to schistosomiasis in living animals.

(ii) *In vivo* mechanisms

Despite the accumulated data on analyses of acquired resistance as it occurs *in vivo*, limited definitive information exists to the exact role of the immune system in providing protection against

schistosomes. However, it has been postulated that acquired resistance in schistosomiasis employs elements of both the humoral and cellular mechanisms of the immune system as well as non-specific effector cells such as eosinophils and macrophages.

A marked increase in the level of IgG has been demonstrated during infections with schistosomes in both human and experimental animals (Smithers and Terry, 1969a). There is evidence that 95% of the increase in serum IgG in rhesus monkey (Freeman, Smithers, Targett and Walker, 1970) and at least 90% of the increase in serum IgG in human patients (Dessaint, Capron, Bout and Capron, 1975) appear to be not parasite-specific. In attempting to define the components responsible for protection in resistant animals, passive transfer of immune serum or cells to normal recipients has been performed in human and experimental animals. Cook, Warren and Jordan (1972) administered large quantities of either normal IgG or hyperimmune anti-schistosome IgG obtained from S. mansoni infected adults into young non-infected children at monthly intervals. This attempt failed to prevent the development of new infections among the previously uninfected children. Similarly, Warren, Cook and Jordan (1972) failed to demonstrate any effect of the transfer of massive doses of IgG on pre-established infection in young children. Using transfer factor, Warren, David, Cook and Jordan (1975) reported an unsuccessful attempt to transfer immunity to children who have an early established infection. Passive transfer of resistance with serum has been achieved in some experimental animals. In a preliminary report, Maddison, Geiger, Botero and Kagan (1970) indicated that the transfer of serum from immune donor rats did not provide protection to the recipient rats. It has also been reported that transfer of homologous immune serum in rhesus monkey

failed to confer significant protection to the normal recipients (Smithers, 1976). Sher, Smithers and Mackenzie (1975) showed that sera from mice which have been infected with S. mansoni for 12-15 weeks transfer to normal recipients partial resistance to subsequent challenge infection. The transfer of immunity was judged by both the reduction in the recovery of adult worms and by the diminished numbers of schistosomula recovered from the lungs. This work was limited by several difficulties; the volume of the serum, the variable immunity level in donor animals and the time of serum transfer with respect to the cercarial challenge. In contrast, transfer of lymphoid cells from resistant donors failed to confer a significant reduction in lung recovery in recipient mice (Sher, Smithers and Mackenzie, 1975). The activity of transferred serum was found to be associated with an IgG fraction in mouse serum (Sher, Smithers, Mackenzie and Broomfield, 1977). In rats, passive transfer experiments have revealed a more complicated system. Phillips, Reid, Bruce, Hedland, Colvin, Campbell, Diggs and Sadun (1975) observed that the transfer of a moderate volume of serum (1.0-3.0 ml) obtained from 3-4 weeks infected animals enhanced the survival of schistosomula in the recipient animals, whereas large volumes conferred a slight degree of immunity. Passive transfer of serum from 7-weeks infected animals caused the greatest degree of protection in normal recipients when transferred prior to cercarial challenge. A delay in transferring immune serum, 3 days after challenge infection diminished its effects, indicating that serum transfer affected the early schistosomula. Furthermore, adoptive transfer of lymphoid cells conferred protection against schistosome infection, but this protection was abolished when immune serum and cells were transferred concomitantly. Smithers (1976) reported evidence that the effector mechanism in rats involves humoral factors and cells.

Acquired resistance to challenge infection in rats was not affected by treating animals with anti-lymphocyte serum, but it was severely reduced by whole body irradiation a few hours before challenge. Resistance to challenge infection was restored by reconstituting the irradiated animals with bone marrow cells from normal rats. All these findings suggested that acquired resistance to re-infection could be mediated *in vivo* by mechanisms which involve some combination of humoral factors and cells. Colley, Magalhas-Filho and Coelho (1972) studied the mechanisms of acquired resistance to schistosome infection by assaying the inflammatory dermal reaction of *S. mansoni* infected mice. The time sequence and cellular composition of the dermal response to a cercarial extract and living cercariae were investigated in either previously infected mice or in naive mice. The response in the re-exposed mice showed an early increase in polymorphonuclear cells (4-8 hours) which later developed into a mononuclear infiltrate (36-48 hours). By about 24 hours after infection, the degenerating schistosomula were evident in the dermis. The responses noted were further followed after passive transfer of immune serum and lymphoid cells from infected donors. Passive transfer of immune serum produced an early polymorphonuclear infiltrate against the cercariae, while lymphoid cells from infected donors resulted in a late mononuclear infiltrate. It was concluded that cooperation between humoral components of the immune system and inflammatory cells could be involved in the development of acquired resistance in mice. Nevertheless, the workers did not support this conclusion by assaying the worm burden or the recovery of schistosomula from the lungs of recipient mice. Von Lichtenberg, Sher, Gibbons and Doughty (1976) studied the inflammatory reactions in the skin of immune and normal mice. A significant increase in the percentage of eosinophils in the inflammatory response to challenge infection in immune

animals was observed. But no evidence of significant reaction against schistosomula arriving in the lungs in immune mice was reported. Von Lichtenberg, Sher and McIntyre (1977) developed a technique to quantitate schistosomula damage and host response to the organisms in more detail. Schistosomula prepared *in vitro* were injected intravenously into the lungs of mice. In normal mice, a small percentage of the injected schistosomula caused focal reactions composed mainly of neutrophils, but with no parasites or parasite remnants. In contrast, in immune mice the inflammatory reactions occurred around large proportions of the intravenously injected schistosomula. The reactions were mainly eosinophilic in nature and the ratio of schistosomula surrounded by the inflammatory foci to those without such foci in histological sections was correlated with the decrease in number of organisms of challenge infection recovered from the lungs and with the reduction in adult worm burden. This inflammatory reaction can be transferred with serum from immune to normal mice. Congenitally athymic mice (Nu/Nu) mice failed to develop the inflammatory foci which formed in the lungs in immunologically intact immune mice. Transfer of serum from infected Nu/Nu mice to normal mice failed to transfer the ability to induce the formation of the inflammatory foci. On the other hand, transfer of serum from immunologically intact immune mice into Nu/Nu mice was associated with the development of the inflammatory foci after challenge (Sher, 1977). Doenhoff and Long (1979) confirmed these results by studying resistance to re-infection with *S. mansoni* in mice deprived of their T-cells by thymectomy. Thymectomized mice failed to resist re-infection to the same extent as immunologically intact mice. All these findings suggested that the effector mechanism involved in the rejection of schistosome challenge appears to be a thymus-controlled humoral factor. As mentioned previously, several *in vivo* studies have

indicated the involvement of cells in addition to the humoral factors in the effector mechanism against schistosomes (Phillips, Reid, Bruce, Hedland, Colvin, Campbell, Diggs and Sadun, 1975; Smithers, 1976; Von Lichtenberg, Sher, Gibbons and Doughty, 1976; Von Lichtenberg, Sher and McIntyre, 1977). Further evidence to confirm this suggestion comes from the work of Sher (1977). Whole body irradiation of either passively or actively immunized mice, 5 days prior to challenge, resulted in the loss of their ability to produce the eosinophilic inflammatory foci in lung tissues (Von Lichtenberg, Sher and McIntyre, 1977) and abolished immunity as measured by the lung recovery assay (Sher, Mackenzie and Smithers, 1974). Immunity was restored by re-constituting the irradiated animals with bone marrow cells from normal donors. Therefore, the effector mechanism of acquired resistance in mice is mediated through antibody-dependent cell-mediated immunity. Several *in vitro* studies have indicated that schistosomula can be damaged by antibodies which cooperate with different types of cells, e.g. eosinophils (Butterworth, David, Franks, Mahmoud, David, Sturrock and Houba, 1977), macrophages (Perez and Smithers, 1977) or neutrophils (Dean, Wistar and Murrell, 1974; Dean, Wistar and Chen, 1975). Mahmoud, Warren and Peters (1975) provided evidence for the involvement of an antibody-mediated eosinophil-dependent mechanism in acquired resistance in mice *in vivo*. *S. mansoni* infected mice were treated with either anti-eosinophil serum or with other antisera prepared against mouse lymphocytes, macrophages, or neutrophils. Intravenous injection of antisera into mice was carried out at 12-32 weeks after primary infection. The treated mice were challenged with cercariae of *S. mansoni* immediately after treatment with antisera. Acquired resistance to the challenge was assayed by the lung recovery technique. It was evident that treatment of mice with anti-eosinophil serum abolished

the resistance of the animals to the cercarial challenge, whereas the other antisera had no effect on immunity. Furthermore, anti-eosinophil serum abolished the resistance conferred by passive transfer of immune serum. These results implied the eosinophil as an important component of immunity in mice.

The evidence presented from all the above mentioned studies deals with one of the many possible mechanisms of acquired resistance operating in different host species. For instance, acquired resistance to challenge infection could be induced through some non-specific factors. Hamsters injected with formalin-killed E. coli, 3 days after primary infection with S. mansoni, showed massive infiltration of the lungs with inflammatory cells (mainly polymorphoneutrophils and some macrophages) associated with resistance to challenge infection. Infected animals which have been treated with bacteria showed an 80% decrease in the recovery of schistosomula from the lungs compared with that in control groups. Decrease in the percentage recovery of adult worms was accounted for elimination of schistosomula at the lung stage (Smith, Clegg, Kusel and Webbe, 1975). Intravenous injection of eggs into the lungs of normal mice induced moderate to high levels of resistance to schistosome infection. Neither intraperitoneal nor intravenous injection of eggs induced such resistance. It was concluded that the inflammatory reaction associated with egg deposition in lung tissues could be responsible for elimination of adult worms and development of resistance, which is a relatively non-specific type (Dean, Minard, Murrell and Vannier, 1978). Thermal inflammation in the tail skin of mice produced by heating at 50°C 1 hour before exposure of the tail to cercariae blocked the infection completely (Gysin and Le Corroller, 1976). Vaccination of mice with BCG (Capron and Lesoin,

1969) or with Toxoplasma gondii (Mahmoud, Warren and Strickland, 1976) induced high level of resistance to schistosome infection.

Cross-immunity between human and animal species of schistosomes has been demonstrated. Primary infection of mice with S. bovis, S. mattheei, or S. rhodhaini conferred a high degree of protection against a challenge infection with S. mansoni (Nelson, Amin, Saoud and Teesdale, 1968). Rhesus monkeys were also partially immunized against S. mansoni by prior exposure to S. bovis or S. mattheei (Amin, Nelson, Saoud 1968). Cross-immunity between schistosomes and Fasciola hepatica has also been reported. F. hepatica and S. mansoni were found to have at least five common antigens (Capron, Biguet, Vernes and Afchain, 1968). Mice and hamsters vaccinated against S. mansoni with antigens prepared from F. hepatica showed some degree of resistance to a challenge with S. mansoni (Hillyer, de Diaz, del Luno and Garcia-Blanco, 1975). Christensen, Nansen, Fraudsen, Bjørneboe and Monard (1978) demonstrated the ability of S. mansoni infected mice to produce heterologous resistance to F. hepatica infection. The mechanism(s) involved in heterologous resistance between different species of schistosomes, or between schistosomes and F. hepatica have not been elucidated yet.

In summary, acquired resistance to schistosome infection has been demonstrated in several experimental animals (Sher, Mackenzie and Smithers, 1974; Perez, Clegg and Smithers, 1974). The effector mechanisms involved appear to be complex and not fully understood yet. Different mechanisms may operate in different host species (Phillips, Reid, Bruce, Hedland, Colvin, Campbell, Diggs and Sadun, 1975; Sher, 1977), or different mechanisms may affect various stages of the parasite in the same host species (Smithers and Gammage, 1980). Some

mechanisms involved in resistance to re-infection appear to be highly specific and mediated through antibody-dependent cell-mediated immunity (Sher, 1980). Specific mechanisms are thymus-dependent (Sher, 1977) and can be transferred with immune serum (Sher, Smithers and Mackenzie, 1975). Using monospecific anti-eosinophil serum have implicated the involvement of eosinophils as effector cells in immunity (Mahmoud, Warren and Peters, 1975). Non-specific forms of acquired resistance, probably involving mechanisms different from that of specific resistance, have been demonstrated (Smith, Clegg, Kusel and Webbe, 1975; Mahmoud, Warren and Strickland, 1976; Gysin and Le Corroller, 1976). Cooperation between specific and non-specific mechanisms in inducing resistance in infected host species can not be discounted.

I.4. Evasion of the Immune Response

Adult schistosomes survive in the blood circulation of their hosts for long periods, despite the host's immune response and the development of resistance to subsequent challenge infection (Smithers and Terry, 1976). Direct transfer of living adult worms into the portal system of rhesus monkeys (Macaca mulatta) initiated a response which killed schistosomula of a challenge infection, but which had apparently no effect on the pre-existing mature worms (Smithers and Terry, 1967). Thus, adult worms are able to escape the consequences of the host's immune attack. Several mechanisms may be operating to enable adult schistosomes to evade the immune response which they provoke.

(i) Surface masking, membrane turnover and intrinsic changes in the tegument

It has been shown that host or host-like antigenic determinants

are present on the surface of adult schistosomes (Smithers, Terry and Hockley, 1969; Clegg, Smithers and Terry, 1971; Goldring, Clegg, Smithers and Terry, 1976; Damian, Greene and Hubbard, 1973; Dean and Sell, 1972). Some of these antigens are of parasite origin, some others are adsorbed from the host. Capron, Biguet, Vernes and Afchain (1968) suggested that the parasite may synthesize proteins which are antigenically identical to those of its host. An example of host-like antigen synthesized by the parasite is the α2-macroglobulin molecules described by Damian, Greene and Hubbard (1973). This antigen can cross-react with mouse α2-macroglobulin and is present on worms recovered from both mice and monkeys. Because of the lack of cross-reactivity between primate and murine α2-macroglobulin and because mouse α2-macroglobulin antigenic determinants are also found on worms from monkeys, it was concluded that these shared host-parasite antigenic determinants are likely to be synthesized by the parasite. The authors also suggested that the parasite by natural selection may have evolved some antigenic determinants similar to those of the host in order to mimic the host. However, the possible role of such determinants in the survival of the parasite in different host species has not been further investigated. Smithers, Terry and Hockley (1969) provided evidence for the presence of molecules of host origin on the surface of adult schistosomes in vivo. Direct transfer of adult worms grown in mice into the hepatic portal system of monkeys previously immunized against mouse cells resulted in rapid destruction of the transferred worms. Such worms survived in non-immunized monkeys. It was postulated that these antigens are synthesized by the host and then incorporated into the surface membrane of schistosomes. The functional role of the acquired molecules could be to mask the antigenic determinants expressed on the schistosome

surface membrane (Smithers, Terry and Hockley, 1969). Several defined host antigens have been described on schistosomes. Clegg, Smithers and Terry (1971) cultured schistosomula *in vitro* in the presence of human blood group A and B erythrocytes. Schistosomula acquired antigens which are common to both A and B erythrocytes. The presence of these antigens was tested by assessing the survival of schistosomula in monkeys immunized against the specific blood groups (Clegg, Smithers and Terry, 1971). Further evidence for the presence of glycolipids of host origin on the surface of schistosomula cultured *in vitro* has been reported (Goldring, Clegg, Smithers and Terry, 1976). Schistosomula of *S. mansoni* cultivated *in vitro* can adsorb Forssman-like antigen from mouse erythrocytes and tissue extracts. These antigens are serologically identical to host antigens found on adult worms (Dean and Sell, 1972). Schistosomula of *S. mansoni* acquire antigens coded for the major histocompatibility complex (MHC) after passage *in vivo* through mice. Products of both the K and the I regions of the mouse MHC could be passively incorporated into the surface membrane of schistosomula during early stages of infection (Sher, Hall and Vadas, 1978). Since the K gene products are glycoprotein in nature, this observation is of interest in providing evidence that molecules other than glycolipids can be acquired by the parasite. In addition, the acquired host molecules appear to share a common feature which is the presence of a sugar residue. Thus, it is conceivable that specific receptors with affinity for the sugar residue of such molecules may be expressed on the parasite surface and that the presence of such receptors may determine the acquisition of host molecules (Sher, 1980). Although it has been suggested that glycolipid molecules of erythrocytes are attached to the schistosome surface membrane via their hydrophobic ceramide end (Clegg, 1972), the possibility of the presence of specific receptors on the

parasite surface could not be discounted. It is evident that antigens shared by the parasite and its host have been demonstrated. Some of these antigens appear to be synthesized by the parasite, some others are apparently acquired from the host. The presence of such antigens on the parasite surface may contribute to the inability of the parasite to be recognized by the immune system. In support of this hypothesis, Clegg and Smithers (1972) demonstrated that schistosomula cultured for 4 days in a medium containing serum and red blood cells, or schistosomula recovered from the lungs of mice 4 days after infection become completely protected against the lethal effect of immune monkey serum (Clegg and Smithers, 1972). Furthermore, antibodies in immune rhesus monkey serum were found to bind to the surface membrane of 3 hour old schistosomula. Whereas, schistosomula recovered from the lungs of mice 4 days after infection showed the presence of mouse erythrocyte antigens on their surfaces and simultaneously lost their susceptibility to the damage by immune serum in vitro (McLaren, Clegg and Smithers, 1975). Sher (1977) studied the requirement of red blood cells as a source of host molecules in vitro for protection against the immune response in vivo. Schistosomula cultured in a medium containing mouse red blood cells, then injected intravenously into the lungs of immune mice were found to be protected against the host's immune response as measured by the lung recovery assay. However, a significant decrease in the percentage recovery of adult worms was reported, indicating that large numbers of the protected worms had been eliminated. Therefore, acquisition of glycolipid molecules from red blood cells provided only partial protection for the parasite against the host's immune attack. Thus, it is reasonable to assume that some other mechanisms could also be involved in providing protection to young schistosomula. For instance, artificially transformed schistosomula incubated in a culture

medium containing serum in the absence of cells, developed protection against the lethal effects of antibodies and complement in vitro (Tavares, Soares, Coelho and Gazzinelli, 1978). The mechanism of this serum-induced protection against schistosomula appeared to be correlated with the metabolic activity of schistosomula. Serum factors acquired by schistosomula stimulated the acquisition of protection against antibody-mediated killing and simultaneously increased the rate of incorporation of labelled amino acids into the tegumental proteins of schistosomula. Addition of the metabolic inhibitor puromycin, decreased the rate of incorporation of labelled amino acids and also increased the susceptibility of schistosomula to the antibody-mediated damage. SDS polyacrylamide gel patterns of tegumental proteins of labelled and unlabelled schistosomula were identical. Thus, exposure to serum factors did not induce any new specific protein synthesis in the tegument, but it may have stimulated the turnover rate of surface proteins. The actual mechanism by which the presence of a serum factor affects the membrane turnover and the development of resistance is unclear. However, it is believed that the continuous shedding and replacement of schistosome surface membrane as shown by electron microscopy (Hockley and McLaren, 1973; Wilson and Barnes, 1974a, 1977) may also be related to the protection acquired by the parasite. Antibodies directed against host antigens on the schistosome surface can initiate complement-dependent cell-mediated killing. Adult worms taken from mice and cultured into monkey anti-mouse serum showed marked increase in the rate of tegumental repair or turnover as demonstrated by electron microscopy (Perez and Terry, 1973). Thus, it is likely that the turnover of schistosome surface membrane could lead to a clearing or sloughing mechanism of the worm for avoiding the lethal attack of the host's immune response.

Some other studies have suggested that intrinsic changes in the surface membrane of developing schistosomula, independent of acquisition of host molecules or membrane turnover, may occur immediately after skin penetration and lead to acquisition of resistance by young schistosomula. Schistosomula cultured in a chemically-defined medium free of serum or cells have been used in such studies. Dean (1977) indicated that skin schistosomula maintained in a chemically-defined medium for 3 days became increasingly resistant to the cytotoxic effects of antibodies and complement. Dessein, Samuelson, Butterworth, Vadas and Davis (1980) indicated that schistosomula incubated in a culture medium in the absence of serum or macromolecules for 24-48 hours, became resistant to damage by human eosinophils in the presence of specific anti-schistosome antiserum. Cultured schistosomula lost their susceptibility to adherence of human eosinophils in the presence of complement. In addition, such cultured schistosomula were rejected less efficiently than uncultured schistosomula when injected into immune mice. Using a different approach, Moser, Wassom and Sher (1980) studied the possibility of an intrinsic change in the tegument of schistosomula as a mechanism for providing resistance to developing schistosomula. 3 hour old skin schistosomula and 5 day old lung schistosomula were surface labelled with TNP-groups. Labelled schistosomula were tested for their susceptibility to damage by anti-TNP antibody-dependent effector mechanism in vivo and in vitro. TNP-labelled skin schistosomula were rejected by mice immunized against TNP-BGG and were highly susceptible to complement-dependent anti-TNP-mediated damage in vitro. On the other hand, TNP-labelled lung schistosomula were found to be resistant to the damage caused by both in vitro and in vivo mechanisms. The authors concluded that intrinsic changes in the tegument of schistosomula may provide an important

mechanism for the evasion of the host's immune response. However, interpretation of this work is complicated by the fact that the binding of anti-TNP-antibodies to TNP-molecules coupled to the schistosomular membrane could be affected by the configuration and distribution of the TNP-molecules on the surface of each of the developmental stages of the parasite. For instance, TNP-molecules which are embedded in the structure of the membrane are more likely to bind antibodies and fix complement than if the molecules stick out some distance from the surface membrane. Nevertheless, one can conclude that acquisition of host-like molecules, membrane turnover and changes in the membrane of developing schistosomula may provide mechanisms which play at least a partial role in acquisition of resistance against immune damage. Clearly, further studies on the exact role of each of these mechanisms in protection should be undertaken.

(ii) Suppression of the immune response

Immunosuppression is known to occur in some parasitic infections as in malarial infection and trypanosomiasis (Cohen, 1976). But it is not clear whether it is a mechanism of parasite survival in the immune host. Nevertheless, immunosuppression occurs during schistosome infection in mice. A marked decrease of the immune response of mice infected with S. mansoni to injected sheep red blood cells (SRBC) was observed (Mota-Santos, Gazzinelli, Ramalho-Pinto, Pellegrino and Dias da Silva, 1976). Using TNP-labelled schistosomula, Ramalho-Pinto, de Souza and Playfair (1976) demonstrated a specific decline in the cooperation between T and B cells in schistosome infected mice as measured by anti-TNP response in normal and infected animals. They also suggested that specific suppression of helper T-cell function may be involved in the later stage of S. mansoni infection in mice

(Ramalho-Pinto, de Souza and Playfair, 1976). The nature of the stimuli which induces immunosuppression during schistosome infection has been investigated. Mota-Santos, Tavares, Gazzinelli and Pellegrino (1977) demonstrated that immunosuppression is induced in mice by adult worms but not by eggs and it can be entirely abolished after treating the animals with a schistosomicidal compound (oxamniquine). Moreover, it was found that immunosuppression can be induced in mice by crude membrane preparations obtained from worms, but not egg extract (Mota-Santos, Tavares, Gazzinelli and Pellegrino, 1977). The proliferation of normal lymphocytes in culture, as measured by DNA synthesis, was shown to be inhibited by factors present in (a) supernatant of schistosome culture, (b) schistosome incubation products, obtained by incubating adult worms in a hypotonic solution, and (c) serum from schistosome infected rats (Dessaint, Camus, Fischer and Capron, 1977). All these studies indicate that materials released by adult worms *in vivo* and *in vitro* may account for the immunosuppressed state found in schistosome infected animals.

(iii) Immunological blockade

Phillips, Reid, Bruce, Hedland, Colvin, Campbell, Diggs and Sadun (1975) have shown that adoptive transfer of thymus-dependent lymphocytes into Fischer rats which have been previously infected with *S. mansoni*, induced protection as measured by the percentage worm recovery. Passive transfer of immune serum enhanced the survival of the worms, and abolished the protective effect of transferred lymphocytes when serum and cells were transferred concomitantly. It was postulated that the enhancing antibody may act to shield the parasite from the immune response of the host, thus promoting the survival of adult worms.

Different classes and subclasses of IgG and IgA and IgM of host origin have been shown to be associated with the tegument of adult S. mansoni (Sogandares-Bernal, 1976; Kemp, Merritt and Rosier, 1978). These workers suggested that the association of immunoglobulins with schistosome tegument may represent an immunological blockade or enhancement similar to that described by Phillips, Reid, Bruce, Hedland, Colvin, Campbell, Diggs and Sadun (1975). The presence of immunoglobulins on the schistosome surface does not entirely support the concept of immunological blockade, since blocking phenomena have usually been associated only with specific IgG antibody. Kemp, Brown, Merritt and Miller (1980) indicated that much of the antibody on the surface of the worm is heterospecific, i.e. non-parasite specific. Torpier, Capron and Ouaissi (1979) presented evidence for the presence of Fc receptors on schistosomula that interact with and bind host IgG. The presence of Fc receptors on the parasite may account for the high quantity of immunoglobulins present on the parasite surface. The significance of the presence of these schistosome associated immunoglobulins has not been elucidated yet. However, it is conceivable that the heterospecific antibodies associated with the tegument may have a protective role by preventing opsonization and subsequent attack by eosinophils or by swamping the cellular Fc binding sites on eosinophils non-specifically.

(iv) Heterogeneity of the cercariae

Smith and Clegg (1979) have shown that different pools of cercariae of S. mansoni can stimulate different levels of immunity in mice. They raised the possibility of the existence of polymorphism among target antigens expressed on the surfaces of cercariae from different clones. However, they could not detect these variable

antigens using an immunofluorescence technique. Yet, it is possible that variations in the metabolic activity among different clones could be responsible for inducing different levels of immunity. Such differences in the rate of metabolism may affect (a) the rate of acquisition of host molecules and (b) the rate of membrane turnover. For instance, the less active clones may get damaged in the skin or on their way to the lungs because they acquire host molecules at slower rates.

In summary, schistosome adults can establish long-term infection in their vertebrate host, in spite of the immune response which they have been shown to provoke (Smithers and Terry, 1976). Several mechanisms have been suggested to explain the longevity of adult worms. The parasite may protect itself against the host's immune system by acquisition of molecules of host origin (Smithers, Terry and Hockley, 1969). Schistosomula recovered from the lungs of infected mice acquired host molecules and simultaneously lost their ability to bind antibody directed against schistosomes in immune monkey serum in vitro (McLaren, Clegg and Smithers, 1975; Clegg and Smithers 1972). Schistosomula acquire A, B, H and Lewis antigens of human erythrocytes (Goldring, Clegg, Smithers and Terry, 1976). Schistosomula adsorb from mice a Forssman-like antigen which is also found on the surfaces of adult worms (Dean and Sell, 1972). Acquired host molecules are mainly glycolipids in nature (Clegg, 1974). Among the adsorbed host molecules are the gene products of the murine major histocompatibility complex (MHC) which are glycoprotein in nature (Sher, Hall and Vadas, 1978). Schistosomula cultured in a chemically-defined medium free of cells or serum acquired resistance against the cytotoxic effects of lethal antibody (Dean, 1977) or lost their susceptibility to adherence of eosinophils in the presence of complement (Dessein,

Samuelson, Butterworth, Vadas and Davis, 1980). Such resistance may result from intrinsic changes in the surface membrane of schistosomula rather than from adsorption of host molecules. Development of the multilayered membrane by young schistosomula immediately after skin penetration (Hockley and McLaren, 1973) and the continuous replacement of the surface membrane (Wilson and Barnes, 1974a) may be mechanisms for avoiding the immune response. Suppression of the host's immune response has been shown to occur during schistosome infection (Mota-Santos, Gazzinelli, Ramalho-Pinto, Pellegrino and Dias da Silva, 1976). Living adult worms and crude membrane extract but not egg antigen stimulated immunosuppression in mice (Mota-Santos, Tavares, Gazzinelli and Pellegrino, 1977). In vitro incubation of mouse lymphocytes with products released by adult schistosomes in culture medium inhibited the proliferation of lymphocytes (Dessaint, Camus, Fischer and Capron, 1977). Passive transfer of immune serum and lymphocytes concomitantly into Fischer rats enhanced the survival of the parasite and obviated the protective capacity of lymphocytes (Phillips, Reid, Bruce, Hedland, Colvin, Campbell, Diggs and Sadun, 1975). Enhancing antibody which may block the immune response against the parasite has been demonstrated in rats. Finally, evidence for the presence of heterogeneity among the infective cercariae has been described (Smith and Clegg, 1979). Yet, it may be another mechanism developed by the parasite to survive the immune reaction of its host.

I.5. The Tegument

I.5.1. General Considerations

The schistosome tegument presents a complex structure which fulfils more than one role in the survival of the parasite. It has

been shown to be involved in the transport of amino acids (Chappell, 1974; Asch and Read, 1975) and glucose (Fripp, 1967; Rogers and Bueding, 1975) through enzyme mediated processes. Numerous sensory organelles on its outer surface may function as mechanoreceptors (Hockley, 1973). The tegument may also release secretions into the environment some of which may be antigenic and some of which may function in the survival of the parasite. It has been shown that surface-associated material from adult worm possesses the capability to inhibit the coagulation system of the host (Tsang and Damian, 1977; Phillips and Colley, 1978). A protein secreted by adult male and transferred through the tegument into the female might function in maintaining the migratory and pairing activities of schistosomes (Atkinson and Atkinson, 1980). The tegument may be a target for the host's immune attack or it may protect the worm against the host's immune system. It is the immunological and protective aspects that will concern us here.

(i) The tegument as a source of released antigenic material

As described previously, the protective immune response against schistosomes appears to be stimulated by antigens associated with living adult worms (Smithers and Terry, 1967). Resistance has also been induced in experimental and domestic animals with living, radiation-attenuated cercariae and schistosomula (Smithers and Terry, 1965b; Hsü, Hsü and Osborne, 1965, 1969; Minard, Dean, Jacobson, Vannier and Murrell, 1978; Eveland and Morse, 1978; Bickle, Taylor, Doenhoff and Nelson, 1979; Taylor, James, Bickle, Doenhoff, Nelson, Hussein and Bushara, 1977). This suggested that stimulation of immunity might be due to the secretion of important antigens into the blood circulation (Smithers and Terry, 1976). There is some *in vitro* evidence indicating that the schistosome tegument is responsible for the release of a number of

antigenic components into the culture medium. The surface membrane of adult worms has been shown to undergo a process of turnover, involving continuous release of membrane antigens into the culture medium (Kusel, Sher, Perez, Clegg and Smithers, 1975; Kusel, Mackenzie and McLaren, 1975). It was suggested that this process may be related to the release of membrane antigens *in vivo*. However, there is little evidence for the release of tegumental antigens *in vivo*. Although the gut antigens (Nash, 1974; Andrade and Sadigursky, 1978) and the egg antigens (Pelley, Pelley, Hamburger, Peters and Warren, 1976; Pelley, Warren and Jordan, 1977) may represent the major sources of circulating schistosome antigens, Houba, Koech, Sturrock, Butterworth, Kusel and Mahmoud (1976) detected the presence of circulating membrane antigens, presumably in the form of immune complexes in infected baboon serum (*Papio anubis*). Materials released from the surface membrane of adult worms and schistosomula in culture can interact with antibodies produced in schistosome infected rhesus monkey (*Macaca mulatta*) (Kusel, Sher, Perez, Clegg and Smithers, 1975). The antibody involvement in protective immunity against schistosomes has been demonstrated by passive transfer of immune serum to normal animals in the rodent system (Sher, Smithers and Mackenzie, 1975; Smithers, 1976). Most of the surface membrane antigens of adult worms have been found to be present in the surface membrane of schistosomula (Kusel, Sher, Perez, Clegg and Smithers, 1975; Ruppel, 1978). Therefore, it is conceivable that antibodies directed against membrane antigens released from adult worms may recognize antigens in the surface membrane of young schistosomula leading to their damage and providing protection against the parasite. This would suggest that schistosome fractions containing membranes might be important in vaccination against the parasite. However, contradictory results have been obtained on attempting to immunize with different schistosome

preparations. Some success has been achieved in immunizing mice with culture antigens from adult worms (Murrell and Clay, 1972). On the other hand, no immunity was found with partially purified membrane antigen from adult worms (Sher, Kusel, Perez and Clegg, 1974). Clearly, careful definition of the different schistosome preparations is essential for future research.

(ii) The tegument as a target for the host's immune response

Several in vitro systems have been established to study the direct damage to the surface membrane of schistosomula by antibodies or antibodies and cells. Clegg and Smithers (1972) and Murrell and Clay (1972) demonstrated the killing of schistosomula by lethal antibodies present in the sera of a number of animals infected with S. mansoni. The lethal antibody was shown to damage the tegument of schistosomula in the presence of complement (McLaren, Clegg and Smithers, 1975). Schistosomula were rapidly killed by rat neutrophils in the presence of complement-dependent antibody from schistosome infected rats (Dean, Wister and Murrell, 1974) and guinea pigs (Dean, Wistar and Chen, 1975). Macrophages from normal rats when sensitized with immune rat serum showed adherence followed by tegumental damage of schistosomula (Perez and Smithers, 1977). Schistosomula were damaged on incubation with macrophages from normal rats activated by serum from schistosome infected rats containing complexes of IgE antibody and circulating schistosome antigens (Capron, Dessaint, Capron and Bazin, 1975; Joseph, Dessaint and Capron, 1977). Normal human eosinophils incubated with sera from schistosome infected patients caused damage to schistosomula (Butterworth, Sturrock, Houba, Mahmoud, Sher and Rees, 1975). However, schistosomula recovered from the lungs of mice 4 days after infection or grown in culture containing normal serum and red blood cells for the

same period, showed no damage by the in vitro antibody-mediated system (Clegg and Smithers, 1972; McLaren, Clegg and Smithers, 1975). Although Hockley and Smithers (1970) demonstrated the destruction of the surfaces of adult worms that had been transferred directly into the portal system of hyperimmune rhesus monkey, there is no firm evidence that the damage to the adult's tegument was a result of the host's immune reaction.

In summary, the schistosome tegument contains specific antigens which can be recognized by antibodies or cells capable of killing the parasite in vitro. Characterization of such antigens is necessary to evaluate their possible role in vaccination against schistosomes.

(iii) The tegument as a source which protects the parasite against the host's immune system

The expression of host-like antigen and the turnover of the surface membrane are essential features of the adult worm tegument. It has been suggested that the masking of surface antigens by host molecules (Clegg and Smithers, 1972) and the rapid turnover of the surface membrane (Wilson and Barnes, 1974a) provide important mechanisms by which the immune attack of the host may be avoided. However, there is still no certain link between these suggested mechanisms by which the schistosome tegument may function to protect the parasite against the host's immune system. The surface of the adult worm is able to adsorb host erythrocyte glycolipids (Clegg, Smithers and Terry, 1971; Goldring, Kusel and Smithers, 1977) which may serve to cover up the antigenic determinants on the surface membrane (Clegg, 1972). There is no evidence to indicate the way in which these molecules are acquired by the worm. Clegg (1972) suggested that the glycolipid molecules may attach themselves to the membrane via their hydrophobic end which can be inserted into lipid-containing structures such as schistosome

tegument. Alternatively, specific membrane receptors for host antigenic material might be expressed on the schistosome outer surface membrane (Cesari, 1976; Cesari and Marchiani, 1977, 1978). The possibility of acquiring host antigen through the gut by the incorporation of the glycolipids from the ingested erythrocytes with the surface membrane, seems to be unlikely since worms can acquire host antigens before they begin feeding on erythrocytes (Clegg, 1974). The continuous synthesis and turnover of the adult worm surface membrane (Kusel, Sher, Perez, Clegg and Smithers, 1975; Kusel and Mackenzie, 1975) may be one way of evading the immune response (Wilson and Barnes, 1974a). Therefore, if the surface membrane is continuously shed and renewed then the host antigen must be continuously replaced. If this is the case, then it is of crucial importance to understand the mechanisms by which this might occur. Perhaps, better understanding of the structural features and chemical properties of schistosome tegument may help to answer many questions concerning these mechanisms.

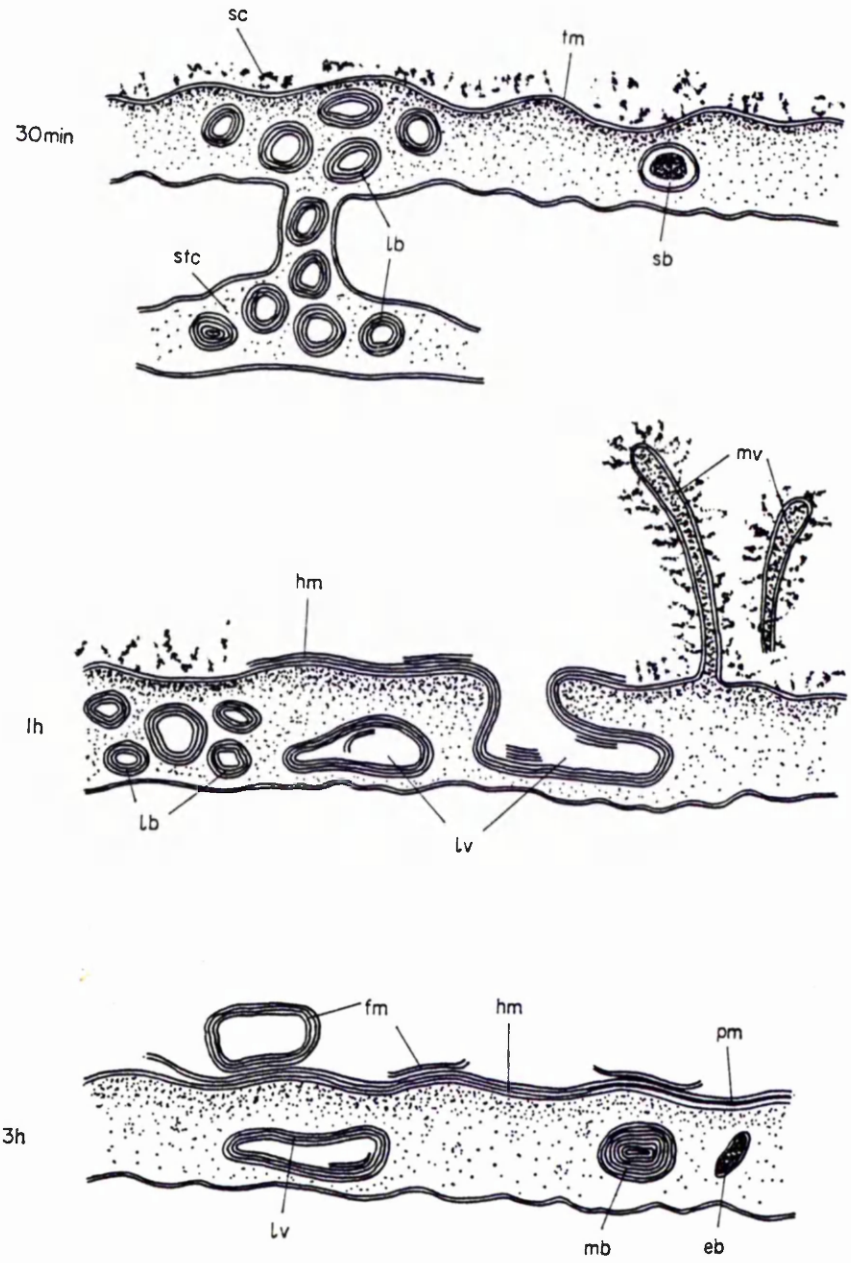
I.5.2. Morphological Studies

The structural features of the schistosome tegument have been well characterized at the level of electron microscopy. In some of the earliest work, Lee (1966) described the typical structure of the tegument in a number of adult Digenea including S. mansoni. It appeared to consist of a syncytial layer of cytoplasm bounded at its outer surface by a trilaminate plasma membrane, and connected to subtegumentary cells. More recently, Hockley and McLaren (1973) have established the ultrastructural features of this tegument at various developmental stages of S. mansoni using uranyl acetate as a fixative in addition to glutaraldehyde and osmium. The cercaria is covered by a trilaminate plasma membrane bearing an outer thick surface coat termed the glycocalyx.

The cercarial glycocalyx is absent in schistosomula and adult worms. Histochemical analysis of the glycocalyx shows its strong positive reaction with periodic acid-Schiff stain (PAS), indicating that it is glycoprotein in nature (Kemp, 1970; Stein and Lumsden, 1973; Stirewalt, 1974). The glycocalyx has been shown to be lost or modified within the first 30 minutes after skin penetration of the vertebrate host. The loss or modification of the cercarial glycocalyx is accompanied by major changes in the permeability (Clegg, 1972) and structure of the tegument of schistosomula (Hockley and McLaren, 1973). One hour after skin penetration, parts of the outer trilaminate membrane become heptalaminate. At the same time the original trilaminate cercarial membrane appears to be formed into microvilli which are apparently cast off from the schistosomula. Scanning electron microscopy of the cercaria and 30 minute old schistosomulum have shown that the surface topography is basically similar except that the schistosomulum shows the presence of microvilli arising from the outer surface of the tegument (McLaren and Hockley, 1976). However, the microvilli represent a temporary feature of the parasite since they disappear about 1 hour after transformation of cercariae to schistosomula. After 3 hours the schistosomula are totally covered by the heptalaminate membrane. Occasionally, some small regions appear with 11-15 layers. This was attributed to the presence of 1 or 2 small pieces of the fragmented trilaminate cercarial membrane on the outer surface of the heptalaminate membrane (Fig. 1.2.). Apart from being more pitted and folded, the tegument of schistosomula after several days resembles that of the 3 hour old schistosomula. Adult worms possess the heptalaminate membrane similar to that of the schistosomula. As the worm matures, the surface membrane increases in complexity and becomes deeply infolded. Freeze-fracture studies have

Figure I.2. Diagram Summarizing Changes in the Tegument of Schistosomula After Penetration of the Vertebrate Host. (Reproduced) from Hockley, D.J. 1973. Ultrastructure of Schistosome Tegument. In "Advances in Parasitology". 11 : 233-305.

30 minutes after penetration the tegument has a thin surface coat (sc) and a trilaminate outer membrane (tm). Subtegumental cells (stc) containing laminated bodies (lb) are connected to the tegument. The tegument contains groups of laminated bodies adjacent to the cell connections and a few spherical, cercarial, inclusion bodies (sb). One hour after penetration parts of the tegument have a heptalaminate outer membrane (hm) and there are microvilli (mv) with a trilaminate membrane and a thin, surface coat. The microvilli appear to be cast off from the tegument. The tegument contains laminated bodies (lb) and large vacuoles (lv) with a heptalaminate limiting membrane. Some large vacuoles open to the tegumental surface. 3 hours after penetration most of the outer membrane is heptalaminate (hm) but parts are pentalaminate and there are fragments of membrane (fm) on the tegumental surface. The tegument contains large vacuoles (lv), also smaller membraneous bodies (mb) and elongate bodies.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revealed that the heptalaminate membrane of the post-cercarial stages of S. mansoni is composed of two closely opposed trilaminate membranes (Hockley, McLaren, Ward and Nermut, 1975). This result is in agreement with the earlier observation of Hockley and McLaren (1973). Although the origin of the inner bilayer (the inner plasmalemma) is uncertain, it is clear that the outer bilayer (the membranocalyx) is formed immediately after cercarial penetration of the vertebrate host. There is evidence that the composition and properties of the membranocalyx are different from that of the underlying plasmalemma. The density of the intramembraneous particles (IMP) which represent the proteins and glycoproteins embedded in the lipid bilayer of the membrane, differs considerably on each of these 2 bilayers forming the schistosome surface (Hockley, McLaren, Ward and Nermut, 1975). In the mature adult worm, the number of the IMP present in the membranocalyx is about twice that in the inner plasmalemma. On the other hand, the newly transformed schistosomula exhibit the 2 bilayered membrane, with the inner plasmalemma very similar to that of the original cercarial trilaminate membrane in having large numbers of the IMP. The membranocalyx appears to have no or very few particles (McLaren and Hockley, 1976). However, the proportion of the IMP in the outermost leaflet of the membranocalyx rises with the maturation of the worm, reaching the peak at the lung stage and remains almost constant during the subsequent stages. The significance of the presence and distribution of the IMP has not been established yet (McLaren, Hockley, Goldring and Hammond, 1978).

A number of suggestions can be made about the origin of the inner plasmalemma:

(i) It is a newly formed membrane synthesized by the parasite to replace the original cercarial membrane which is cast off from the schistosomula.

(ii) It is partially derived from the cercarial membrane. Because of the fluidity of membranes (Singer and Nicolson, 1972), protein components (IMP) of the cercarial membrane might move into the newly formed membrane while the other components might be lost in the form of microvilli.

(iii) It represents the retained cercarial membrane which becomes exposed after the loss of glycocalyx upon skin penetration of the vertebrate host. Thus, the newly formed membranocalyx may serve to mask the exposed membrane. But evidence for the presence of microvilli originating from parts of cercarial membrane and casting off from schistosomula (Hockley and McLaren, 1973; McLaren and Hockley, 1976) argues against this suggestion. Nevertheless, there is as yet no conclusive evidence for any of the above hypotheses.

The mechanism of the formation of schistosome tegument is not yet understood. However, a number of cytoplasmic inclusions have been revealed by ultrastructural studies (Smith, Reynolds, Von Lichtenberg, 1969; Hockley and McLaren, 1973; Wilson and Barnes, 1974a, 1977, 1979). One type of inclusion is thought to be important in the formation of the tegument. It has been termed the membraneous body by Hockley and McLaren (1973) and the multilaminate vesicle (MLV) by Smith, Reynolds and Von Lichtenberg (1969) and Wilson and Barnes (1974a). The MLV appear as a mass of loosely or tightly packed and concentrically arranged membranes. They are occasionally found close to or associated with the surface membrane of the parasite. Smith, Reynolds and Von Lichtenberg (1969) considered that the MLV might be concerned with phagocytosis and absorption of nutrients through the surface membrane. But, evidence presented by Hockley and McLaren (1973) and Wilson and Barnes (1974a, 1977) has precluded this view. Hockley and McLaren (1973)

suggested that the possible function of the MLV is to contribute their contents to the outer surface of the worm. They based their conclusion on two major observations; firstly, some of the MLV appear close to the tegumental pits in the ultrathin sections of fixed worms, and secondly, the MLV and the multilaminate membrane are unique features of schistosomes compared with the other trematodes. Further evidence on the involvement of the MLV in the formation of schistosome tegument comes from the work of Wilson and Barnes (1977). They demonstrated the occurrence of side channels branching from the base of the tegumental pits and fusing with the MLV. This raised the possibility that the side channels could be the site of fusion of the MLV with the inner plasmalemma as they release their contents to its outer surface, and consequently forming the membranocalyx.

Both biochemical and morphological evidence have shown that the surface membrane of S. mansoni is in a continuous process of turnover. This process was first described by Kusel, Sher, Perez, Clegg and Smithers (1975). These workers have studied the membrane turnover and the release of membrane antigens into the culture medium by using radioactive labelling techniques. This is discussed in more detail in later sections. Wilson and Barnes (1977) demonstrated the phenomenon of membrane turnover directly by labelling adult worms with cationized ferritin then assessing by electron microscopy the presence and distribution of the label on the worm surfaces after a certain period of incubation. The ultrastructural micrographs show clearly the uniform labelling of the membranocalyx but not the inner plasmalemma. Partial loss of the label bound to the membranocalyx was noted 2 hours after incubation in the chase medium without ferritin. The total shedding of the membranocalyx into the culture medium 4 hours after

chase incubation indicates that the membranocalyx has a half-life of 2-3 hours. Kusel and Mackenzie (1975) postulated that membrane material may be released into the culture medium as a result of two processes: a rapid secretory process and a slower membrane turnover. Therefore, the shedding process of the membranocalyx demonstrated by Wilson and Barnes (1977) is likely to represent the rapid secretory process suggested by Kusel and Mackenzie (1975). However, there is no direct in vivo evidence to support this view. Radiolabelling of proteins specific to the outer membrane and following their appearance in the blood circulation could be the ideal approach for obtaining such evidence.

I.5.3. Biochemical Studies

A number of attempts have been made by several investigators to characterize and analyse schistosome surface membrane components. Kusel (1970, 1972) has established methods for isolating the surface membrane of S. mansoni. He carried out preliminary studies on the protein composition of the surface membrane of adult worms, cercariae and schistosomula. Living adult worms were incubated with radioactive precursors under suitable culture conditions. The labelled surfaces were isolated and analysed by SDS polyacrylamide gel electrophoresis. A high degree of identity was noticed between the banding patterns of the different developmental stages examined. A high molecular weight component described as being unique to the cercariae might be associated with the cercarial surface coat (the glycocalyx). This finding was confirmed in later studies (Ruppel, 1978; Cordeiro and Gazzinelli, 1979). Analysis of adult worm surfaces by SDS polyacrylamide gel electrophoresis revealed 7 major protein peaks. It also showed the presence of a low molecular weight component which migrated very

rapidly on electrophoresis. This component is stained by periodic acid-Schiff stain but not coomassie blue stain which indicated that it is glycolipid in nature. The absence of this material from the cercarial and schistosomular preparations suggested that it might be incorporated into the surface membrane of adult worm during development. There is evidence that red blood cell glycolipids are acquired from the host and incorporated into the surface membrane of schistosomes (Goldring, Clegg, Smithers and Terry, 1976). In another work, Kusel and Mackenzie (1975) incubated adult worms with 2 different isotopes, ^{14}(C) and ^{3}(H) of the same amino acid (leucine). The molecular weights of the labelled proteins were estimated by SDS polyacrylamide gel electrophoresis. Nine labelled protein peaks with molecular weights ranging between 18,000-90,000 were described with each of the radioactive isotopes. The information obtained by other investigators has extended the results of Kusel (1972) and Kusel and Mackenzie (1975) using improved gel systems. A comparative analysis of schistosome surface membrane from worms of different host origin, sex and age was undertaken by Cordeiro and Gazzinelli (1979). The number of protein bands resolved from adult worms and schistosomula was approximately 25. This number is larger than that reported by Kusel (1972) and Kusel and Mackenzie (1975). This variation could be due to the improvement in gel separation and modification of membrane isolation methods. No variations in components were obtained with tegumental preparations of schistosomes grown in different hosts. The electrophoretic patterns of male and female teguments resolved some qualitative differences. A similar result was obtained by Ruppel (1978) working with total extract of adult worms. Analysis of the surface membranes isolated from adult worms at different ages showed that there is a gradual increase in the proportion of high molecular weight proteins during maturation. This is compatible with

the observation that there is a qualitative increase of the intramembraneous particles (IMP) in the membranocalyx during development (McLaren, Hockley, Goldring and Hammond, 1978). Cordeiro and Gazzinelli (1979) reported the presence of a high degree of identity between the coomassie blue and periodic acid-Schiff stained bands on SDS-gels. They concluded that most of the tegumental components are glycoproteins. In contrast, Hayunga, Murrell, Taylor and Vannier (1979a) detected the presence of a single band with a high molecular weight (more than 100,000) by staining with periodic acid-Schiff stain. In further studies, the carbohydrate constituents of schistosome surface membrane were demonstrated using a lectin binding assay (Bennet and Seed, 1977; Murrell, Taylor, Vannier and Dean, 1978; Simpson, Cesari and Evans, 1980) and radiolabelling techniques (Wilson and Barnes, 1979). Thus, it appears that glycoproteins constitute major components of schistosome surface membrane, but particular emphasis should be given to the fact that different methods were used by different workers for labelling and extracting surface membrane of schistosomes. Ruppel (1978) reported a detailed analysis of the surface membrane proteins labelled with lactoperoxidase. Several iodinated proteins with molecular weights ranging between 17,000-120,000 were shown by SDS polyacrylamide gel electrophoresis. He found that incubation of adult worms in the iodinating medium (usually phosphate buffered saline, PBS) resulted in the release of the labelled proteins which can be re-adsorbed to fresh adult worms. This could be due to the leakage of internal proteins into the medium, where they become labelled and subsequently re-adsorbed by the tegument (Ruppel, 1978). Indeed, the damaging effect of the phosphate buffered saline (PBS) on the integrity of schistosome tegument has been recently reported (Simpson, Cesari and Evans, 1980). Hayunga, Murrell, Taylor

and Vannier (1979a) failed to label significant amounts of surface membrane proteins using the lactoperoxidase method. They investigated conditions required for the direct iodination of living adult worms using the Bolton-Hunter reagent. They determined the molecular weights of labelled proteins by SDS polyacrylamide gel electrophoresis. 10 identifiable protein peaks were resolved, 4 of them with molecular weights corresponding to those shown by Kusel and Mackenzie (1975) and Ruppel (1978). The authors concluded that the Bolton-Hunter labelled proteins represent at least some of the surface membrane proteins. Although there is evidence that the Bolton-Hunter reagent is capable of penetrating the human erythrocyte membrane and labelling internal proteins (Hayunga, Murrell, Taylor and Vannier, 1979a), the authors argued that the multilaminate tegument of schistosomes is much more complex than the erythrocyte plasmalemma and therefore the direct comparison in this regard is not valid.

The rate of synthesis in the surface membrane of adult worms and schistosomula was studied by Kusel (1972). The rate of incorporation of radioactive amino acids into the surface of adult worms and schistosomula was used as a parameter of the rate of synthesis of surface membrane proteins. Adult worms incubated with ^{14}(C)-leucine showed progressive uptake of radioactivity into the surface membrane. This process was inhibited by low temperature and puromycin. Tavares, Cordeiro, Mota-Santos and Gazzinelli (1980) obtained progressive incorporation of radioactive arginine into the surface proteins of schistosomula cultured in a medium deficient in unlabelled arginine.

A series of studies by Kusel; Kusel, Sher, Perez, Clegg and Smithers; Kusel, Mackenzie and McLaren; Kusel and Mackenzie (1975) were concerned with the turnover of schistosome surface membrane.

Kusel, Sher, Perez, Clegg and Smithers (1975) labelled worms with ^{3}(H)-leucine and followed the appearance of labelled proteins during subsequent incubation in fresh, non-radioactive medium. They found that there is a progressive loss of radioactivity from the surface membrane accompanied by an increase in the trichloroacetic acid (TCA) precipitable radioactive leucine in the culture medium. Radioactive culture medium antigens could be partially precipitated by hyperimmune schistosome infected monkey serum. Isolated surface membranes from adult worms competed with the precipitation. This evidence suggested that schistosomes release proteins into the culture medium due to membrane turnover. In subsequent work, Kusel and Mackenzie (1975) used a different approach to study the surface membrane turnover. They applied double labelling of adult worms with ^{14}(C) and ^{3}(H)-leucine, then analysed the ^{3}(H)/^{14}(C) ratios in various worm fractions and in the culture medium antigens. It was found that the ^{3}(H)/^{14}(C) ratios of most of the surface proteins were about the same. Therefore, the majority of membrane proteins turnover at the same rate. Culture medium proteins appeared to have a turnover higher than the majority of the proteins in the surface membrane fractions. Since some of these culture proteins shared antigenic determinants with the schistosome surface membrane, the culture antigens may be released by two main processes; a rapid secretory process and a slower membrane turnover.

Analysis of secreted culture antigens by immunochemical methods has been the subject of several studies. Murrell, Vannier and Ahmed (1974) studied what they termed the secreted-excreted antigens of adult worms in culture medium. Analysis on polyacrylamide gel revealed the presence of at least 15 proteins, 6 of which reacted with antisera from immune mice and monkeys. Comparison of secreted antigens with

extract of adult worms by immunodiffusion analysis showed that most of the secreted material could be obtained by either freezing and thawing or by extraction with 3M KCl. These two methods have been described by Kusel (1972) and Vannier, Fireman, Chestnut and Murrell (1974) as effective procedures in isolating schistosome surface membrane. Kusel, Mackenzie and McLaren (1975) showed that about 36% of the TCA precipitable counts of culture medium antigens can interact with hyperimmune monkey serum and 22% with specific anti-schistosome surface membrane serum. It is clear that a considerable quantity of the culture antigens do not react with either immune monkey serum or antisera artificially prepared in rabbits against schistosome surface membrane. This may indicate that most of the proteins secreted by adult worms *in vitro* are non-immunogenic, or that most of the material released by adult worms under *in vitro* conditions are antigenically different from that released in the host. Clegg and Smith (1978) stated that at present very limited information is available on culture antigens which are worth further examination as possible functional antigens in schistosomiasis.

I.6. Aims of This Thesis

Despite the fact that schistosome tegument is central to many unsolved problems concerning its structure, functions and relationship with the host, little is known about the composition and synthesis of schistosome surface antigens. The morphology of schistosome tegument is studied in detail at the ultrastructural level, but knowledge is still lacking at the molecular level. This is owing partly to the absence of refined methodology necessary for detailed investigations. In addition, the low yield of proteins obtained on isolating schistosome surface membrane hampered the investigations for their immunochemical nature. However, the utilization of isotope labelling

technique has provided a useful tool for studying the composition and synthesis of schistosome surface membrane proteins by using small amounts of proteins. Also, the application of a variety of analytical methods for the study of radiolabelled membrane components should be useful in elucidating information about the properties and functions of protein molecules associated with schistosome surface membrane.

To date, a detailed study of schistosome surface membrane with respect to:

(a) Isolation and characterization of protein components

(b) Identification of specific membrane proteins

(c) Determination of the synthetic and secretory capacity of membrane protein components as a mechanism for the survival of the parasite has not been undertaken. It was the aim of this thesis to understand some of these aspects.

In the present work, radiolabelled proteins in different schistosome preparations will be compared on polyacrylamide gels (SDS and IEF gels). Protein patterns are presented which were obtained after electrophoresis of various fractions from adult worms and schistosomula. Immunodiffusion analysis and immunoelectrophoresis were also performed. In addition, the rate of synthesis of surface membrane proteins in individual clones of the parasite was studied using the radiolabelling techniques.

These analyses were undertaken in view of the need to obtain knowledge about specific antigens of the surface membrane, and also to develop ways to define such antigens. This kind of information is relevant as attempts to use purified worm components will become necessary in various fields of experimental schistosomiasis.

CHAPTER II

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Biological Materials

Parasite ; A Puerto Rican strain of Schistosoma mansoni was maintained in Biomphalaria glabrata and in a variety of strains of mice (Parkes, BALB/C, CBA). This strain of S. mansoni and the snail were originally obtained from stocks at the NIMR, Mill Hill, London.

Hamsters ; Syrian Golden hamsters supplied by Wrights of Essex, England.

Mice ; Parkes, BALB/C, CBA and C_3H strains of mice. All strains were bred in the animal house, Department of Biochemistry, University of Glasgow.

Rabbits ; Dutch or Half-lop strain purchased from MRC accredited source.

All animals were obtained through the animal house, Biochemistry Department, University of Glasgow.

2.2. General Materials

2.2.1. Tissue Culture Materials

Eagle's Medium

Foetal Calf Serum — Gibco-Bio-Cult, Paisley.

Penicillin, Streptomycin — Vestric and Co. Ltd., Hatrick Branch, Glasgow.

Sterile petri dishes, flasks, tubes and pipettes were supplied by the Tissue Culture Unit, Biochemistry Department, University of Glasgow.

2.2.2. Radiochemicals

Radiochemicals were supplied by the Radiochemical Centre, Amersham, England.

Amino Acids	Specific Activity
L-(^{35}S)-Methionine	850-1300 Ci/mmol
L-(4,5-^{3}H)-Leucine	100-150 Ci/mmol
L-(^{14}C)-Amino Acids	780 mCi/mAtom
(^{125}I)-NaI supplied by Western Infirmary, Glasgow.	carrier free

2.2.3. Liquid Scintillation Spectrophotometry Materials

Triton-X-100	Sigma Chemical Co., Poole, England.
2,5-diphenyloxazole (PPO)	Koch-Light Laboratories Ltd., Colnbrook, England.
Toluene AR Grade	" "
Protosol	New England Nuclear, South Wonston, England.

2.2.4. Photographic Materials

Kodak-FX-40-X-Ray Liquid Fixer	Kodak (U.K.) Ltd., London, England.
Kodak-DX-80-X-Ray Developer	" "
Kodak-X-O-Mat-Royal Film	Kodak Canada Ltd., Toronto, Ontario.

2.2.5. Fine Chemicals

The following chemicals were supplied by Sigma Chemical Co., Poole, Dorset, England.

Agarose

Bovine Serum Albumin (BSA)

Haemoglobin

α-Methyl-D-Glucoside

Myoglobin

Ovalbumin

Tris (Hydroxymethyl)aminomethane (Trizma Base).

The following chemicals were supplied by BDH Chemicals Ltd., Poole, Dorset, England.

Acrylamide

L-Amino Acids, A Grade

Ammonium Persulphate

Coomassie Blue Brilliant Stain R250

Folin Ciocalteu's Phenol Reagent

N,N', Methylene Bisacrylamide

Polyethylene Glycol 6000 (PEG)

Sodium Barbitone

N,N,N',N tetramethylethylenediamine (TEMED)

Urea AR Grade

The following chemicals were supplied as indicated:

Acquacide II	Calbiochem Ltd., Bishops-Stortford, England.
Ampholine Carrier Ampholytes	LKB (G.B.), South Croydon, England.
Dimethyl Sulfoxide (DMSO)	Koch-Light Laboratories Ltd., Colnbrook, Bucks.
Freund's Incomplete Adjuvant (FIA)	Difco Laboratories, West Molesey, Surrey.

Freund's Complete Adjuvant (FCA)	Difco Laboratories, West Molesey, Surrey.
Heparin	Vestric and Co. Ltd., Glasgow.
2-Mercaptoethanol	Koch-Light Laboratories Ltd., Colnbrook, Bucks.
Repelcote	Hopkins and Williams, Chadwell Heath, Essex.
Sagatal	May and Baker Ltd., Dagenham, England.
Sucrose, AR Grade	Fisons Scientific Apparatus, Loughborough, England.
Trichloroacetic Acid (TCA)	Koch-Light Laboratories Ltd., Colnbrook, Bucks.

2.2.6. Chromatographic Materials

QAE Sephadex A50	Pharmacia (G.B.) Ltd., London, England.

All other chemicals, whenever possible "Analar" Grade reagents.

2.3. Standard Solutions

2.3.1. Media

(i) Eagle's medium

The composition of Eagle's Medium is shown in Table 2.1.

The medium was prepared before use as follows:

10% by volume	IF
4% " "	$NaHCO_3$
10^4 units/litre	Penicillin
100 mg/litre	Streptomycin

Foetal calf serum (FCS) was added at 10% (by volume) when required. Lactalbumin hydrolysate (Difco Laboratories) was added at 5 gm/litre in certain individual experiments.

Table 2.1.

Composition of Eagle's Medium

(a) IF (10x concentrated)

Inorganic Salts	mg/litre
$MgSO_4 \cdot 7H_2O$	200.0
KCl	400.0
NaCl	6400.0
$NaH_2PO_4 \cdot 2H_2O$	140.0
$CaCl_2 \cdot 2H_2O$	264.9

Amino Acids	mg/litre
L-Arginine (HCl)	126.4
L-Cystine Disodium	28.42
L-Glutamine	584.6
L-Histidine (HCl)	21.0
L-Isoleucine	52.46
L-Leucine	52.46
L-Lysine (HCl)	73.06
L-Methionine	14.92
L-Phenylalanine	33.02
L-Threonine	47.64
L-Tryptophan	8.16
L-Tyrosine	36.22
L-Valine	46.86

Vitamins	mg/litre
D-Ca Pantothenate	2.00
Choline Chloride	2.00

Table 2.1. (continued)

Vitamins	mg/litre
Folic Acid	2.00
i-Inositol	4.00
Nicotinamide	2.00
Pyridoxin HCl	2.00
Riboflavin	0.20
Thiamine HCl	2.00

Other Components	mg/litre
D-Glucose	4500.0
Ferric Nitrate $9H_2O$	0.10
Phenol Red Sodium	17.00

(b) Sodium Bicarbonate (5.6%)

Components	litre
$NaHCO_3$	56.0 gm
Phenol Red 1%	1.5 ml
Distilled Water to 1 litre.	

(c) Antibiotics (Penicillin and Streptomycin)

Components	litre
Penicillin	10^6 units
Streptomycin	10.0 gm
Distilled Water to 1 litre.	

The pH was adjusted to 7.0. The medium was sterilized by Millipore filtration using a G.S. membrane (0.22 μ), and stored at $4^{\circ}C$.

(ii) RPMI 1640 medium (Gibco-Bio-Cult Catalogue, 1974-1975)

The composition of RPMI 1640 medium is shown in Table 2.2.

The medium was prepared before use as follows:

90% by volume	RPMI 1640 medium
10% " "	FCS
10^4 units/litre	Penicillin
100 mg/litre	Streptomycin

2.3.2. Solutions for Scintillation Spectrophotometry

(1) Toluene - PPO

4 gm/litre PPO in toluene.

(2) Triton-Toluene-PPO

33% by volume	Triton-X-100
67% " "	Toluene
4 gm/litre	PPO

2.3.3. Buffers

(1) Phosphate Buffered Saline pH 7.4 (PBS)

Solution A 0.5M PO_4 Buffer, pH 7.4

0.5M	Na_2HPO_4
0.5M	NaH_2PO_4

Solution B 0.9% Physiological Saline

0.15M	NaCl.

PBS consists of:

10% by volume	Solution A
90% " "	Solution B.

Table 2.2.

Composition of RPMI 1640 Medium

Inorganic Salts	mg/litre
$Ca(NO_3)_2 \cdot 4H_2O$	100.0
KCl	400.0
$MgSO_4 \cdot 7H_2O$	100.0
NaCl	6000.0
$NaHCO_3$	2000.0
$Na_2HPO_4 \cdot 7H_2O$	1512.0
Amino Acids	**mg/litre**
L-Arginine (free base)	200.0
L-Asparagine	50.0
L-Aspartic Acid	20.0
L-Cystine	50.0
L-Glutamic Acid	20.0
L-Glutamine	300.0
Glycine	10.0
L-Histidine (free base)	15.0
L-Hydroxyproline	20.0
L-Isoleucine (Allo free)	50.0
*L-Leucine (Methionine free)	50.0
L-Lysine HCl	40.0
*L-Methionine	15.0
L-Phenylalanine	15.0
L-Proline (Hydroxy-L-Proline free)	20.0
L-Serine	30.0
L-Threonine (Allo free)	20.0

Table 2.2. (continued)

Amino Acids	mg/litre
L-Tryptophan NaOH	5.0
L-Tyrosine	20.0
L-Valine	20.0

Vitamins	mg/litre
Biotin	0.2
D-Ca Pantothenate	0.25
Cholin Chloride	3.0
Folic Acid	1.0
i-Inositol	35.0
Nicotinamide	1.0
Para-amino benzoic Acid	1.0
Pyridoxine Hcl	1.0
Riboflavin	1.0
Thiamine HCl	1.0
Vitamin B12	0.005

Other Components	mg/litre
Glucose	2000.0
Glutathione (reduced)	1.0
Phenol Red	5.0

*Prepared separately at 10x concentration and added when required. For instance, in some labelling experiments, incubation in leucine or methionine free medium was essential.

(2) Barbitone Buffer pH 8.4

0.04M Na-Barbitone

(3) Tris-HCl Buffer pH 6.5

0.1M Trizma Base

(4) Sorenson's Buffer

Solution A

0.05M KH_2PO_4

Solution B

0.06M Na_2HPO_4

Sorenson's buffer consists of:

5% by volume Solution A

95% " " Solution B

2.3.4. Staining Solutions

2.3.4.1. Solutions for Immunoelectrophoresis and Immunodiffusion Analyses

(1) 0.1% (w/v) Coomassie Brilliant Blue for 15-30 minutes

10% (v/v) Acetic Acid

45% (v/v) Ethanol.

(2) Destain Solution for 10-20 minutes

10% (v/v) Acetic Acid

25% (v/v) Ethanol

2.3.4.2. Solutions for SDS Polyacrylamide Gel

(1) 0.2% (w/v) Coomassie Brilliant Blue for 30 minutes at 60°C

50% (v/v) Methanol

7% (v/v) Acetic Acid (added just before use).

(2) Destain Solution — for 2-3 hours at 60°C

5% (v/v) Methanol

7% (v/v) Acetic Acid

2.3.4.3. Solutions for IEF Gel

(1) 0.1% (w/v) Coomassie Brilliant Blue — for 45-60 minutes

10% Acetic Acid

45% Ethanol

(2) Destain Solution — for 1 hour

5% Acetic Acid

30% Ethanol

2.4. Serological Reagents

2.4.1. Immunoglobulins

The following antisera against mouse immunoglobulins were obtained as indicated:

Rabbit-anti-mouse IgG_1	Miles Laboratories Research Products
Rabbit-anti-mouse IgG_3	Ltd., Stoke Poges, England.
Rabbit-anti-mouse IgA (Myeloma)	A kind gift from Mrs. L. Blakely,
Rabbit-anti-mouse IgM (")	Biochemistry Department, University
Rabbit-anti-mouse IgG_{2a} (")	of Glasgow.
Rabbit-anti-mouse IgG_{2b} (")	

In addition, several antisera were raised in rabbits to crude preparations of mouse immunoglobulins and to different fractions of adult Schistosoma mansoni (2.8).

2.5. Maintainance of the Parasite

Schistosoma mansoni was maintained in Biomphalaria glabrata species of snail and in several strains of mice.

2.5.1. Maintainance and Infection of Snails

Stock snails were maintained in plastic aquaria exposed to day-light at 25°C. Snails were fed on dried lettuce at regular intervals. The small intestines of 8-weeks schistosome infected mice were used as a source of eggs to produce miracidia for infecting snails. The guts were cleaned with physiological saline (0.15M NaCl) and homogenized in 50.0 ml Sorenson's buffer (2.3.3). The homogenate was transferred into a conical flask containing a further 50.0 ml of Sorenson's buffer. A small quantity of trypsin (Difco Laboratories, U.S.A.) was added (1 mg/gut) to the homogenate and the suspension was incubated at 37°C for 2 hours while beingrocked gently on a shaker. After digestion, the homogenate was strained through 2 layers of muslin cloth, the filtrate was washed and centrifuged twice at 800x g for 10 minutes. The supernatant was decanted and the final sediment of packed eggs was transferred into a conical flask containing approximately 1500 ml/6 guts of aquarium water (Copper free), and the eggs were allowed to hatch under a light source at room temperature. The miracidia from hatched eggs were used to infect snails. Infection of snails was undertaken in two different ways:

(i) Infection with few (10-15) miracidia for routine work.

(ii) Infection with a single miracidium for production of a clone of single sex cercariae.

(i) Routine infection. Snails (4-8 mm in diameter) were placed individually in small bottles (4 x 2 cm) containing about 2.0 ml of aquarium water. Aliquots of water containing about 10-15 miracidia were added into each bottle using a Pasteur pipette. The snails were left for 24 hours at 25°C before being pooled together and transferred

to the plastic aquarium. Snails were kept in the dark for 6 weeks. Thereafter, snails were shed in approximately 20.0 ml of aquarium water under bright light source. Cercariae were collected and used for infecting mice.

(ii) Infection of snails with a single miracidium. Multi-welled trays, 140 x 100 x 25 mm (Linbro Scientific Co. Inc., Hamden, London) were used for this purpose. An aliquot of water containing a single miracidium was transferred into each well (10 mm in diameter) with a fine Pasteur pipette. Subsequent checks of the number of miracidia in each well was made under a dissecting microscope (Olympus, SZ-III). Individual snails were placed in each well and a further 2.0 ml of aquarium water was added to each well. The trays were covered and snails were kept at 25°C for 24 hours. Infected snails were transferred individually into Universal bottles (R. and J. Wood, Paisley) of 20.0 ml capacity and capped with muslin cloth to allow aeration. Snails were manipulated and maintained at 25°C for 6 weeks. Snails were shed individually and cercariae were recovered 2 hours after exposure to the light source.

2.5.2. Infection of Animals

(i) Percutaneous method of infection

The percutaneous method of exposure to cercariae described by Smithers and Terry (1965a) was used. Mice were anaesthetized using 10% (v/v) Sagatal in distilled water:ethanol, 9:1 by volume. The dosage rate was 0.9 ml anaesthetic/100 gm body weight. Anaesthetized mice were shaved and layed on their backs in polystyrene trays. Metal rings of 300 µl capacity were placed on the shaved abdomen of anaesthetized

animals. 50 µl aliquots of the cercarial suspension were fixed and stained with small volumes of diluted iodine solution, then counted under a dissecting microscope (Olympus, SZ-III). Aliquots of the cercarial suspension containing 120-200 cercariae were introduced into the metal ring placed on the mice. Mice were left undisturbed for about 10-20 minutes to allow the cercariae to penetrate into the skin.

(ii) Intraperitoneal infection

This method was used mainly to obtain high yields of recovery of adult worms from mice and hamsters. Cercariae were allowed to sediment on ice bath for 30 minutes, after sedimentation, the supernatant fluid was removed and the concentrated cercariae were left in a total volume of approximately 1 ml. Penicillin/streptomycin was added and aliquots (containing 500-1000 cercariae) from the concentrated, well-mixed cercarial suspension were injected into the peritoneal cavity of each animal. Adult worms were recovered from infected animals 5-6 weeks after infection.

2.5.3. Perfusion of Animals for Recovery of Adult Worms

Perfusion of infected animals was performed 6-8 weeks after infection. The perfusion method was essentially that of Smithers and Terry (1965a). Mice received an injection of 10^3 units of heparin followed by a lethal dose of undiluted sagatal (1 ml/100 gm body weight). The abdomenal and thoracic cavities of the dead animal were opened. The animal was held by rubber stripes into a vertical perspex stand. The animal was perfused with Eagle's medium containing 2×10^2 units of heparin. The hepatic portal vein was incised and a 50 ml disposable syringe (Plastipak-B-D, Becton, Dickinson & Co., Ireland) containing

warm Eagle's medium was put into the heart. The medium was flushed into the heart, through the circulation and worms from the opened hepatic portal vein were collected in a glass bowl. The worms were allowed to sediment in 50 ml centrifuge tubes, then washed thoroughly with Eagle's medium. Thereafter, the washed worms were treated in different ways as required for individual experiments.

2.6. Preparation of Adult Worm Fractions

2.6.1. Collection of Adult Worms

Worms were obtained from the hepatic portal vein of schistosome infected animals by perfusion technique (2.5.3). After perfusion, worms were washed extensively with Eagle's medium then stored in small volumes (1-2 ml) PBS at -20°C for at least 24 hours before being used.

2.6.2. Fractionation of Adult Worms

Freezing and thawing method described by Kusel (1972) was used. Frozen worms (approximately 1000 worms/preparation) were allowed to thaw at room temperature. On thawing, the surface membrane partially separated away from the bodies of the worms but leaving worm bodies intact. The separated surface membranes appeared as small bits and sheets under the light microscope. Worms were sieved through muslin cloth then washed twice in PBS pH 7.4. Here, the intact worm bodies remained on the muslin, whereas the surface membranes with soluble proteins passed through with the washing buffer. The worm bodies were transferred into a Potter glass homogenizer containing 1 ml PBS, and homogenized then suspended in PBS. The worm bodies suspension and the membrane filtrate were centrifuged at 40k for 2 hours. After centrifugation, 4 fractions were collected:

(a) Frozen-thawed supernatant (FTS) - contains soluble membrane proteins and other proteins.

(b) Membrane pellet (MP) - contains insoluble membrane proteins.

(c) Worm homogenate supernatant (WHS) - contains soluble proteins from the whole worm body without membrane.

(d) Worm homogenate pellet (WHP) - contains insoluble proteins from the whole worm body without membrane.

Pellets were solubilized in 1% (v/v) solution of Triton-X-100. Fractions were concentrated using acquacide II when required. All fractions were dispensed into 100-200 µl aliquots and stored at -20°C.

2.6.3. Determination of Protein Concentration (Lowry Method)

Protein concentration was determined following Lowry method (1951), using bovine serum albumin (BSA) as standard protein (Figure 2.1.).

2.7. Radioactive Labelling of the Parasite

2.7.1. Internal Labelling of Schistosomula with Radioactive Amino Acids and Preparation of Labelled Fractions (Figure 2.2)

2.7.1.1. Obtaining Schistosomula

Schistosomula were prepared by the mechanical transformation method described by Ramalho-Pinto, Gazzinelli, Howells, Mota-Santos, Figueiredo and Pellegrino (1974) with some modification. Cercariae were collected from infected snails (2.5.1). 10 ml aliquots of the cercarial suspension were collected in Universal bottles (20 ml capacity) and cooled on ice bath to reduce mobility of the organisms. After 20 minutes, the supernatant was decanted and the sedimented cercariae were resuspended in 10 ml of warm Eagle's medium. Transformation of

Figure 2.1. Calibration Curve for Standard Protein (Bovine Serum Albumin, BSA) Used in Lowry method (2.6.3).

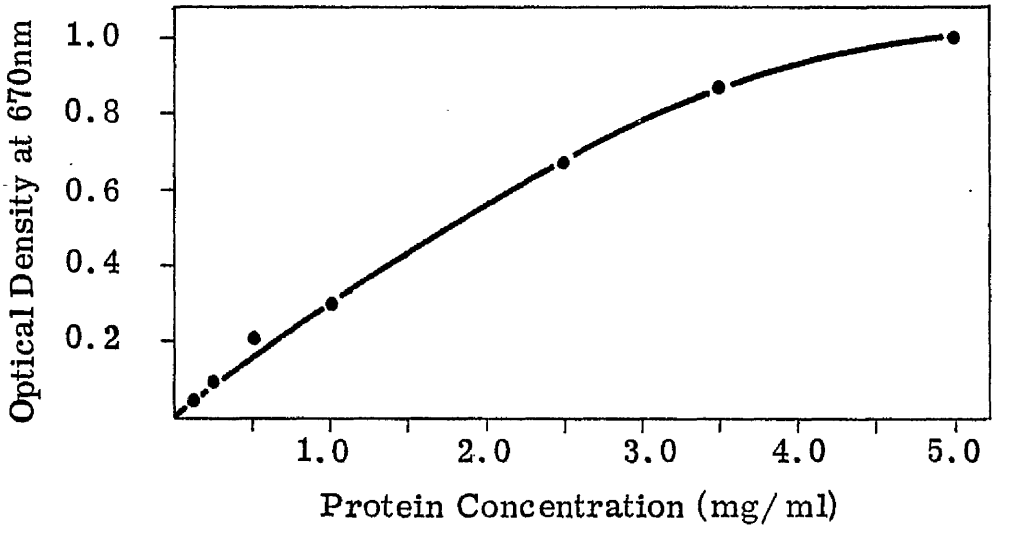


Figure 2.1. Calibration Curve for Standard Protein (Bovine Serum Albumin, BSA) Used in Lowry Method (2.6.3.).

cercariae into schistosomula was achieved by passing the cercarial suspension through 10 ml syringe (Plastipak-B-D, Becton, Dickinson & Co. Ltd., Ireland) five times. The transformed schistosomula were washed thoroughly by slow centrifugation (about 500x g for 1 minute) in sterile Eagle's medium. The tail-rich supernatant was decanted and the sedimented bodies were cultured in Eagle's medium containing 10% foetal calf serum (FCS) and 0.5% lactalbumin hydrolysate (E/Lac) (Table 2.1). 3-4% of the preparation usually consists of "broken cercarial tails".

2.7.1.2. Labelling of Schistosomula with L-(^{35}S)-Methionine

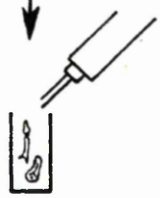
The procedure was adapted from the method described by Ruppel (1978). All stages were performed in a sterile cabinet (Bassaire, John Bass Ltd., Southampton, England) under sterile conditions. 0.1 ml aliquots of Eagle's medium containing 5000-7000 schistosomula were transferred into sterile test tubes (Falcon, Becton, Dickinson and Co., USA). Schistosomula were allowed to sediment and the culture medium was sucked off and replaced by 5 ml of fresh medium (E/Lac) containing 10% FCS. Cultured schistosomula were kept at 37°C under 8% CO_2 in air (Distillers Company, Glasgow) for 24 hours. After this time, cultures were examined under the light microscope, the damaged schistosomula were removed whereas all the remaining viable organisms were maintained for labelling. The radioactive labelling was carried out through 2 successive stages of incubation. During the first stage, schistosomula were incubated in 5 ml of RPMI 1640 medium (labelling medium) deficient in methionine (Table 2.2) and containing 10% FCS at 37°C for 3 hours. During the second stage, the labelling medium was decanted and a mixture of L-(^{35}S)-methionine (250 µCi/ml medium) and 2 ml labelling

Figure 2.2. Diagrammatic Illustration of the Radioactive Labelling of Schistosomula of S. mansoni with L-(^{35}S)-Methionine and Preparation of Labelled Fractions (2.7.1).

6 weeks infected snails shedding under a light source for 2 hrs.

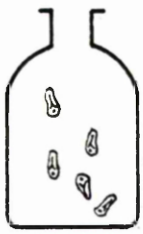
Cercariae

Sedimentation on ice bath for 20 minutes



Transformation into schistosomula by passing through 10ml syringe containing 10ml Eagle's medium at 37°C

Washing transformed schistosomula 5 times in sterile Eagle's medium at 37° C



Incubation at 37° C in :-

a. Eagle's medium + 10% FCS + 0.5% E/Lac 24hrs
b. Labelling medium + 10% FCS - Methionine 3hrs
c. Labelling medium + 10% FCS + ^{35}S Methionine 18hrs

^{35}S-labelled schistosomula

Homogenization in 1ml PBS, and centrifugation at 40k for 2 hrs

Soluble Fraction (The supernatant) (S.F.)

Fast Pellet (The pellet) (F.P.)

medium + 10% FCS was added to the culture tubes. After 18 hours incubation at 37°C, the incorporation of L-(^{35}S)-methionine was terminated by washing schistosomula 5 times in 10 ml labelling medium containing 30 µg unlabelled methionine/ml medium. Labelled schistosomula were suspended in PBS pH 7.4 and stored at -20°C if not being used instantly.

2.7.1.3. Fractionation of Labelled Schistosomula

The method of Sher, Kusel, Perez and Clegg (1974) for preparing schistosome antigens was used. Labelled schistosomula were taken up in 1 ml PBS, pH 7.4 and disrupted in a Potter glass homogenizer. The homogenate was next centrifuged at 40k for 2 hours at 4°C. Two fractions were collected, (a) the soluble fraction (SF), i.e. the supernatant and (b) the fast pellet (FP), i.e. the pellet. The pellet was washed twice by centrifugation in cold PBS, then solubilized in 1% (v/v) solution Triton-X-100. Both fractions were dialysed against PBS containing non-radioactive methionine (30 µg/ml) at 4°C for 24 hours.

2.7.2. Internal Labelling of Adult Worms and Preparation of Labelled Fractions (Figure 2.3)

2.7.2.1. Obtaining and Labelling Adult Worms

Adult worms were obtained from infected animals by perfusion technique as described previously (2.5.3). Immediately after perfusion, worms were cultured in Eagle's medium containing 10% FCS and incubated at 37°C for 24 hours. The subsequent steps were exactly the same as those described for labelling schistosomula with L-(^{35}S)-methionine (2.7.1.2) (Figure 2.2), except for the following stages:

Figure 2.3. Diagrammatic Illustration of the Radioactive Labelling of Adult S. mansoni with L-(^{35}S)-Methionine and Preparation of Labelled Fractions (2.7.2).

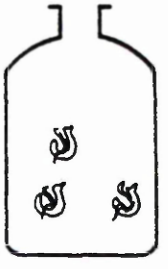
8 weeks infected mice

↓

Perfusion (Smithers & Terry, 1965a)

Adult worms

↓



Incubation at 37° C in:-

a. Eagle's medium + 10% FCS — 24hrs
b. Labelling medium - Met. + 10% FCS — 3hrs
c. Labelling medium + ^{35}S-Met + 10% FCS — 18hrs

↓

^{35}S - labelled worms

↓

Washing extensively in labelling medium + Met.

Freezing and thawing

"Membrane" fraction — Worm bodies minus membranes

Centrifugation at 40K for 2 hrs

A — B — C — D

A: Frozen-thawed supt (contains soluble membrane proteins) (FTS)

B: Membrane pellet (MP)

C: Worm homogenate supt (WHS)

D: Worm homogenate pellet (WHP)

(a) During labelling procedure, each culture vessel contained up to 50 pairs of worms (average 100 worms/2 ml culture medium). Worms were examined and the culture medium was degased several times during the incubation period.

(b) Eagle's medium without lactalbumin hydrolysate was used at the primary stages of incubation.

(c) In some experiments, radioactive labelling of living adult worms with (^{14}C)-amino acids or (^{3}H)-leucine, instead of (^{35}S)-methionine was carried out. Isotopes were added as 30 µCi (^{14}C)-amino acids/ml culture medium or 200 µCi (^{3}H)-leucine/ml culture medium.

2.7.2.2. Fractionation of Labelled Worms

The same procedure described for fractionation of unlabelled worms (2.6.2) was followed (Kusel, 1972). Thus, four fractions from labelled worms were obtained; (a) ^{35}S-frozen-thawed supernatant (^{35}S-FTS); (b) ^{35}S-membrane pellet (^{35}S-MP); (c) ^{35}S-worm homogenate supernatant (^{35}S-WHS) and (d) ^{35}S-worm homogenate pellet (^{35}S-WHP). All fractions were treated as described before (2.7.1.3).

2.7.3. Determination of the Amount of Incorporated Amino Acids into Labelled Fractions

2.7.3.1. Trichloroacetic Acid Precipitation (TCA Precipitation)

10 µl aliquots of labelled fractions were treated with 5.0 ml of ice-cold 10% (v/v) TCA containing 10 mM non-radioactive amino acid. The precipitates were centrifuged at 1,400 x g for 10 minutes and the supernatant fluids were decanted. The pellets obtained were washed 3 times by resuspension and centrifugation. The pellets were dissolved in 0.5 ml protosol. Toluene-PPO scintillation fluid (2.3.2) was then

added and samples were counted on a Beckman LS 335 scintillation counter. FCS was used as a carrier protein.

2.8. Preparation of Serological Reagents

2.8.1. General Preparation Methods

2.8.1.1. Preparation of Schistosome Fractions

Different adult worm fractions were prepared as described previously (2.6.2). Protein concentration was measured (2.6.3) and appropriate amounts were used for immunization (2.8.2).

2.8.1.2. Preparation of Mouse Immunoglobulins

Serum samples were obtained from normal BALB/C strain of mice. Saturated ammonium sulphate solution (55% w/v) was added slowly with constant stirring to the serum sample until 30% saturation was obtained. After standing for 1 hour, the suspension was centrifuged at 1400x g. The precipitate was washed twice in 55% (w/v) solution of saturated ammonium sulphate, dissolved in PBS pH 7.4 then dialysed against the same buffer.

2.8.2. Raising of Antisera

2.8.2.1. Primary Immunization

The concentration of protein in all preparations used for immunization was estimated by Lowry method (2.6.3). 0.5-1.0 mg protein was used per inoculum. Preparations for immunization (1.0-2.0 ml) were added to equal volume of Freund's complete adjuvant (FCA), then the mixture was sonicated at room temperature. Rabbits were injected at several sites subcutaneously.

2.8.2.2. Booster Inoculation

Booster inoculations were given 5 weeks after primary injection. 1.0-2.0 mg of protein antigens were used.

2.8.2.3. Collection of Antisera

Antisera were obtained by bleeding rabbits from the ear vein 5 days after the booster and at 1-2 weeks intervals afterwards. Approximately 20-40 ml of blood was collected at each bleeding. Blood was allowed to clot at room temperature for 1 hour and at 4°C for 24 hours. The clot was removed by centrifugation at 800x g for 10 minutes. Serum samples were dispensed into aliquots and stored at -20°C.

2.8.2.4. Preparation of Immune Serum to Schistosomes in Mice

Groups of twenty mice weighing 20-25 gm were infected with S. mansoni by percutaneous exposure to 60 cercariae (2.5.2.). Sub-groups, each containing 5 animals, were anaesthetized, bled and killed every 20 days for 120 days after schistosome infection. Sera were collected separately, pooled then stored at -20°C.

2.8.2.5. Antibody Titre of Antisera

Titration of antisera was carried out using the immunodiffusion analysis (2.9.1.). Ouchterlony technique (2.9.1.1) was conducted by placing the antigenic proteins in the central well surrounded by several-fold dilutions of the antiserum (Figure 2.4.B).

2.9. Analysis of Schistosome Preparations

2.9.1. Immunodiffusion Analysis and Immunoelectrophoresis

2.9.1.1. Ouchterlony Plates

This method was adapted from the method described by Ouchterlony (1958). 1.5% (w/v) agarose solution containing 2% (w/v)

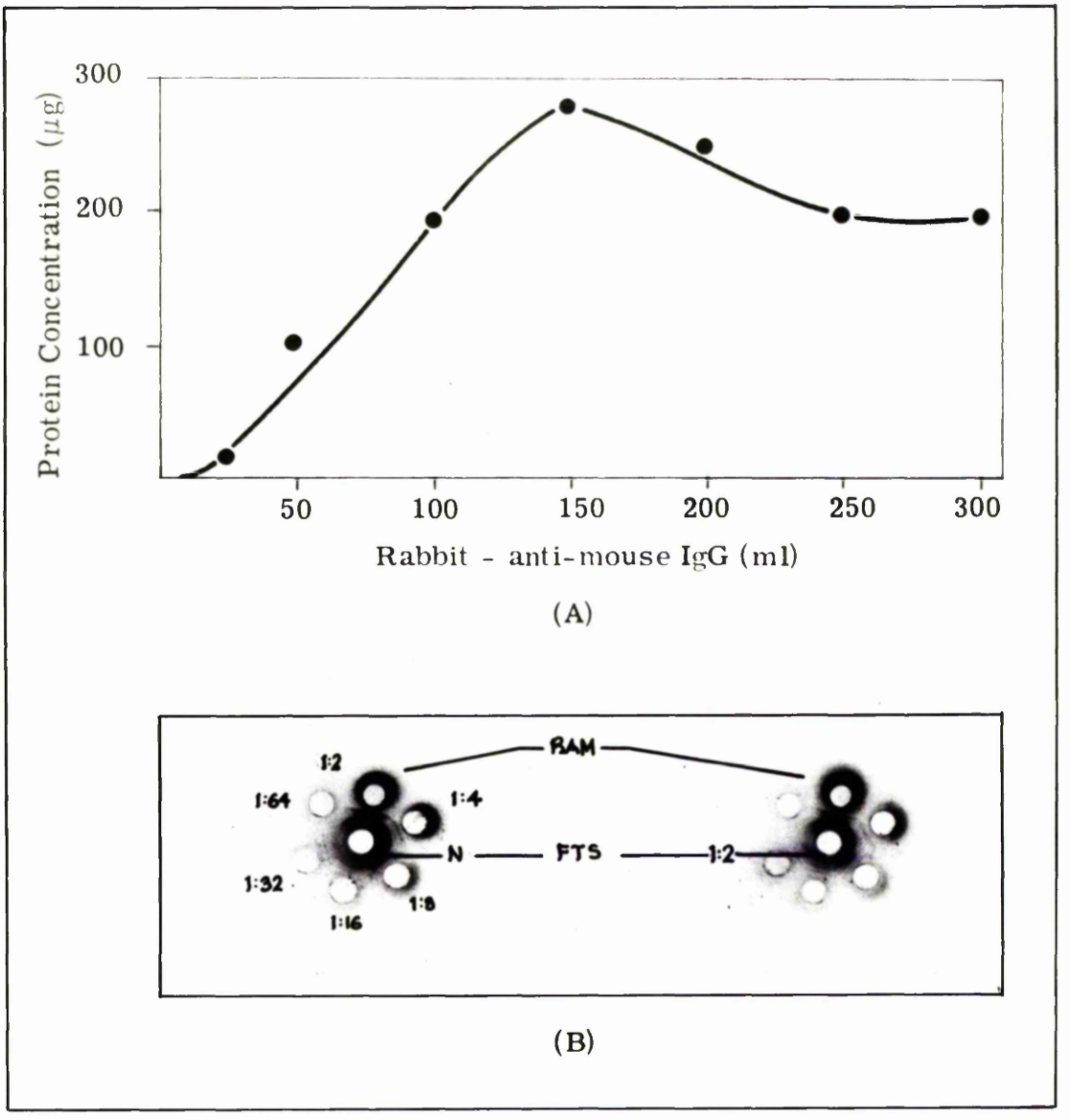
Figure 2.4. Titration of antisera using:

A. Precipitin Curve

To a fixed amount of mouse IgG (15 µg) various amounts of rabbit-anti-mouse IgG (0-300 µl) were added. After a certain period of incubation (37°C for 1 hour and 4°C for 24 hours) the amount of antibody precipitated was measured by determining the amount of proteins in the precipitates using Lowry method (2.6.3). The point of equivalence was reached at 150 µl, of the added antisera. This volume was used in the coprecipitation assays (2.9.3.2).

B. Ouchterlony Analysis

5 µl of frozen-thawed supernatant (FTS) containing 5 mg/ml proteins were placed in the central wells. 2 dilutions of FTS were used; neat (N) or 1:2. Serial dilutions of rabbit anti-schistosome surface membrane antiserum (RAM) were placed in the surrounding wells. The dilutions were 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. Optimal precipitin lines developed with neat (N) frozen-thawed supernatant (FTS) against 1:2 dilution of rabbit anti-membrane antiserum (RAM).


300
200
100
Protein Concentration (μg)
50
100
150
200
250
300
Rabbit - anti-mouse IgG (ml)
(A)
RAM
1:2
1:64
1:4
N
FTS
1:2
1:32
1:8
1:16
(B)

polyethylene glycol (PEG) was poured hot onto washed microscope slides (76 x 26 mm). The volume required was 2.0 ml agarose/slide. The gels were allowed to set at room temperature. Wells were punched in the gel using a template. The punched wells were filled with the antigen and antibody samples to be tested as required. The gels were incubated in a humid sandwich box at room temperature for 24 hours. After this time, gels were examined for the precipitin lines, then washed in 3 changes of PBS pH 7.4, followed by a rinse in distilled water. Gels were dried and next stained in 0.1% (w/v) coomassie brilliant blue (2.3.4.1).

2.9.1.2. Immunoelectrophoresis (IEP) (Hudson and Hay, 1976)

A solution of 1.2% (w/v) agarose in sodium barbitone buffer 0.04M, pH 8.4 (2.3.3) was pipetted into microscope slides (2 ml agarose/slide). Wells were made in the middle of the slide using the cutting template. The wells were filled with antigens to which small quantity (1 µl) of 1% solution of bromophenol blue was added. Electrophoresis was carried out at 8 mA/slide and 180 volts for 60 minutes. After electrophoresis, longitudinal troughs (70 x 2 mm) were cut about 3 mm from the original circular wells and parallel to the direction of the electrical poles. Troughs were filled with sera. Gels were incubated in a humid chamber at room temperature for 24 hours. Precipitin arcs were examined visually. Washing and staining steps were similar to those used for Ouchterlony plates (2.9.1.1).

2.9.1.3. Cross — Over-Immunoelectrophoresis (CIE) (Hudson and Hay, 1976)

The gel solution and buffer are essentially the same as those described for IEP (2.9.1.2). Four wells were made on each slide, each well was separated lengthwise from the other by 8 mm distance.

The wells on the anode side were filled with antisera and the wells on the cathode side were filled with antigens. Electrophoresis, washing and staining gels were performed as described for IEP (2.9.1.2).

2.9.2. Polyacrylamide Gel Electrophoresis Assays

2.9.2.1. Isoelectric Focusing (IEF) (Williamson, 1976)

(1) Stock Solutions

10 M	Urea
30% w/v	Acrylamide
1% w/v	Methylene Bisacrylamide
2% w/v	Ammonium Persulphate
40% w/v	Ampholine carrier Ampholytes

(2) Gel Preparation

3.0 gm	Sucrose
13.8 ml	10 M urea
3.3 ml	30% Acrylamide
3.9 ml	1% Bisacrylamide
1.0 ml	1% Ammonium Persulphate
1.2 ml	40% Ampholine
5.0 µl	TEMED

80 x 80 mm glass plates (Ilford Research Labs, Essex) were washed in chromic acid, rinsed very thoroughly in distilled water then air dried. Clean glass plates were siliconized using repelcote, some other plates were gelatinized in 0.1% (w/v) gelatin solution. The siliconized plate with two rubber bands (1 mm in thickness), each lying along one edge, was placed under the gelatinized plate and the sandwich was secured by double rubber bands. The gel mixture was introduced as a

1 mm thick layer between the two plates using 10 ml syringe (Gillette, U.K.). The gel was allowed to polymerize for 10-15 minutes. All gel plates were kept in a humid chamber at 4°C for 24 hours before being used.

(3) Sample Preparation and Focusing

Siliconized plates and the spacers (rubber bands) were removed gently from the gelatinized plates (and the gel) as the gel adhered to the latter. Gels were placed face down onto carbon electrodes in an airtight, humified box and pre-focused at a constant power of 300 mW for 1 hour. Electrodes were previously moistened with phosphoric acid solution (5% v/v) for the anode, and ethanolamine solution (5% v/v) for the cathode. Small volumes of sample (10-20 µl) were soaked into 5.0 x 1.0 mm filter papers (Whatman 3 MM) and applied to the surface of the pre-focused gel at its anodic end. Focusing was allowed to proceed for 5 hours with a constant power of 300 mW/gel at 4°C. After focusing is completed, the filter papers were removed and the pH gradient was measured with a flat dual microelectrode (Pye Unicam, U.K.).

(4) Fixing and Processing Gels

Gels were fixed in 10% w/v TCA for 24 hours at room temperature, then washed in distilled water for 2-4 hours. Gels were either stained for protein bands in 0.1% coomassie blue (2.3.4.3.) or processed for detecting radioactive proteins (2.10.2).

2.9.2.2. SDS Polyacrylamide Gel Electrophoresis (Laemmli, 1970)

(1) Stock Solutions

Solution A

30% w/v Acrylamide

0.8% w/v Methylene Bisacrylamide

Solution B

1.5 M Tris-HCl Buffer, pH 8.8.

Solution C

0.5 M Tris-HCl Buffer, pH 6.8.

(2) Gel Preparation

(a) Sealing gel preparation

The bottom of the gel casting mould was sealed by immersing it in a gel solution prepared as follows:

2.5 ml Solution A

7.5 ml Solution B

0.25 ml TEMED

0.25 ml 10% Ammonium Persulphate

(b) Separating gel preparation

Gel solutions for 7.5% separating gel were prepared as follows:

3.3 ml Solution A

5.0 ml Solution B

0.2 ml 10% SDS

0.01 ml TEMED

0.2 ml 10% Ammonium Persulphate

Distilled water to 20 ml.

The gel was poured onto 65 x 88 mm glass plates and left to polymerize at room temperature for 1 hour.

(c) Stacking gel preparation

Gel solution for 3% polyacrylamide stacking gel was prepared as follows:

1.0 ml Solution A

2.5 ml Solution C

0.1 ml 10% SDS

5.0 µl TEMED

0.15 ml 10% Ammonium Persulphate

Distilled water to 10 ml.

The gel solution was poured on the top of separating gel and left to polymerize around a well template with 7 sample application wells.

(3) Sample Preparation and Electrophoresis

Protein samples to be electrophoresed were dissolved in equal volume of sample buffer and heated at 100°C for 2 minutes.

Sample Buffer

2.0 ml 10% SDS

0.5 ml Mercaptoethanol

0.5 ml Glycerol

few granules Bromophenol Blue

The samples were loaded into the sample wells, under the running buffer. Electrophoresis was carried out with 15-20 mA/gel constant current for about 1-2 hours.

Running Buffer, pH 8.8

3.0 gm Trizma-Base

28.8 gm Glycine

20.0 ml 10% SDS

Distilled water to 2 litres.

(4) Fixing and Staining Gels

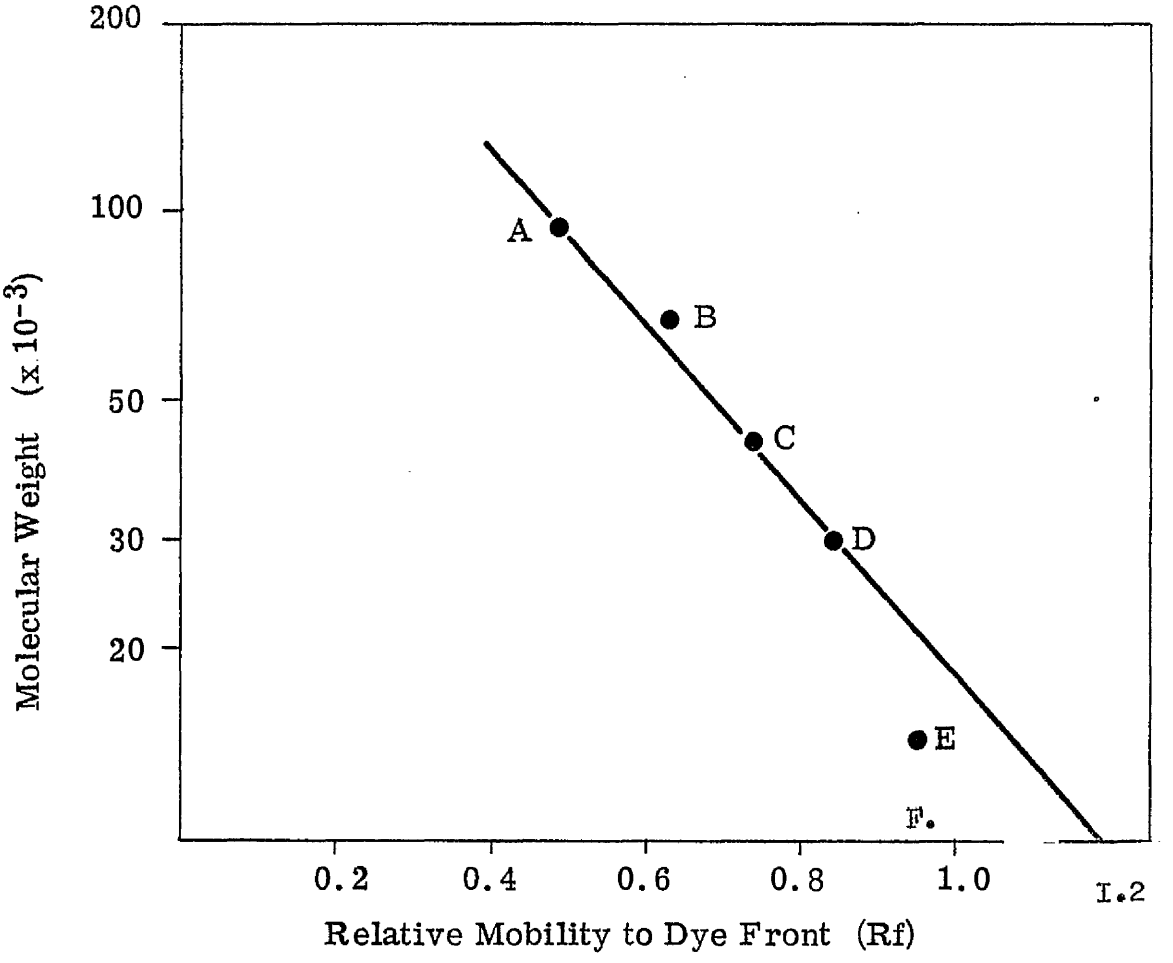
Gels were removed from the glass plates and fixed and stained according to Maizel (1971) (2.3.4.2.).

(5) Molecular Weight Determinations of Proteins

The molecular weights of polypeptides run as standard proteins on the 7.5% SDS gel are shown in Figure 2.5.

Figure 2.5. Calibration Curve for the Standard Proteins for SDS Polyacrylamide Gel (2.9.2.2). The mobilities of the following markers are plotted versus their molecular weights on a logarithmic scale.

A	Phosphorylase	94,000
B	Bovine Serum Albumin	68,000
C	Ovalbumin	43,000
D	Carbonic anhydrase	30,000
E	Lysozyme	14,700
F	Soya bean trypsin inhibitor	4,000


200
100
50
30
20
Molecular Weight (x 10-3)
A
B
C
D
E
F.
0.2
0.4
0.6
0.8
1.0
1.2
Relative Mobility to Dye Front (Rf)

2.9.3. Coprecipitation of Radioactive Proteins from Labelled Fractions

2.9.3.1. Titration of Rabbit-Anti-Mouse-IgG (RAMIgG) (Figure 2.4.A)

In order to establish the most efficient precipitation conditions, titration of RAMIgG was carried out. Increasing amounts of RAMIgG were added to a series of tubes containing fixed amounts of mouse IgG (15 µg). Tubes were incubated at 37°C for 1 hr and at 4°C for 24 hours. The precipitates formed were centrifuged at 1,400 x g for 15 minutes and washed 3 times with ice-cold PBS. The precipitates were dissolved in 1 ml of 0.2M NaOH and protein concentration was determined by Lowry method (2.6.3).

2.9.3.2. Coprecipitation Assay

The method of Kusel, Sher, Perez, Clegg and Smithers (1975) was used to detect the presence of free antibodies in mouse serum infected with S. mansoni against labelled adult worm fractions. 5 µl of mouse serum was added to duplicate samples of labelled antigens. After 2 hours incubation at 37°C, 150 µl of RAMIgG (2.9.3.1) was added to each sample. All samples were incubated at 37°C for 1 hour, then at 4°C for 24 hours. The coprecipitated samples were diluted with 5 ml of ice-cold PBS, pH 7.4 and centrifuged at 1,400 x g for 10 minutes. The pellets were washed three times with PBS. The final pellets were dissolved in 0.5 ml protosol. Toluene-PPO scintillation fluid was added and samples were counted for ^{35}(S) or ^{14}(C) or ^{3}(H) on a scintillation counter (Beckman LS 335).

2.9.4. Lectin Assay

2.9.4.1. Lectin Over-Lay Technique

The method described by Tanner and Anstee (1976) was used. Non-

labelled samples from adult worms were separated by isoelectric focusing in polyacrylamide gel (2.9.2.1). The gels were washed in distilled water, then in PBS, pH 7.4 for 24 hours. The gels were transferred to 500 ml chambers containing a mixture of: 30 ml PBS + 60 mg haemoglobin + 0.05% (w/v) sodium azide. The labelled lectin (^{125}I-Con A) was added to the gels, and the mixture was shaken gently for 3 days. A control gel was treated in the same way but with α-methyl-D-glucoside added as a monosaccharide inhibitor. After incubation the solution was decanted and the gels were washed extensively with PBS, pH 7.4 for 3 days. The gels were stained for proteins with 0.1% coomassie blue stain (2.3.4.3). The labelled bands were detected by fluorography (2.10.2). Con A was iodinated by the method of Hunter and Greenwood (1962).

2.9.5. Separation Technique

2.9.5.1. QAE-Sephadex Chromatography (Hudson and Hay, 1976)

The sample was dialysed extensively against 0.1M Tris-HCl buffer, pH 6.5 (2.3.3) at 4°C. The column was poured using QAE-Sephadex swollen beads after being degased and adjusted to pH 6.5. The column was washed thoroughly with 0.1M Tris-HCl buffer, pH 6.5. The sample was added to the column and 5 ml fractions were collected at about 1 ml/minute. Fractions were monitored by their absorbancy at 280 nm. The appropriate peak fractions were pooled on the bases of absorbance (A280) measurements. Pooled samples were concentrated using acquacide II if required.

2.10. Detection of Radioactivity

2.10.1. Scintillation Counting of Labelled Materials

Triton-toluene-PPO scintillation fluid (2.3.2) (10 ml) was

added to labelled samples and the radioactivity was counted on a scintillation counter (Beckman LS 335). The amount of radioactivity in TCA precipitated proteins (2.7.3.1) or in immunoprecipitates (2.9.3) was detected by solubilizing the pellets in 0.5 ml protosol, then Toluene-PPO scintillation fluid was added. Samples were counted on a Beckman LS 335 scintillation counter.

2.10.2. Fluorography of Gels (Bonner and Laskey, 1974)

Slab gels were given 3 successive washes in 200 ml dimethyl sulphoxide (DMSO) for a total of 90 minutes with gentle shaking. Gels were then immersed in 100 ml of 20% (w/v) PPO in DMSO for 45 minutes. PPO-DMSO solution was decanted and gels were flooded with water for 1 hour. Gels were dried onto Whatman 3MM filter paper under vacuum, then placed in contact with Kodak-X-O-mat-Royal films and exposed at $-70^{\circ}C$.

CHAPTER III

ELECTROPHORETIC ANALYSIS OF FRACTIONS FROM SCHISTOSOMULA AND ADULT WORMS OF *SCHISTOSOMA MANSONI*

3. ELECTROPHORETIC ANALYSIS OF FRACTIONS FROM SCHISTOSOMULA AND ADULT WORMS OF SCHISTOSOMA MANSONI

3.1. Introduction

Attempts have been made by several investigators to analyse and compare various schistosome preparations. Much of the previous work on the composition of schistosome extracts has utilized gel electrophoresis as an analytical tool for such purpose. This technique has been used to compare protein components in complex preparations of the entire parasite (Sodeman, 1967, 1968), of parasite secretions and excretions (Murrell, Vannier and Ahmed, 1974) or of crude extract of the different developmental stages of the parasite (Ruppel and Cioli, 1977). Kusel (1970, 1972), Cordeiro and Gazzinelli (1979) and Hayunga, Murrell, Taylor and Vannier (1979a, 1979b) have reported studies on the composition of schistosome surface membrane proteins in which SDS-gel electrophoresis was used.

While all of the studies referred to above have demonstrated a marked heterogeneity of schistosome extracts, the nature of specific schistosome antigens (whether stage specific or fraction specific antigens) is still not well-defined. This investigation presents a comparison by electrophoretic methods, with emphasis on the technique of isoelectric focusing (IEF) of different extracts prepared from adult worms and schistosomula of S. mansoni. The technique described by Kusel (1972) and Kusel and Mackenzie (1975) to prepare different fractions from adult worms and schistosomula has been further developed and used to study the protein patterns of such fractions after electrophoresis on SDS and isoelectric focusing in polyacrylamide gels. One important reason for using the IEF technique is its ability to differentiate

native protein components in a heterogeneous mixture. In addition, although this technique provides a useful tool for fractionating and characterizing protein components, its use in the field of schistosomiasis has been very limited but has been used with great value in fractionating trypanosome antigens (Cross, 1975).

3.2. SDS Polyacrylamide Gel Electrophoretic Patterns of Schistosome Fractions Extracted from Adult Worms

Fractions from adult worms were prepared as described previously (2.6.2) and electrophoresed on thin-layer SDS polyacrylamide gels (2.9.2.2). Protein bands were detected by staining in coomassie brilliant blue stain R250 (2.3.4.2). The patterns are presented in Figure 3.I. The FTS fraction (the supernatant of membrane fraction) and the WHP fraction (the pellet of the whole worm without membrane) each had 28 bands, whereas the MP fraction (the pellet of the membrane fraction) had only 18 bands, if faint and closely associated bands were counted. Fractions gave similar patterns on SDS-gel except for the following qualitative differences: (a) a diffused band (S_1) with a high molecular weight (112,000) was identified only in the MP fraction, (b) a band with 94,000 molecular weight (S_2) was present in the FTS and WHP fractions but absent in the MP fraction. S_2 appeared to split into 2 bands in the WHP fraction which may represent a separate band, and (c) a major band (S_5) with 60,000 molecular weight was unique to the FTS and MP fractions. Additional quantitative differences could be detected (Table 3.I). There are also minor differences in the relative intensities of some bands in different worm preparations.

Results obtained with SDS-gel indicated two main points; first, the complex nature of the patterns shown by each of the adult worm fractions examined. Second, the presence of some bands (S_1, S_5)

Figure 3.I. 7.5% SDS Polyacrylamide Gel of Schistosome Fractions Extracted from Adult Worms of S. mansoni

The gel contains the following fractions (from left to right): Frozen-thawed supernatant (FTS), membrane pellet (MP), worm homogenate pellet (WHP) and the molecular weight standard proteins phosphorylase (94 k), bovine serum albumin (68 k), carbonic anhydrase (30 k) and soya bean trypsin inhibitor (4 k). The gel was stained with coomassie blue stain R250 (2.3.4.2).

S_1-S_{10} present major bands resolved on SDS gel. For molecular weights of these bands see Table 3.I.

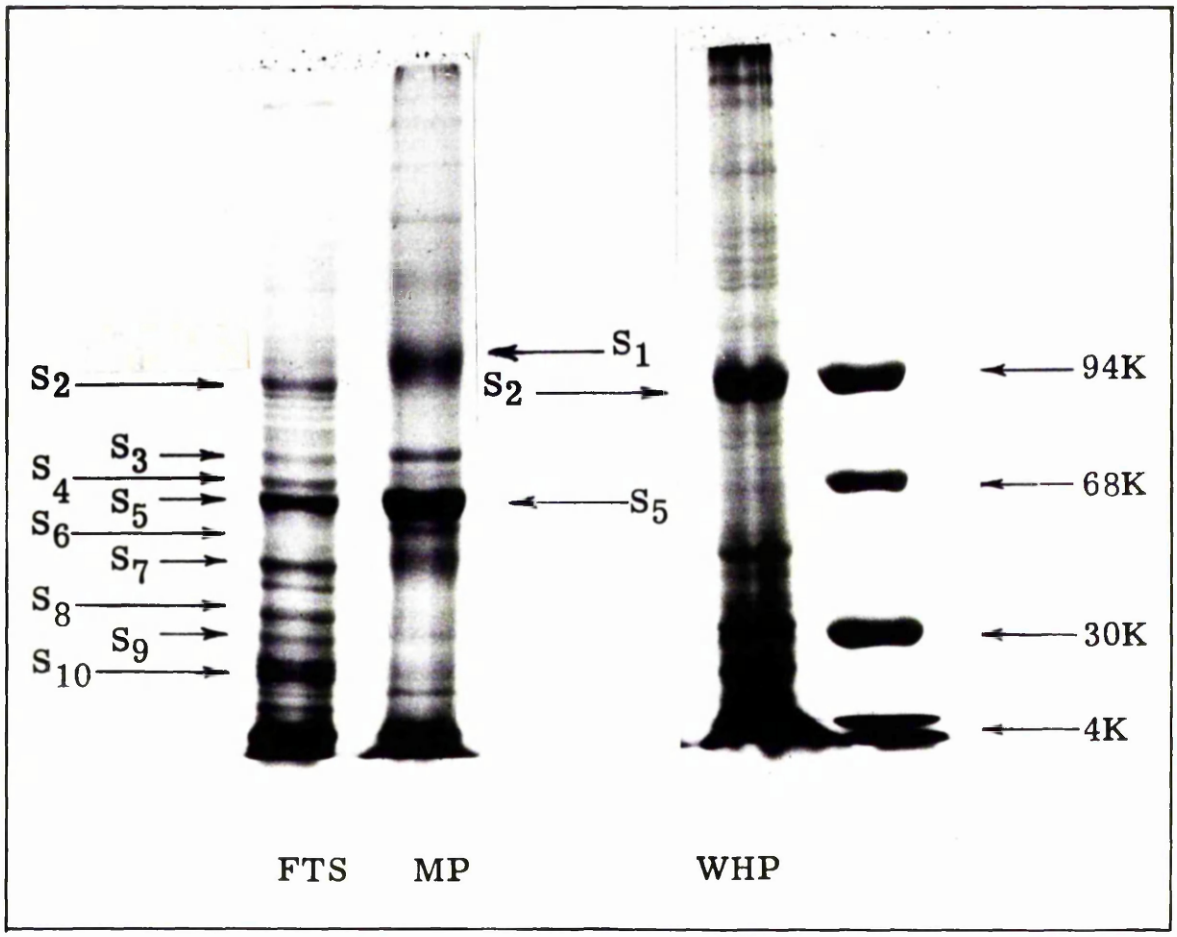

S2
S3
S4
S5
S6
S7
S8
S9
S10
S1
S2
S5
94K
68K
30K
4K
FTS
MP
WHP

Table 3.I

* The Molecular Weights of Electrophoretic Bands Indicated in Figure 3.I.

Electrophoretic Band	Fractions FTS	MP	WHP	Molecular Weight (Daltons)
S_1	-	++	-	112
S_2	+	-	++	94
S_3	++	++	+	74
S_4	++	+	+	70
S_5	+	++	-	60
S_6	+	+	+	54
S_7	++	++	++	46
S_8	+	+	+	36
S_9	++	+	++	34
S_{10}	++	+	++	28

* Protein bands were obtained by SDS-gel electrophoresis of 3 schistosome fractions extracted from adult worms.

++ Prominent band

+ Less prominent band

- No band.

in the membrane fractions (FTS and MP) which are absent in the fraction obtained from the whole worm without membrane (WHP). To characterize further the nature of protein bands revealed by the SDS-gels with emphasis on the membrane fractions, isoelectric focusing in thin-layer polyacrylamide gel (IEF) was carried out as described below.

3.3. IEF Patterns of Schistosome Fractions Extracted from Adult Worms and Schistosomula

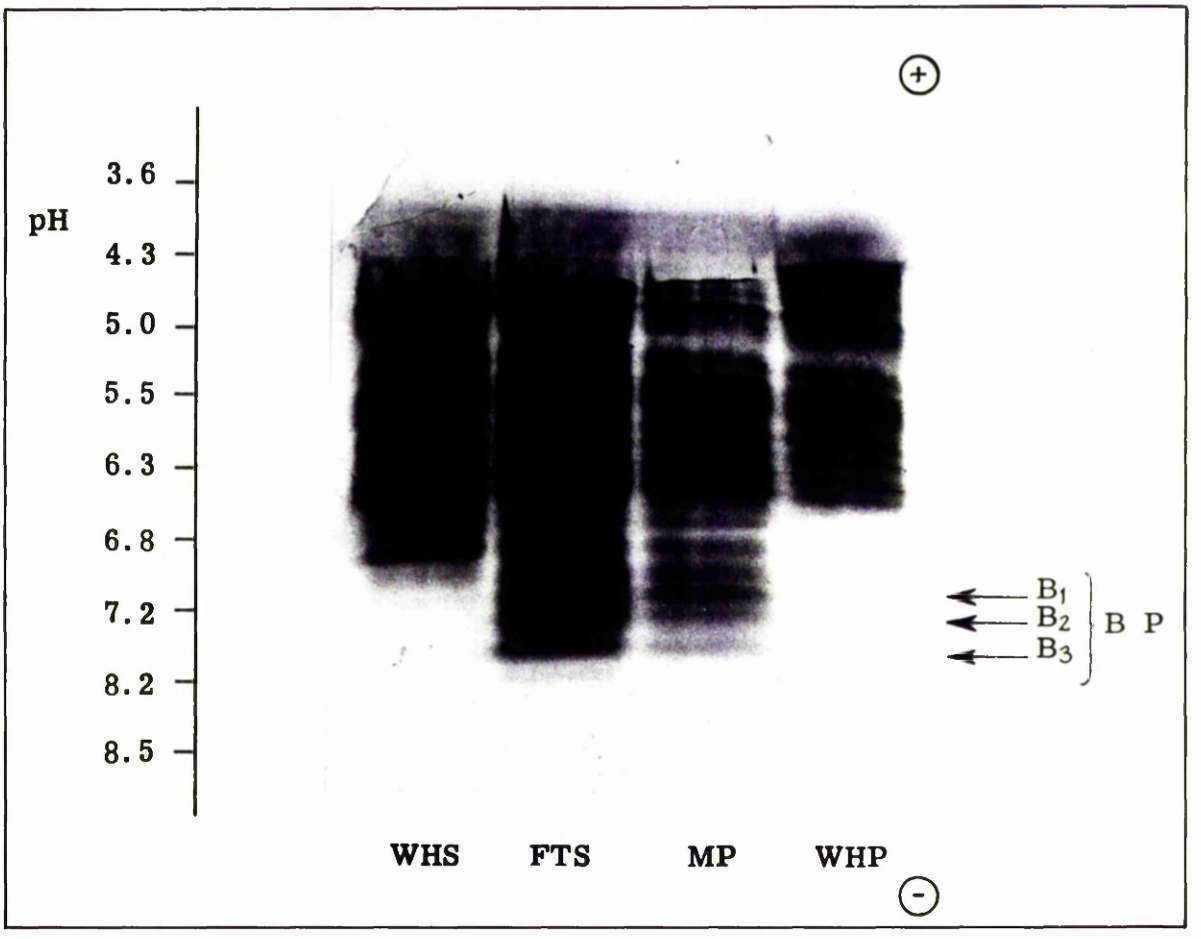
3.3.1. IEF Patterns of Non-Labelled Fractions Extracted from Adult Worms

Isoelectric focusing in thin-layer polyacrylamide gel (2.9.2.I) was carried out with 4 schistosome fractions extracted from adult worms of S. mansoni (2.6.2). The fractions were WHS, WHP, FTS and MP. The WHS and WHP represented the supernatant and the pellet obtained from the whole worm without membrane respectively. Whereas the FTS and the MP fractions were the supernatant and the pellet of the membrane fraction isolated by the freezing and thawing method of Kusel (1972). All fractions were focused in thin-layer polyacrylamide gels. Focusing in a gel containing 6 M urea gave an identical pattern to that observed on focusing in polyacrylamide gel alone, but with better resolution of the focused bands. Thus, urea gels were used throughout the present work. Pellets were solubilized in Triton-X-100 before being electrophoresed on gels. Approximately 16 components focused between pH 3.9-8.2 were obtained on focusing the solubilized pellets in gels containing 6 M urea. A similar number of components was obtained on solubilizing pellets in Triton-X-100 and focusing in gels containing 1% Triton-X-100 and 6 M urea. However, the use of 6 M urea gel seemed better suited for displaying protein bands in all fractions. The patterns of stained bands are shown in Figure 3.2. It clearly

Figure 3.2. Isoelectric Focusing Patterns of Various Schistosome Fractions Extracted from Adult Worms

Reference fractions applied are (from left to right): worm homogenate supernatant (WHS), frozen-thawed supernatant (FTS), membrane pellet (MP) and worm homogenate pellet (WHP). The gel was stained in coomassie brilliant blue stain R250 (2.3.4.3). The scale along the plate shows the pH values along the gel (pH 3.5-10.0).

B_1, B_2, B_3 (BP) indicate the protein bands (at pH 7.2-8.2) present in both FTS and MP fractions.


pH
3.6
4.3
5.0
5.5
6.3
6.8
7.2
8.2
8.5
WHS
FTS
MP
WHP
B1
B2
B3
B P

indicates some variations between different fractions extracted from adult worms. In addition, although equal amounts of proteins from each fraction were applied to the gel, the relative intensities of the bands of the pellet fractions (MP and WHP) appeared less than that in the bands of the supernatant fractions (FTS and WHS). By direct comparison, approximately 16 protein bands could be identified in all fractions with 3 exceptional bands present in both the MP and FTS fractions, but absent in the WHS and WHP fractions. These bands focused between pH 7.2-8.2 and were termed the basic protein (BP). A number of conclusions could be drawn from the above data; (a) some schistosome fractions show different protein banding patterns on IEF gels, (b) patterns from the surface membrane pellet (MP) and the FTS fraction show the presence of protein bands (BP) with isoelectric points at pH 7.2-8.2, and (c) this BP is absent in the fractions derived from the whole worms without membranes (WHS and WHP).

The presence of BP in the stained membrane fractions suggested that it may be either a membrane protein synthesized by the parasite or it may represent protein molecules associated with the tegument such as proteins found in mouse serum where adult worms were grown *in vivo*. In order to determine the origin of this BP observed in membrane fractions, radioactive labelling of adult worms with (^{35}S)-methionine *in vitro*, followed by focusing of labelled fractions on IEF gels was undertaken as described in later sections.

3.3.2. IEF Pattern of Labelled Adult Worm Fractions

Kusel, Sher, Perez, Clegg and Smithers (1975) and Ruppel (1978) labelled living adult worms with radioactive amino acids *in vitro*. This method served as the basis for the labelling experiments applied in the present work. Some variables involved in such experiments were investigated in order to establish the optimal culture conditions for labelling schistosomes *in vitro*.

3.3.2.1. Determination of the Labelling Conditions

(i) Effect of incubation period on the rate of incorporation of (^{35}S)-methionine into adult worms and schistosomula

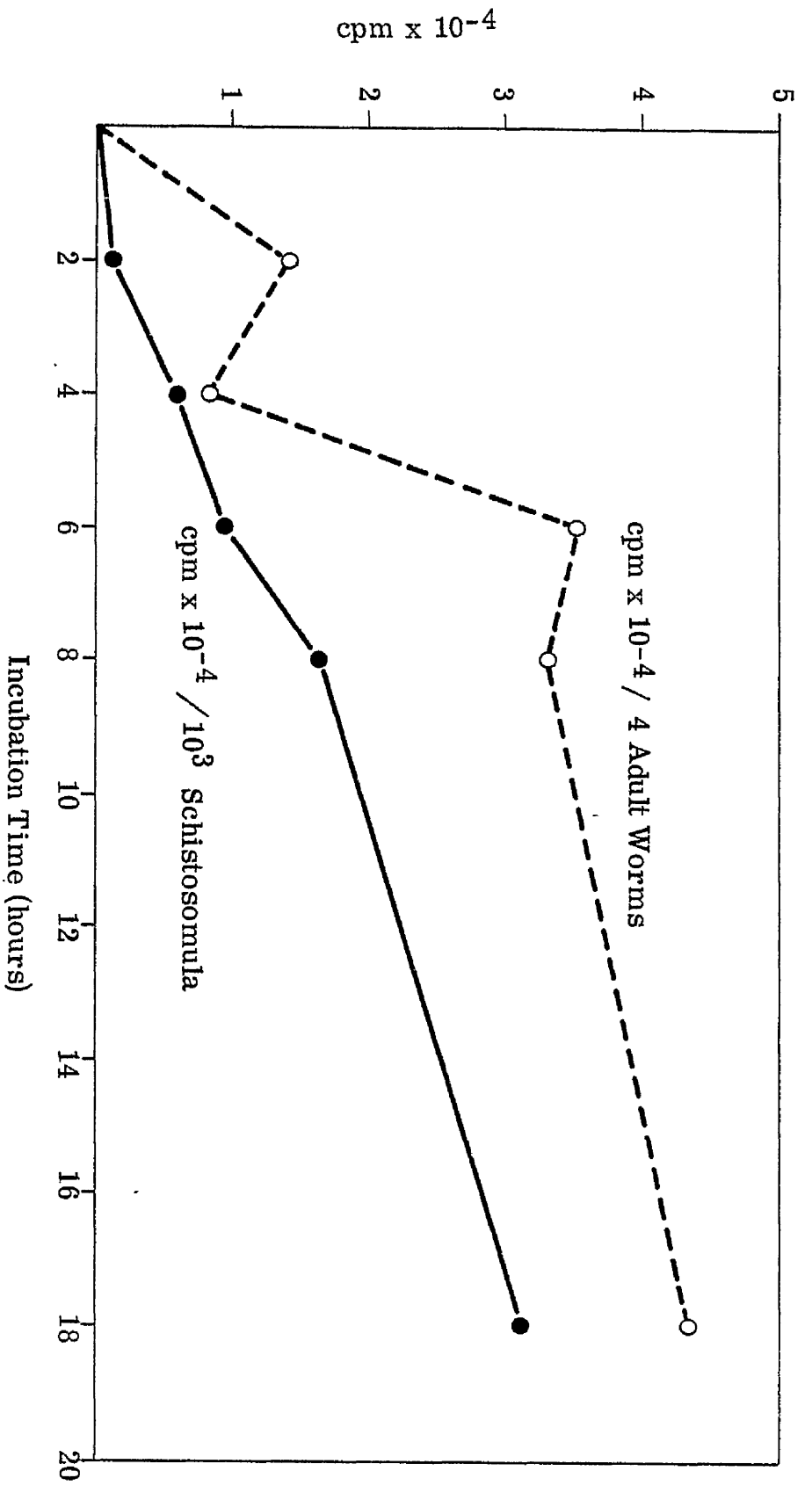
Adult worms or schistosomula of S. mansoni were internally labelled with (^{35}S)-methionine in vitro. The labelling procedure described previously (2.7.1 and 2.7.2) was followed. The incorporation of ^{35}(S)-methionine into adult worms or schistosomula was compared by labelling triplicate samples of 10 pairs of adult worms or 500 schistosomula/ml labelling medium with 500 µCi of ^{35}(S)-methionine. At 2 hourly intervals, 2 pairs of adult worms or 200 µl aliquots of medium containing approximately 100 schistosomula were collected from each culture tube. The incorporated radioactivity was determined by TCA precipitation of the FTS fractions obtained by freezing and thawing labelled adult worms or schistosomula. Results are shown in Figure 3.3. The incorporation of ^{35}(S)-methionine increased progressively in both adult worms and schistosomula until the end of incubation period, i.e. after 18 hours incubation at 37°C. Such incubation time was applied in subsequent labelling experiments.

(ii) Effect of serum on the IEF patterns of labelled fractions

Ruppel (1978) indicated that incubation of internally labelled adult schistosomes in a medium deficient in serum resulted in a significant increase in the amount of radioactive proteins released from adult worms into culture media. Simpson, Cesari and Evans (1980) suggested that incubation of adult worms of S. mansoni in a chemically defined medium (RPMI medium) without serum has damaging effects on the parasite surface membrane. In order to investigate this possibility, an experiment was carried out in which adult worms were labelled under the

Figure 3.3. Incorporation of ^{35}(S)-Methionine into Adult Worms and Schistosomula of S. mansoni

Schistosomes were radiolabelled with ^{35}(S)-methionine (2.7.I, 2.7.2). The incorporated radioactivity was determined by TCA precipitation of the frozen-thawed supernatant fraction. Results are expressed as count/minute per 4 adult worms or count/minute per 1000 schistosomula. (See 3.3.2.I for detail).


cpm x 10^{-4}
1
2
3
4
5
2
4
6
8
10
12
14
16
18
20
Incubation Time (hours)
cpm x 10^{-4} / 4 Adult Worms
cpm x 10^{-4} / 10^3 Schistosomula

same culture conditions described before (2.7.2) but in the absence of serum. Surface membrane fractions were prepared by freezing and thawing, then focused on IEF gels. Results are shown in Figure 3.4. Comparison of the results obtained in this experiment (Figure 3.4) with that obtained on labelling adult worms in the presence of serum (Figure 3.5) indicated that the protein pattern was complex in both cases, but not identical. In addition, although the patterns of the 2 fractions examined (FTS and MP fractions) appeared to be similar within each experiment, there are clear differences in the patterns of both fractions from one experiment to another, i.e. in the presence or in the absence of serum. This result is compatible with the observation reported by Ruppel (1978) who suggested that the release of proteins from adult worms into such culture medium (deficient in serum) may be due to lysis of the schistosome tegument.

One interpretation of the results described above could be that damage in the surface membrane of labelled worms might occur in the absence of serum which might be accompanied by the release of internal proteins into culture media. Thus, freezing and thawing of such damaged worms may result in the leakage of the exposed internal proteins of the parasite along with the isolated surface membrane. A more conclusive experiment regarding this result would be to obtain the IEF patterns of labelled proteins released from adult schistosomes into culture medium in the presence and in the absence of serum.

3.3.2.2. IEF Pattern of Labelled Fractions Extracted from Adult Worms

One of the main aims of the labelling experiments was to determine whether the BP observed to be confined to the FTS and MP fractions in stained gels is synthesized by the parasite. To examine this possibility, adult worms of *S. mansoni* were labelled with ^{35}(S)-

Figure 3.4. Isoelectric Focusing Spectra and the Corresponding Densitometric Tracings of 2 Radiolabelled Schistosome Fractions Extracted from Adult Worms Incubated with ^{35}(S)-Methionine in the Absence of Serum

Adult worms of S. mansoni were incubated with ^{35}(S)-methionine in the absence of serum. 2 fractions were prepared: ^{35}(S)-frozen-thawed supernatant (^{35}S-FTS) and ^{35}(S)-membrane pellet (^{35}S-MP). The IEF spectra and their corresponding densitometric tracings indicate changes in the pattern compared with that obtained on radiolabelling in the presence of serum. (See 3.3.2.I for detail.)

Dotted lines indicate faint bands shown on gels by fluorography.

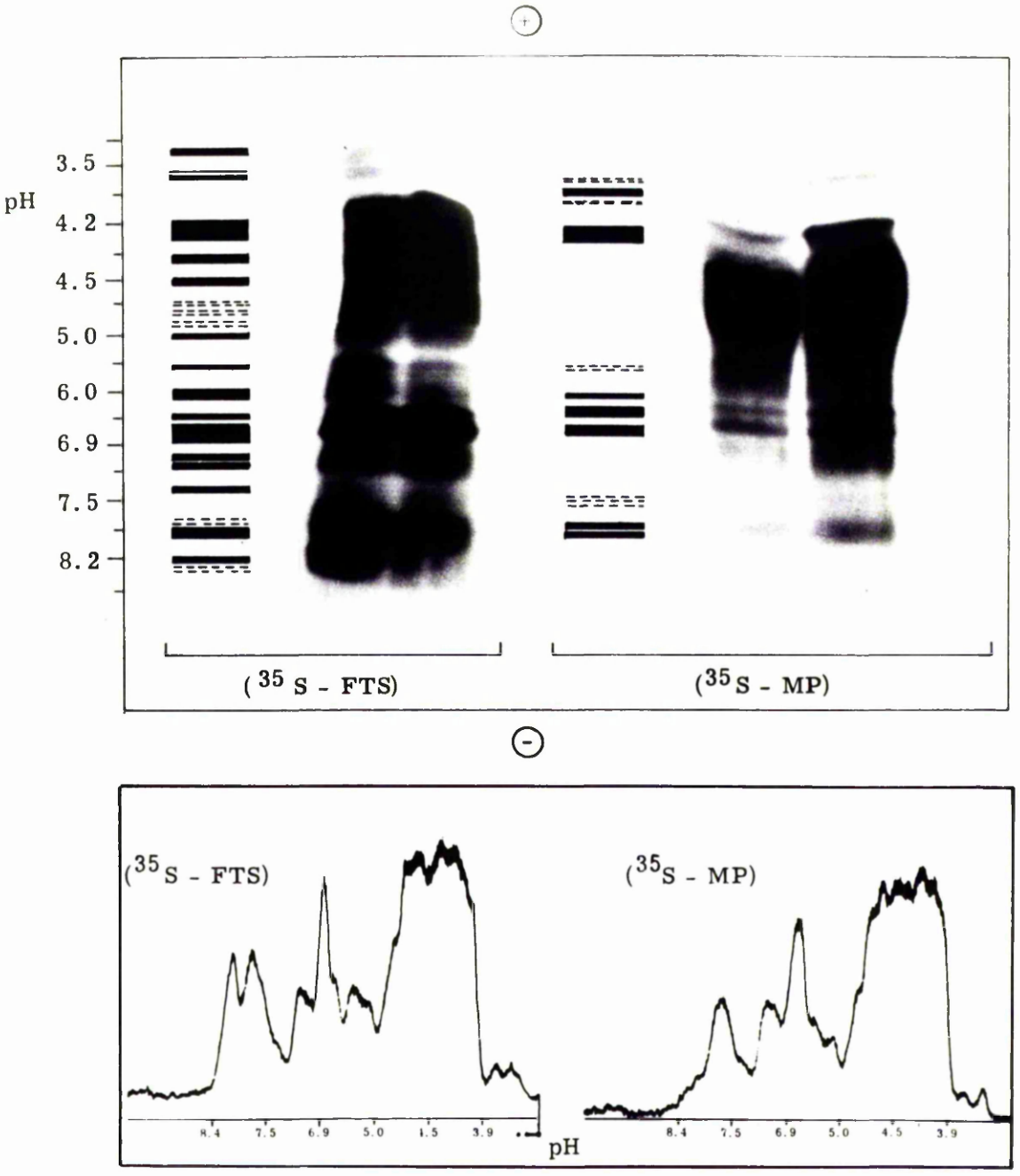
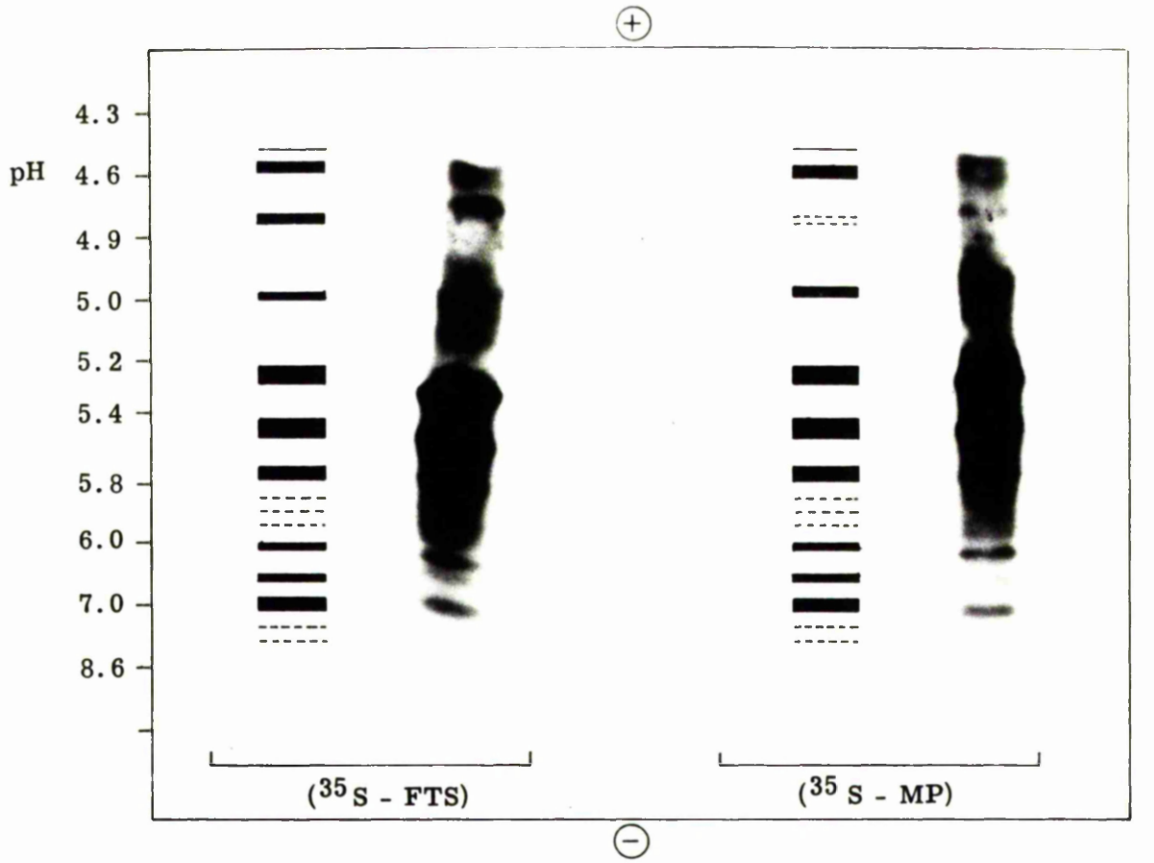

pH
3.5
4.2
4.5
5.0
6.0
6.9
7.5
8.2
(35 S - FTS)
(35 S - MP)
(35S - FTS)
(35S - MP)
8.4
7.5
6.9
5.0
4.5
3.9
pH

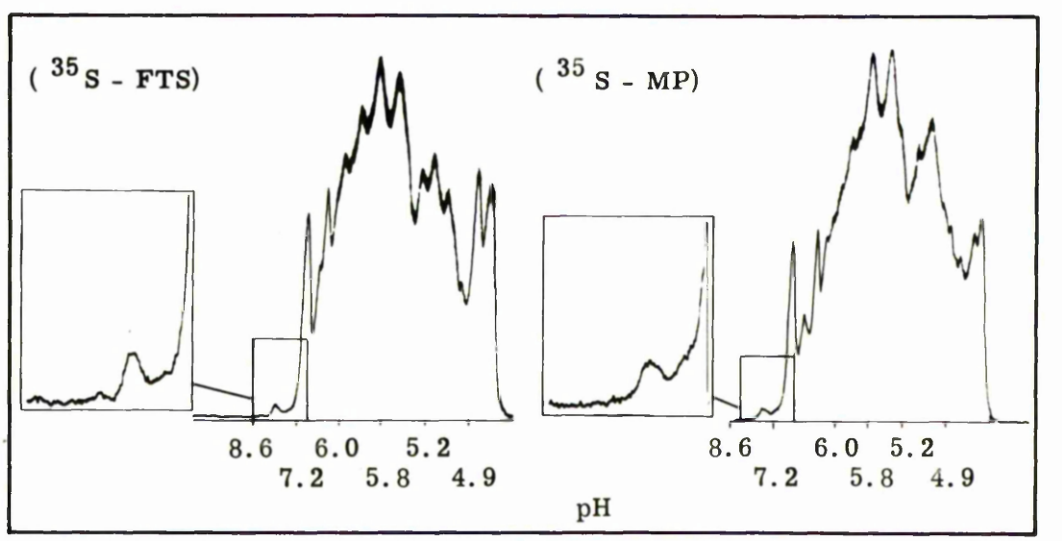
Figure 3.5. Isoelectric Focusing Spectra and the Corresponding Densitometric Tracings of 2 Radiolabelled Schistosome Fractions Extracted from Adult Worms Incubated with ^{35}(S)-Methionine in the Presence of Serum

Adult worms of S. mansoni were incubated with ^{35}(S)-methionine in the presence of serum. 2 membrane fractions were prepared: ^{35}(S)-frozen-thawed supernatant (^{35}S-FTS) and ^{35}(S)-membrane pellet (^{35}S-MP). The IEF spectra and their corresponding densitometric tracings indicate changes in the pattern compared with that obtained on radiolabelling in the absence of serum. (See 3.3.2.I for detail.)

Dotted lines indicate faint bands shown on gels by fluorography.

Inset: 5 times magnification of the radiolabelled peak at pH between 7.2-8.2. This peak appeared to be unique to the membrane fractions on IEF gels.


⊕
pH
4.3
4.6
4.9
5.0
5.2
5.4
5.8
6.0
7.0
8.6
(35S - FTS)
(35S - MP)
⊖


(35S - FTS)
(35S - MP)
8.6
6.0
5.2
7.2
5.8
4.9
pH

methionine under suitable culture conditions (3.3.2.1). 4 labelled fractions were prepared; ^{35}(S-FTS), ^{35}(S-MP), ^{35}(S-WHS) and ^{35}(S-WHP). Approximately 20,000 counts of TCA precipitable proteins from each fraction were focused on IEF gels containing 6 M urea. Figure 3.6 shows the IEF spectra of the 4 labelled fractions with major protein bands being sequentially numbered A_1 - A_{10} from pH 3.5-10.0. Densitometric tracing of the labelled bands was performed on Joyce Loebl microdensitometer (Model Mk 3) and shown in Figure 3.7. Comparison of the banding patterns of the 4 fractions as detected by fluorography (Figure 3.6) and densitometric tracing (Figure 3.7) showed the following characteristics: (a) 10 major protein components (A_1 - A_{10}) were obtained with each fraction. The relative distribution of radioactivity within all fractions appeared to be similar; (b) spectra of (^{35}S-MP) and (^{35}S-FTS) showed the presence of a minor component at pH 7.2-8.2. It is believed that this component represents the BP observed to be present in the same 2 fractions in stained gels (3.3.1). The (^{35}S-WHS) and (^{35}S-WHP) showed total lack of this component as revealed by both fluorography (Figure 3.6) and densitometric tracing (Figure 3.7); (c) a few quantitative variations between different fractions were clear. For instance, A_3 appeared to be more evident in both the WHS and WHP than in the FTS. Also, A_3 appeared as a minor component in the MP fraction compared with that in the other 3 fractions (FTS, WHS and WHP).

When the stained gel (Figure 3.2) is compared with the labelled gel (Figure 3.6), it can be seen that most of the 10 major components correspond to prominent coomassie blue stained bands. This result indicates that the majority of proteins in adult worms were labelled under the applied culture conditions. Here, it should be noted that the amount of BP detected in the labelled membrane fractions appeared to be much less than that observed in stained gels.

Figure 3.6. Isoelectric Focusing Spectra of Various Schistosome Fractions Extracted from Radiolabelled Adult Worms of S. mansoni

Radiolabelled fractions applied are (from left to right): ^{35}(S)-frozen-thawed supernatant (^{35}S-FTS), ^{35}(S)-worm homogenate supernatant (^{35}S-WHS), ^{35}(S)-membrane pellet (^{35}S-MP) and ^{35}(S)-worm homogenate pellet (^{35}S-WHP). The radiolabelled proteins were detected by fluorography (2.10.2).

A_1-A_{10} indicate the major protein bands shown on IEF gel. Dotted lines indicate faint bands shown on IEF gel by fluorography. The bold arrow indicates the radiolabelled basic protein bands (BP) at pH between 7.2-8.2 present in both (^{35}S-FTS) and (^{35}S-MP) but absent in (^{35}S-WHS) and (^{35}S-WHP). Insets at bottom indicate over-exposed fluorograms of the same region of the gel (pH 7.2-8.2) of the 4 radiolabelled fractions to show the presence of BP in (^{35}S-FTS) and (^{35}S-MP) which could not be revealed clearly on the under-exposed gels shown in the figure.

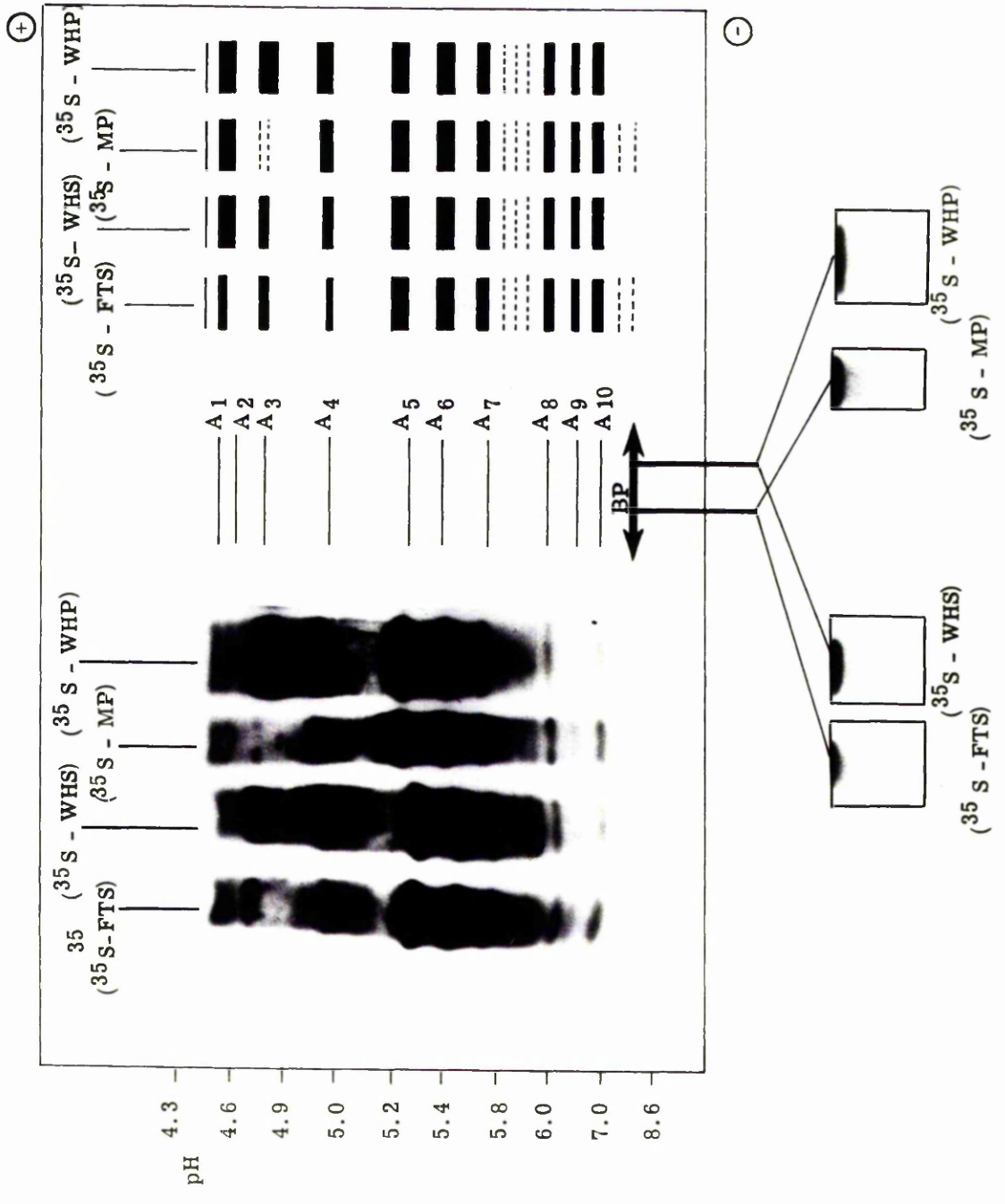
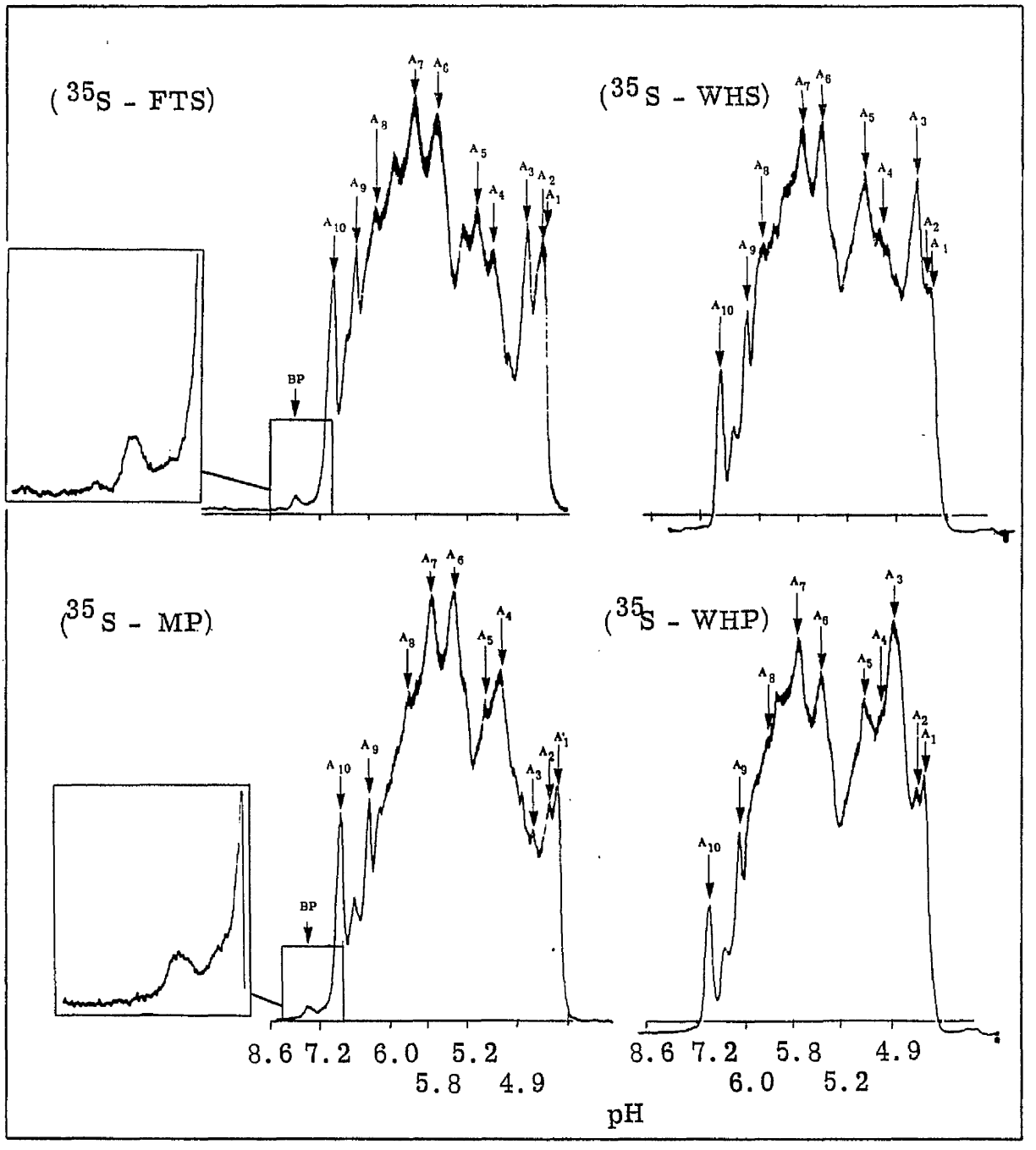

⊕
⊖
35 (35S-FTS)
(35S - WHS)
(35S - MP)
(35S - WHP)
(35S - FTS)
(35S- WHS)
(35S - MP)
(35S - WHP)
A 1
A 2
A 3
A 4
A 5
A 6
A 7
A 8
A 9
A 10
BP
pH
4.3
4.6
4.9
5.0
5.2
5.4
5.8
6.0
7.0
8.6
(35S - FTS)
(35S - WHS)
(35S - MP)
(35S - WHP)

Figure 3.7. Densitometric Tracings of 4 Radiolabelled Schistosome Fractions Extracted from Adult Worms of S. mansoni

^{35}(S)-labelled schistosome fractions were focused in IEF gels. Focused radiolabelled bands were scanned on microdensitometer. The radiolabelled fractions used are: ^{35}(S)-frozen-thawed supernatant (^{35}S-FTS), ^{35}(S)-worm homogenate supernatant (^{35}S-WHS), ^{35}(S)-membrane pellet (^{35}S-MP) and ^{35}(S)-worm homogenate pellet (^{35}S-WHP). (See 3.3.2.2. for detail.)

A_1-A_{10} indicate major radiolabelled peaks in each fraction examined.

Inset: 5 times magnification of the minor peak (at pH 7.2-8.2) present in both (^{35}S-FTS) and (^{35}S-MP) fractions.


(^{35}S - FTS)
(^{35}S - WHS)
(^{35}S - MP)
(^{35}S - WHP)
BP
BP
8.6 7.2 6.0 5.2
5.8 4.9
8.6 7.2 5.8 4.9
6.0 5.2
pH

3.3.3. IEF Pattern of Labelled Schistosomula

Schistosomula represent the immature stage of the parasite which develop into adult worms in the vertebrate host. Having identified the IEF pattern of protein components expressed at the mature adult stage (3.3.2.2), it would be of importance to know whether such proteins are expressed at the schistosomular stage. For this reason, schistosomula were labelled with ^{35}(S)-methionine as described before (2.7.1). Since very small quantities of schistosomular surfaces could be extracted by freezing and thawing, it was necessary to homogenize labelled schistosomula to prepare 2 fractions, (^{35}S-SF) (^{35}S-soluble fraction) and (^{35}S-FP) (^{35}S-fast pellet). Hence, it should be emphasized that these 2 fractions are different from the 4 fractions extracted from adult worms and used throughout current experiments in terms of the method of preparation. (^{35}S-SF) and (^{35}S-FP) were focused on IEF gels and labelled proteins were detected by fluorography. Results are shown in Figure 3.8. 11 major protein components were resolved at pH 3.9-7.0 (Sc_1 - Sc_{11}) in addition to a minor component at pH 7.2-8.2 (BP). It can be seen that these 2 fractions differ in the relative quantities of labelled proteins at pH between 3.9-5.2 (Sc_1-Sc_5). However, no appreciable difference could be detected in the amount of BP (pH 7.2-8.2) present in both fractions. This finding is of interest, since it shows that this particular component is expressed at the schistosomular stage as well as the adult worm stage.

3.4. Discussion

In view of the importance of developing ways to define schistosome antigens, analysis of schistosome proteins by utilizing the technique of electrophoresis has been performed. Analysis included SDS and IEF polyacrylamide gel electrophoresis. At least 10 discrete

Figure 3.8. Isoelectric Focusing Spectra and the Corresponding Densitometric Tracings of 2 Radiolabelled Schistosome Fractions Extracted from Schistosomula of S. mansoni.

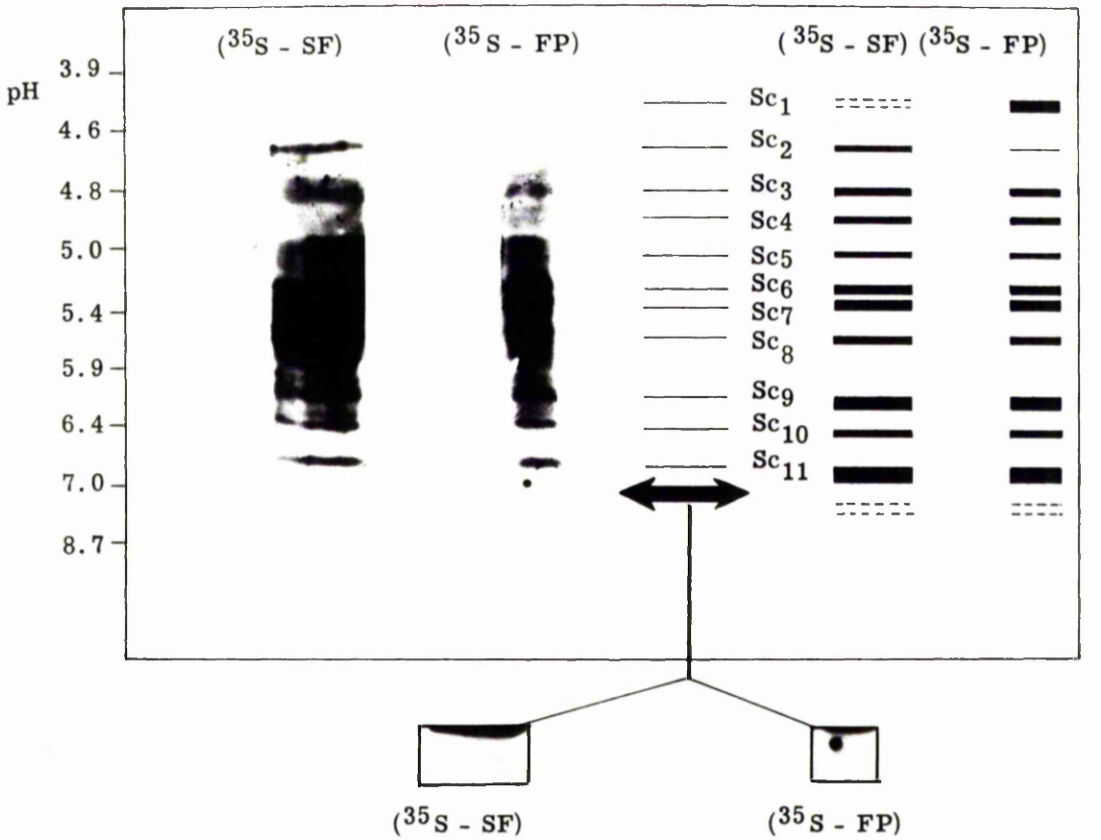
(upper) Schistosomula were radiolabelled with ^{35}(S)-methionine (2.7.I). 2 radiolabelled fractions were extracted then focused on IEF gels; ^{35}(S)-soluble fraction (^{35}S-SF) and ^{35}(S)-fast pellet (^{35}S-FP). Radiolabelled bands were detected by fluorography (2.10.2).

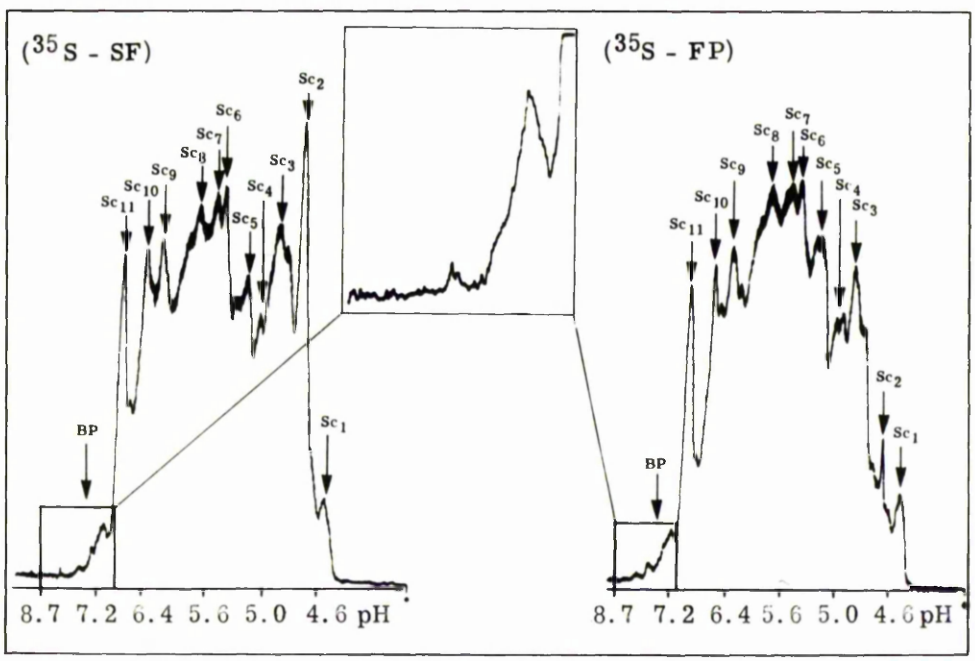
A_1-A_{10} indicate major radiolabelled protein bands focused at pH between 3.5-10.0.

Dotted lines indicate faint radiolabelled bands shown on gels by fluorography.

The bold arrow indicates radiolabelled basic protein bands at pH between 7.2-8.2 present in both fractions. Insets at bottom show over-exposed fluorograms of the basic region of the gel (pH 7.2-8.2).

(lower) Densitometric tracings of the two radiolabelled fractions shown above. Inset shows the densitometric tracing of the basic region of IEF gel (pH 7.2-8.2) with 5X magnification to clarify the presence of basic protein (BP) in both fractions.


(35S - SF)
(35S - FP)
(35S - SF)
(35S - FP)
pH
3.9
4.6
4.8
5.0
5.4
5.9
6.4
7.0
8.7
Sc1
Sc2
Sc3
Sc4
Sc5
Sc6
Sc7
Sc8
Sc9
Sc10
Sc11
(35S - SF)
(35S - FP)


(35S - SF)
(35S - FP)
Sc11
Sc10
Sc9
Sc8
Sc7
Sc6
Sc5
Sc4
Sc3
Sc2
Sc1
BP
8.7 7.2 6.4 5.6 5.0 4.6 pH
8.7 7.2 6.4 5.6 5.0 4.6 pH

protein components with molecular weight range of approximately 112,000-28,000 were identified by SDS-gel (Table 3.2). Many of these components were shared by the 3 fractions electrophoresed on gel, the MP, FTS, and WHP fractions (Figure 3.1). Some components, notably those of molecular weight 70,000, 60,000 and 28,000 appeared to correspond to labelled surface proteins obtained by Kusel and Mackenzie (1975). Several bands appeared to be unique to a given preparation. These include (a) a high molecular weight component (S_1) of 112,000 molecular weight present in the MP fraction only. It may be associated with the insoluble proteins of the surface membrane. While this protein (112,000 molecular weight) was detected in the MP fraction in the present study, Cordeiro and Gazzinelli (1979) reported the presence of a protein component with a similar molecular weight in the FTS fraction extracted from adult schistosomes. This discrepancy of the results might be due to differences in the methods of extracting schistosome fractions or in the gel system employed; (b) a protein component of 94,000 molecular weight present in both the FTS and WHP fractions but absent in the MP fraction; (c) the most noticable feature of the pattern obtained by SDS-gel is the presence of a prominent band with molecular weight of 60,000 in both the FTS and MP fractions, but absent in the WHP fraction. The presence of this component is of interest since these 2 fractions have been shown to contain membrane material by electron microscopic examination (Kusel, 1972; Ramalho-Pinto, Goldring, Smithers and Playfair, 1976). This result raised the possibility that there may be proteins present in the membrane which are not found in the rest of the worm, and therefore they are specific for the surface membrane.

In order to examine the above hypothesis, further analysis of the same adult worm fractions by IEF gel was performed. When the membrane fractions (MP and FTS) were analysed on IEF gel (Figure 3.2),

Table 3.2

A Comparison Between Molecular Weights of Surface Membrane Protein Components as Indicated by SDS-Gel Electrophoresis by Various Investigators

Molecular Weights (Daltons)				
(A) Present Thesis	(B) Ruppel (1978)	(C) Hayungaet al (1979)	(D) Kusel & Mackenzie (1975)	(E) Cordeiro & Gazzinelli (1979)
112	120	100	90	112
94	78	78	70	98
74	55	68	60	92
70	44	60	48	94
60	39	43	38	82
54	30	36	31	
46	25	30	28	
36	20	26	26	
34		21	18	
28				

Methods used for detecting surface membrane proteins:

A - Freezing and thawing, staining with coomassie blue stain.

B - Surface iodination with ^{125}I by the aid of lactoperoxidase.

C - Surface labelling in Bolton-Hunter reagent.

D - Internal double labelling with $^{3}(H)/^{14}(C)$-amino acids, freezing and thawing.

E - Freezing and thawing, staining with coomassie blue stain.

3 discrete bands at pH 7.2-8.2 were detected. These bands were termed the BP and appeared to be absent in both the WHS and WHP fractions. Radiolabelling of adult worms showed that BP in the MP and FTS fractions focused at the same region of the gel (pH 7.2-8.2) in both fractions. However, the amount of BP resolved in the labelled gels (Figure 3.6 and Figure 3.7) appeared to be less than that in the stained gels (Figure 3.2). There may be a greater rate of synthesis of membrane proteins in the normal environment (the blood stream of vertebrate host) than under the applied culture conditions. Alternatively, immunoglobulins which have been shown to be associated with schistosome tegument (Sogandares-Bernal, 1976; Kemp, Merritt and Rosier, 1978; Kemp, Brown, Merritt and Miller, 1980) may contribute to the intensity of the stained bands at that region of the gel (pH 7.2-8.2). However, this does not invalidate the finding that there is a protein component(s) specific to the surface membrane fractions synthesized by adult worms of S. mansoni.

On comparing the protein patterns of different adult worm fractions obtained by IEF gel electrophoresis, it was shown that apart from the presence of BP in the MP and FTS fractions, there was a high degree of similarity between the 4 fractions examined. This is surprising because of the great structural differences between these fractions. For instance, components from the gut and the muscular layers of the parasite should be extracted mainly in the WHS or WHP fractions rather than in the FTS or MP fractions. This may reflect similarity in the protein components of different structures of the parasite. A similar observation was reported by Ruppel (1978) working with the total extracts from different developmental stages of S. mansoni. However, the finding that protein components present in the MP fraction could also be resolved in the FTS fraction from adult worms appears to offer a more practical approach to the studies of the properties of the surface membrane of adult worms, since these are soluble membrane proteins.

It was demonstrated that there is a specific protein component(s) in the membrane fractions of adult worms of S. mansoni. This raised the question whether such protein is expressed at different developmental stages of the parasite. Since there is evidence that schistosomula represent the target for the host's immune response (see I.3.2.2), it would be of interest to detect the presence of BP at the schistosomular stage. IEF spectra of labelled schistosomula (Figure 3.8) confirmed the presence of BP in the schistosomular fractions electrophoresed on gels.

During the course of this investigation, attempts were made to establish the optimal culture conditions for labelling adult worms and schistosomula of S. mansoni. Radioactive methionine incorporation into the surfaces of adult worms and schistosomula incubated in vitro increased progressively up to 18 hours after incubation. On the other hand, the lack of serum in the incubation medium appeared to cause changes in the surface membrane of adult worms as shown on IEF gels (Figure 3.4). There was an increase in the number of bands resolved by the membrane fractions (MP and FTS) mainly in the FTS fraction. In addition, 2 major peaks at pH 7.2-8.2 were observed compared to minor peaks resolved by the same fractions extracted from adult worms labelled in the presence of serum (Figure 3.5). Ruppel (1978) reported that the amount of proteins released from labelled worms incubated in the absence of serum is 5 times greater than the corresponding amount released by labelled worms incubated in the presence of serum. He also indicated that the process of protein release could be interrupted immediately by the addition of serum to the incubation medium. Thus, the absence of serum in the incubation medium may lead to structural damage in the parasite surface membrane accompanied by an increase in the rate of membrane turnover to repair the damaged membrane (Simpson, Cesari and

Evans, 1980). This may explain changes in the IEF patterns of FTS and MP fractions obtained in the present work. Therefore, the IEF patterns may have included some internal proteins leaked out after the damage of the tegument as well as the surface membrane proteins. The suggestion of an increase in the membrane turnover may offer an explanation for the increase in the amount of BP (pH 7.2-8.2) which has been shown to be a membrane protein synthesized by the parasite. Tavares, Cordeiro, Mota-Santos and Gazzinelli (1980) indicated that the presence of serum in incubation medium stimulates the incorporation of labelled amino acids (^{3}H-arginine) in the proteins of the parasite tegument and also the acquisition of protection against cytotoxic effects of antibodies in the presence of complement. They suggested that schistosomula may acquire serum factor(s) which may stimulate the turnover rate of tegumental proteins. Although this result was interpreted as a mechanism of protection, it may well be another demonstration of the effect of serum on the synthesis of surface membrane proteins. However, the mechanism in which serum affects this metabolic process is not clear yet.

The present investigation has given evidence for the presence of a protein component(s) specific to the surface membrane fractions of adult worm and schistosomula. There are a number of unanswered questions about the structure and functions of schistosome surface membrane. First, living adult worms stimulate the immune response against young schistosomula of a challenge infection (Smithers and Terry, 1969b). Adult worms and schistosomula share several antigenic determinants on their surface membranes (Kusel, Sher, Perez, Clegg and Smithers, 1975). Proteins which are antigenically identical to the surface membrane proteins are released from adult worms into culture medium. The release of these proteins was considered to represent the phenomenon of membrane turnover in schistosomes (Kusel, Sher, Perez, Clegg and Smithers, 1975).

This phenomenon may occur in vivo and may be the way in which (a) the immune response against young schistosomula is stimulated (Kusel, Sher, Perez, Clegg and Smithers, 1975) and/or (b) the immune response against adult worms is evaded (Wilson and Barnes, 1974a, 1977). However, there is no evidence to confirm these 2 suggestions. Demonstration of the presence of a component(s) specific to the surface membrane fraction may give such evidence. The use of this component(s) as a specific marker for the surface membrane proteins may help in understanding some metabolic processes associated with the surface membrane and may demonstrate its significance in immunity. Second, it has been shown that the membrane fractions from schistosome tegument possess haem-agglutinating activity (Cesari, 1976). This activity was found to be due to some acidic phospholipid components present in schistosome tegument (Cesari and Marchiani, 1978). Also, it was found that a haemagglutinin inhibitory component occurs in the tegumental fraction of adult worms (Cesari and Marchiani, 1977). It was suggested that the inhibitory component might be associated with the haemagglutinating phospholipids and thus inhibit their activities in adult worms. No further analysis of this component has been reported yet. The BP presented in our study is specific to the membrane fractions of adult worms and is positively charged (pH 7.2-8.2). Thus, it is conceivable that it might represent the inhibitory component suggested in the above mentioned studies. Also, histochemical studies have indicated that the surface membranes of adult worms and schistosomula are electronegatively charged (Stein and Lumsden, 1973). Then, it is probable that the presence of BP in the membrane fractions may contribute to the integrity of schistosome tegument by associating with the acidic phospholipids demonstrated by Cesari and Marchiani (1978) and thus providing a frame-work more suitable for the survival of the parasite in the host environ-

ment. Nevertheless, no evidence was found in the present work to support either of the above hypotheses. Clearly, more information will be required to reconcile these hypotheses.

CHAPTER IV

A STUDY OF SOME CHARACTERISTICS OF THE FROZEN-THAWED SUPERNATANT FRACTION EXTRACTED FROM ADULT WORMS OF *S. MANSONI*

4. A STUDY OF SOME CHARACTERISTICS OF THE FROZEN-THAWED SUPERNATANT FRACTION EXTRACTED FROM ADULT WORMS OF S. MANSONI

4.I. Introduction

Several studies have indicated the importance of the surface membrane antigens of S. mansoni. The schistosome surface membrane may present a potential source of released antigenic material (Kusel, Sher, Perez, Clegg and Smithers, 1975). It may also act as a target for the host's immune attack (Smithers, McLaren and Ramalho-Pinto, 1977), or it may protect the parasite against the host's immune system (Clegg and Smithers, 1972). (See I.5.I. for detail).

The surface membrane of S. mansoni can be isolated by freezing and thawing of adult worms (Kusel, 1972). The supernatant obtained after removing the membrane pellet is termed the frozen-thawed supernatant fraction (FTS). In the present investigation the FTS fraction was used to study the surface membrane proteins of adult worms of S. mansoni. There is evidence that this fraction contains soluble membrane proteins. Electron microscopic examination of the pellet obtained by ultracentrifugation of the FTS fraction showed the presence of the membraneous bodies and multilaminate vesicles which are the major cytoplasmic inclusions of schistosome tegument. Further freezing and thawing of the FTS fraction resulted in the formation of aggregated material which contained membrane-like structures as revealed by ultrastructural examination (Ramalho-Pinto, Goldring, Smithers and Playfair, 1976). Immunodiffusion analysis of culture antigens (material released from adult worms into the culture medium), membrane antigens (membrane material extracted by 3 M KCl solution) and FTS antigens (frozen-thawed supernatant) showed that these 3 preparations have common antigens which can be recognized by antibodies in antisera from schistosome infected mice

or from rabbits immunized against culture antigens. These observations suggested that the FTS fraction contains soluble membrane antigens which might be released into culture media (Murrell, Vannier and Ahmed, 1974). A finding reported in this thesis indicated that the membrane pellet fraction (MP fraction) and the FTS fraction gave similar patterns on SDS-gels. Moreover, a protein component(s) present in the MP fraction could also be resolved in the FTS fraction on IEF gels. This component was termed the basic protein (BP), and considered as a specific protein of the surface membrane of the parasite.

This chapter is presented in 2 parts: the first deals with the definition of antigens in the FTS fraction extracted from adult worms (4.2-4.4), the second describes the attempted isolation and characterization of BP obtained from this particular fraction (4.5- 4.6).

4.2. Electrophoretic Analysis of Frozen-Thawed Supernatant Fractions (FTS) in Thin-Layer Polyacrylamide Gels (SDS and IEF Gels)

FTS fractions extracted from adult worms of S. mansoni were electrophoresed on SDS thin-layer polyacrylamide gels. At least 28 bands were identified with 10 major bands of molecular weights ranging between 28,000 - 112,000 (Table 3.I). Both the FTS fraction and the MP fraction showed similar patterns on SDS-gels, with a prominent band of 60,000 molecular weight. Isoelectric focusing of these 2 fractions (MP and FTS fractions) showed a minor component focused at pH between 7.2-8.2 (See 3.2 and 3.3 for detail).

4.3. Recognition of Frozen-Thawed Supernatant Antigens by Antibodies in Mouse and Rabbit Sera

4.3.1. Double Immunodiffusion Analysis (Ouchterlony Plates), Cross-over Immunoelectrophoresis (CIE) and Immunoelectrophoresis (IEP)

Immunodiffusion analysis of the FTS fraction and other 2

fractions extracted from adult worms (the worm homogenate supernatant WHS, and the soluble fraction SF) against antisera from schistosome infected mice and rabbit anti-schistosome antisera is shown in Figure 4.1.C. Each of these antigen preparations formed precipitin lines by Ouchterlony analysis when tested against antisera either from artificially immunized rabbits or from mice infected with S. mansoni. Comparison between sera from rabbit and mouse can be made on the basis of the relative numbers of precipitin lines formed. The largest number of precipitin lines was noted when different antigen preparations (WHS, SF, FTS) were tested against antisera from artificially immunized rabbits. Precipitin lines against these antigen preparations appeared to be fewer in sera from schistosome infected mice (BALB/c strain). No reactions were seen in sera from infected mice collected at early stages of infection (10, 20, 40 days), while only 1-2 precipitin lines were detected with sera collected at late stages of infection (60, 80, 120 days).

Cross-over immunoelectrophoretic analysis of the FTS fraction developed with the same serum pools (rabbit anti-schistosome antiserum and antisera from schistosome infected mice) showed at least 6 discernable precipitin lines with rabbit antiserum and 3 precipitin lines with mouse sera collected at late stages of infection (80, 120 days) (Figure 4.1.A).

Immunoelectrophoresis of the FTS fraction with rabbit antiserum raised specifically against the membrane fraction and the FTS fraction respectively, revealed at least 6 precipitin arcs. The electrophoretic mobilities of the reacting antigens appeared to be distinctly towards the anodic pole of the gel (Figure 4.1.B). This result may indicate that most of the reacting components have acidic groups.

Results obtained by immunodiffusion analysis and immunoelectro-

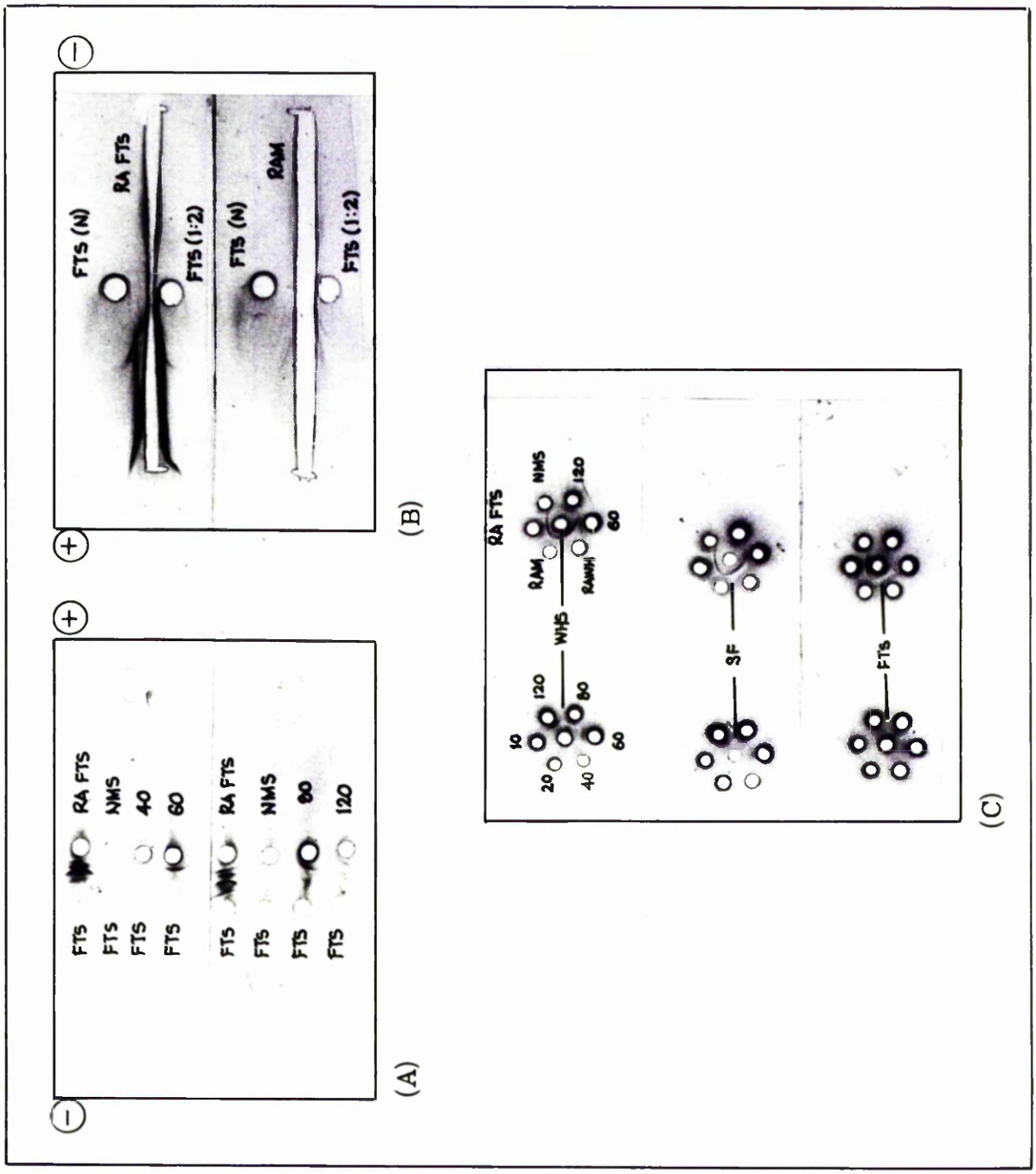
Figure 4.I. Immunoelectrophoresis and Immunodiffusion Analysis of Various Schistosome Antigen Preparations Against Rabbit and Mouse Anti-Schistosome Antisera

(A) Cross-over immunoelectrophoresis of the frozen-thawed supernatant fraction (FTS) against: rabbit anti-frozen thawed supernatant antisera (RAFTS), normal mouse serum as a control (NMS), and antisera from schistosome infected BALB/c mice collected at different times after infection (40, 60, 80, 120 days after infection). Precipitin lines developed with rabbit antisera and also with each of 60, 80 and 120 days infected mouse sera.

(B) Immunoelectrophoresis of the frozen-thawed supernatant fraction (FTS) against rabbit antisera specifically raised in rabbit against frozen-thawed supernatant (RAFTS) and against schistosome membrane pellet (RAM). 6 precipitin arcs developed in each case.

N = Neat, 1:2 = Preparation diluted 1:2.

(C) Immunodiffusion analysis of worm homogenate supernatant (WHS), soluble fraction (SF) and frozen-thawed supernatant (FTS) prepared from adult schistosomes against: antisera from schistosome infected BALB/c mice (10, 20, 40, 60, 80, 120 days after infection). Also against rabbit anti-frozen-thawed supernatant (RAFTS), rabbit anti-surface membrane (RAM), rabbit anti-worm homogenate supernatant (RAWH). Large number of precipitin lines developed with rabbit antisera compared to mouse sera.


(A)
(B)
(C)
FTS
RA FTS
NMS
40
80
120
FTS (N)
FTS (1:2)
RAM
10
20
40
60
SF

phoresis of different schistosome preparations including the FTS fraction provided another demonstration of the heterogeneity of the extracts composition. However, these results gave an indication for the presence of antibodies in rabbit and mouse sera against components in the FTS fraction.

With 3 schistosome preparations from adult worms, the FTS, SF and WHS fractions, and a preparation of egg antigen (EA), Ouchterlony analysis demonstrated that antibodies in pooled sera collected from 3 different strains of mice infected with S. mansoni were directed against several antigens in each preparation (Figure 4.2). Differences were detected in both the time of appearance of the antibody response and the number of precipitin lines which could be seen. There were also strain differences in antibody response to the antigen preparations. Further work was carried out to identify such strain differences in schistosome infected mice with respect to other responses to infection, including: body weight changes, worm burden, splenomegaly and distribution of eggs in tissues (See Appendix for detail).

4.3.2. Coprecipitation Test

From the above results, it is clear that immune mice (120 days infection) had detectable levels of antibody that recognized some antigens in the FTS fraction. In order to assess the amount of these antigens which had complexed with antibodies in immune mouse serum, the coprecipitation technique of Kusel, Sher, Perez, Clegg and Smithers (1975) has been used (2.9.3). Radiolabelled FTS fractions were extracted from adult worms which have been internally labelled with ^{35}(S)-methionine, or (^{3}H)-leucine or ^{14}(C)-amino acids (2.7.2). Thus, 3 radiolabelled FTS fractions were used in this experiment: (^{35}S-FTS), (^{3}H-FTS) or (^{14}C-FTS). Known amounts of TCA precipitable counts from

Figure 4.2. Immunodiffusion Analysis of 4 Different Schistosome Antigen Preparations Against Mouse Sera Collected After Different Periods of Infection with S. mansoni .

FTS = Frozen-thawed supernatant

SF = Soluble fraction

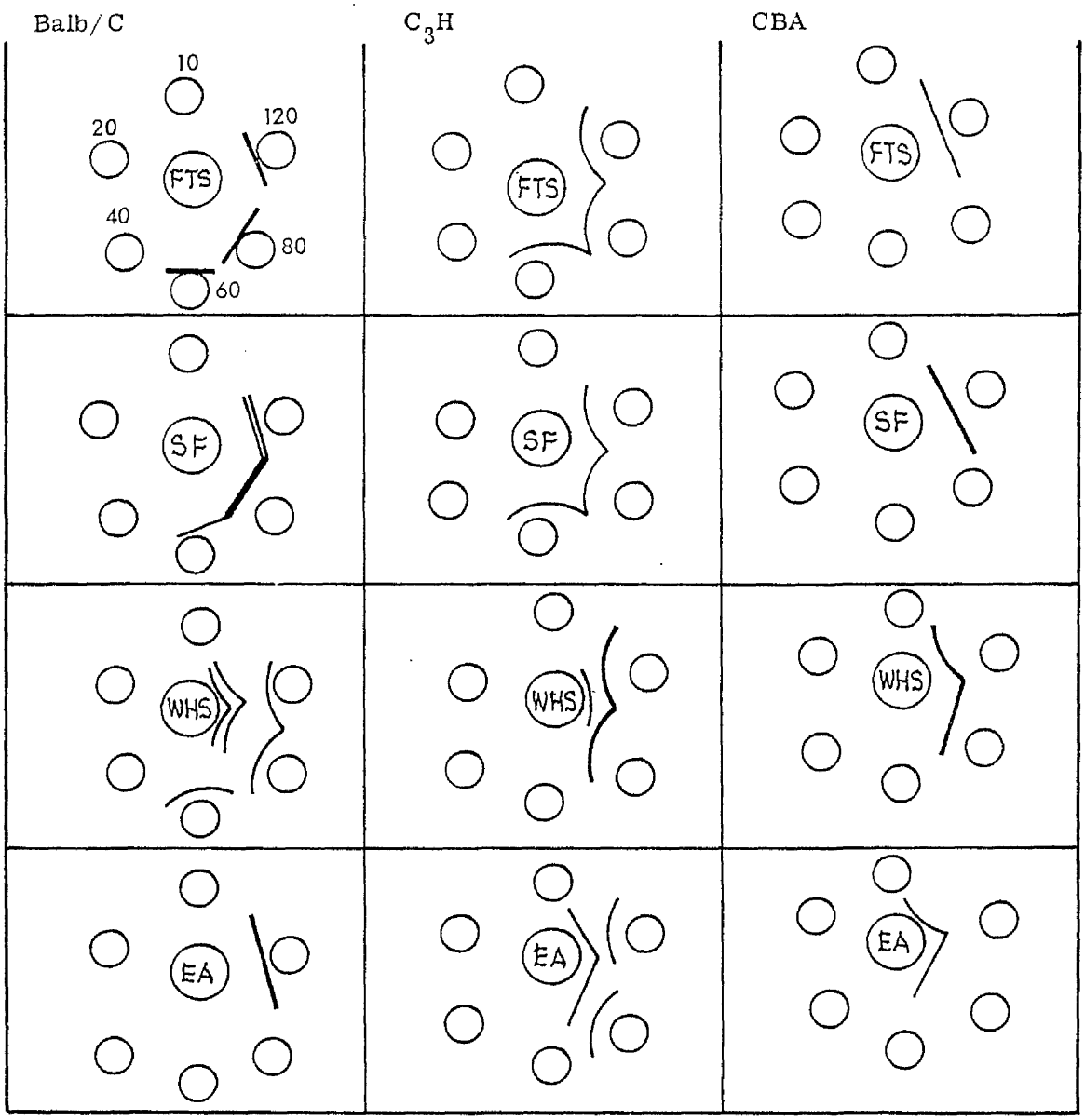
WHS = Worm homogenate supernatant

EA = Egg antigen

10, 20, 40, 60, 80, 120 = Days after infection with S. mansoni

BALB/c, C_3H, CBA = Strains of mice used as a source of antisera.

Differences could be detected in: the time of appearance of the antibody response and the number of precipitin lines developed.


Balb/C
C_3H
CBA
10
20
120
40
80
60
FTS
SF
WHS
EA

each of these 3 fractions were incubated with normal mouse serum (control) or immune mouse serum (experimental) obtained from schistosome infected BALB/c mice (120 days). The antigen-antibody complexes were coprecipitated with rabbit anti-mouse immunoglobulins antiserum (RAMIg antiserum). The coprecipitates were counted for radioactivity. Results are shown in Table 4.I. It can be seen that although appreciable quantities of radioactive antigens were coprecipitated in both (^{35}S-FTS) and (^{3}H-FTS), there was insignificant difference between the labelled material coprecipitated with both normal and immune mouse serum, whereas with (^{14}C-FTS), a significant amount of radioactive antigens was specifically coprecipitated. The results were consistent in 2 sets of experiments, each repeated twice with duplicate samples (Table 4.I).

One interpretation of this result was that components from mouse serum (mainly immunoglobulins) usually associated with schistosome tegument (Sogandares-Bernal, 1976; Kemp, Merritt and Rosier, 1978) might compete with the antigen-antibody complex (^{35}S-labelled antigens and specific antibodies) for the binding sites of RAMIg. Indeed, immunodiffusion analysis revealed precipitin lines between RAMIg and the FTS fraction. However, substituting RAMIg for the protein A (Staphylococcus aureus, Cowan I strain) did not improve the results. Furthermore, the use of a highly specific rabbit anti-mouse IgG (rabbit anti-myeloma protein) which showed no precipitin lines with the FTS fraction and which gave a single distinct line with mouse IgG gave similar results to those shown in Table 4.I.

No clear conclusion could be drawn from the data presented above. However, it would be of interest if the coprecipitated material in both experimental and control groups was electrophoresed on SDS-gels for comparison with the radiolabelled FTS fractions, i.e.

Table 4.I

Coprecipitation of Radiolabelled Schistosome Antigens (FTS Fraction) Using Immune Mouse Serum (120 Days Infection)

	Antigen	Percentage of counts precipitated(± S.D.)	Number of sera tested	P*
E	a. (^{35}S-FTS)	23.22 ± 1.6	4	> 0.1 N.S.
C		14.95 ± 2.8	4	
E		44.75 ± 1.4	4	> 0.1 N.S.
C		32.55 ± 4.2	4	
E	b. (^{3}H-FTS)	36.82 ± 2.0	4	< 0.5 N.S.
C		16.0 ± 0.8	4	
E		47.17 ± 2.0	4	< 0.5 N.S.
C		28.15 ± 2.0	4	
E	c. (^{14}C-FTS)	75.47 ± 1.6	4	< 0.05
C		25.55 ± 3.3	4	

E = Experimental

C = Control

Total No. of counts used

a. 3400 cpm

b. 2600 cpm

c. 1400 cpm

*Values obtained from student's t-test. Differences between control and experimental groups not significant at the $P < 0.05$ are designated N.S.

specific antigens in (^{35}S-FTS) recognized by antibodies in immune mouse or rabbit sera would be identified according to their molecular weights.

4.4. Definition of Contaminants in the FTS Fraction

(i) Immunoglobulin classes and subclasses

Sogandares-Bernal (1976) indicated the presence of immunoglobulins of mouse origin associated with the tegument of S. mansoni. He suggested that the presence of these immunoglobulins may protect the parasite by blocking other surface antigenic sites. This finding was further confirmed and extended by Kemp, Merritt and Rosier (1978) and Kemp, Brown, Merritt and Miller (1980). Using the immunofluorescence technique, they detected the presence of the following classes and subclasses of immunoglobulins on the tegument of adult worms grown in mice; IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA and IgM.

In view of the above cited evidence, it was decided to examine the immunoglobulin classes and subclasses that might be present in the FTS fraction, since it represents the tegumental extract of adult worms. Immunodiffusion analysis and immunoelectrophoresis were used to detect the presence of IgM, IgA, IgG_{2a}, IgG_{2b} in the FTS fraction. Results are shown in Figure 4.3.A,B. No IgM was detected. But all other classes and subclasses of immunoglobulins tested for were found to be present in the FTS fraction. IgA, IgG_{2a}, IgG_{2b} were revealed by both immunoelectrophoresis and Ouchterlony analysis.

Attempts to remove immunoglobulins from the FTS fraction by treating this fraction with conjugated rabbit anti-mouse Ig and Staphylococcus aureus Cowan I strain (protein A) were undertaken. For the preparation of the conjugate, an appropriate volume of RAMIg was mixed with S. aureus Cowan I strain. The mixture was stirred gently for 1 hour at 37°C, then washed 4 times in PBS by centrifugation at

Figure 4.3. Demonstration of the Presence of Mouse Immunoglobulins and the Absence of Schistosome Egg Antigen in the Frozen-Thawed Supernatant Fraction

(A) Immunodiffusion analysis of frozen-thawed supernatant (FTS) against rabbit anti-mouse IgA (RAIgA), rabbit anti-mouse IgM (RAIgM), rabbit anti-mouse IgG_{2a} ($RAIgG_{2a}$) and rabbit anti-mouse IgG_{2b} ($RAIgG_{2b}$). 3 different dilutions of FTS were used: neat (N), 1:2 and 1:4.

NMS = Normal mouse serum (control)

NRS = Normal rabbit serum (control)

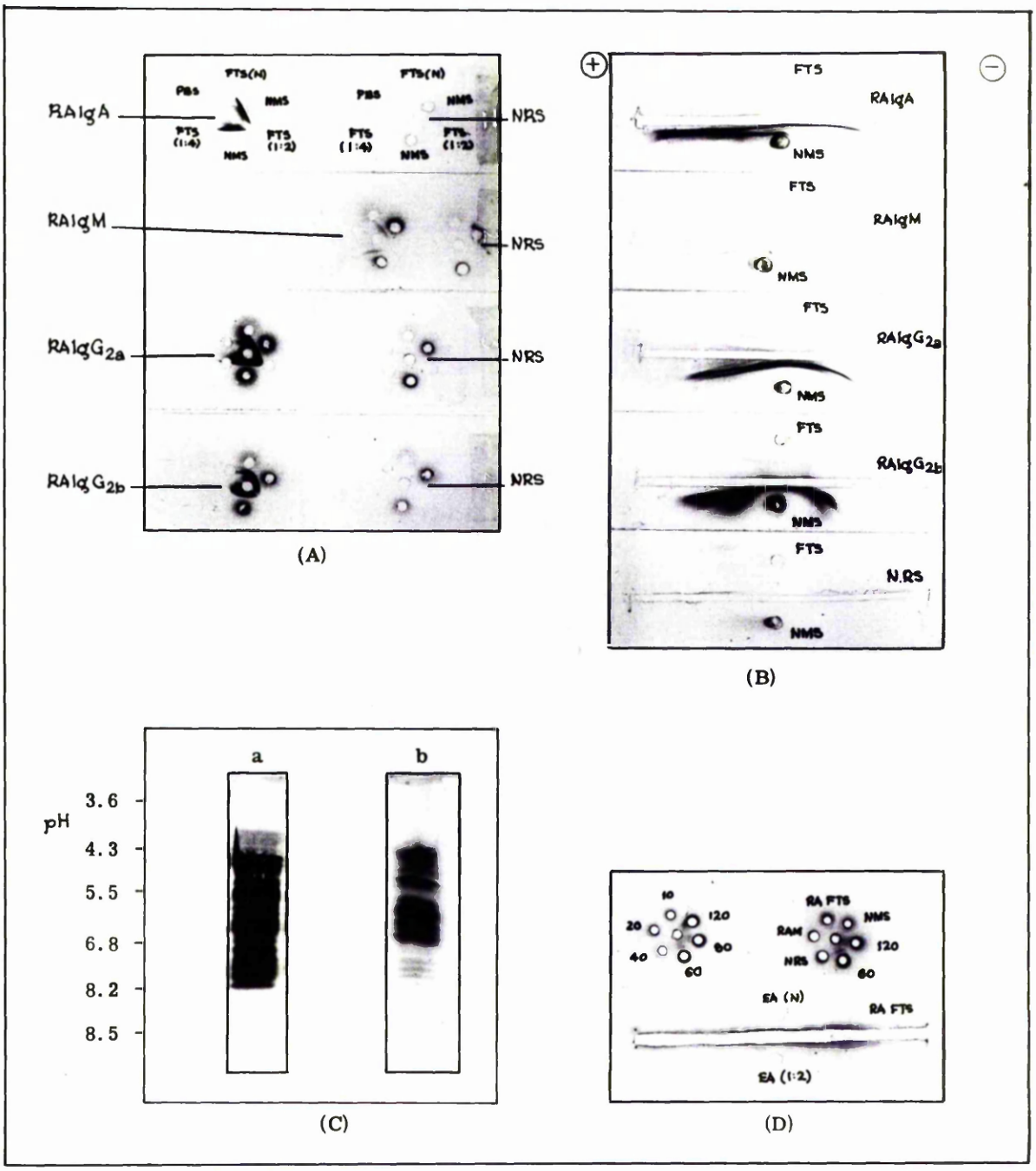
PBS = Phosphate buffered saline (control).

Precipitin lines developed with RAIgA, $RAIgG_{2a}$, $RAIgG_{2b}$.

(B) Immunoelectrophoresis (IEP) of the FTS fraction against the same antisera described in (A). Results are similar to (A).

(C) Isolectric focusing pattern of the FTS fraction before (a) and after (b) the removal of mouse immunoglobulins. Most bands became faintly stained in (b).

(D) Immunodiffusion analysis and IEP of egg antigen against antisera from schistosome infected mice (10, 20, 40, 60, 80, 120 days after infection) and against rabbit antisera specifically raised against schistosome fractions. Abbreviations are the same as those used in (A).


RAIgA
RAIgM
RAIgG2a
RAIgG2b
NRS
NRS
NRS
NRS
(A)
FTS
RAIgA
NMS
FTS
RAIgM
NMS
FTS
RAIgG2a
NMS
FTS
RAIgG2b
NMS
FTS
NRS
NMS
(B)
a
b
pH
3.6
4.3
5.5
6.8
8.2
8.5
(C)
RA FTS
NMS
RAM
NRS
120
60
EA (N)
RA FTS
EA (1:2)
(D)

about 300 g for 5 minutes. The final supernatant was tested for cross-reaction with sheep anti-rabbit immunoglobulins using sheep anti-rabbit IgG and sheep anti-rabbit normal serum. The pellet (the conjugated antiserum with bacteria) was resuspended to 10% in PBS, then packed in a column and washed thoroughly to assure total removal of free immuno-globulins. The FTS fraction was passed slowly through the column. The collected fraction was tested by immunodiffusion analysis against different classes of immunoglobulins. Assay results were consistently negative and therefore were not shown.

Having assured the removal of immunoglobulins from the FTS fraction, it would be of interest to obtain the IEF pattern of such fraction for comparison with the corresponding pattern shown before passage through the column. The IEF patterns of the FTS fraction with and without immunoglobulins are shown in Figure 4.3.C. It gives clear indication that the relative intensities of most protein bands became less evident in the FTS fraction treated with the conjugated RAMIg—S. aureus. In particular, protein bands at pH 7.0-8.2 (BP). This result indicated that (a) most of immunoglobulins detected in the FTS fraction before passage through the column were removed, and (b) protein bands at pH 7.2-8.2 (BP) were partially derived from the host.

(ii) Egg antigen

Immunodiffusion analysis and cross-over immunoelectrophoresis of the FTS fraction against mouse sera collected from schistosome infected mice at different times of infection showed the appearance of precipitin lines only with 60-120 days sera (4.3.I). There is evidence that antibodies against egg antigen could be detected in the mouse circulation at this time after infection, i.e. 60-120 days (Pelley, Pelley, Hamburger, Peters and Warren, 1976). Thus, it is possible that

precipitin lines detected above may be due to the presence of contaminants of egg antigen in the FTS fraction, or the FTS fraction may contain antigens that cross-react with antibodies against egg antigen. To examine these possibilities a crude preparation of egg antigen was prepared by the method described by Boros and Warren (1970). Eggs were obtained from the intestines of schistosome infected mice. Crude egg antigen (EA) was prepared by homogenization and centrifugation, the supernatant was collected, concentrated and used in current experiments. Egg antigen was tested by immunodiffusion analysis and immunoelectrophoresis against rabbit antisera specifically raised against the FTS fraction and the membrane fraction (RAFTS and RAM respectively). Results are shown in Figure 4.3.D. No precipitin lines were obtained with either of the antisera tested. However, a diffused and intense precipitin line was noted on testing EA against infected and immune mouse sera (60, 80 and 120 days).

4.5. Isolation of Basic Protein (BP) from the FTS Fraction Using Ion-Exchange Chromatography

It has been demonstrated that there is a specific component(s) in both the FTS and MP fractions extracted from adult worms and schistosomula of *S. mansoni* (Chapter 3). The isoelectric points of this component(s) was found to be between pH 7.2-8.2 as shown by isoelectric focusing in thin-layer polyacrylamide gel (3.3).

Attempts were made to isolate this component(s) by fractionation of the FTS fraction using a strong basic anion exchanger. QAE-sephadex was used for this purpose. QAE-sephadex column was equilibrated in Tris-HCl buffer 0.1M (pH 6.5). 20 mg of the FTS fraction (after being treated with conjugated RAMIg - *S. aureus*) was applied to 2.0 x 10.0 cm QAE-sephadex column. Material was eluted with Tris-HCl

buffer (pH 6.5) at room temperature. 5 ml fractions were collected. Based on the absorbance at 280 nm, fractions 1-10 were pooled (Figure 4.4.A), concentrated and assayed for protein activity and protein banding patterns on SDS and IEF gels. The total amount of protein yielded was about 2.0 mg/ml, i.e. about 10% of the total amount applied to the column. Attempts to elute the material binding to the column by lowering the pH to 3.5 have failed. Also application of known amounts of radiolabelled FTS fraction to the column has met with limited success.

4.6. Characterization of Material Eluted from QAE-Sephadex Column (BP)

(i) Immunodiffusion analysis and immunoelectrophoresis

The antigenic activity of material eluted from the QAE-sephadex column was tested by Ouchterlony analysis and immunoelectrophoresis (Figure 4.4.A). Immunoelectrophoresis revealed a single arc in the middle of the gel. Whereas, immunodiffusion analysis showed an intense precipitin line which formed a line of identity with the FTS fraction when both were tested against RAFTS antiserum.

(ii) SDS-gel electrophoresis

SDS polyacrylamide gel electrophoresis of the eluted peak revealed about 20 minor components with one major component of 60,000 molecular weight (Figure 4.4.C). Demonstration of such large number of bands reflects limitation of the separation method used.

(iii) IEF gel electrophoresis

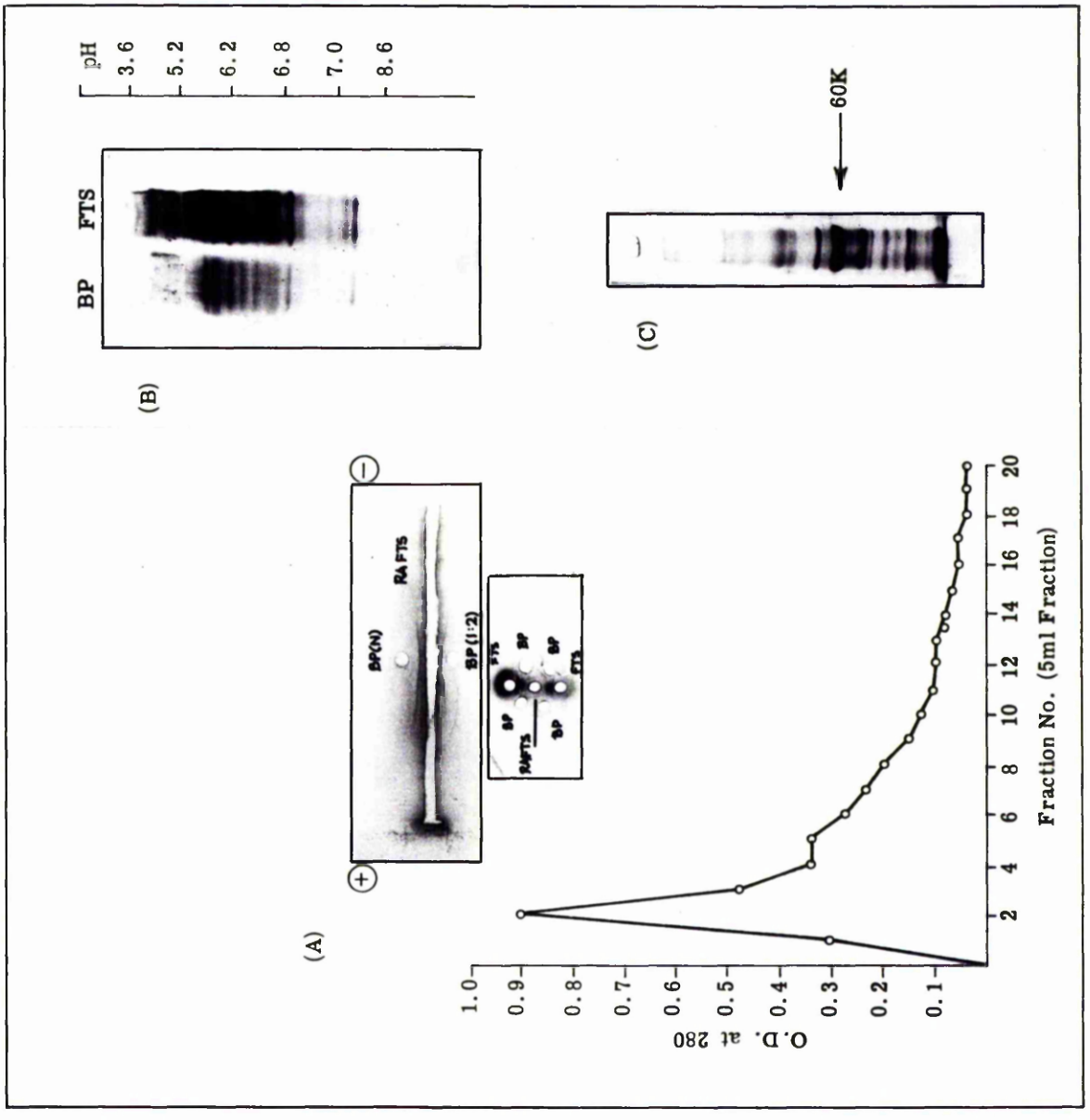
The peak material was subjected to IEF in thin-layer polyacrylamide gel containing 6 M urea. The IEF pattern showed the presence of protein bands at pH 7.2-8.2 (BP), but was significantly contaminated with other protein components of the FTS fraction. Most

Figure 4.4. Fractionation of the Frozen-Thawed Supernatant Fraction by Ion-Exchange Chromatography and Characterization of the Eluted Peak Proteins

(A) 20 mg of the frozen-thawed supernatant fraction was applied to QAE-sephadex column. 5 ml fractions were collected. This is a profile of the protein peak eluted from the column. (See 4.5 for detail). The top insets demonstrate analysis of antigenic activity of the eluted peak proteins by IEP and Ouchterlony analysis. Precipitin lines developed on testing the eluted peak proteins (BP) against rabbit anti-frozen thawed supernatant antiserum (RAFTS). Two dilutions of peak proteins were used; neat (N) and 1:2.

(B) Isoelectric focusing patterns of the eluted peak proteins (BP) and the frozen-thawed supernatant (FTS) on polyacrylamide gel. Most of protein bands could be seen in both preparations. (See 4.6 for detail).

(C) SDS-gel pattern of the eluted peak proteins electrophoresed in 7.5% polyacrylamide gel. Large number of protein bands could be seen with a prominent band with 60,000 molecular weight. (See 4.6 for detail).


(A)
(+)
(−)
BP(N)
RA FTS
BP(1:2)
RAFTS
BP
FTS
O.D. at 280
Fraction No. (5ml Fraction)
(B)
BP
FTS
pH
3.6
5.2
6.2
6.8
7.0
8.6
(C)
60K

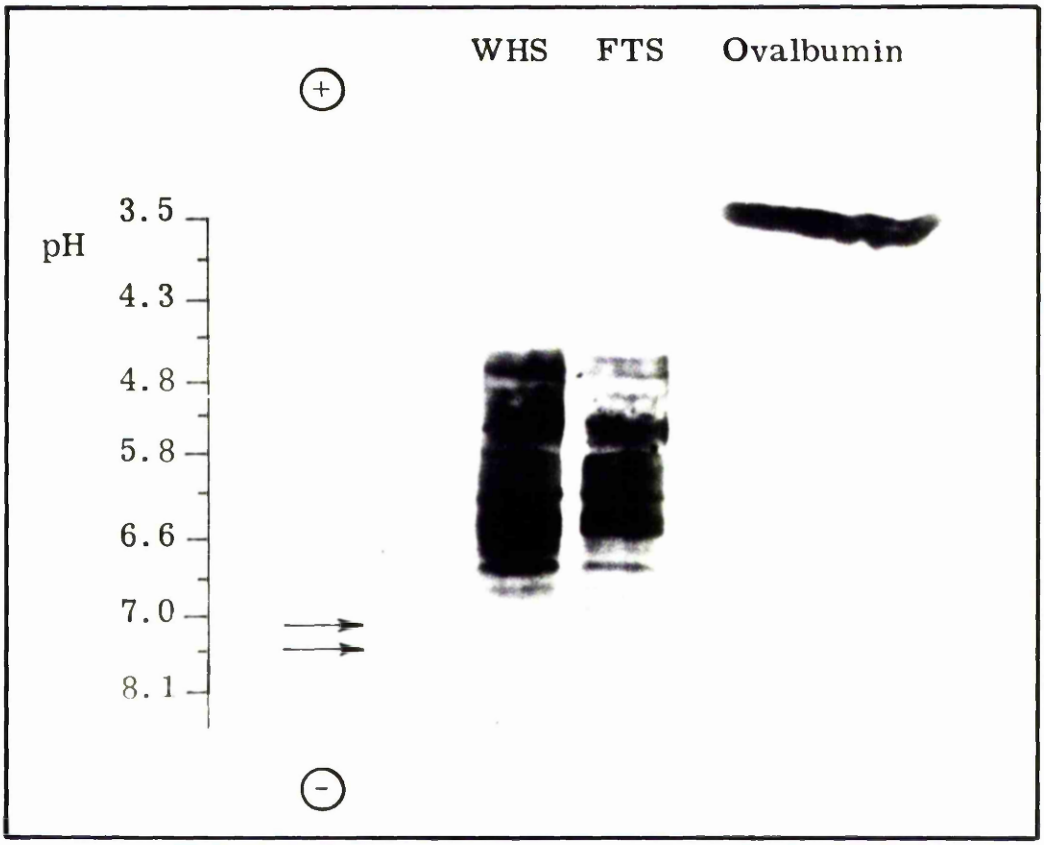
of the bands at pH between 3.9-7.0 have not been removed by the column separation method. This experiment basically confirmed the results obtained on SDS gels in terms of the limitation of the column method for isolating BP from the other components of the FTS fraction. Nevertheless, the presence of protein bands at pH 7.2-8.2 indicated the presence of BP in the eluted material (Figure 4.4.B).

(iv) Chemical nature of BP

Although the specific membrane component(s) with isoelectric points at pH 7.2-8.2 was termed basic protein (BP) and stained distinctly with coomassie blue stain, it was considered possible that carbohydrate groups may be associated with this protein. An attempt was made to investigate this assumption by using the lectin-overlay technique of Tanner and Anstee (1976) (2.9.4). Because of the small quantity of material that could be eluted from the QAE-sephadex column, the FTS fraction was used in this experiment. Non-labelled FTS fraction and WHS fraction (as reference fraction) were focused in IEF gel. The focused bands were overlayed by ^{125}I-Con A. After appropriate incubation and washing (2.9.4), the labelled bands were tested by fluorography (2.10.2). Results are shown in Figure 4.5. No bands were detected at pH 7.2-8.2 in the FTS fraction, therefore, these bands are not detectably glycoprotein in nature. But it should be emphasized that the failure to demonstrate binding at pH 7.2-8.2 may reflect low sensitivity of the method rather than the absence of glycoproteins at that region of the gel. It was of interest to note that most of the bands at pH between 4.3-6.8 showed binding to the labelled lectin. This might indicate that (a) glycoproteins may constitute the major components of the surface membrane of the parasite. Cordeiro and Gazzinelli (1979) reported a similar finding, or (b) immunoglobulins of host origin which have been shown to be present in the FTS fraction

Figure 4.5. A Fluorogram of 2 Schistosome Fractions Focused on IEF Gels and Overlayed by ^{125}I-Con A

2 non-labelled schistosome fractions; the frozen-thawed supernatant (FTS) and worm homogenate supernatant (WHS) and ovalbumin (as a control) were focused on IEF gels. The focused bands were overlayed by ^{125}I-Con A. Binding to the radiolabelled lectin could be seen at pH between 4.3 - 6.8. Arrows indicate the absence of binding at pH between 7.2 - 8.2.


WHS
FTS
Ovalbumin
+
pH
3.5
4.3
4.8
5.8
6.6
7.0
8.1
-

may have contributed to the lectin binding shown on IEF gel. However, the use of the FTS fraction with no immunoglobulins (after being treated with RAMIg - S. aureus), and mouse immunoglobulins (as control) in such experiment would have clarified this point.

(v) Presence of proteins eluted from the column on the schistosomular surface membrane

Despite the impure nature of BP eluted from the column (as shown by IEF and SDS gel electrophoresis), immunization of rabbits with this material was undertaken as described previously (2.8.2). Rabbit anti-basic protein (RABP) antiserum was prepared in order to determine the presence or absence of its respective antigens on the parasite's surface membrane. Schistosomula prepared by mechanical transformation (2.7.I.I) were incubated with RAWH antiserum (positive control), RAFTS antiserum (reference antiserum), RABP (experimental antiserum) or NRS (negative control) for 60 minutes at 37°C. After 2 washes in Eagle's medium (Table 2.I), schistosomula were incubated for 30 minutes with a suspension of S. aureus Cowan I strain. Schistosomula were washed twice in Eagle's medium and examined under the light microscope. Results are shown in Figure 4.6. Schistosomula treated with RAWH antiserum showed positive binding (Figure 4.6.A) as indicated by the clustered bacteria all over the schistosomular surface membrane. Whereas, schistosomula treated with RAFTS antiserum showed patchy binding (Figure 4.6.B). Schistosomula treated with RABP antiserum or NRS showed no bacterial binding (Figure 4.6.C, D). Therefore, the proteins eluted (including BP) may not be expressed on the outer surface of schistosomula. However, further experiment to support this point would be to absorb the prepared antiserum (RABP) with a homogenate from worm bodies without membranes, then detect the binding of the absorbed antiserum to the

Figure 4.6. Demonstration of the Binding of Antibodies in Rabbit Anti-Schistosome Antisera to the Surface Membrane of Schistosomula of *S. mansoni*

(A) Schistosomula incubated with rabbit anti-worm homogenate antiserum (RAWH) then treated with a suspension of *Staphylococcus aureus* Cowan I strain. Even distribution of the bacteria bound to the schistosomular surface membrane is clear (arrows).

(B) Schistosomula treated as described in (A) but with rabbit anti-frozen-thawed supernatant antiserum (RAFTS) as test serum. Patchy binding (arrows) of the bacteria to the surface membrane is shown.

(C) Schistosomula treated as described in (A) but with rabbit anti-basic protein antiserum (antiserum raised in rabbit against peak proteins eluted from the QAE-sephadex column) as test serum. No binding is demonstrated.

(D) Schistosomula treated as described in (A) but with normal rabbit serum as a control serum. No binding is demonstrated.


(A)
(B)
(C)
(D)

surface membrane of schistosomula and adult worms using more sensitive techniques such as the immunofluorescence test.

4.7. Discussion

In view of the likely importance of schistosome surface membrane antigens in stimulating immunity in the vertebrate host, investigations were carried out to establish the identity of tegumental proteins present in a particular fraction extracted from the surface membrane of adult schistosomes. This fraction was obtained as a supernatant after freezing and thawing of adult worms (Kusel, 1972). It is termed the frozen-thawed supernatant (FTS). Previous studies have indicated that this fraction contains soluble membrane material as revealed by electron microscopy (Ramalho-Pinto, Goldring, Smithers and Playfair, 1976) and immunodiffusion analysis (Murrell, Vannier and Ahmed, 1974). Evidence presented in this thesis has shown that the FTS fraction shares some characteristics with the surface membrane fraction as shown by electrophoresis on SDS and IEF gels. Further, a specific component(s) confined to fractions derived from the surface membrane (MP) and the frozen-thawed supernatant (FTS) has been demonstrated (Chapter 3). For all these reasons, it was decided to characterize further the antigens in the FTS fraction extracted from adult worms of S. mansoni.

Immunodiffusion analysis and immunoelectrophoresis have been utilized to demonstrate the presence of antibodies in mouse serum infected with S. mansoni, and also in rabbit serum specifically raised against different schistosome fractions (Figure 4.I). The number of precipitin lines detected by immunodiffusion analysis of the FTS fraction against mouse sera from schistosome infected mice appeared to be fewer than that observed with rabbit antisera. This might reflect species

differences in susceptibility to immunization with S. mansoni. However, mouse serum was obtained from mice harbouring living adult worms, while rabbit antiserum was obtained by immunization with the frozen-thawed supernatant material extracted from adult worms. Thus, it is possible that some of the macromolecules found in the FTS fraction may not be released by adult worms in vivo. The finding reported by Murrell, Vannier and Ahmed (1974) may argue against this suggestion. They indicated that material released from adult worms into culture media (culture antigen) and material obtained by freezing and thawing of adult worms (FTS fraction) or by treating worms with a hypertonic salt solution 3 M KCl (membrane fraction) appeared to have common antigenic determinants as shown by immunodiffusion analysis. Moreover, absorption of rabbit anti-culture antigen antiserum with the FTS or with the membrane fraction removed most but not all of the precipitin lines against culture antigen. This result was considered as a demonstration of the presence of membrane material in the FTS fraction which could be released into culture media.

Immunoelectrophoresis of the FTS fraction against rabbit antisera (RAFTS, RAM) showed at least 6 precipitin arcs. The anodic migration of the arcs may indicate that many antigens in this fraction may have acidic groups. This is compatible with the observation reported in the present work on the IEF pattern of the FTS fraction (Chapter 3). This result may also indicate that BP found to be specific to the FTS fraction is either a poor immunogen or it might be present in minute amounts so that it could not be detected by this technique.

Immunodiffusion analysis of 4 schistosome antigen preparations, FTS, WHS, SF and EA against antisera collected from mice infected with S. mansoni showed differences in the pattern of precipitin lines formed and also in the time of appearance of these lines, i.e. the appearance

of the antibody response (Figure 4.2). Whether the differences were due to qualitative differences in the antigenic components of the 4 preparations or to the relative amounts of common antigens could not be determined with certainty. Also, strain differences in the antibody response to each of these antigen preparations were observed. This observation was followed up with respect to other responses such as worm burden, splenomegaly and distribution of eggs in tissues (See Appendix for detail).

Results of immunodiffusion analysis gave clear indication of the presence of antibodies in sera from schistosome infected mice (60-120 days) against antigens in the FTS fraction. Several studies have indicated the appearance of antibodies against egg antigen after a similar time of infection with S. mansoni (Carter and Colley, 1978; Pelley, Pelley, Hamburger, Peters and Warren, 1976). Then, it is possible that the FTS fraction may contain contaminants of egg antigen that reacted with anti-egg antibodies present in immune mouse serum. Alternatively, antigens in the FTS fraction may cross-react with specific antibodies against egg antigen in mouse serum. However, the finding that Ouchterlony analysis and immunoelectrophoresis of rabbit antiserum specifically raised against the FTS fraction gave negative results invalidated these possibilities (Figure 4.3.D).

Attempts were made to assess the amount of antigens in the FTS fraction that would react with antibodies in immune mouse serum (120 days). Using the coprecipitation test showed that a significant amount of the FTS fraction extracted from adult worms labelled with ^{14}(C)-amino acids could be coprecipitated with RAMIg after being treated with immune mouse serum (Table 4.I). On the other hand, the FTS fractions obtained from worms labelled with ^{35}(S)-methionine or ^{3}(H)-leucine showed less amounts of coprecipitated proteins as judged by the percentage of the number of

counts in the coprecipitates. There are several explanations for this result (a) most of the antibodies against FTS antigens may be present in the form of immune complexes. Evidence to support this suggestion comes from the work of Houba, Koech, Sturrock, Butterworth, Kusel and Mahmoud (1976); Phillips and Draper (1975); Bout, Santoro, Carlier, Bina and Capron (1977). These workers indicated the presence of circulating immune complexes of schistosome material and specific antibodies in both experimental animals and patients at late stages of infection with S. mansoni. But, the large number of counts of ^{14}(C)-labelled FTS that coprecipitated may argue against this suggestion. (b) The presence of immunoglobulins in the FTS fraction has been demonstrated by both immuno-electrophoresis and Ouchterlony analysis (Figure 4.3, A,B). It was assumed that the presence of immunoglobulins in the fraction might compete with the immune complex (the labelled antigen and specific antibodies in mouse serum) for the binding sites of RAMIg used for the coprecipitation. However, neither the substitution of RAMIg for protein A nor the use of a highly specific RAMIg (rabbit anti-myeloma protein) altered the results.

In view of the finding that BP is present in both the FTS fraction and in the membrane fraction, an attempt was made to isolate this BP from the FTS fraction. Ion-exchange chromatography (QAE-sephadex column) has been utilized for this purpose. This attempt failed to yield a purified BP. Both IEF and SDS gels revealed the presence of large number of components along with BP in the peak material eluted from the column. However, characteristics of this eluted material as shown on IEF and SDS gels were consistent with that shown by the whole FTS fraction (Figure 4.4). Lectin over-lay technique showed no binding of BP to the labelled lectin on IEF gel indicating that this component is not detectably glycoprotein in nature.

Binding experiments to detect the presence of BP on the outer surface of schistosomula gave negative results. This does not preclude the possibility that this protein might be expressed on the outer surface of the parasite as a minor component. The immunogenicity of this BP in normal infection with schistosomes is questionable.

In summary, our initial definition of the FTS fraction indicated the following: It is a complex mixture of components containing both proteins and glycoproteins as revealed by coomassie blue staining of IEF and SDS gels and lectin over-lay assay. The appearance of precipitin lines in sera collected from mice infected with S. mansoni may indicate the release of this material into the blood circulation in vivo. This fraction contains contaminants of host origin, mainly immunoglobulins usually found to be associated with schistosome tegument, but not egg antigen. A component(s) with isoelectric points at pH between 7.2-8.2 is a consistent feature of this fraction. However, further investigations involving detailed studies of the stage specificity of antigens in this fraction, the use of these antigens as specific markers of the surface membrane of the parasite, and quantitative analyses of the ability of purified antigens from this particular fraction to elicit the immune response are necessary. The results of such studies will define the true significance of these antigens.

CHAPTER V

A STUDY OF SOME CHARACTERISTICS OF INDIVIDUAL CLONES OF *S. MANSONI* WITH EMPHASIS ON MEMBRANE PROTEIN SYNTHESIS

5. A STUDY OF SOME CHARACTERISTICS OF INDIVIDUAL CLONES OF S. MANSONI WITH EMPHASIS ON MEMBRANE PROTEIN SYNTHESIS

5.1. Introduction

A recent study by Smith and Clegg (1979) gave evidence that variation in the levels of immunity to S. mansoni is related to variations between different pools of cercariae rather than to variations in the immune response of the host. This evidence was based upon the finding that individual clones of cercariae derived from snails infected with a single miracidium showed a high level of susceptibility to immunity stimulated by a bisexual infection or were not susceptible at all. Since target antigens may occur on the schistosome surface membrane (see section 1.5), the presence of variable antigen(s) on the surfaces of different clones of S. mansoni is the most likely explanation for such variations in immunity. However, the immunofluorescence technique showed that schistosomula derived from different clones share at least one common surface antigen. This result does not preclude the presence of clone-specific antigen(s) as minor determinants on the parasite surface.

During the course of our investigations (Chapters 3 and 4) of the synthesis and characterizations of different schistosome fractions, 3 features of particular interest were observed. Firstly, surface membrane fractions (the FTS and MP) showed the presence of protein bands with isoelectric points at pH 7.2-8.2. Secondly, this protein is synthesized by the parasite, and thirdly this protein is absent from the pellet prepared from the worm without membrane. If this protein represented the variable antigen on schistosome surface membrane, it would be expected that (a) some clones might express such protein

while others might not or (b) the isoelectric points of this protein might vary from one clone to another. In order to distinguish between these possibilities, several clones of S. mansoni have been established as described below. In addition, investigations into some other characteristics of these individual clones have been undertaken.

5.2. Establishment of the Clones of S. mansoni (Figure 5.I.)

Single miracidia were used to infect individual snails (2.5.I). Snails were shed individually and cercariae were used to infect separate groups of mice percutaneously (2.5.2). All experiments in the sections below were performed on single-sex parasites derived from individual clones of S. mansoni. Individual clones were coded according to the batch of snails from which the clone was derived and the snail number in that particular batch. For instance, $C_1(14)$ is the 14th snail from batch number one.

5.3. Susceptibility of Biomphalaria glabrata to Infections with a Single Miracidium of S. mansoni

The susceptibility of B. glabrata to infections with a single miracidium of S. mansoni was assessed. Batches of 20-30 snails were exposed individually to a single miracidium of S. mansoni (Table 5.I). A total infection rate of 40.9% (90/220) occurred among the snails which received single miracidia. The rate of infection in different batches of snails varied from 25%-57%. The figures in Table 5.I indicate that the proportion of snails that shed cercariae increased gradually from 7.7% during the first 8 weeks to 18.6% within 12 weeks after infection. Results were similar in several batches of snails. Mortality varied between 16% to 38%, most of the deaths having occurred during the first 8 weeks after infections. Cercariae were obtained from snails

Figure 5.I. The Establishment of the Clones of *S. mansoni* and the Labelling of Adult Worms with ^{35}(S)-Methionine

Single miracidia were used to infect individual snails of *B. glabrata*. Snails were shed and cercariae were collected and used to infect separate groups of BALB/c mice. Adult worms were collected by perfusion of infected mice. Adult worms were cultured then labelled with ^{35}(S)-methionine. Fractions from radiolabelled worms were focused on IEF gels.

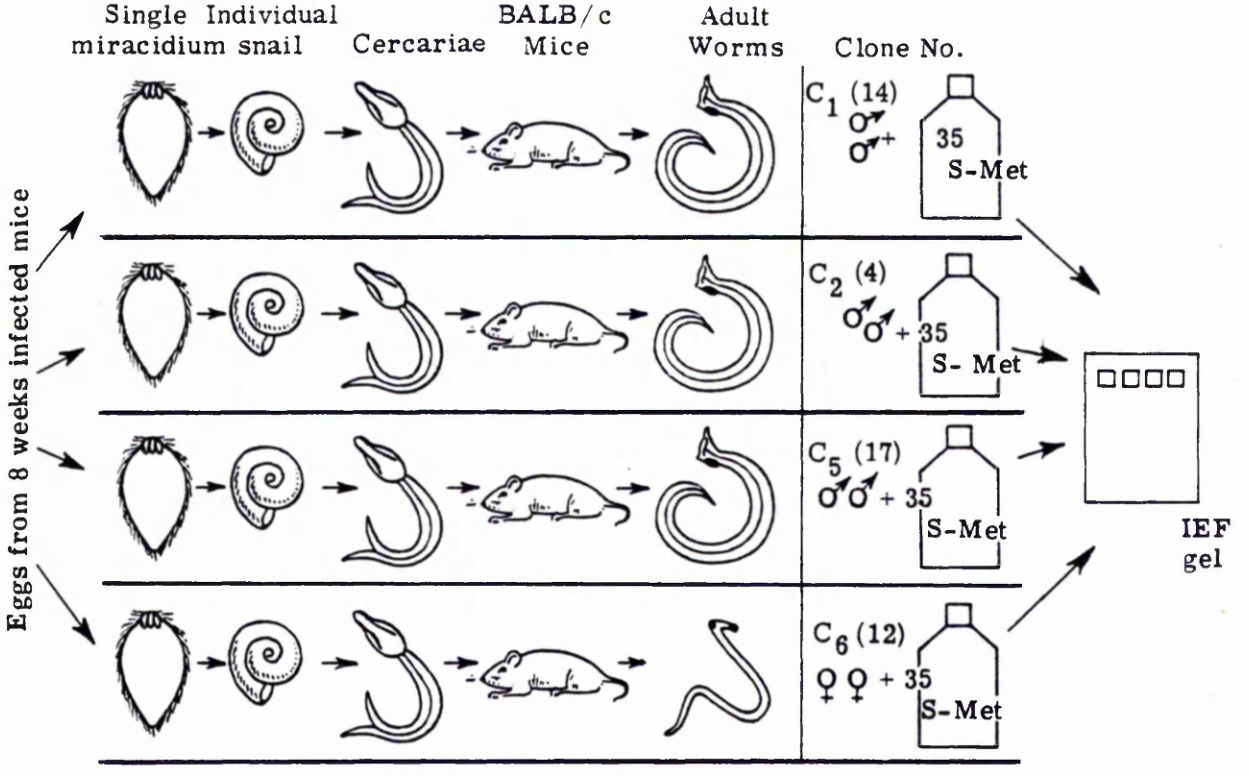

Single miracidium
Individual snail
Cercariae
BALB/c Mice
Adult Worms
Clone No.
Eggs from 8 weeks infected mice
C1 (14)
♂♂+
35 S-Met
C2 (4)
♂♂ + 35 S- Met
C5 (17)
♂♂ + 35 S-Met
C6 (12)
♀♀ + 35 S-Met
IEF gel

Table 5.1.

The Percentage of Infection and the Percentage of Death in Snails After Exposure to A Single Miracidium of S. mansoni

Batch	Weeks after exposure to a single miracidium							% (Total)		
	8			10		12				
	No. exposed	No. having shed cerc.	No. died	No. having shed cerc.	No. died	No. having shed cerc.	No. died	Snails shedding cerc.	Snails died	Snails surviving but not shedding cerc.
I	21	2	2	0	0	10	6	57.1	38.0	4.9
II	24	3	2	7	0	3	2	54.1	16.6	16.8
III	24	3	9	1	1	8	3	50.0	33.3	16.7
IV	39	3	10	1	0	6	2	25.6	30.7	43.7
V	38	1	6	5	1	1	0	26.3	18.4	55.3
VI	38	2	9	3	3	6	2	28.9	36.8	34.3
VII	36	3	7	7	4	7	1	47.2	33.3	19.5

8 weeks after infection. As a rule the number of emerging cercariae was approximately 50 cercariae per snail in the first 8 weeks. Thereafter, it was gradually increased and fluctuated widely through the remaining period. A group of 10 snails which showed the highest numbers of cercarial yields (average 500-2000/shed) were selected to obtain individual clones used in subsequent experiments.

5.4. Susceptibility of Mice to Infections with Different Clones of S. mansoni

(i) General observations

In order to determine the sex of worms derived from single pools of cercariae, separate groups of mice (BALB/c and Parkes) were infected with different clones of cercariae. 8 weeks after exposure to cercariae, infected mice were perfused (2.5.3) and worms were collected. Of 10 selected snails, each exposed to a single miracidium, 8 (80%) were found to have male infection, and the remaining snails (20%) with female infection. Mice infected with female clones showed considerable pigment deposition in the livers. Eggs were not found in the intestines or livers of infected animals. Female worms recovered from 8 weeks infected mice measured approximately 5.25 mm in length (range 3.5-7.0 mm), i.e. slightly less than half of the average length of the corresponding female worms from bisexual infection (6.5-15.0 mm, average 10.75 mm). Great variations in size were noted among adult males in unisexual infection. The average length of the males from 8 weeks infection, varied between 2.0-16.0 mm (average 9.0 mm). Homosexually paired males were occasionally observed in infections with single male clones in the absence of females. On the other hand, a few females obtained from mice 12 weeks after unisexual infection

developed a ventrolateral groove similar to the gynaecophoric canal usually found in adult males.

(ii) Recovery of adult worms from infected mice

Although it has been shown that mice infected with S. mansoni vary widely in their susceptibility to challenge infection with individual clones of the same parasite (Smith and Clegg, 1979), the nature of the differences between individual clones is obscure. As a first step to understand such differences, the infectivity of individual clones in the BALB/c strain of mice was studied. The degree of infectivity in mice was assayed by the recovery of adult worms from the portal system 8 weeks after infection (2.5.2). 4 individual clones of S. mansoni were obtained by infecting individual snails with a single miracidium (5.2). Assay of the level of infectivity by portal perfusion required the use of 5 groups of mice. In each of 3 experiments, 4 groups of 5-10 BALB/c mice were given a primary infection of 80-100 cercariae/mouse using the percutaneous method of exposure (2.5.2). An additional group of mice was infected with cercariae obtained from a pool of snails infected with several miracidia. The size of each primary infection was dependent upon the yields of cercariae from individual snails and was kept between 80-100 cercariae/mouse in each experiment. Portal perfusion was carried out 8 weeks after infections. The ratio of the number of worms recovered from the animals in a group to the total number of cercariae to which the group had been exposed was expressed as the mean percentage recovery of adult worms. The results are summarized in Table 5.2 and Figure 5.2. The mean percentage of adult worms recovered from the portal veins of mice infected with an individual clone in each of the 4 groups of clones vary slightly in different experiments. For instance, in clone $C_1(14)$, the mean

Table 5.2.

The Proportion of Adult Worms Recovered from BALB/c Mice Infected with Single Clones and with a Mixed Population of *S. mansoni*

Experiment No.	*Mean Percentage of Recovered Adult Worms (mean ± S.D.)				
	C_1(14)	C_5(17)	C_6(12)	C_2(4)	m.p.
I	13.3±8.0	1.6±2.1	6.6±3.8	10.7±5.5	25.2±9.7
	**(10)	(8)	(5)	(7)	(5)
II	10.2±5.2	3.1±3.2	8.2±6.7	10.9±3.1	35.7±10.4
	(7)	(5)	(5)	(5)	(5)
III	12.1±9.8	3.7±5.1	7.4±3.2	8.3±4.7	45.0±16.3
	(5)	(6)	(5)	(6)	(5)

*Adult worms recovered from groups of 5-10 mice 8 weeks after exposure to 80-120 cercariae. The average number of worms recovered from mice infected with individual clones varied between 0-20 worms/mouse.

**Number in parenthesis indicates number of mice assayed/group.

Table 5.2 continued

*Statistical Analysis of the Data Shown in Table 5.2 (Student's t-test)

Clone No.		Experiment No. I	Experiment No. II	Experiment No. III
$C_1(14)$ Vs $C_5(17)$	S	$P < 0.01$	$P < 0.01$	$P < 0.05$
$C_1(14)$ Vs $C_6(12)$		$P = 0.05$	$P = 0.57$(N.S.)	$P > 0.05$(N.S.)
$C_1(14)$ Vs $C_2(4)$		$P > 0.05$(N.S.)	$P = 0.74$(N.S.)	$P > 0.05$(N.S.)
$C_1(14)$ Vs m.p.		$P = 0.05$	$P < 0.003$	$P < 0.008$
$C_5(17)$ Vs ($C_6(12)$	S	$P < 0.04$	$P < 0.05$	$P < 0.05$
$C_5(17)$ Vs $C_2(4)$		$P < 0.04$	$P < 0.003$	$P < 0.05$
$C_5(17)$ Vs m.p.		$P < 0.005$	$P < 0.002$	$P < 0.005$
$C_6(12)$ Vs $C_2(4)$		$P > 0.1$ (N.S.)	$P > 0.3$ (N.S.)	$P > 0.7$(N.S.)
$C_6(12)$ Vs m.p.	S	$P < 0.001$	$P < 0.002$	$P < 0.007$
$C_2(4)$ Vs m.p.		$P < 0.02$	$P < 0.006$	$P < 0.008$

*Statistical analysis was performed on ICL 2976 computing service available in Univ. of Glasgow.

N.S., Difference not statistically significant.

S, Difference is statistically significant.

Figure 5.2. Percentage of Recovery of Adult Worms from BALB/c Mice Infected with Individual Clones and with a Mixed Population of S. mansoni

Each histogram represents the proportion of adult worms recovered from a group of 5-10 mice 8 weeks after infection with:

clone $C_1(14)$ males

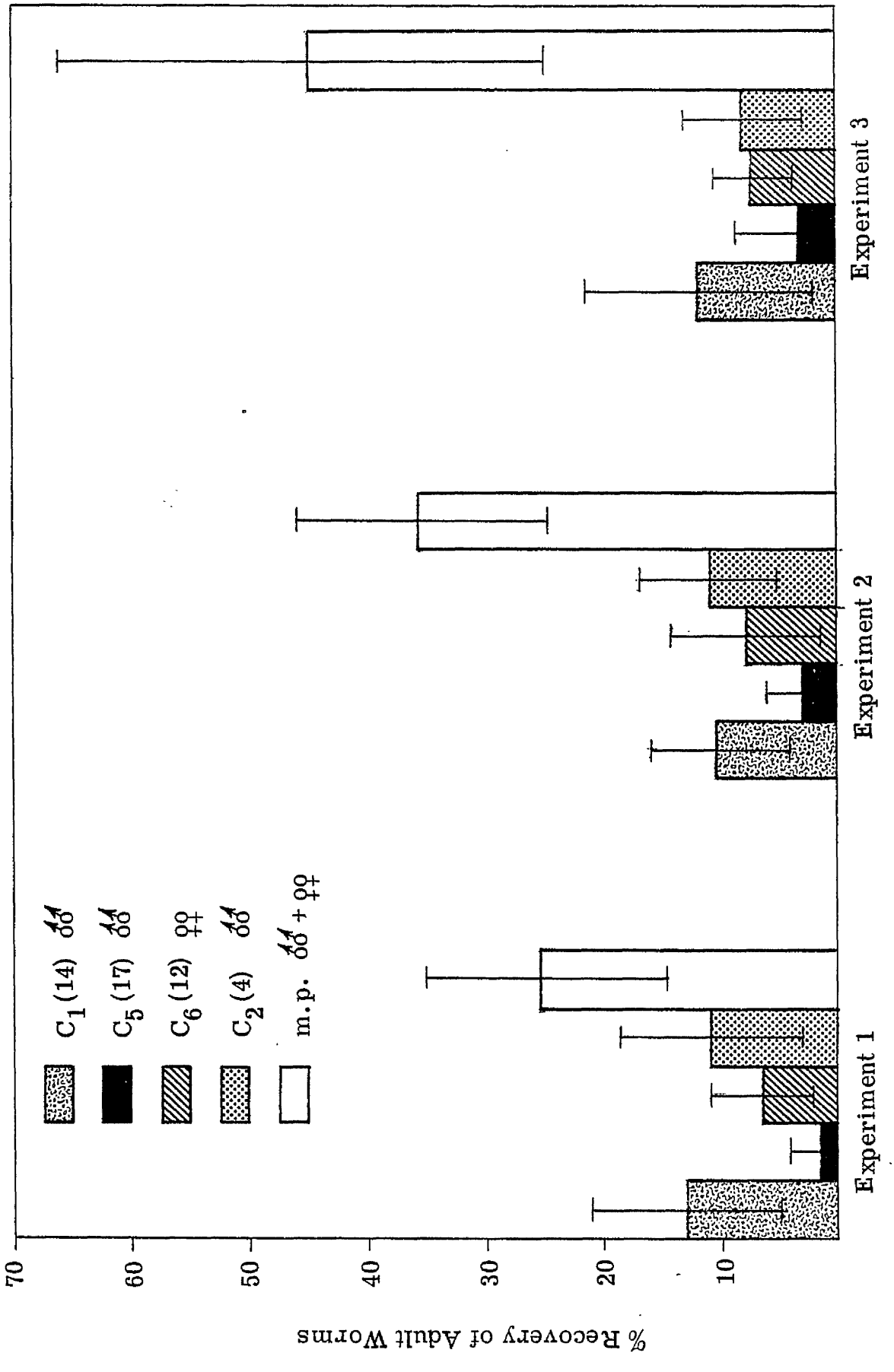
clone $C_5(17)$ males

clone $C_6(12)$ females

clone $C_2(4)$ males

mixed population (m.p.) males and females.

Bars indicate standard deviations within each experimental group. (See 5.4 for detail).


% Recovery of Adult Worms
70
60
50
40
30
20
10
C1 (14) ♂♂
C5 (17) ♂♂
C6 (12) ♀♀
C2 (4) ♂♂
m.p. ♂♂ + ♀♀
Experiment 1
Experiment 2
Experiment 3

percentage recovery ranged between 10.2%-13.3% in the three experiments, although these experiments were performed at 3 different intervals with cercariae drawn from different sheddings but of the same individual clone. On the other hand, the mean percentage recovery of adult worms from mice infected with cercariae obtained from pools of snails (mixed population, m.p.) varied considerably (25.2%-45.0%) in different experiments. The mice were all of the same sex, age and strain (BALB/c), suggesting that there is variation between different batches of cercariae. However, in each of the 3 experiments, the mean percentage of adult worms recovered from mice infected with a mixed population was significantly higher than the mean percentage recovery of adult worms obtained from infections with individual clones ($P < 0.05$). Variations in the mean percentage recovery of worms within the same group of mice infected with individual clones was noted. The average number of worms recovered from a group of mice infected with a single clone varied between 0-20 worms/mouse. However, the degree of infectivity appeared in the adult recovery assay of each of $C_1(14)$, $C_6(12)$ and $C_2(4)$ was found to be significantly higher than the infectivity shown by clone $C_5(17)$ in all of the 3 sets of experiments. $C_1(14)$, $C_5(17)$ and $C_2(4)$ represent male clones, whereas $C_6(12)$ is the only female clone used in the study. No significant difference in the mean percentage recovery of worms was obtained between the male and the female clones.

The basic conclusion to be drawn from the results of the portal perfusion is that the primary infection with different individual clones of S. mansoni leads to various degrees of infectivity in mice. This conclusion would be greatly strengthened, if it could be shown that: (a) variation in the proportion of cercariae derived from individual clones which die in the skin during penetration is related

to the proportion of cercariae recovered as a result of portal perfusion, and (b) a mixture of cercariae pooled from 2 individual clones resulted in a different degree of infection than that obtained from infection with each of the 2 clones separately. For instance, a mixture of cercariae from $C_1(14)$ and $C_2(4)$ might show a different degree of infection than an infection with $C_1(14)$ or $C_2(4)$ alone, i.e. The effect of individual male clones on one another would be assessed. This would be of importance particularly as some studies have indicated that the presence of male worms affects the survival of female worms of S. mansoni in the vertebrate host (Armstrong, 1965; Floyd and Nollen, 1977). These 2 approaches could not be used for such further studies because the maintainance of an individual clone was limited by two main factors (a) the survival period of the infected snail and (b) the persistence of the cercarial yield emerging from the infected snail.

5.5. Radioactive Labelling of Adult Worms and Schistosomula Obtained from Individual Clones of S. mansoni

In the preceding chapter (Chapter 4), the observation was made that the supernatant of the membrane fraction from adult worms (FTS) contained the protein with isoelectric points at pH 7.2-8.2 which we termed the basic protein (BP). Here, the observation on basic protein will be studied in some further detail for two main reasons: (a) A specific protein present in the membrane fraction might represent the variable antigen(s) expressed by various individual clones of S. mansoni i.e. the existence of antigenic variations among individual clones of S. mansoni could be established as suggested by Smith and Clegg (1979). (b) The fact that this basic protein (BP) from the FTS fraction have been shown to be synthesized by the parasite poses the possibility that certain individual clones might synthesize such protein at a higher rate

than the other clones, i.e. differences in the rate of synthesis of specific membrane proteins might reflect differences in the rate of the metabolic activities of individual clones and consequently provide an important factor for differentiating individual clones from one another.

5.5.I. The Isoelectric Focusing Spectra of FTS Fractions Obtained from Adult Worms of Individual Clones of S. mansoni

Adult worms were internally labelled with L-(^{35}S)-methionine as described previously (2.7.2). 3 groups of worms obtained from mice infected with individual clones and a group of worms from mice infected with a mixed population of S. mansoni were incubated separately in Eagle's medium + FCS at 37°C for 24 hours. After incubation, 50 worms were selected from each group and incubated in labelling medium deficient in methionine (Table 2.2). L-(^{35}S)-methionine was added to each culture tube as 250 µCi/ml medium. The incorporated radioactivity was determined by TCA precipitation (2.7.3) of the FTS fraction prepared from each group of labelled worms. The labelling experiment was carried out twice under the same culture conditions, but at different intervals. Results are shown in Table 5.3. Under the applied culture conditions, the amount of radioactivity incorporated into the FTS fraction extracted from individual clones varied considerably. A relatively high rate of incorporation of radioactivity was shown by C_1(14) and C_2(4) compared with C_5(17) and C_6(12). The rate of incorporation of ^{35}(S)-methionine into the female clone C_6(12) was about 10 times less than that observed with the male clones C_1(14), C_2(4) and C_5(17). The high number of counts detected in the TCA precipitable proteins from the mixed population worms (males and females together) indicated the high rate of incorporation of the radioactive amino acid into the surface membrane

Table 5.3

TCA Precipitation of FTS Fractions Extracted from Individual Clones and from a Mixed Population of S. mansoni

Experiment No.	TCA precipitable counts/minute/one worm				
	$C_1(14)$	$C_5(17)$	$C_6(12)$	$C_2(4)$	m.p.
	(males)	(males)	(females)	(males)	(males + females)
I	41020	20440	5848	54804	79160
2	33412	25364	5016	54104	71382

proteins of these worms. However, these results were considered as preliminary observations and examined in further detail in later experiments (5.5.2).

Isoelectric focusing of FTS fractions in polyacrylamide slab gels was carried out. FTS fractions were extracted from individual clones, $C_1(14)$, $C_2(4)$ and $C_5(17)$ or from a mixed population (m.p.). The fact that $C_6(12)$ which is the female clone, showed very low number of TCA precipitable counts has limited the attempts to obtain the isoelectric focusing pattern of this clone. However, clear resolution of protein components of all clones tested as well as that of the mixed population was obtained after fluorography. Indications of the isoelectric points of the focused protein bands were obtained by measuring the pH gradients. For convenience in discussion, the bands were numbered sequentially (A_1-A_{10}) from the pH 3.5-10.0. The IEF spectra of FTS fractions obtained from different clones consist of a regular pattern of 10 major bands (A_1-A_{10}) ranging in their isoelectric points from pH 3.9-7.0 (Figure 5.3). The presence of protein bands (BP) which have been shown to be specific to the FTS fraction at pH between 7.2-8.2 is also clear. This spectrum is consistent with that obtained from the FTS fraction of a mixed population except in some quantitative respects. Densitometric tracing on Joyce Loebl microdensitometer (Model Mk 3) gave an indication of quantitative differences between the FTS fraction extracted from individual clones and that obtained from the mixed population. Although the amount of labelled proteins applied to the gels from each preparation was the same, the FTS fraction from the mixed population showed more heavily labelled bands than those from individual clones (Figure 5.4).

One of the main aims of this experiment was to assess the presence of variations in the isoelectric points of protein bands which

Figure 5.3. Isoelectric Focusing Spectra of Radiolabelled Frozen-Thawed Supernatant Fractions Obtained from Adult Worms of Individual Clones of S. mansoni

Adult worms of clone $C_1(14)$, clone $C_2(4)$, clone $C_5(17)$ or of a mixed population (males and females) were incubated with $^{35}(S)$-methionine. Radiolabelled frozen-thawed supernatant fractions were extracted from each group of worms and focused on IEF gels. (See 5.5.I for detail).

A_1 - A_{10} indicate major radiolabelled bands in each fraction.

Dotted lines indicate radiolabelled protein bands shown by fluorography.

The bold arrow indicates basic protein bands (BP) focused in all fractions at pH 7.2-8.2. Insets at bottom present over-exposed fluorograms of the same region of the gel (pH 7.2-8.2) of each fraction showing quantitative differences in the radiolabelled BP in each of the individual clones and the mixed population examined.

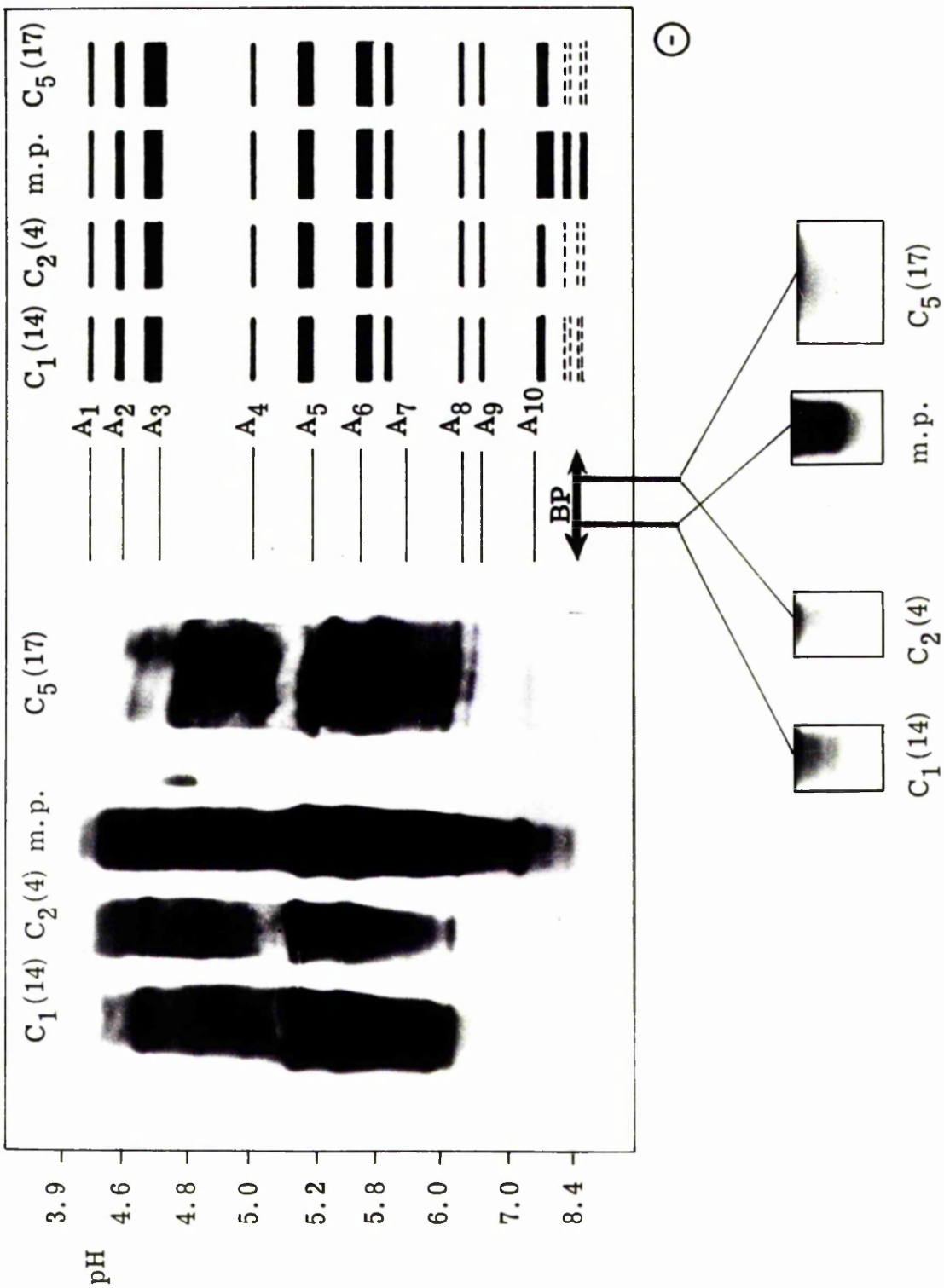
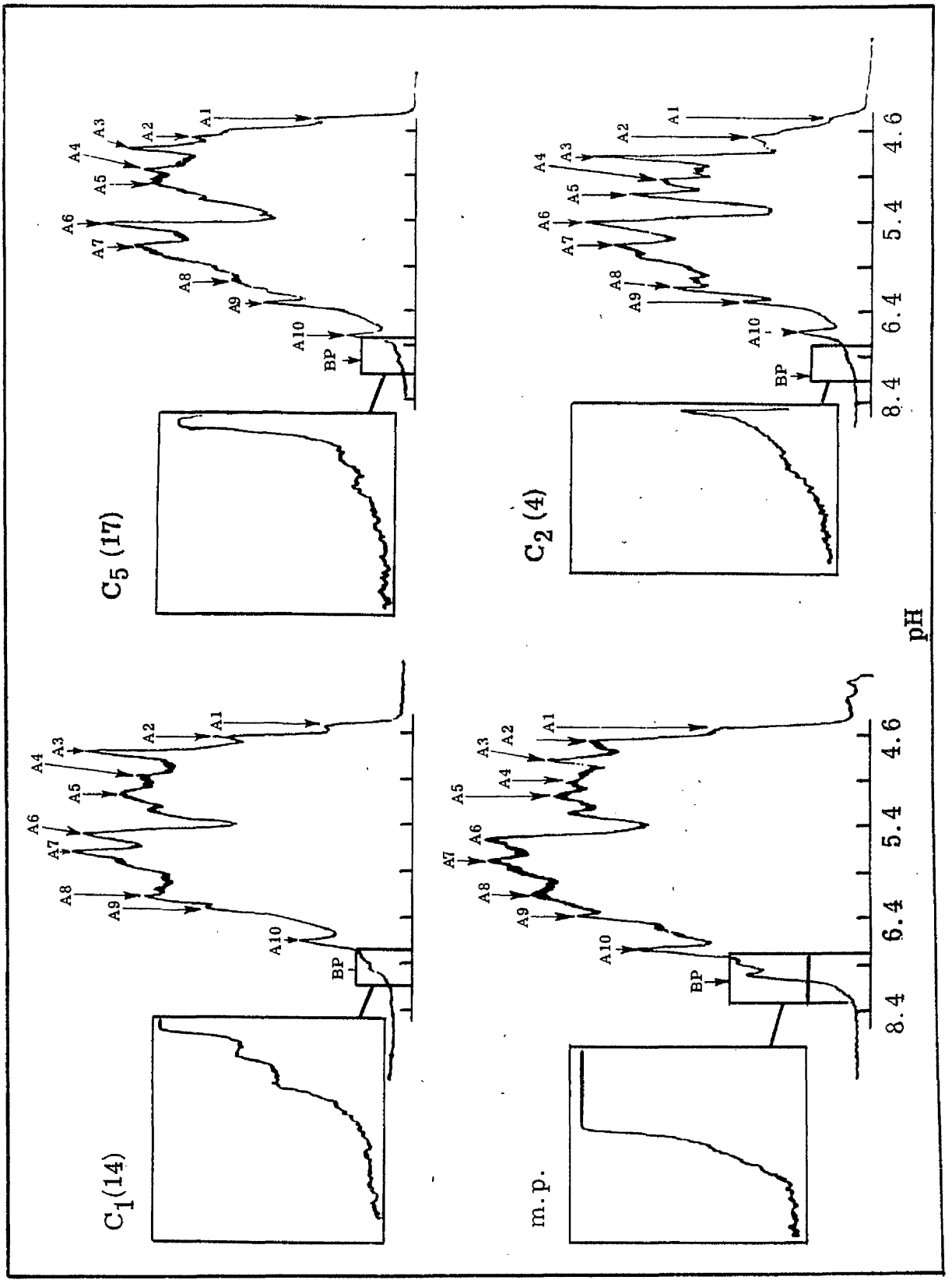

C1(14) C2(4) m.p. C5(17)
C1(14) C2(4) m.p. C5(17)
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
BP
pH
3.9
4.6
4.8
5.0
5.2
5.8
6.0
7.0
8.4
⊖
C1(14) C2(4) m.p. C5(17)

Figure 5.4. Densitometric Tracings of Radiolabelled Frozen-Thawed Supernatant Fractions Extracted from Adult Worms of Individual Clones and from a Mixed Population of S. mansoni

^{35}(S)-labelled frozen-thawed supernatant fractions (^{35}S-FTS) from adult worms of: clone $C_1(14)$, clone $C_5(17)$, clone $C_2(4)$ and a mixed population (m.p.) were focused on IEF gels (5.5.I). A_1-A_{10} indicate the major protein peaks in each preparation.

Insets: 5 times magnification of the basic region of the gel (pH 7.2-8.2) indicating quantitative differences in the labelled basic protein bands (BP) present in all clones and in the mixed population.


C5 (17)
C2 (4)
C1(14)
m.p.
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
BP
8.4
6.4
5.4
4.6
pH

have been shown previously to be specific to the FTS fraction (pH 7.2-8.2). Quantitative differences in the amount of this protein in each of the FTS fraction extracted from different individual clones was observed. It appeared that this protein is more heavily labelled in some clones than in the others (as revealed by densitometric tracing, Figure 5.4). For instance, protein bands focused at pH 7.2-8.2 in the FTS fraction prepared from $C_1(14)$ appeared to be more heavily labelled than that derived from $C_5(17)$ and focused at the same region. Quantitative differences in the labelling of the same component shown to be present in different clones means that this component could have been synthesized by all clones but at different rates or it could be present in variable amounts in different clones but synthesized at the same rate. However, this result and Table 5.3 pointed to the possibility of differences in the rate of metabolism among individual clones. The concept of differences in the rate of metabolism of different clones was supported in subsequent experiments (5.5.2).

5.5.2. The Incorporation of L-(^{35}S)-Methionine into Adult Worms and Schistosomula Obtained from Individual Clones of S. mansoni

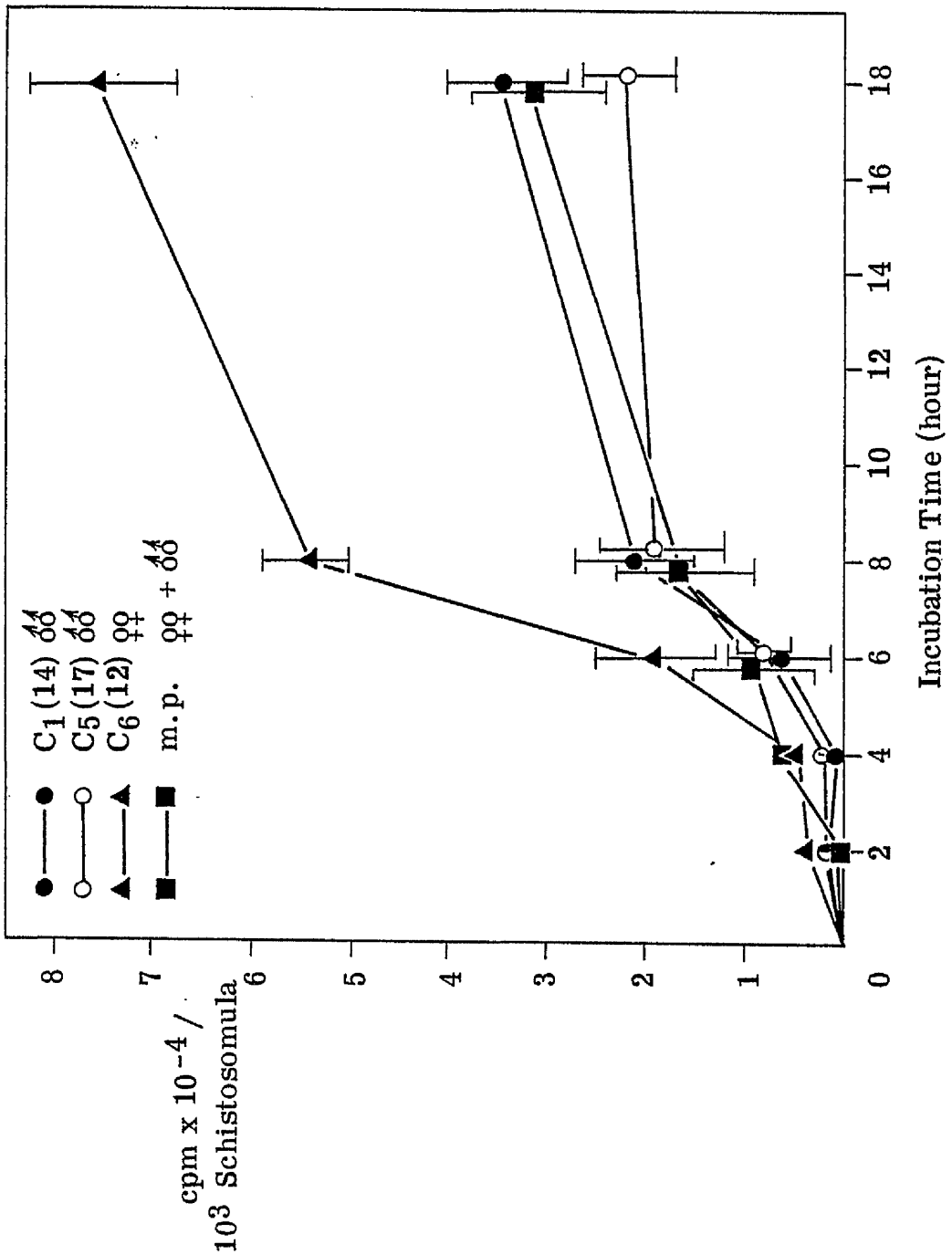
(i) Schistosomula

Schistosomula were prepared by the mechanical transformation method (2.7.I.I). 4 schistosomular preparations were used in this experiment, 3 of them were derived from individual clones $C_1(14)$, $C_5(17)$ and $C_6(12)$ and one was from a mixed population. Triplicate samples of 0.3 ml aliquots of the schistosomular suspensions (containing 800-1000 schistosomula in Eagle's medium) from each preparation were placed in sterile culture tubes. 1 ml Eagle's medium containing 0.5% E/Lac + 10% FCS was added to each tube. After 24 hours incubation at

37°C, 1 ml fresh labelling medium (Table 2.2) containing (^{35}S)-methionine (specific activity 870 Ci/mmol) at 66 μCi/ml medium was added to each culture tube. 0.2 ml aliquots of the incubation medium (containing 123-153 schistosomula) were taken at 2 hour intervals. The schistosomula were thoroughly washed in labelling medium, then resuspended in 0.2 ml PBS and stored at -10°C. Surfaces of schistosomula were removed by the freezing and thawing method and the FTS fractions were prepared (Kusel, 1972). 50 μl aliquots of the FTS fractions were tested for the total radioactivity. Triplicate aliquots were tested for TCA precipitable radioactivity. The counts were expressed as counts per minute per 1000 schistosomula. Figure 5.5 shows that there is progressive incorporation of the radioactive methionine into the surfaces of schistosomula in all preparations. The rate of incorporation of (^{35}S)-methionine increased significantly 6 hours after incubation in all preparations of schistosomula. One striking observation obtained with the female clone $C_6(12)$ was the significant increase in the amount of radioactivity incorporated. A maximum was after 18 hours incubation. On comparing the amount of TCA precipitable radioactivity present in the FTS fractions obtained from $C_1(14)$ and (m.p.) with the one shown by $C_6(12)$ a significant difference was consistently found. $C_5(17)$ showed the least amount of incorporation of (^{35}S)-methionine among the 4 schistosomular preparations tested. Statistical analysis indicated that after 18 hours incubation, there is a significant difference in the rate of incorporation of (^{35}S)-methionine into the FTS fraction obtained from $C_6(12)$ compared to each of $C_1(14)$, (m.p.) and $C_5(17)$. Each of $C_1(14)$ and (m.p.) are significantly different from $C_5(17)$ after 18 hours incubation time (Student's t-test). The results of this experiment show that schistosomula derived from

Figure 5.5. Incorporation of ^{35}(S)-Methionine into Schistosomula Obtained from Individual Clones and from a Mixed Population of S. mansoni

Schistosomula derived from individual clones, C_1(14), C_5(17), C_6(12) and from a mixed population (m.p.) were radiolabelled with ^{35}(S)-methionine. The amount of incorporated radioactivity in the frozen-thawed supernatant fractions was determined by TCA precipitable counts. Variation in the extent of incorporation of ^{35}(S)-methionine into individual clones is clear. (See 5.5.2 for detail).


C1 (14) ♂♂
C5 (17) ♂♂
C6 (12) ♀♀
m.p. ♀♀ + ♂♂
cpm x 10-4 / 10³ Schistosomula
Incubation Time (hour)
0
1
2
3
4
5
6
7
8
2
4
6
8
10
12
14
16
18

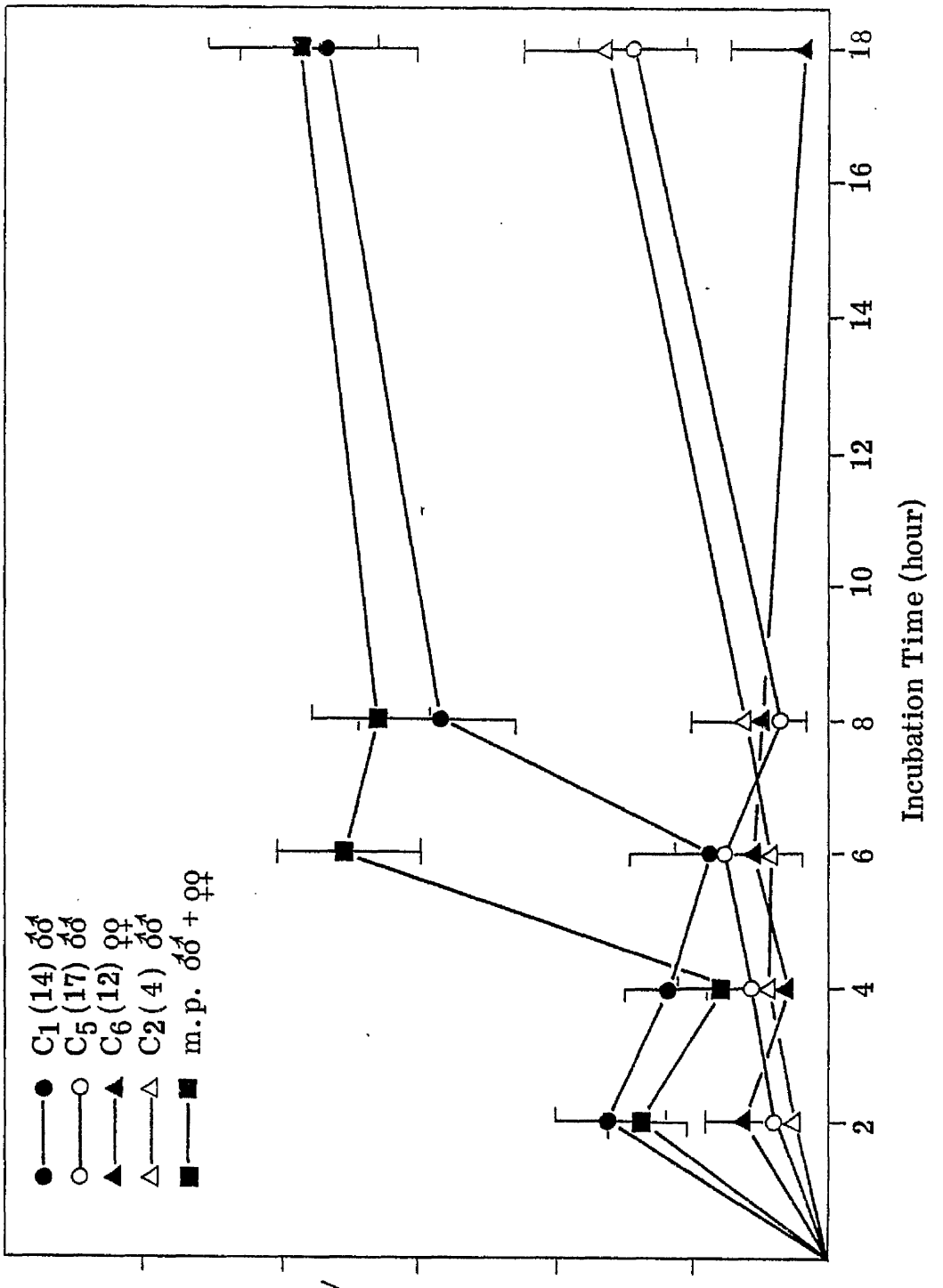
individual clones of S. mansoni incorporate methionine into membrane proteins yet at different rates. This indication of variability in the rate of synthesis between schistosomula derived from individual clones raised the question whether adult worms of the same clones would demonstrate such variations after maturation and sexual differentiation into males or females. In order to answer this question, the rate of incorporation of (^{35}S)-methionine in groups of adult worms obtained from individual clones was examined as described below.

(ii) Adult worms

Adult worms were recovered from groups of mice infected separately with 4 different clones: $C_1(14)$, $C_2(4)$, $C_5(17)$ and $C_6(12)$. All clones examined were male clones except $C_6(12)$ which is a female clone. A group of worms obtained from a bisexual infection with a mixed population of S. mansoni was used as a control group. In this group, worm pairs were used as mixed populations of males and females. After 24 hours incubation at 37°C, 60 viable worms were collected from each group, then worms were distributed into sterile culture tubes. Each culture tube contained 20 worms in 1 ml labelling medium + 10% FCS. All experimental and control groups were assayed in triplicate. (^{35}S)-methionine (specific activity 850 Ci/mmol) was added at 60 µCi/ml to each culture tube. 4 adult worms were removed from each incubation tube at 2 hour intervals. Labelled worms were treated as described before (2.7.2.2). The amount of radioactivity in FTS fractions prepared from each sample was detected by TCA precipitation. Results are shown in Figure 5.6. The incorporation of radioactivity in all groups of worms was detectable at 2 hours after incubation, whereas incorporation was detected in schistosomula only after 6 hours. One interpretation of this observation is that the rate of protein synthesis in adult

Figure 5.6. Incorporation of ^{35}(S)-Methionine into Adult Worms Obtained from Individual Clones and from a Mixed Population of S. mansoni

Adult worms derived from individual clones $C_1(14)$, $C_5(17)$, $C_6(12)$, $C_2(4)$ or from a mixed population were internally radiolabelled with ^{35}(S)-methionine. The amount of incorporated radioactivity in the frozen-thawed supernatant fractions was determined by TCA precipitable counts. Variation in the extent of incorporation of ^{35}(S)-methionine into individual clones is demonstrated. (See 5.5.2 for detail).


C1 (14) ♂♂
C5 (17) ♂♂
C6 (12) ♀♀
C2 (4) ♂♂
m.p. ♂♂ + ♀♀
cpm x 10⁻⁴ / 4 worms
5
4
3
2
1
2
4
6
8
10
12
14
16
18
Incubation Time (hour)

worms was higher than that in schistosomula. A similar observation has been reported by Chappell (1974) working with paired males and females and schistosomula from mixed populations. A significant increase in the amount of radioactivity incorporated into the FTS fraction from the mixed population was clear 6 hours after incubation. After that time, a steady increase in the amount of ^{35}(S)-methionine incorporated was shown by each of C_1(14) and m.p. ^{35}(S)-methionine was incorporated at approximately the same rate by C_2(4) and C_5(17) and at no time was there a significant difference in the incorporation of methionine shown by these 2 clones. The female clone C_6(12), showed a consistent low rate of incorporation of radioactivity throughout the total labelling period. This result is of great interest when it is considered that the data obtained with schistosomula derived from the same clone C_6(12) indicated a significant increase in the amount of incorporated radioactivity during the same labelling period and under the same culture conditions. The data show that the rate of metabolism of the parasite is not necessarily the same during different developmental stages. However, results obtained with the other clones C_1(14) and C_5(17) and even with the mixed population (m.p.) showed a consistent increase in the amount of radioactivity incorporated after 18 hours incubation at both the schistosomular and mature adult stages. These results together with those in the previous section showed that variations in the rate of metabolism can be demonstrated with different clones of S. mansoni either at the schistosomular or at the mature adult stage.

5.6. Discussion

Previous investigators (Smith and Clegg, 1979) have indicated that the variable levels of immunity to a challenge infection with S. mansoni in mice is due to variations in the parasite rather than in

the host. This evidence was based upon *in vivo* experiments employing individual clones of *S. mansoni* obtained from infection of individual snails with single miracidia. While the existence of heterogeneity has been established, the nature and the number of factors controlling the expression of such heterogeneity by individual clones is still unknown. It was the aim of the present work to determine whether such heterogeneity could be demonstrated by individual clones of *S. mansoni* with respect to:

(i) The infectivity for snails.

(ii) The infectivity for mice.

(iii) The isoelectric focusing spectra of a particular schistosome fraction extracted from adult worms.

(iv) The rate of synthesis of membrane proteins of schistosomula and adult worms.

The establishment of infections in *B. glabrata* appears to be affected by the number of miracidia to which the snails have been exposed. There is evidence indicating the presence of a direct relationship between the number of miracidia used for infecting snails and the proportion of snails producing cercariae as a result of infections (Schreiber and Schubert, 1949a). Our data (Table 5.I) indicate that a relatively high rate of infection occurs among snails receiving single miracidium (40.9%) compared with the data reported by other investigators; 14% (Schreiber and Schubert, 1949a), 35% (Stirewalt, 1954) and 12-30% (Smith and Clegg, 1979). However, this proportion is still much lower than that reported for infecting snails with several miracidia; 70-85% (Schreiber and Schubert, 1949a), 78% (Stirewalt, 1954) and 70-80% (Smith and Clegg, 1979). Snails infected with a single miracidium yielded a relatively high number of emerging cercariae. This compares well with the number reported from snails infected with several miracidia

(Schreiber and Schubert, 1949b). Data are not available for the number of daughter sporocysts produced by a single mother sporocyst inside the snail tissues. Such data would help to explain the high yield of cercariae in both single and multiple miracidial infections.

Variable levels of infectivity as a result of primary infections with individual clones were recorded in BALB/c mice (Table 5.2). This finding indicates that variations between cercariae derived from individual clones could result in variations in the level of infections in mice. It also suggests the possibility raised by Smith and Clegg (1979) of the presence of variable antigens, expressed by cercariae from individual clones. In order to relate this result to the previous finding reported in the present work that protein with isoelectric points between pH 7.2-8.2 is specific to one fraction extracted from schistosomes, the isoelectric focusing spectra of labelled adult worms from individual clones were examined by means of fluorography of IEF gels. Patterns of FTS fraction extracted from individual clones were similar on IEF gels. While the various extracts were qualitatively similar, a quantitative variation was evident. Densitometric tracing of each extract indicated that the bands representing the BP at pH 7.2-8.2 were more heavily labelled in certain clones than in the others (Figure 5.4). Furthermore, the FTS fraction from the mixed population showed more heavily labelled bands than those obtained from individual clones. It is possible that this protein is synthesized by all clones but at different rates, or it is synthesized at the same rate, but present in various amounts. Also, the presence of large amounts of this protein in the mixed population compared to the individual clones suggested that it might be synthesized more rapidly as a result of the presence of the 2 sexes together. The IEF pattern from the individual female clone $C_6(12)$ could not be obtained owing to the low number of

counts demonstrated on labelling adult worms derived from this clone. Thus, the contribution of the females to the BP from the mixed population could not be assessed.

Since the rate of incorporation of radioactive amino acids into the surface of schistosomes has been used as a measure of the rate of incorporation of freshly synthesized proteins into the surface (Kusel, 1972), this method was used to compare the rate of synthesis of membrane proteins in adult worms and schistosomula derived from individual clones. We shall consider 2 clones used in all experiments; $C_1(14)$ and $C_5(17)$. Both were male clones. $C_1(14)$ showed the highest percentage of recovery of adult worms and the highest rate of incorporation of ^{35}(S)-methionine compared with the other clones. Whereas, $C_5(17)$ showed the least percentage of recovery of adult worms and the least rate of methionine incorporation. This suggests the following hypothesis; the high rate of metabolism shown by an individual clone may account for the enhanced survival of cercariae derived from that clone during penetration of the skin and migration through the host.

A result which did not support the above hypothesis was that obtained with the female clone $C_6(12)$. It showed a high percentage of recovery of adult worms as judged by the portal perfusion assay. Whereas during labelling experiments, a very low level of methionine incorporation was shown by mature adult worms from this clone. A level of methionine incorporation by schistosomula significantly higher than other clones was observed. There are 2 possible explanations for the apparent discrepancy in the results obtained with $C_6(12)$. (a) Non-optimal culture conditions may have inhibited the metabolic process of the parasite at the adult worm stage rather than at the schistosomular stage. But, if this is the case, such results should have been obtained with all other clones cultured at the same time under the same

conditions. The results obtained with all other clones as well as with the mixed population make this suggestion the least likely explanation. (b) Several studies have indicated that the rate of metabolism among adult females is much higher than that in the males (Zussman, Bauman and Petruska, 1970; Lawrence, 1973). It was suggested that there is a direct relationship between the rate of metabolism and the high rate of egg production in female worms. It is well known that in S. mansoni, females do not mature sexually unless in the presence of males (Armstrong, 1965). Female worms transplanted into the hepatic portal system of hamsters showed degenerative changes in their reproductive systems, whereas male worms developed normally during transplantation and no degenerative abnormalities were detected (Floyd and Nollen, 1977). Although in the present work no attempts were made to investigate the development of the reproductive system in adult worms of the female clone $C_6(12)$, observations reported by other investigators could explain the results obtained with this particular clone. The high rate of metabolic activity (as shown by methionine incorporation) of schistosomula derived from this clone may explain the high yields of recovery of adult worms. It is probable that most of the cercariae from $C_6(12)$ would penetrate host skin due to their high rate of metabolism. As these schistosomula establish themselves successfully in the host and mature into adult females, the absence of males may have led to lack of development of their reproductive systems accompanied by reduction in their metabolic activities, as has been demonstrated in the labelling experiments. Although this explanation seems to be more acceptable, no firm evidence was found to support either one of these hypotheses. A detailed comparative study on a wider scale

between the incorporation of radioactive amino acids in paired and single clones should provide such evidence.

The present study has led to the view that individual clones of S. mansoni vary with respect to some of their characteristics (Table 5.4). Individual clones of S. mansoni with a high rate of metabolism could establish better infection in mice than those with a low rate of metabolism. This finding may indicate an important mechanism utilized by schistosomes in penetration of the tissues of host skin. Also, it is possible that such variations in the metabolic activities of individual clones could be responsible for protecting the parasite from the immune attack of its host. For instance, the more active clones may acquire host molecules or may shed the surface membrane faster than the less active clones. Further studies into the different biological aspects of individual clones of S. mansoni should yield information that could be of great value in the field of schistosomiasis.

Table 5.4.

Summary of Worm Recoveries, the Amounts of BP on IEF Gels and the Rates of Incorporation of ^{35}(S)-Methionine Shown by Individual Clones of S. mansoni

Clone Number	Adult worms recovery	Amount of BP shown on gel	Rate of incorporation of ^{35}(S)-methionine	
			Adults	Schistosomula
C_1(14)	High	High	High	High
C_5(17)	Very low	Relatively low	Low	Low
C_2(4)	Relatively high	Low	Relatively low	-
C_6(12)	High	-	Very low	Very high
m.p.	High	High	High	High

CHAPTER VI

GENERAL DISCUSSION

6. GENERAL DISCUSSION

The primary purpose of this study was to identify and characterize specific schistosome antigens present in the surface membrane of the parasite. Given the complexity of surface membrane antigens (as revealed by SDS-gel electrophoresis), 3 factors were of importance for the success of such study: (a) a sensitive assay system for fractionating membrane proteins should be developed. In the present work, this involved the isoelectric focusing in thin-layer polyacrylamide gel, (b) membrane preparations should be obtained from the parasite by a method which is least likely to result in contamination with irrelevant antigenic or chemical materials. Thus, instead of using complex procedures such as extraction by saponin-calcium chloride treatment (Kusel, 1970, 1972) or by 3 M KCl treatment (Murrell, Vannier and Ahmed, 1974), the surface membrane was isolated by freezing-thawing technique (Kusel, 1972), (c) owing to the fact that very small quantities of surface membrane proteins could be extracted by freezing and thawing, an efficient radiolabelling system should be established. The internal labelling of adult worms with (^{35}S)-methionine has been utilized for such purpose (Ruppel, 1978).

Our initial definition procedures, which involved IEF of radiolabelled adult worm proteins, have revealed the presence of a protein component(s) in the membrane fractions which could not be found in the rest of the worm. It has been termed the basic protein (BP). The isoelectric point of BP was found to be between pH 7.2-8.2. The patterns of radiolabelled and non-labelled surface membrane fractions, obtained by IEF gel electrophoresis, showed that the amount of BP appeared to be less in the labelled than in the non-labelled fraction. The possibility that these proteins (BP) rather than of being of parasite origin may represent immunoglobulins (shown to be associated with schistosome

tegument, Sogandares-Bernal, 1976; Kemp, Merritt and Rosier, 1978) was discarded for 2 main reasons: (a) BP bands could be detected on stained gels at pH between 7.2-8.2, after specific removal of immunoglobulins, and (b) the presence of BP in radiolabelled fractions indicated that it is synthesized by the parasite. The synthesis of such protein by adult schistosomes is of interest for several reasons. Firstly, adult schistosomes have a complex surface membrane with a multilaminate appearance as revealed by ultrastructural studies (Hockley and McLaren, 1973). This complex membrane appears to be formed immediately after penetration of host's skin, i.e. both schistosomula and adult worm possess the complex surface membrane. It has been suggested that the development of such membrane may present a mechanism utilized by the parasite for its survival in the blood circulation (Clegg, 1972). The outer layer of schistosome surface membrane is negatively charged (Stein and Lumsden, 1973). The presence of acidic phospholipids in schistosome membranes has been demonstrated (Cesari and Marchiani, 1978). Thus, it is possible that a positively charged protein component is synthesized by the parasite to maintain the integrity of this complex membrane by associating with the negatively charged acidic phospholipids. Demonstration of the presence of BP in schistosomula as well as in adult worms supports this suggestion. However, the synthesis of such protein at the cercarial stage of the parasite is unknown. Radiolabelling of cercariae and fractionation of surface membrane proteins on IEF gels may help to clarify this point. Also, studies in the rate of growth of schistosomula in the presence of antibodies specifically raised against BP might give an indication whether this suggestion is valid or not. Secondly, the synthesis of specific membrane proteins by the parasite, could be of value to study some metabolic processes shown to be associated with schistosome surface membrane. For instance, the

phenomenon of membrane turnover is shown to occur in vitro (Kusel, Sher, Perez, Clegg and Smithers, 1975), and believed to be an important mechanism utilized by the parasite to evade the host's immune response (Wilson and Barnes, 1974a, 1977). Yet, no firm evidence to show the occurrence of this phenomenon in vivo exists. Thus, radiolabelling of specific membrane proteins (such as BP) and following their appearance in the blood circulation could be used to obtain evidence of membrane turnover rates in normal and immune hosts. Thirdly, although the immunogenicity of BP is unclear, it is possible that it might play a role in inducing the immune response against membrane proteins. Investigations utilizing antibodies either from animals specifically immunized against BP, or from schistosome infected animals may help to obtain information about its significance in immunity.

Internal radiolabelling of schistosomes was performed under conditions which were assumed to permit incorporation of ^{35}(S)-methionine into adult worms and schistosomula of S. mansoni. A significant incorporation of radioactivity has been achieved after 18 hours incubation in Eagle's medium containing calf serum (as judged by TCA precipitation of radiolabelled fractions). When the labelling was performed in Eagle's medium deficient in serum, the pattern of surface membrane fractions on IEF gels was markedly changed. The absence of serum may have affected the increased rate of synthesis of surface membrane proteins. Alternatively, structural damage of schistosome tegument may have occurred and resulted in alteration of the membrane pattern observed on IEF gels. This observation may be seen in context with the findings reported by other investigators. Smith, Reynolds and Von Lichtenberg (1969) observed changes in the tegument of adult worms on incubation in PBS. These changes included disappearance of the multilaminate vesicles (MLV) from the epithelial layer accompanied by

vacuolar transformation of the tegument. Wilson and Barnes (1974b) indicated that transient structural changes occurred in schistosome tegument after incubation in Hank's saline for 30 minutes. Such changes included the development of vacuoles in the tegument. The mechanisms of these changes are still unknown. But, it is conceivable that protein could be released from the surface membrane in all these circumstances as a result of an increase in membrane turnover to repair the damaged membrane, or because of the leakage of proteins from the damaged areas of the membrane. Studies at the ultrastructural level on the integrity of adult worms after certain incubation periods without serum could explain these results (Simpson, personal communication). Also, electrophoretic analysis of proteins released from worms labelled in the presence or absence of serum may provide another approach to understand the mechanisms of these changes.

A fraction extracted by freezing and thawing of adult worms was used in this work to study some properties of surface membrane proteins of the parasite. It is termed the frozen-thawed supernatant (FTS). Electron microscopic studies (Ramalho-Pinto, Goldring, Smithers and Playfair, 1976) and immunodiffusion analysis (Murrell, Vannier and Ahmed, 1974) have indicated the presence of membrane proteins in this fraction. The studies reported herein, added further evidence that the FTS fraction does share some characteristics with the surface membrane fraction from adult worms. In addition, the presence of BP described previously in both the FTS and membrane fraction has encouraged the use of this fraction for such studies. The procedures used in identifying proteins in this fraction have provided the following information: It contains a complex mixture of proteins and glycoproteins, which might be released with other surface membrane protein components into the blood circulation, perhaps through the membrane turnover phenomenon.

Immunoglobulins of host origin are found to be present in the FTS fraction. The most noticable feature of this fraction is the presence of BP focused at pH between 7.2-8.2 on IEF gels.

These observations outline the general characteristics of protein components in the FTS fraction studied. Further studies are required for better understanding of the significance of antigens in the FTS fraction in immunity to schistosome infection. These include:

(a) quantitative determinations of the specific antigens in the FTS fraction that would be recognized by the host on infection with schistosomes.

(b) Determination of the relationship between membrane proteins released from adult worms as a result of membrane turnover and those present in the FTS fraction.

(c) Studying the expression of specific antigens in this fraction at different developmental stages of the parasite.

Purification of BP from the FTS fraction was attempted to obtain further information about this specific membrane protein. The use of QAE-sephadex column failed to yield purified BP. This failure could be due to the presence of BP bound to other proteins to form complex mixtures in the FTS fraction. However, some characteristics of the eluted peak proteins were consistent with that of the FTS fraction. Antisera against eluted proteins were prepared in rabbits, but showed no binding to the surface membrane of schistosomula of S. mansoni. This might indicate that proteins in that preparation (including BP) could not be expressed on the outer surface of schistosomula or they form minor components of the schistosomular surface membrane.

The hypothesis that there exists antigenic polymorphism among individual clones of S. mansoni was first suggested by Smith and Clegg (1979). This hypothesis was based on evidence that individual clones

of S. mansoni showed high levels of susceptibility to immunity stimulated by a primary bisexual infection, or were not susceptible at all. The finding reported herein, that primary infections of mice with individual clones of S. mansoni have led to variable levels of infection (as judged by the portal perfusion assay) strengthened the above hypothesis. The possibility that BP demonstrated in the membrane fraction might represent the variable antigen was tested by examining the IEF spectra of the FTS fractions from individual clones of the parasite. It was evident that BP is synthesized by all clones tested for, but either present in different amounts or synthesized at different rates. Further investigations into the rate of synthesis of surface membrane proteins in adult worms and schistosomula derived from individual clones revealed an interesting finding. Some clones have a high rate of metabolism compared to the others. In addition, clones which showed high levels of radioactive methionine incorporation at the schistosomular stage appeared to yield high percentage of recovery of adult worms when used for infecting mice. This observation could be compatible with the findings reported by other investigators (Rai and Clegg, 1968; Bruce, Weiss, Stirewalt and Lincicome, 1969; Smithers, 1976). All these studies have indicated that the rate of metabolism of the cercariae is an important factor for successful penetration of host's skin and probably establishment of the infection. The high yield of recovery of adult worms from clones with high metabolic activities may suggest the following:

(a) Acquisition of host or host-like molecules by schistosomes has been shown to occur at the same time as the parasite becomes inaccessible to the host's immune response (Clegg, 1974; McLaren, Clegg and Smithers, 1975). The mechanism of the acquisition of host antigens and evasion of the immune response has not been clarified yet. Thus, it is conceivable that clones with high rate of metabolism may be less susceptible to the immune damage because they acquire host antigens at higher rates. Therefore, clones with high metabolic activities

establish better infection than those with low metabolic activities. (b) It is believed that the continuous turnover of schistosome surface membrane could be related to the protection acquired by the parasite against the host's immune response (Wilson and Barnes, 1974a, 1977). Since membrane turnover is a metabolic process (Kusel, 1970, 1972), it is likely that the high rate of metabolism would result in high rate of membrane turnover and consequently better protection against the host's immune attack.

These kind of explanations are possible, but further careful investigations into the nature of differences between individual clones with respect to these aspects, i.e. acquisition of host antigen and membrane turnover, would possibly help to select clones likely to be susceptible to immunity. Such studies might present a new approach to some of the unanswered questions concerning immunity to schistosome infection.

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Appendix

This work has been undertaken in collaboration with Dr. Janet Jones. It had been presented orally by Dr. Janet Jones in the spring meeting of the British Society for Parasitology, 1980. The paper has been sent for publication during the time of writing this thesis.

Schistosoma mansoni: Genetic variation in murine responses to infection

Janet T. Jones, Batool El Adhami, and John R. Kusel.

Biochemistry Department
University of Glasgow
Glasgow G12 8QQ

ABSTRACT

A comparison has been made of the responses of seven inbred strains of mice to primary infection by Schistosoma mansoni. The strains used, including two different substrains of C57BL, were CBA/Ca, BALB/c, DBA/2J, C3H/He, A/Ola, NIH, C57BL/Tb, and C57BL/6/Ola. Differences were observed between strains in mortality, the distribution of eggs in the tissues, splenomegaly, and faecal egg counts. The differences between strains in worm burden were not significant, and the pattern of weight change showed little variation. The rank order of the strains in each response showed some similarities; the CBA/Ca mice for instance had a high mortality, the faecal egg count and splenomegaly were high, and there were a great many eggs in the tissues. In contrast, C57BL/Tb and NIH mice had a low mortality, low faecal egg counts, and fewer eggs in their tissues. Strains which differ markedly in responses to infection will be useful in investigating the host-parasite interaction, and particularly in analysing the host immune responses.

Index Descriptors: Trematode, Schistosoma mansoni, mice, inbred strains, faecal egg count, splenomegaly, mortality.

INTRODUCTION

Animals which show inherited differences in their response to infection provide useful models for investigating the mechanisms required for resistance to that infection. Different inbred strains of mice have been shown to vary in their responses to a variety of parasitic infections (for review, see Wakelin, 1978). Recent evidence indicates that inbred strains of mice vary in their ability to resist a secondary (challenge) infection by Schistosoma mansoni (Bickle et al, 1980; Murrell et al, 1980). Responses to schistosomiasis of mice which differ in the genes of the major histocompatibility complex have been described by Claas and Deelder (1979). Stocks of mice which have inherited differences in their levels of antibody production (Biozzi mice) have been infected with S. mansoni, and their responses have been shown to vary by Blum and Cioli (1978). However, no systematic study of the course of a primary chronic infection, and of its effects in different inbred strains of mice has been described. Accordingly, we undertook the present study in the form of two strain surveys, which included seven inbred strains of mice, including two different substrains of C57BL mice, the BALB/c strain being included in both experiments. The parameters of infection described here were chosen to represent what might be called the clinical symptoms of the disease; in a subsequent paper we shall describe the correlation of these parameters with antibody levels in these mice.

Materials and Methods

Mice

Table 1 gives the details of the mice used in the two strain survey experiments. They were all males. Some of the groups were rather small, but it was felt important to cover as many strains as possible in these initial experiments, so that extreme strains could be selected for further study.

Parasites

The cercariae used for infection were from a Puerto Rican strain of *Schistosoma mansoni* maintained in this laboratory in *Biomphalaria glabrata* and several different mouse strains. The experimental mice were infected subcutaneously between the scapulae with 81 $\pm$ 6 cercariae in either 0.1 ml or 0.15 ml of copper-free water, to which 100 iu/ml of penicillin and streptomycin had been added.

Weight change, worm burden, splenomegaly, and distribution of eggs in tissues

During the course of the infection, the mice were weighed and bled at fortnightly intervals. At the end of the experiments, 120 days after infection, or at death if they were moribund, the mice were assessed for worm burden, splenomegaly, and the distribution of eggs in the tissues. Adult worms were perfused from the portal and mesenteric veins in the manner described by Smithers and Terry (1965), and the number of male and female worms counted. The spleen was weighed and the ratio of spleen weight to total body weight was calculated. A small sample of tissue from each of six organs, the gut (small intestine), liver, spleen, lung, kidney, and brain, was taken and squashed between two microscope slides. The same part of the organ was sampled in every

case. Each of these squashes was scored for numbers if eggs present on a scale of one to four by two observers independently.

Faecal egg counts

Faeces for egg counts were collected three times during the course of the first experiment, at 80 days, 98 days, and 119 days after infection, and five times during the second experiment, at 44, 62, 72, 86, 100, and 114 days after infection. One to three faecal pellets, weighing usually between 10 and 100 mg were collected from each mouse between 9 and 11 am and were immediately weighed and dispersed in 5 ml of normal saline. The suspension was allowed to sediment for 15 min, and then washed with a further 5 ml of normal saline. After the second sedimentation, the faecal material was washed through two layers of cotton gauze supported on a stainless steel mesh, onto a 5.5 cm circle of Whatman No.54 filter paper in a Buchner funnel. The filter papers were stained by dipping the back of the paper into an 0.25% aqueous solution of ninhydrin. They were then dried. The eggs were counted using a dissecting microscope after dampening the filter papers with saline to flatten them onto a glass slide.

The number of eggs per gram was calculated, and a log transformation was done on all of the counts. For each mouse, an average count and a maximum egg count were calculated. Because of the variation in egg output during the course of the infection, the maximum reached seemed to be more characteristic of an individual animal's infection than the average output. The maximum egg counts for the animals of a strain also showed a more homogeneous variance than the average egg counts so this measure was used in a one-way analysis of variance to detect a strain effect in each of the experiments. Sheffe's method for multiple comparison of means (Miller, 1966) was used to assess significance.

Results

Mortality

There were large differences between strains in the numbers of deaths caused by the infection, as indicated in Table 2. The strains seemed to fall into three groups. The first, CBA/Ca and A/Ola, had a high mortality. In the second, consisting of BALB/c, DBA/2J and C3H/He, about half the mice died, and the third group, consisting of NIH and the two C57Bl substrains, had a very low mortality. Considering the animals which died of the infection, the number of days survived after infection was highly variable within strains, and there were no significant differences between strains.

Worm Burden

The mean total numbers of worms obtained by perfusion are shown in Table 3. The within strain variation was high, and there are no significant differences between strains. There is a possible bias in the worm burden data which arises from the methodology of the experiment. Only animals which survived until the end of the experiment, or which were seen to be moribund, could be perfused; if animals were found dead in the cage, the blood had clotted in the veins, and perfusion could not be done. Animals which were perfused before the end of the experiment had, on average, higher worm burdens. (The mean for 12 animals of all strains perfused before the end of the experiment because they were moribund was 21.7 ± 15.1. The mean for 46 animals of all strains which were perfused at the end of the experiments was $9.1 \pm 9.$) The missing data thus tend to be from high mortality strains, and possibly from animals with higher worm burdens.

Tissue distribution of eggs

The numbers of animals which had eggs in various tissues, and the scores for the tissues, are shown in Table 4. In almost all of the animals of all of the strains, the scores in the liver and gut were high. In contrast, relatively few animals had eggs in the kidney and brain. The strain in which eggs were most often found in these sites was CBA/Ca. One A/Ola mouse had an egg in the brain, one DBA/2/J had an egg in the kidney, and two BALB/c's had an egg in each of these organs.

The organs which seemed to reflect the greatest variability between strains were the spleen and the lung. In the case of the lung, CBA/Ca, C3H/He, and BALB/c mice showed the most frequent and heaviest deposition of eggs; in the A/Ola and C57Bl/Tb mice eggs were infrequently found in the lung. The strains in which eggs were most frequently found in the spleen were C57BL/6/Ola, CBA/Ca, and DBA/2J, with C57BL/Tb being the lowest strain in this respect.

It is notable that the two C57BL substrains differed in the degree to which eggs were found in the tissues, with C57BL/Tb mice having fewer eggs deposited in their tissues than C57BL/6/Ola mice.

Weight change

The mean weights of all infected animals of each strain are shown in Figure 1. It is apparent from this graph that weight loss does not seem invariably to accompany infection. Furthermore, the average weight of the surviving mice of each strain was similar to the average weight of normal controls of the same strain, age, and sex. The data as presented in Figure 1, however, fail to emphasize the following factors: 1) for those strains in which deaths occurred, the mean weights are calculated for decreasing numbers of animals during the course of the experiments, and 2) the pattern of weight change in those animals

which survived the infection was different from that of animals which died from the infection. Figure 2 shows the pattern of weight change for three individual mice of different strains, indicating that a drop in weight often preceded death from the infection. The general pattern seemed to be that those animals which survived the infection maintained a steady weight; those which died of the infection often, but not always, showed a drop in weight. The pattern was not different for the different strains of mice.

The fact that two of the heavier strains of mice (NIH and C57BL/Tb) had a low mortality suggested that perhaps the weight of the mouse at the time of the infection would influence its chances of survival. Figure 3 clearly indicates that within strains, the body weight of the mouse at the time of infection does not influence its chances of survival.

Splenomegaly

The degree to which spleen weight increased over normal varied in the different strains, as shown in Table 5. The CBA/Ca strain showed the greatest increase in the spleen weight to body weight ratio, and of the strains for which there were adequate controls, the C3H/He strain showed the lowest increase. The data for four of the strains are incomplete, because of the lack of suitable controls, i.e. mice of the same age and sex which were in our animal house at the same time. The DBA/2J control animal indicated was one from the experimental group which was found at the end of the experiment not to have been infected. The control values indicated for the NIH mice are from females, and those for the A strain mice are for males of a different substrain.

It is also apparent from the table that the within strain variance of the spleen weight to body weight ratio of the infected mice was much higher than that of the normal mice.

Faecal Egg counts

The numbers of eggs per gram faeces were measured for each animal three times during the first experiment, and five times during the second experiment. During the course of each experiment animals were dying, so the egg counts for the strains in which deaths occurred were representing only the survivors. The pattern which successive egg counts followed was different both for individual animals and for different strains. In some strains (CBA/Ca and BALB/c) the pattern seemed to be that some animals maintained a fairly steady output of less than 1000 eggs per gram, and these animals were more likely to survive. Other animals of these strains had a very high egg output, more than 1000 eggs per gram, and these animals usually died. In other strains, (A/Ola and C3H/He) death was less frequently preceded by high faecal egg counts; in fact two of the A/Ola strain mice died at 57 and 61 days after having shown no faecal egg output at 44 days, and while egg counts of other animals of the strain were still very low. The NIH and C57BL/Tb strains had consistently low faecal egg counts and no deaths occurred.

Because of the missing egg counts from the mice which died, and because of high variation between counts done at different times on the same animal, analysis of the egg count data was based upon only one parameter of output. As the counts had been made during the course of the infection when egg counts were rising from nil to a maximum, and in some cases falling again, the variance of the average egg counts was high, and was significantly different for different strains. When the maximum egg count was taken as the characteristic of each animal, the variances were smaller, and were similar enough between strains to allow a one-way analysis of variance to be done. The strain means of the maximum log egg counts for each animal are shown in Table 6, and

values which are significantly different are indicated. It should be noted that in this respect, as in others, the two C57BL substrains are somewhat different.

Discussion

These experiments were designed to look for genetic variation in responses to infection by S. mansoni. Seven different strains of mice were used, and we report in this paper the results of the measurement of six different responses to the infection: weight change, worm burden, splenomegaly, faecal egg counts, mortality, and tissue distribution of eggs.

Weight loss has sometimes been observed in animals infected with schistosomes. Doenhoff et al (1979) reported that CBA mice which had been infected with 200 cercariae of S. mansoni lost weight from about 40 days after infection. Preston, Dargie and McLean (1973) reported weight loss in sheep infected with S. mattheei. Our observation is that those animals which survive the infection (which might be called chronically infected) do not seem to lose weight, whereas those animals which are more severely affected by the disease, either of a high worm burden, or because of their genetic constitution, sometimes, but not always, lose weight before they die. The pattern of weight loss in our mice was similar to that observed by Dargie et al (1977) in sheep with S. mattheei infections. In their experiments, animals with chronic infection did not show weight loss, but the two sheep which died did lose weight before death. The CBA mice used by Doenhoff et al (1979) had received a dose of cercariae which would have been lethal for all the mice, so the pattern of weight loss was similar to that shown in our experiment and that with sheep reported by Dargie et al (1977).

Fadl (1971) noted that when rats of two different strains were infected percutaneously with S. mansoni, there was a significant correlation between the body weight of the rat at the time of infection, and the numbers of worms recovered. In his study it was unclear whether this correlation was due to the body weight differences, or to the age of the rats, which also correlated with the body weight. Our mice were all of similar age, but varied in weight both within and between strains. We found no correlation between weight at the time of infection and the worm numbers recovered either within strains or between strains. Our data also indicate that within strains, the body weight at the time of infection does not seem to influence the animals chances of survival. It should be noted, however, that rats and mice differ in their responses to S. mansoni; adult worms in rats can be killed by the immune response, so chronic infection is less easily established (Phillips et al, 1975).

We did not find evidence in these experiments of strain differences in the number of adult worms which establish after a subcutaneous injection of cercariae. There have been three studies in which the numbers of adult worms recovered was different in mice of different genetic constitution. Stirewalt, Shepperson and Lincicombe (1965) studied parasite penetration and maturation in mice of one inbred strain, an outbred stock, and two stocks carrying mutations. They found that these stocks of mice differed in the number of cercariae which penetrated, and in the maturation of the parasites which penetrated. Colley (1972) found that there were differences in worm burden when he infected different inbred strains. Blum and Cioli (1978) infected two stocks of outbred mice which differ in their level of antibody responses (Biozzi high and low responders) and found that the stocks differed in the numbers of worms recovered. It seems likely that our study failed

to show differences in worm burden for two reasons: 1) We deliberately used a relatively low dose of cercariae in order to induce a chronic infection, so the numbers recovered were relatively low and had a high variance, and 2) we used subcutaneous injection as a route of infection, deliberately to eliminate strain differences in the penetrability of the skin. It is possible that a differently designed experiment would demonstrate either differences in the penetrability of the skin of different strains of mice, or if a higher dose of parasites were to be given subcutaneously differences in worm maturation might be found.

We have found that the different strains of mice vary markedly in the degree of splenomegaly developed in response to schistosome infection. Other studies on the splenic response have shown that it can be influenced by at least two different host factors, T-cell function and histocompatibility type. Doenhoff et al (1979) found that CBA mice which had been T-cell depleted by thymectomy and treatment with anti-thymocyte serum did not show an increase in spleen weight to body weight ratio up to fifty days after infection, while the values for normal controls increased two to three fold. Phillips et al (1977) studied the response of congenitally athymic (T-cell deficient) mice to schistosomiasis, and showed that the athymic mice developed considerably less splenomegaly than either normal controls or athymic mice which had been artificially reconstituted with T-cells.

Claas and Deelder (1979) used H-2 congenic strains of mice, that is, animals of a similar genetic constitution except for a difference at the major histocompatibility locus, to study responses to schistosome infection. They found that the two strains differed significantly with respect to splenomegaly, the animals of $H\text{-}2^{k}$ type showing a greater degree of splenomegaly than the animals of $H\text{-}2^{b}$ type. In the human response to schistosomiasis, Salam, Ishaac and Mahmoud (1979)

have found that the presence of splenomegaly correlates with the presence of two HLA determinants, A1 and B5. Our results seem to indicate that there are other factors in the genetic background as well as the histocompatibility type which influence the splenic response. The strain which showed the greatest degree of splenomegaly, CBA/Ca, was an H-2^k strain, but so was the C3H/He strain which showed a rather lower degree of splenomegaly.

There are several mechanisms which may contribute to the increase in spleen weight to body weight ratios which is observed in animals with schistosome infections. These are 1) granuloma formation around the eggs which have been deposited in the spleen, 2) increased portal pressure which is observed in infected animals, and 3) germinal centre formation and white pulp hyperplasia due to the immune response to a) the worms and b) the eggs. In our mice the degree of splenomegaly did not seem to correlate with the actual number of eggs found in the spleen, i.e. it was probably not to any great extent due to granuloma formation around the eggs themselves. Dumont et al (1975) studied the increase in size of pieces of spleen transplanted to subcutaneous pockets in infected mice and concluded that increased portal pressure did not play a large part in the increase in spleen weight, and that it was mainly due to germinal centre formation and white pulp hyperplasia. Both Doenhoff et al (1979) and Claas and Deelder (1979) have shown that the spleen weight to body weight ratios begin to increase at about 40 days after infection in mice, which is about the time that eggs begin to appear in the faeces. We have observed that in mice with single sex infections, the degree of splenomegaly is very much less than in animals carrying worms of both sexes and showing excretion of eggs (unpublished data). We conclude that a large part of the splenomegaly is related to the immune response to the eggs.

The strains of mice used in this study showed significant differences in the faecal egg counts resulting from the infection. The faecal egg count is presumably influenced by several factors: the number of pairs of worms, the rate at which eggs are being laid by them, the degree or rate of sequestration of the eggs in the gut wall and other tissues, the degree of hydration of the faeces, and the total faecal output of the mouse. These factors are influenced by a number of different responses in the host. One of these is T-cell function, as reported by Doenhoff et al (1979) who found that T-cell deficient mice showed much lower faecal egg counts in the early stages of infection than did normal controls. The factors underlying the strain variation which we observed may be related to such things as T-cell function and the rate of granuloma formation; the mechanism behind these differences requires further investigation. The faecal egg count has been shown to be of great importance to the clinical manifestation of the disease: in sheep with similar numbers of worms, animals with lower faecal egg counts have less severe clinical symptoms (Dargie et al, 1977).

The numbers of mice which died during the course of the experiment was widely variable between strains. Claas and Deelder (1979) in their study of mice of H-2^b and H-2^k types on a C3H background found that the H-2^b mice had a higher mortality. This contrasts with our findings, that both of the H-2^b substrains used (C57BL/Tb and C57BL/6/Ola) had a low mortality. We conclude that there must be other factors in the C57 genetic background which must override the effects of the H-2^b locus. The time at which animals died after infection was quite variable within strains, but the A/Ola mice tended on average to die rather earlier.

It should be noted that in many animals, but not all, we noticed what seemed to be a threshhold pattern of response leading to

death. That is, the animal showed a high faecal egg count, then started to lose weight, became anaemic, and then died. These animals were often those with high worm numbers. This pattern was less often observed in A/Ola mice, suggesting that the pathology of the disease may be different in that strain.

The sequestration of eggs in the tissues is clearly a response which is worth investigating in more detail. Doenhoff et al (1979) have shown that animals which are T-cell deprived have a lower degree of sequestration of eggs in the liver, and that it starts later than in normal controls. This may be related to either the rate at which the worms are laying eggs, or to the mechanism of trapping in the tissues. Our findings indicate that especially in the lungs and spleen there are clear strain differences in egg sequestration, and more accurate measurement of the numbers of eggs trapped, and the timing of their sequestration, are underway in this laboratory.

The experiments described in this paper are a pilot study which we undertook in order to find out which strains of mice differ most markedly in their responses to schistosomiasis, and which aspects of the response to primary infection are most variable. It is interesting that there were some similarities in the ranking of the different strains in those responses which were variable. In CBA/Ca mice mortality and faecal egg counts were high, splenomegaly was pronounced, and there were many eggs in the tissues. In contrast, C57BL/Tb and NIH mice had a low mortality and faecal egg counts, with fewer eggs in the tissues. It is hoped that the strains which are most distinct will be of use in investigating the mechanisms of the immune response to the disease. The practical value of these investigations lies in two areas. Firstly, knowledge of what constitutes an effective immune response, that is, one which is protective against reinfection, which is damaging to the worms,

and which is not deleterious to the host will be of use in designing a vaccine to the disease. Secondly, elucidation of the genetic factors influencing responses is of possible application in the field of livestock breeding, as schistosomiasis is a disease of great agricultural importance.

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Table 1. Numbers, age, and origins of mice of different strains used for infection with Schistosoma mansoni in the two experiments.

Strain	Number	Expt.	Age at Infection (da)	Source	H-2 type
BALB/c	10	I	42	Biochemistry Physiology Animal House, Glasgow Univ.	d
CBA/Ca	9	I	43-57	Bantin and Kingman	k
C57BL/Tb	5	I	58-71	Institute of Genetics Glasgow Univ.	b
DBA/2J	7	I	50-62	Institute of Genetics Glasgow Univ.	d
NIH	10	I	53	Anglia	q
A/Ola	11	II	70-84	Olac, 1976 Ltd.	a
BALB/c	10	II	40	Biochemistry Physiology Animal House, Glasgow Univ.	d
C3H/He-mg	10	II	70-84	Olac, 1976 Ltd.	k
C57BL/6/Ola	10	II	70-84	Olac, 1976 Ltd.	b

Table 2. Numbers of mice of each strain which died from the Schistosoma mansoni infection. The average survival time of these mice is also given.

Strain	Expt.	Mice which died from the infection: Number		%	Mean number of days between infection and death ± s.d.
CBA/Ca	I	7/9	1)	78	92 ± 18
A/Ola	II	8/11	2)	73	79 ± 23
BALB/c	I	5/10		50	85 ± 12
	II	3/8		38	108 ± 15
DBA/2J	I	3/7		43	91 ± 17
C3H/He	II	4/10		40	99 ± 17
C57BL/6/Ola	II	1/6		17	81
C57BL/Tb	I	0/5		-	-
NIH	I	0/9		-	-

1) Significantly different from NIH P 0.01

2) Significantly different from C57BL/6/Ola P 0.01

Chi squared test with Yates' correction.

Table 3. Mean number of adult worms perfused from mice of different strains after infection with Schistosoma mansoni

Strain	Expt.	Number of mice perfused	Mean total number worms ($\sigma^{\prime} + \varphi$) ± s.e.m.
A/Ola	II	5	18.0 ± 8.5
C57BL/6/Ola	II	7	15.6 ± 3.7
CBA/Ca	I	4	15.5 ± 3.6
DBA/2J	I	5	14.0 ± 8.7
NIH	I	10	11.3 ± 3.0
C3H/He	II	8	10.3 ± 2.4
C57BL/Tb	I	5	7.2 ± 1.9
BALB/c	I	9	7.6 ± 0.9
	II	5	4.2 ± 1.8

Table 4. Numbers of mice of each strain which had eggs in different tissues after infection with Schistosoma mansoni. For those with eggs present, the average score in the squash is indicated.

Strain	Liver		Gut		Spleen		Lung		Kidney		Brain	
	No.	Aver. Score	No.	Aver. Score	No.	Aver. Score	No.	Aver. Score	No.	Aver. Score	No.	Aver. Score
CBA/Ca	5/5	4	5/5	3.6	4/5	2.3	3/3	3.7	3/5	1.7	3/5	1.3
A/Ola	3/4	3.3	3/4	3.7	3/4	1.3	1/4	1	-	-	1/4	1
C57BL/6/Ola	5/5	4	5/5	4	5/5	2.6	3/5	2.3	-	-	-	-
C3H/He	8/8	4	8/8	3.5	5/8	1.8	6/8	3.0	-	-	-	-
BALB/c I	8/8	4	7/8	3.4	3/8	1.7	7/7	2.3	2/8	1	2/8	1
II	2/2	3.5	2/2	3.5	1/2	2	-	-	-	-	-	-
DBA/2J	4/4	3.3	4/4	3.3	3/4	2	3/4	2.3	1/4	2	-	-
NIH	8/9	3.9	7/9	3.9	3/9	2.7	3/9	1.3	-	-	-	-
C57BL/Tb	5/5	3.6	5/5	3.0	-	-	1/5	1	-	-	-	-

Table 5. Spleen enlargement in mice of different strains after infection with *Schistosoma mansoni*

Strain	Spleen wt (mg) / Body wt (gm) ± s.e.m. (no mice)		Mean, Infected Mice / Mean, Uninfected Mice
	Infected	Uninfected	
CBA/Ca	16.02±2.33 (5)	2.39±0.05 (6)	6.69
BALB/c	18.47±2.59 (10)	4.67±0.36 (9)	3.96
C57BL/6/Ola	10.23±1.66 (7)	2.80±0.17 (5)	3.65
C3H/He	16.56±4.10 (10)	4.69±0.29 (10)	3.53
DBA/2J	13.58±2.45 (4)	3.93 (1)	3.48
NIH	11.94±2.75 (8)	4.19±0.61 (6) (♀)	2.85
A/Ola	6.72±0.76 (4)	5.51±0.42 (8) (A/Nimr)	1.22
C57BL/Tb	6.36±0.41 (5)		

Table 6. Strain Averages of the maximum log faecal egg counts for mice infected with Schistosoma mansoni.

Experiment I

Strain	No. Mice	Mean of Max Log Egg Counts ± s.d.
CBA/Ca	8	7.101 ± 0.730
BALB/c	8	6.783 ± 0.577
DBA/2J	6	6.377 ± 1.046
NIH	9	5.357 ± 1.138
C57BL/Tb	5	5.221 ± 0.408

Brackets enclose values not significantly different from each other

BALB/c is significantly different from NIH

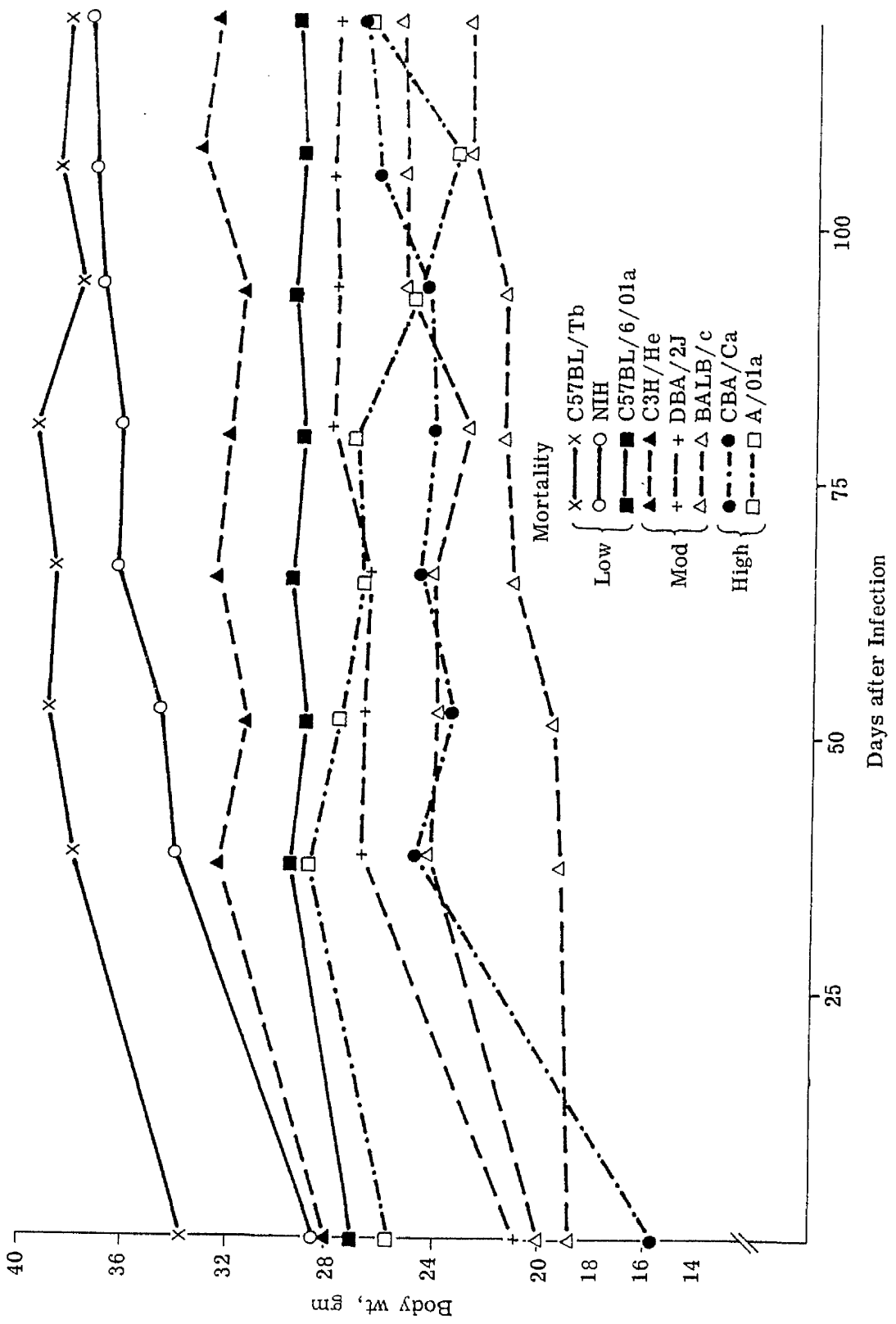
Experiment II

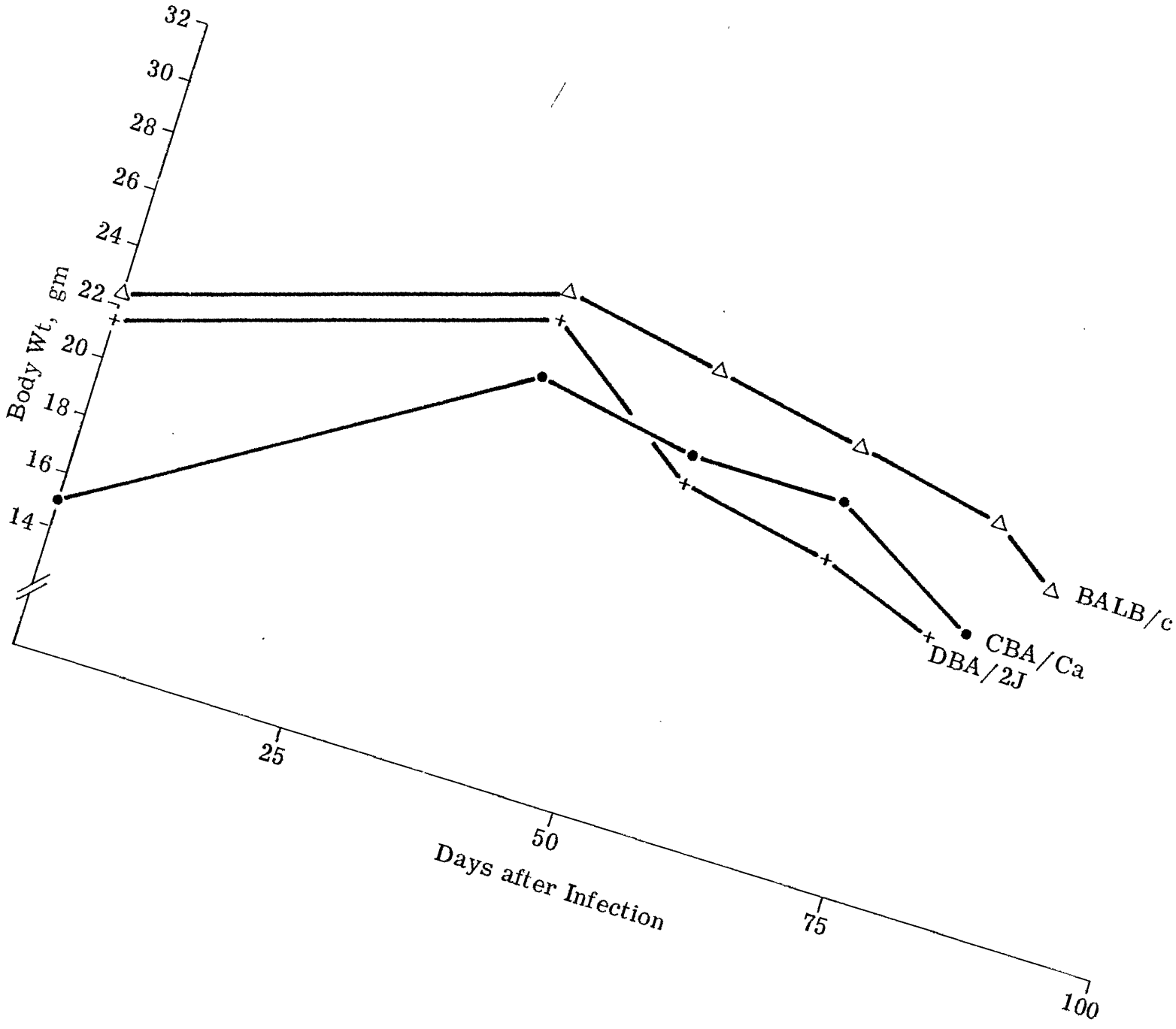
BALB/c	5	7.480 ± 0.676
A/Ola	6	6.734 ± 0.851
C57BL/6/Ola	6	6.594 ± 0.580
C3H/He	9	6.193 ± 0.908

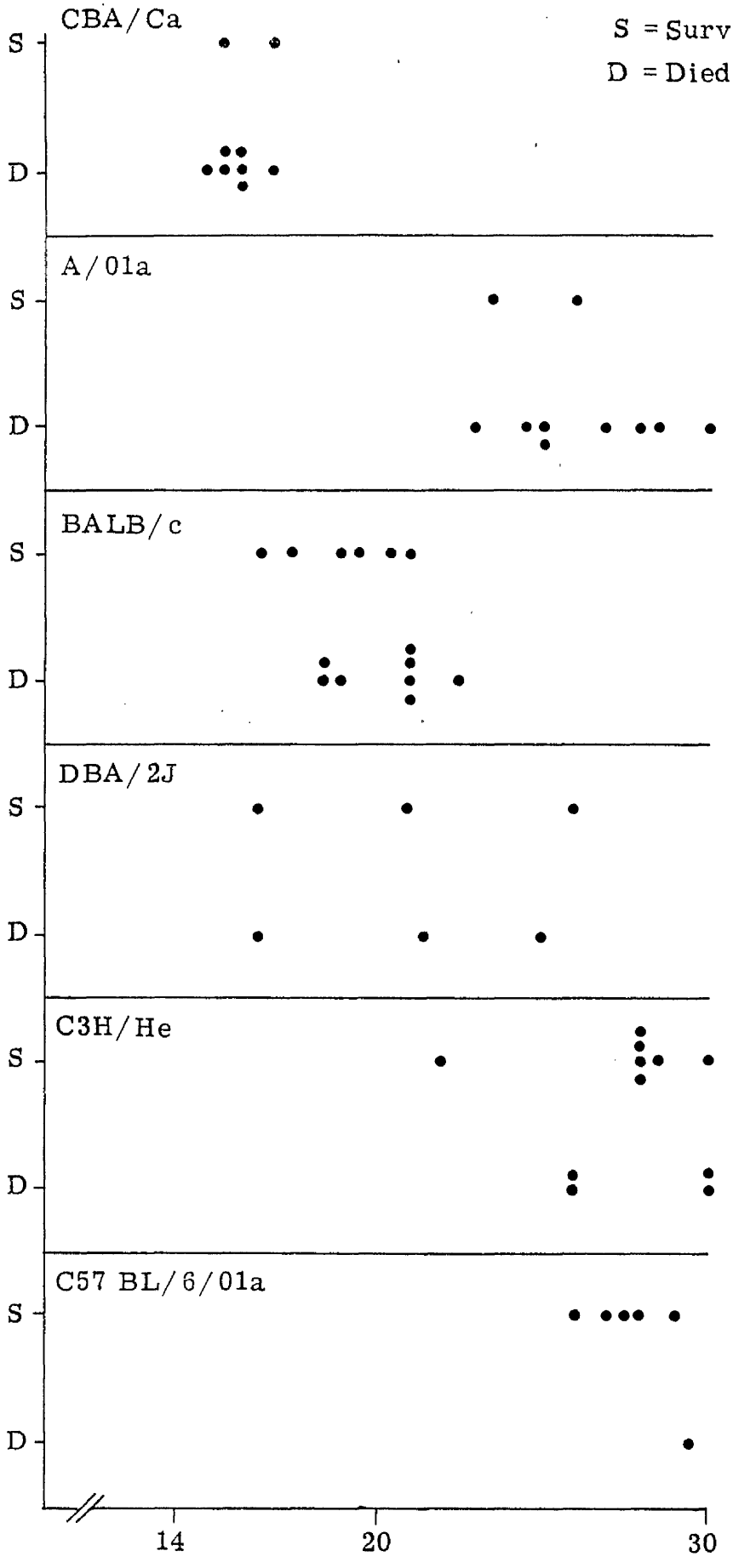
Fig 1 Schistosoma mansoni : Average weights of mice of different inbred strains during the course of infection. Strains which had low, moderate, or high mortality are indicated.

Fig 2 Weight change in three individual mice of different strains which died after infection with Schistosoma mansoni.

Fig 3 Relationship between survival and body weight at time of infection with Schistosoma mansoni in mice of different inbred strains.


Body wt, gm
40
36
32
28
24
20
18
16
14
25
50
75
100
Days after Infection
Mortality
Low
Mod
High
C57BL/Tb
NIH
C57BL/6/01a
C3H/He
DBA/2J
BALB/c
CBA/Ca
A/01a


Body Wt, gm
32
30
28
26
24
22
20
18
16
14
25
50
75
100
Days after Infection
BALB/c
CBA/Ca
DBA/2J


CBA/Ca
S = Survived
D = Died
S
D
A/01a
S
D
BALB/c
S
D
DBA/2J
S
D
C3H/He
S
D
C57 BL/6/01a
S
D
14
20
30
Body wt. at time of Infection, gm

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