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INDUCTION OF CHANGES IN ANTIGEN EXPRESSION AT THE SURFACE MEMBRANE OF ADULT MALE SCHISTOSOMA MANSONI

by

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ABBREVIATIONS

Abbreviations used in this thesis follow the Biochemical Journal Instructions to Authors (revised 1981) with the following additions :

BSA	bovine serum albumin
DHP	delipidated human plasma
DMSO	dimethyl sulphoxide
EMS	Eagles medium
EM + CS	Eagles medium + 10% (v/v) newborn calf serum
EM + DHP	Eagles medium + 10% (v/v) delipidated human plasma
EM + NHP	Eagles medium + 10% (v/v) normal human plasma
FITC	fluorescein isothiocyanate
HDL	high density lipoproteins
HSA	human serum albumin
IFAM	indirect fluorescent antibody method
IMP	intramembraneous particle
IRAM	indirect radiolabelled antibody method
LDL	low density lipoproteins
MHC	major histocompatibility complex
Na_2 EDTA	ethylenediaminetetra-acetic acid
NHP	normal human plasma
NMS	normal mouse serum
NP 40	Nonidet P-40
NRS	normal rabbit serum
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMSF	phenyl methyl sulphonyl fluoride
PPO	2,5-diphenyloxazole

PPP	PBS containing 100μM-PMSF and 1μM-pepstatin
RBC	red blood cell(s)
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylenediamine
TNP	trinitrophenyl
VLDL	very low density lipoproteins
WGA	wheat germ agglutinin

CONTENTS

SUMMARY

The adult schistosome surface membrane exposes a low density of parasite antigens to the immune system of an infected host. In an attempt to increase the expression of parasite antigens, schistosomes were incubated in the presence of a number of reagents and culture media each of which has the potential to interact with the parasite surface membrane with some specificity. By comparing the results obtained with the different treatments it was hoped to gain information relating both to the organisation of parasite antigens, particularly with respect to host antigens, within the membrane and to the properties required by drugs to increase expression of parasite antigens *in vivo*.

Reagents considered for use in the investigation were initially examined for their ability to perturb the surface membrane as measured by (a) transmission electron microscopy, or (b) their effect on membrane permeability (^{51}Cr release) and release of surface macromolecules (^{125}I-wheat germ agglutination release). Reagents which were considered to be causing gross membrane damage were rejected.

Changes in antigen expression were measured by a newly developed indirect antibody method. This technique is able to provide quantitative data by virtue of employing an ^{125}I-labelled second antibody as an indicator system. Parasite antigen expression was measured using an antiserum raised in rabbits against mechanically transformed schistosomula. This antiserum was shown by coprecipitation and SDS-PAGE to recognise a number of parasite membrane polypeptides. Host antigen expression was measured using an antiserum raised in rabbits

against mouse RBC. Attempts to characterise the antigen(s) detected by this antiserum at the schistosome surface indicated that it was (a) shared by worms derived from several mouse strains and hamsters; (b) not subject to coprecipitation employing anti-mouse RBC serum as first antibody.

It was concluded on the strength of this latter observation that the antigen was unlikely to be protein in nature.

Formalin, glutaraldehyde and trypsin - reagents which interact with protein molecules, were each found to increase the expression of parasite antigens at the schistosome surface. The effect of trypsin could be increased by raising the concentration of the enzyme to which the worms were exposed. Formalin and glutaraldehyde conversely promoted maximum increase in antigen expression at a concentration of 0.1% (w/v) and 0.01% (w/v) respectively. Raising the concentration of either aldehyde resulted in a reduced alteration in antigen expression. This result is discussed in relation to different concentrations of the aldehydes varying in their ability to form cross-links between and within proteins, and also to alter protein charge.

Lipophilic retinol (1mg/ml) and the non-ionic detergents Tween 40 (2.5% (v/v)) and Tween 80 (2.5% (v/v)) were each unable to alter the expression of parasite antigens at the schistosome surface. Tween 20 conversely, promoted an increase in expression of parasite antigens and a decrease in expression of host antigens. This reagent in contrast to the other three was found to disrupt the surface membrane as measured by release of ^{51}Cr and ^{125}I-WGA from pre-labelled

worms. It was therefore suggested that amphiphiles which are able to insert themselves into the lipid phase of the surface membrane may have to cause a critical amount of perturbation in order to promote changes in antigen expression.

Incubation of worms for 24h in medium containing calf serum or normal human plasma was found to cause an increase in expression of parasite antigens. By using purified lipoproteins, it was possible to show that the increase was in part lipoprotein-mediated. High density and low density lipoproteins were found to be more effective than very low density lipoproteins and it was therefore suggested, based on consideration of lipoprotein composition and function, that the detected increase might be due to transfer of cholesterol or phospholipid between the lipoproteins and the worms.

A report of the ability of praziquantel (10µg/ml) to increase the susceptibility of cestodes to host proteases *in vivo* prompted the idea that it might increase the expression of membrane antigens at the schistosome surface. This hypothesis was found to be correct. The mechanism involved in altering antigen expression and the implications of this result in relation to the mode of action of praziquantel *in vivo* are discussed.

Formalin and glutaraldehyde were both found to decrease the expression of host antigens at the parasite surface, maximum reduction taking place when using concentrations which were most effective at increasing parasite antigen expression. Retinol, Tween 40, Tween 80, praziquantel, mammalian plasma and serum had no effect on host antigen expression. An effect

of trypsin on host antigen expression is suggested by the finding that second antibody differs in its affinity for first antibody bound to trypsin treated and control worms. This change in antibody affinity was only observed when employing concentrations of enzyme which were able to alter parasite antigen expression (200-400µg/ml).

The finding that the concentration of both aldehydes and possibly trypsin which was most able to change parasite antigen expression was also most effective in altering host antigen expression was considered as evidence that the two antigen species were in close association or in close proximity to each other within the membrane. Other evidence supporting this idea is discussed. These results were considered to be consistent with and discussed in relation to the hypothesis that host antigens may mask parasite antigens at the surface of the adult schistosome thereby allowing the worm to evade the immune response of the parasitised host. The ability of reagents such as praziquantel to expose parasite epitopes in the absence of a simultaneous decrease in host antigen expression may indicate :

(a) that not all membrane parasite antigens are masked by host antigens ; or

(b) that host antigens can in certain cases be relocated within the membrane such that their reactivity for antibody is not diminished.

Finally, the implications of the results of this investigation are discussed in relation to promoting antibody-dependent rejection of worms <u>in vivo</u>.

CHAPTER 1

General Introduction

1. General Introduction

1.1 The Disease

1.1.1 Prevalence and socioeconomic importance

Schistosomiasis is a chronic debilitating disease which ranks second only to malaria in terms of public health and socioeconomic importance in tropical and subtropical regions of the world. In 1981, Iarotski and Davis on examining information obtained from 103 countries in reply to a questionnaire circulated by the WHO in 1976, calculated that almost 500 million people in 73 countries were exposed to the disease. In the African countries of Chad, Madagascar, Sudan and Togo, the proportion of the population considered to be at risk was greater than 80%.

Estimates as to the number of people at risk who are actually infected vary from 125 million (Wright, 1972) to 300 million (Jordan, 1975). Iarotski and Davis (1981) however, consider that any estimate of the prevalence of the disease is bound to be inaccurate as a consequence of unrepresentative sampling and the uneven distribution of the disease in the countries concerned. Wright (1972) in fact considers his calculated figure to be an underestimate.

An estimation of the total number of persons partially or totally disabled as a consequence of schistosomiasis was made by Wright in 1972. The figures obtained were 24,782,805 and 2,650,260 respectively. Wright also calculated that the annual loss in productivity as a consequence of this disability was equal to US $ 641,790,130.

This figure may be inaccurate but it is believed to be on the conservative side. In addition it does not take into account the sums required for medical care, public health programmes or compensation for illness.

1.1.2 Life-cycle of the parasite

The causative agent of schistosomiasis is a parasitic flatworm; a digenetic trematode, belonging to the genus Schistosoma. A number of distinct species exist, the three primarily responsible for human schistosomiasis being Schistosoma haematobium which is widely distributed in Africa and is also present in the Middle East; S. mansoni which is found in Africa, the Middle East and parts of South and Central America; and S. japonicum which is restricted to the Far East. Each species has a similar, complex, life cycle (Fig. 1), involving a number of free living and parasitic stages; sexual and asexual reproduction; and two distinct parasitised hosts. The species employed in this investigation is S. mansoni and for this reason its life cycle will be described in some detail.

Adult S. mansoni live in the mesenteric veins of the infected mammalian host. The worms are dioecious, the female being held in a groove in the ventral region of the male body, referred to as the gynaecophoral canal, during copulation and oviposition. The male worm varies between 6 and 12mm in length and the longer more slender female between 7 and 17mm. Both sexes have oral and ventral suckers which may assist in attaching the worms to the blood

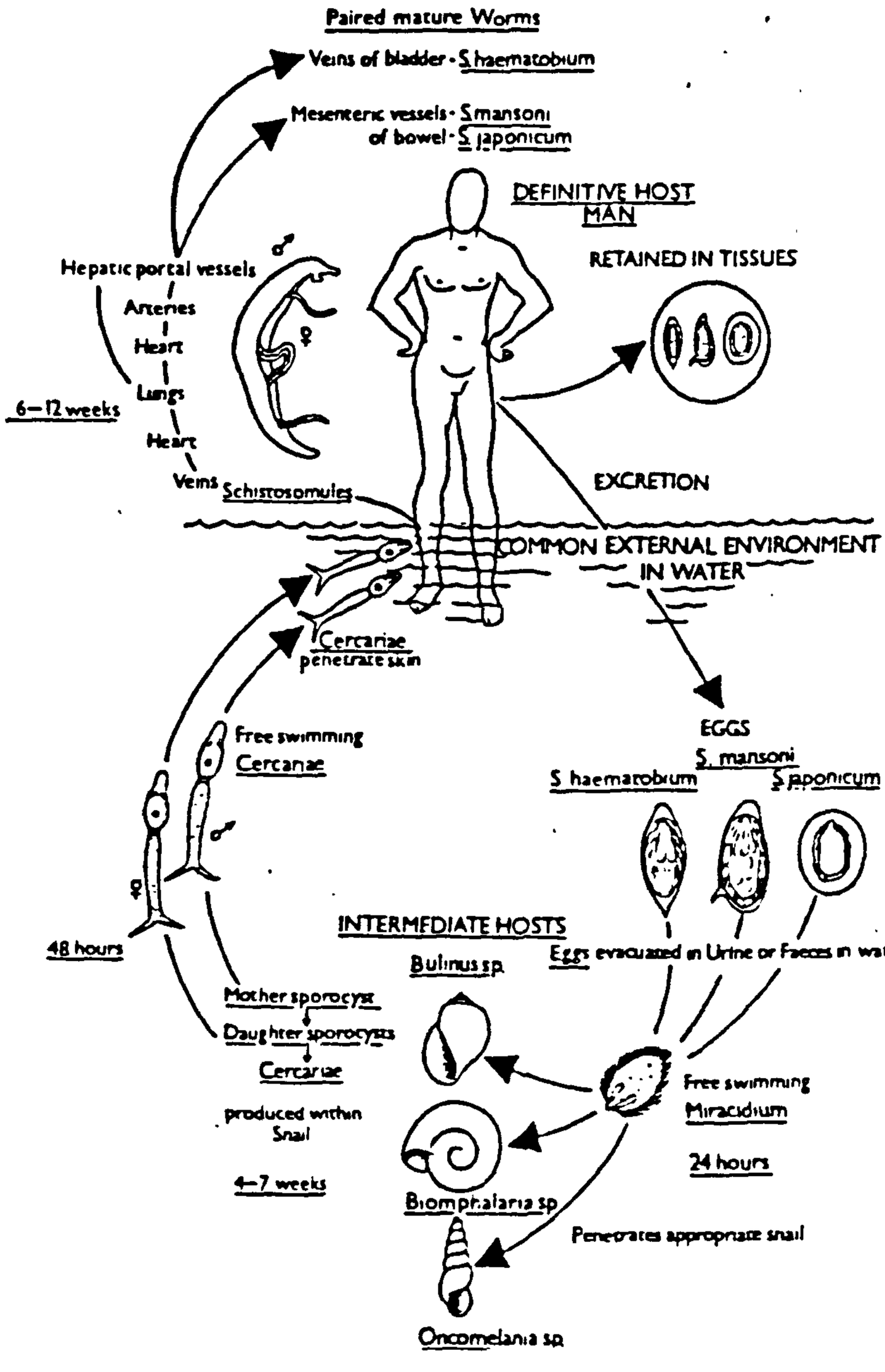


FIG. 1. The Life-cycle.

vessel wall.

Each female S. mansoni produces between 100 and 300 immature eggs per day. These eggs are about 114-175µm in length by 45-68µm in diameter and have a large characteristic lateral spine. The released eggs, perhaps aided by the spine and proteases, pass through the blood vessel wall to enter the wall and finally the lumen of the intestine. From here, by now usually mature, they are excreted in the faeces. Not all eggs are excreted in this way however as many become entrapped in the intestine and other tissues of the body to which they may be carried by the movement of blood. The immune response of the host to such eggs is the initial step in the development of most of the pathological consequences and clinical manifestations of schistosomiasis. The immunopathology of the disease has recently been reviewed by Smithers and Doenhoff (1982).

Introduction of eggs to water under suitable environmental conditions results in their hatching and the release of a free living larval stage known as a miracidium. The miracidium is equipped to find and penetrate the soft tissues of the intermediate host, a fresh water snail, which in the case of S. mansoni is a member of the genus Biomphalaria. Following penetration, the miracidium transforms into a mother sporocyst which then proceeds to asexually produce a number of daughter sporocysts. The daughter sporocysts migrate to the liver or ovotestes of the snail where, following a further period of asexual reproduction, they give rise to the second free living stage of the parasite, the cercariae.

The cercaria is the stage of the life cycle which is infective for man. Following their shedding from snails cercariae move through water with the aid of a locomotory tail until they make contact with the surface of human skin. The cercariae then attach to the skin and force their way through it by a combination of physical abrasion, lytic secretions and mechanical disruption (Bruce et al, 1970; Stirewalt and Dorsey, 1974). Upon entry to the mammalian host the tail is shed and the parasite is now referred to as a schistosomulum. This newly transformed organism differs from the cercariae in being unable to survive in water (Clegg and Smithers, 1968); being able to survive in saline or serum (Stirewalt, 1963); being receptive to staining with acid colloidal iron (Stein and Lumsden, 1973); having affinity for concanavalin A and wheat germ agglutinin (WGA) (Murrel et al, 1978a); and having a surface which is insensitive to the action of urea (Kusel, 1970). These changes are no doubt associated with the organism becoming physiologically adapted to its new environment.

Schistosomula remain in the skin for one to several days before migrating via the blood vessels, heart, lungs and liver to the portal vein where maturation to the adult form is completed. Mating between males and females then takes place before parasites proceed to their final habitat in the mesenteric veins. The worms may survive in the mesenteric veins for many years during which, the female worm will continuously secrete eggs.

1.1.3 Origin and spread of schistosomiasis

The finding by Ruffer (1910) of large numbers of calcified eggs in the kidneys of Egyptian mummies of the Twentieth Dynasty (1250-1000 BC) indicates that schistosomiasis has been present in Egypt for thousands of years. The disease is not considered to have originated in Egypt however but rather to have arisen in the Great Lakes region of Central Africa (Wright, 1961; Nelson et al, 1962). From here it has become so widespread that it is present in all African states with the exception of Lesotho.

The presence of the disease in Egypt is presumed to be due to movement of the schistosomes and their intermediate hosts down the Nile. Entrenchment of the disease in other areas of the Arab world is presumably due to movement of infected individuals along trade and religious routes : the western and southern dissemination of the disease is considered to be due to the migration habits of various Bantu tribes during the last few centuries. The presence of S. mansoni in the Western Hemisphere is thought to be a consequence of the slave trade during the sixteenth to eighteenth centuries.

In 1981, Iarotski and Davis reported that schistosomiasis is continuing to spread in intensity and prevalence of infection in certain areas of the world including Ghana, Nigeria and Sudan in Africa; Brazil in South America, and the Phillipines in South-East Asia. The main reason for this is the creation of irrigation systems, man-made lakes and other types of project involving water supplies which are required for food production and the generation of hydro-

electric power. Since the economic progress of many Third World countries is dependent on the extensive use and development of water resources, it is likely that the spread of the disease will continue.

1.1.4 Control of schistosomiasis

The most common methods employed to control schistosomiasis include chemotherapy, health education, installation of water supplies or improvement of existing supplies, molluscicíding, and provision of sanitary facilities. Almost 50% of countries in which the disease is known to be endemic use more than three different methods of control in combination (Iarotski and Davis, 1981). That such combinations can be successful is shown by the reduction in prevalence of the disease in countries such as Japan (Yokogawa 1976a; Yokogawa 1976b), Puerto-Rico (Negran-Apante and Jobin, 1979) and Venezuela (Rey, 1978). A reduction in prevalence has also been reported in the Fayum oasis region of Egypt using a simple combination of molluscicides and drugs (Mobarak, 1978).

Although existing methods of control are, as shown above, attributed with a certain degree of success, they are also associated with a number of problems which prevent them from being totally satisfactory. Chemotherapy, the most common control measure in use, is hindered by the fact that schistosomicidal drugs are generally toxic, producing undesirable side-effects. For example niridazole and hycanthone - the most widely used drugs, are associated with adverse

neuropsychiatric side effects and acute hepatic necrosis respectively. Molluscicíding, another common control method, has the disadvantages of a) being very expensive;b) requiring a degree of special expertise ;and c) possibly upsetting the ecosystem in areas under treatment. In addition it has recently been reported that snails may develop resistance to molluscicides (Jelnes, 1977).

1.2 The target for host immunity

1.2.1 Introduction

The problems associated with existing forms of control ensure that the need for alternative methods such as immunological control is great. During the past two decades therefore the immunology of the disease has come under increasing scrutiny. As a consequence, it is now known that the longevity of schistosomes in the bloodstream of certain experimental hosts such as the mouse, hamster and rhesus monkey is not a product of an inactive or unstimulated host immune system as all three species have been found to develop resistance to reinfection, the completeness of which varies with the species (for a review, see Phillips and Colley, 1978) and in the case of the mouse, the strain (Dean et al, 1981a). Immunity in the presence of a living infection has been termed concomitant immunity by Smithers and Terry (1969) and in addition to being associated with non-human hosts, is likely, on the basis of epidemiologic evidence (Bradley and McCullough, 1973; Phillips and Colley, 1978) to exist in the natural host, man. Thus, although schistosomes may survive in man for up to 30 years (Berberian et al, 1952), their presence is likely to prevent or reduce additional infection.

The mechanism(s) by which the adult worm is able to evade the immune response of the host has been the subject of much investigation and this will be described in sections 1.4 and 1.5. However, as a first step, the nature and target of hostimmunity will be considered as this information is relevant to gaining an understanding of the evasion mechanism. Information relating to the nature of the response will be considered in section 1.3; this section will be concerned with the target.

1.2.2 The early schistosomulum,

Sher (1977) has shown that transfer of immune mouse serum, which normally donates resistance to recipients, has no effect when administered later than 17 hours after a challenge infection. *In vitro* studies suggest that schistosomula develop an ability to evade the host immune response with maturation, as witnessed by the susceptibility of newly transformed schistosomula, but not four day lung schistosomula, to the cytotoxic effects of antibody plus complement (Clegg and Smithers, 1972), antibody and/or complement plus eosinophils (Ramalho-Pinto *et al*, 1978), or complement alone (Santoro *et al*, 1979). Indeed McLaren and Incani (1982) have shown that schistosomula are significantly less vulnerable to *in vitro* immune mechanisms as early as three hours after skin penetration. Taken together, these results suggest that immunity *in vivo* may only be active as the parasites migrate through the skin or perhaps also, from the skin to the lungs. Evidence for and against this proposal is discussed below.

1.2.2.1 Histological studies

The existence of active immunity against reinfection in the skin of a previously infected animal was originally demonstrated by Magalhoes -Filho and Barras-Coelho in 1957. These workers showed that schistosomula were subjected to a damaging cell-dependent immune response as they traversed the skin of mice which had been previously exposed to cercariae but not control mice. This result was confirmed by Colley *et al*, (1972), who reported the presence of schistosomula in various stages of degeneration in the skin of previously exposed mice. A different result was presented by Lichtenberg *et al*, (1976) however: these workers found very few (1 in 30 or 40) damaged schistosomula in the skin of previously exposed mice. Similarly Lichtenberg and Ritchie (1961) had previously found no difference in the survival rate of schistosomula in the skin of normal and resistant rhesus monkeys. A study by Davis *et al*, (1963) using *S. japonicum* however has shown that most of the schistosomula migrating through the dermis of immune but not non-immune rhesus monkeys are attacked and engulfed by host cells. Taken together, these results present a rather confusing picture. This may be due to the problems associated with estimating schistosomulum damage or schistosomulum numbers using histological techniques (Lichtenberg *et al*, 1976).

1.2.2.2 Recovery of worms from the skin

Smithers and Gammage (1980) have investigated whether schistosomula are killed in the skin of a previously infected mouse by comparing the number of schistosomula recoverable from the skin of normal and infected mice following cercarial penetration. This work has shown that by day two after penetration, 65% of the migrating schistosomula have died in the skin of both normal and previously infected mice, but an additional number equal to about 30% of the surviving parasites have also been killed in the skin of mice infected 15 to 17 weeks previously. This latter value represents a relatively small decrease in surviving parasites and in fact was found to be variable and not always statistically significant.

In a different type of study, Doenhoff and Long (1979) compared the ability of cercariae to penetrate isolated abdominal skin of mice infected 7 weeks previously or of control mice. The results of this work showed that the number of schistosomula recoverable from the skin of infected animals after 3 hours was significantly lower. The authors concluded however that this result was likely to be a consequence of a delay rather than a reduction in migration since an earlier study (Doenhoff *et al*, 1978) employing mice with a similar infection had shown the existence of a delay in migration of schistosomula to the lungs. A similar result has been independently described by Sher *et al*, (1974a) and Smithers and Gammage (1980).

1.2.2.3 Autoradiographic tracking experiments

Dean and Mangold (1983) have recently shown using autoradiographic tracking experiments that the number of schistosomula reaching the lungs by day 6 after a challenge infection is equal in naive and 6- or 25- week infected mice. In animals carrying a 16-week infection however a slight reduction in migration was recorded. This dependence for an effect on the age of the initial infection is also witnessed in the work of Smithers and Gammage (1980) described earlier: these workers were unable to find a reduction in the number of living schistosomula recoverable from the skin of previously infected mice unless the initial infection had been present for at least fifteen weeks.

1.2.2.4 Recovery of worms from lungs

Schistosomula can be recovered from an infected host by allowing them to crawl out of isolated minced lung fragments (Oliver, 1952). This technique has frequently been used as a method of parasite recovery when investigating resistance to reinfection. With respect to the target for host resistance however, it should be remembered that a decrease in recovery of schistosomula from the lungs may reflect killing which has taken place before or after arrival of the parasites at this site. The likelihood of the latter possibility is perhaps lessened by the finding that killing measured at the lung stage in CBA mice can be entirely attributed to death of schistosomula in the skin (Smithers and Gammage, 1980). As described in section 1.2.3,

however, killing in the lungs of previously infected hosts has been demonstrated by some workers.

Studies concerned with quantitating the degree of resistance to reinfection acquired by immune animals, as measured by recovery of schistosomula from the lungs, have produced variable results. For example, Perez et al, (1974) were able to show a much greater level of immunity in the rat than Mangold and Knopf (1978). Differences in results obtained by different workers would perhaps appear to be primarily due to differences in technique, as Mangold and Knopf (1978) were able to produce highly variable results by simply altering such assay conditions as the time allowed for recovery of parasites, or the medium in which the lungs are incubated. In spite of this and other problems associated with the assay (see Philips and Colley, 1978; Blum and Cioli, 1981; Dean, 1983) a reduction in recovery of parasites from immune animals has been reported in almost all studies performed (for review see Dean, 1983). In general therefore, it would appear that the results obtained indicate the existence of a killing stage before or in the lungs. The possibility that host immunity may interfere with the ability of the parasites to crawl out of the lungs rather than actually kill them should perhaps not be forgotten however.

1.2.3 The lung-stage schistosomulum

1.2.3. Histological studies

Lichtenberg and Ritchie (1961) have found that a

proportion of a challenge infection of S. mansoni are killed in the lungs of resistant rhesus monkeys. Similarly, Magalhoes-Filho (1959) reported the presence of dead schistosomula in the lungs of previously infected, but not naive mice. This latter result however could not be confirmed by Lichtenberg et al, (1976).

1.2.3.2 Worm recovery experiments/autoradiographic tracking experiments

One piece of information which was initially considered as being consistent with the idea that killing probably takes place in the lungs was provided by Dean et al, (1978_a). These workers showed that the reduction in the number of schistosomula retrievable from the lungs of mice infected at least twelve weeks previously was proportional to the number of parasite eggs in the lungs. Furthermore, it was found that intravenous injection of eggs into the lungs of previously uninfected mice induced moderate to high levels of resistance to reinfection. Conversely subcutaneous or intraperitoneal injection was without effect. In a later study employing autoradiography and adult worm recovery however it was shown that most of the schistosomula which could not be recovered from egg infested lungs were not dead and would eventually make their way to the liver.

1.2.3.3 Injection of lung stage schistosomula intravenously into an immune animal

The fate of lung stage schistosomula (obtained from infected donor mice) injected into the tail vein of previously infected or control mice has been investigated by Blum and Cioli (1981). These workers reported a reduction in the number of 5- to 9-day (post transformation) parasites which survive in previously infected animals as measured by recovery of worms from the mesenteric veins. Results obtained with 10- and 11-day old lung stage schistosomula were however variable as were results obtained with 10- and 11-day worms obtained from the liver of the donor mice. Worms recovered from the liver on day 12 or later and injected into the mesenteric veins of recipient mice were in general found to be able to evade immunity. These results are consistent with the idea that lung stage worms are susceptible to host immunity but that this vulnerability diminishes around the time at which the worms are moving from the lungs to the liver. An alternative explanation put forward by the authors however is that killing is dependent on parasites traversing the lungs of an infected animal.

Dean *et al* (1981b) also reported that 5-9-day lung schistosomula, injected into the tail vein of previously infected mice, are in part eliminated as compared to parasites introduced to control animals. In addition, however, it was also shown that both 11- and 20- day worms which had been injected into the mesenteric veins of recipient immune mice suffered a similar level of attrition.

It is therefore possible that in this study, the lung stage schistosomula were not killed in the lungs, but at some later stage in migration.

1.2.4 The post-lung worm

The existence of a post lung killing stage has been demonstrated by a number of workers employing the mouse model. Preliminary evidence was provided by Sher (1977) who showed that 44hr cultured schistosomula injected intravenously into immune mice showed similar levels of recovery from the lungs, but a reduced level of recovery from the portal system to schistosomula injected into control mice. A similar result was noted by Dean *et al* (1978) when comparing the fate of a challenge population of cercariae allowed to penetrate the skin of 6-8 week infected or control mice. This latter result was confirmed by Smithers and Gammage (1980) who, by employing an early liver perfusion technique (Doenhoff *et al*, 1978) were able to show that post-lung killing took place between 6 and 14 days after challenge. These workers did not establish whether killing took place before or after entry to the liver but recent studies (Dean and Mangold, 1983) employing a combination of autoradiography of squashed liver preparation and portal perfusion, have indicated that the majority of a challenge population which leave the lungs reach but do not leave the liver.

Additional evidence for and against a post-lung killing stage, obtained from studies investigating the fate of

intravenously injected parasites, has been discussed in section 1.2.3.3.

A different form of post lung killing has been described in the immune rhesus monkey by Lichtenberg and Ritchie (1961). These workers demonstrated that a substantial number of schistosomula are killed in the lungs of an immune animal but that an equal number are able to complete their migration. This latter group however do not undergo further maturation on leaving the lungs and are also eventually killed.

1.3 The mechanism of host immunity

1.3.1 *In vitro* studies employing the early schistosomulum

Since the discovery by Clegg and Smithers in 1972 that newly transformed schistosomula are killed by immune rhesus monkey serum plus complement *in vitro*, the juvenile stage of the mammalian parasite has been employed as a target to investigate the possible role in immunity to schistosomiasis of a number of components of the host immune system. This has led to the discovery during the past decade of a range of *in vitro* effector mechanisms of perhaps quite remarkable diversity (Table 1). The relevance to immunity *in vivo* of many of the reported *in vitro* findings has yet to be established however and these studies will therefore not be discussed in any detail. Further reference to some of this work will however be made in subsequent discussion on the role played by the various components of the immune response *in vivo*.

Table 1. Effector mechanisms active in vitro against newly transformed schistosomula

Effector mechanisms	Original reference(s)
IgG + complement	Clegg and Smithers (1972)
IgG + complement + neutrophils	Dean et al (1974)
IgG + eosinophils	Butterworth et al (1975)
IgG + macrophages	Perez and Smithers (1977)
complement	Santoro et al (1979)
complement + eosinophils	Ramalho-Pinto et al (1978)
IgE/antigen complexes + macrophages	Capron et al (1975;1977)
IgE + platelets	Joseph et al (1983)
monocytes	Ellner and Mahmoud (1979)
activated macrophages	Mahmoud et al (1979)

The above list is not complete in that only the reference to the initial observation has been included. In addition the source of the various immune components has not been stated as several of the effector mechanisms have been described using antibody from different species. Further information can be obtained from McLaren et al (1980) or Smithers and Doenhoff (1982).

1.3.2 The role of antibody in vivo

1.3.2.1 Passive transfer experiments

Most studies concerned with the role of antibody in passive transfer of resistance to reinfection have been undertaken using the mouse model. The first successful transfer was demonstrated by Sher et al (1975a)who reported that naive mice given an intravenous injection of serum from infected donorswere able to show a resistance to re-infection equal to about half of that demonstrable in the donor animals. Further experiments (Sher et al, 1977) indicated that the factor responsible for the transfer of immunity was IgG. Successful attempts to transfer resistance by intravenous injection of immune serum have since been recorded by other workers (for example, Mahmoud et al, 1975; Smith et al, 1982). In addition intravenous injection of serum from immune mice also confers on recipients the ability to a) mount an accelerated inflammatory response, similar to that observed in an immune animal, to schistosomula injected into the lungs (Olveda et al, 1981) and b) kill Millipore chamber-enclosed schistosomula implanted in the peritoneal cavity of normal mice to a level equal to 65% of that seen in infected mice (Kassis et al, 1979).

In contrast to the above results, studies by Doenhoff and Long (1979) and Maddison and Kagan (1979) have failed to demonstrate that intravenous injection of immune serum into naive mice can alter their ability to reject a challenge infection of cercariae. In addition studies

employing parabiotic mice have indicated that the ability to resist a challenge infection is not passed from immune to naive partners (Hunter et al, 1967; Dean et al, 1981c). Murrel et al, (1978b) have also been unable to demonstrate passive transfer of immunity by intravenous injection of immune serum but intravenous injection of immune serum in combination with intradermal injection of reaginic antibody was found to be effective:reaginic antibody used on its own was without effect however. Doenhoff and Long (1979) have also demonstrated that intradermal injection of immune serum at the site of a percutaneous cercarial challenge has little effect on parasite survival.

Attempts to transfer resistance to reinfection by intravenous injection of immune serum have also produced successful (Smithers, 1976; Phillips et al, 1975) and unsuccessful (Maddison et al, 1970) results when employing the rat model. The study performed by Phillips and co-workers (1975) indicated that the age of the infection influenced results in that only serum obtained from rats harbouring a 6-7 week infection was effective. The factor responsible for transfer of immunity was found to be IgG.

A role in immunity for skin-sensitising antibodies in the rat was demonstrated by Ogilvie et al (1966). These workers showed that an intradermal injection of serum transferred immunity to a recipient providing that the injection was given at the site which would be exposed to cercariae. The active ingredient in the serum was reported as being IgE.

Attempts to transfer resistance to reinfection by injection of serum from infected donors have proved unsuccessful in the rhesus monkey (Smithers, 1976; Maddison et al, 1976) and in man (Cook et al, 1972). Some success has been obtained with the former species however if the serum is employed in combination with dialysable transfer factor from normal donors (Maddison et al, 1976).

1.3.2.2 Additional studies

A comparison has been made by Blum and Cioli (1978) of the immune response to S. mansoni mounted by mice genetically selected for either high or low humoral antibody responses (Biozzi). This work has shown that although the high responders produce higher levels of antibody to schistosome antigens, they are not more able, and indeed may even be less able, to resist a challenge infection. These results therefore cast doubt on the idea that antibody plays a role in resistance to reinfection. A similar conclusion is obtained from results provided by Maddison et al, (1980) ; these workers showed that mice suffering a deficiency of B cells due to injection of anti-mouse μ chain serum were able to resist a challenge infection to the same extent as control mice.

Mota-Santos et al, (1981) have shown that mice exposed to bisexual cercariae produce levels of lethal antibody which are related to resistance to reinfection as measured by the lung recovery technique. Mice exposed to a unisexual infection showed no immunity as measured by this technique

but in some cases significant resistance to reinfection could be detected when liver perfusion was employed as the method of assessment. The fact that unisexually infected mice have low antibody titres even when immunity can be detected by liver perfusion has led Mota-Santos and co-workers to suggest that immunity detectable at the lung stage is antibody dependent and immunity detectable at the liver stage is not. This latter hypothesis is consistent with the idea that post lung killing may not be dependent on a specific form of immune response but may arise as a consequence of pathological changes in the host (Dean et al, 1981a).

The study by Mota-Santos et al (1981) has shown that resistance to reinfection as measured by the lung recovery technique would appear to be related to the in vivo level of an antibody which is lethal in the presence of complement (lethal antibody) for schistosomula in vitro. The presence of detectable anti-schistosome antibody in vivo and lethal antibody activity in vitro has not correlated with immunity in some other studies however. For example, Sher et al, (1974b) were able to raise a lethal antibody in rats by immunising them with adult membrane material. Such rats however were as susceptible as untreated controls to a challenge infection of cercariae. In addition, Perez et al, (1974) have shown that lethal antibody levels remain high in infected rats even at a time when their resistance to reinfection has waned. Additional studies employing the rat model however have shown the existence of a correlation between the degree of resistance to reinfection in infected

animals and their levels of both an IgG antibody which promotes eosinophil mediated damage of schistosomula *in vitro* (Mackenzie *et al*, 1977) and an IgE antibody which may induce killing of schistosomula by macrophages *in vitro* (Rousseaux-Prevost *et al*, 1978).

1.3.3 Complement

The following results suggest that the complement system may be of importance in protecting infected mice from a challenge infection of cercariae :

i) mice genetically deficient in C5 are apparently less able than mice with an intact complement system to prevent reinfection (Sher *et al*, 1975b).

ii) the effectiveness of immunity in mice harbouring a chronic infection is reduced by treating them with cobra venom factor (Tavares *et al*, 1978a).

iii) cobra venom factor inhibits the antibody-dependent killing of schistosomula enclosed in Millipore chambers in the peritoneal cavity of infected mice (Kassis *et al*, 1979).

The evidence in support of complement having a role *in vivo* is not unequivocal however as the result obtained by Tavares and fellow workers (1978a) could not be repeated by Doenhoff and Long (1979). It should be pointed out however that in this latter study, C3 levels in infected animals were not reduced to zero, but remained at a value equal to 11% of the normal concentration.

1.3.4 Thymus-dependent immunity

1.3.4.1 Cell-transfer experiments

Phillips et al (1975) have been able to promote resistance to re-infection in uninfected rats by giving them an injection of spleen or peritoneal exudate cells obtained from infected animals. The cell responsible for transfer of immunity was found to be a T-lymphocyte as shown by its susceptibility to the cytotoxic effects of anti-thymocyte serum plus complement. Only cells recovered from animals 3-4 weeks after infection were able to confer resistance : cells recovered earlier or later were without effect. Cells obtained from the spleen or lymph nodes of infected mice have also been utilised in transfer experiments but a successful transfer of immunity in the absence of antibody has not been recorded (for review see Dean, 1983).

1.3.4.2 Measurement of resistance in T-cell deficient animals

Measurement of resistance in T-cell deficient animals has been undertaken using both the rat and the mouse models. Working with the rat, Cioli et al (1980) have reported that animals deprived of T-cells are unable to demonstrate an enhanced destruction of cercarial challenge following immunization by two previous exposures : normal rats conversely show a significant degree of immunity. Employing the mouse, Doenhoff and Long (1979) have demonstrated that T-cell deprived animals are inferior to control animals in resisting a challenge infection. Similarly Phillips et al (1980) have obtained results which indicate that nude mice (nu/nu) are less protected against reinfection than animals with intact thymus-dependent immunity.

1.3.4.3 Role of thymus-dependent immunity in resistance to reinfection

Although a role would appear to exist for the thymus-dependent arm of the immune response in resistance to reinfection, the exact nature of this role has yet to be elucidated. Several possible functions can be considered based on results obtained from a comparison of the immune response to schistosomiasis mounted by T-cell deficient or depleted and control animals. These are :-

i) Two studies have indicated that the humoral immune response of the mouse to schistosomiasis is under thymus control : Mussalam et al (1980) have shown that an antibody response to schistosome antigens is lacking in T-cell deficient mice: Hsu et al (1976) have demonstrated that athymic nude mice (nu/nu) are unable to respond to infection in a manner similar to their heterozygote (nu/+) litter mates by producing specific skin-sensitizing antibody. It could be argued therefore that a major function of T-cell immunity in resistance to reinfection is to aid in antibody production.

ii) T-cell deficient mice (Phillips et al 1977) and rats (Cioli et al, 1980) appear to produce a lower peripheral blood eosinophilia than intact animals in response to infection with S. mansoni. In addition Hsu et al (1976) have observed an impairment in the ability of eosinophils to migrate to the site of a challenge infection in athymic nude mice. The results therefore suggest that thymus dependent immunity may have a role in influencing or determining the extent of the eosinophil response mounted by the infected host. As discussed in section 1.3.5, the eosinophil may play an important role in resistance to reinfection.

iii) Athymic nude mice (Hsu et al, 1976; Phillips et al, 1977) and mice depleted of T-cells by thymectomy and anti-thymocyte serum (Pepys et al, 1980) develop significantly smaller hepatic granulomas following infection, than are found in immunologically intact animals. Since resistance in mice has been shown to increase in proportion to the extent of pathological change associated with the disease (Dean et al, 1981a) it is reasonable to propose that T-cells are of importance in immunity as a consequence of their involvement in the formation of granulomas. A similar argument has been put forward by Cioli et al (1980) to explain the low resistance to reinfection associated with T-cell depleted rats, as such animals are known to be defective in producing granulomas.

iv) T-cell depleted mice, unlike normal animals, do not develop a marked elevation of C3 as a consequence of a 6-7 week infection with S.mansoni (Pepys et al, 1980). It is therefore possible that T-cells may act in resistance to reinfection by in some way controlling or influencing the activity of the complement system. As discussed in section 1.3.3., complement may be of importance in enabling an infected host to resist further infection.

Which, if any, of the above mechanisms is active in vivo has yet to be established, but it is likely that T-cells do not function as cytotoxic cells as pre-treatment of infected mice with anti-thymocyte serum before challenge does not abrogate resistance (Mahmoud et al, 1975; Doenhoff and Long, 1979).

1.3.5 Polymorphonuclear leucocytes

Both neutrophils and eosinophils are active components in a number of in vitro anti-schistosomulum cytotoxicity assays (reviewed by McLaren, 1980). The role which these two cells play in resistance to reinfection in vivo however has yet to be clearly defined. Studies by

Mahmoud et al (1975) have shown that previously infected and passively immunized mice lose their ability to resist a challenge infection of cercariae if they are pre-treated with anti-eosinophil serum but not anti-neutrophil, anti-macrophage or anti-lymphocyte serum. In addition, histological studies indicate that cercariae penetrating the skin of previously uninfected mice attract an influx of host neutrophils (Colley et al, 1972; Lichtenberg et al, 1976) but that penetration of previously infected animals induces an eosinophil enriched response (Lichtenberg et al, 1976). These results are therefore consistent with the idea that the eosinophil may be an important component of the immune response which enables infected mice to resist a challenge infection: the neutrophil conversely may simply play a role in the non-specific inflammatory response to invading schistosomula.

1.4 Comparison of the properties of the outer membranes of schistosomula and adult worms

1.4.1 Introduction

The evidence described in section 1.2 would appear to indicate that migrating schistosomes can be killed at more than one site in an immune host. In the chronically infected mouse two killing stages would appear to exist : an early stage which takes place before parasites arrive in the lungs and a later stage whose target has yet to be clearly defined (Smithers and Gammage, 1980). Recent studies indicate that the latter stage of killing may not be dependent on humoral factors but may be related to liver pathology (Dean et al, 1981a, b). In contrast, although some conflicting and paradoxical results have been demonstrated, the early stage of killing is generally considered to be antibody dependent (Sher et al, 1974a; Sher, 1977; Mota-Santos et al, 1981). This anti-

body-dependent mechanism is considered to arise as a consequence of stimulation of the immune response by adult worm antigens (Smithers and Doenhoff, 1982). Thus, adult worms can be regarded as inducing an immune response against which they appear insensitive. Since schistosomula appear to be susceptible to killing by this immunity, they must possess properties which are lacking in the adult worm. The target of the immune response to which the schistosomulum is sensitive would appear to be the parasite surface membrane (McLaren, 1980). It has therefore been postulated that the adult worm is insensitive to the immune response which it itself elicits by virtue of some change which takes place in its surface during maturation (McLaren, 1980).

The surface of the adult schistosome is in the form of a tegument consisting of a syncytium of cytoplasm enclosed by a plasma membrane. This plasma membrane is involved in nutrient uptake as witnessed by the active transport and/or diffusion across the membrane of hexoses (Isseroff et al, 1972; Rogers and Bueding, 1975; Cornford and Oldendorf, 1979), amino acids (Asch and Read, 1975a, b; Cornford and Oldendorf, 1979) and purines and pyrimidines (Levy and Read, 1975a,b), and may also have other as yet undefined functions. In order to determine if one of these functions is to permit parasite survival, it is relevant to compare the properties of the surface membrane of the susceptible skin stage schistosomulum with those of the mature parasite surface membrane. Section 1.4 will therefore be concerned with discussing the changes in the nature and organisation of membrane components which accompany maturation: section 1.5 will then go on to discuss how some of these changes may enable the adult worm to resist host immunity.

1.4.2 Development and maintenance of the surface membrane

The development of the surface membrane of the schistosome tegument has been investigated using transmission electron microscopy by Hockley and McLaren (1973). Examination of the cercarial surface reveals a typical trilaminate plasma membrane, 8.5nm thick, being below a surface coat of loosely stranded material. This coat, termed the glycocalyx is generally lost within one hour of penetration. Three hours after penetration the newly formed schistosomulum shows a very different surface in that the original trilaminate membrane of the cercaria has been almost entirely replaced by a seven layered structure. This new structure is maintained in the adult schistosome : it has the appearance of two contiguous conventional trilaminar membranes and a total thickness of approximately 17nm.

In addition to acquiring a heptalaminate structure, the developing surface membrane also greatly increases in surface area. Invaginations of the membrane appear within the first four days of development leading to the formation of surface pits, which become deeper as maturation progresses.

The formation of the new heptalaminate membrane would appear to depend on multilaminate membrane bound vacuoles. These bodies, formed in subtegumental cells, are passed into the tegument where they enlarge, possibly by fusion, before joining the trilaminate membrane and opening to the exterior. At the same time, much of the old membrane is cast off. The new membrane is thus formed from the heptalaminate limiting membranes possessed by the vacuoles and perhaps some of the old cercarial membrane. On completion of the new membrane the large vacuoles disappear to be replaced by smaller membraneous bodies. Hockley and

McLaren (1973) considered that the latter bodies might continuously contribute to the membrane in a process of constant renewal.

The role played by the small membraneous bodies in tegument structure in the adult worm was later investigated by Wilson and Barnes (1974a). These workers concluded :- i) that the tegument possesses a normal three layered surface membrane to which the bounding membranes of the small membraneous bodies (or "multilaminate vesicles" - author's term) fuse, and ii) that fusion results in the lamellate contents of the membraneous bodies pouring out and over the original membrane to form a second. In this way a structure is formed which appears heptalaminate when examined by transmission electron microscopy. Further work (Wilson and Barnes, 1977) has suggested that the site where fusion occurs is at the base of the surface pits and that deposition of new membrane here, results in the outer of the two bilayers being pushed up the sides of the pits, before being sloughed off at the tegument surface.

1.4.3 Comparison of the nature and organisation of proteins in the surface membrane of juvenile and mature parasites

Studies in which a comparison was made of either the population of molecules present at the surface of the schistosomulum and adult worm which can be radiolabelled *in situ* (Snary *et al*, 1980; Shah and Ramasamy, 1982) or the antigenic composition of solubilised surface membrane preparations isolated from both stages (Kusel *et al*, 1975), indicate that the nature of antigens in the schistosome surface membrane is conserved with maturation. Interestingly however, Cordeiro and Gazzinelli (1979) have shown that maturation of the mammalian parasite appears to be accompanied by a general increase in the molecular weight of tegument antigens. In addition, in a recent study by Norden *et al*,(1982)

it was demonstrated that a schistosome protein found both in cercariae and in the surface membrane at the tegumental spines of the adult worm had a molecular weight of 120,000 in the former and 170,000 in the latter. This protein was also found at the spines of the schistosomulum surface although the authors did not calculate (or did not state) its molecular weight. It is therefore unknown as to whether the change in molecular weight had already taken place during the first few hours post-penetration or whether it occurred at a later stage in development. It is perhaps more likely in view of the results of Cordeiro and Gazzinelli that the latter possibility is correct. In addition Tavares et al, (1980) have demonstrated that the protein content of the tegument of the newly transformed schistosomulum does not change during the first three hours culture in vitro.

An increase in the size of membrane antigens is also indicated by a freeze fracture study which showed that migration of schistosomula from the skin to the lungs is accompanied by an increase in intramembraneous particle (IMP) size (McLaren et al, 1978). This increase is restricted to the outer leaflets of both the inner and outer bilayers, the extent of the increase being greater on the latter (9.5-14 as against 7-12nm in diameter. The IMP in the 3hr skin-stage schistosomulum membranes are 6-9.5nm in diameter). The adult worm also has larger IMP than the skin-stage schistosomulum on the outer leaflet of both bi-layers but the size (ranging between 7 and 11nm in diameter on both leaflets) is not as large as is measured for the lung stage worm. McLaren (1980) has suggested that enlarged

IMP may represent a combination of parasite antigens and antigens acquired from the host (see section 1.4.6). A similar hypothesis has been put forward by Torpier and co-workers to explain the increased size of the IMP of the adult surface membrane (Torpier et al, 1977) but these workers have also suggested that an increase in IMP size recorded with lung-stage parasites may reflect aggregation of particles (Torpier et al, 1982).

Freeze fracture studies have also demonstrated that both the number and the distribution of IMP alter as maturation progresses. Two relevant examples of this phenomenon are firstly, that the adult worm has a smaller total number of IMP per unit area than the 3 hr schistosomulum and secondly that the adult worm has the majority of its IMP in the outer bilayer whereas the greater part of the IMP in the 3hr schistosomulum membrane are located in the inner bilayer. These observations are consistent with the idea that differences in membrane antigen organisation exist between the two parasite stages. This hypothesis is also supported by the finding that membrane antigens are more readily labelled at the surface of the schistosomulum than at the surface of the adult worm (Snary et al, 1980).

1.4.4 Changes in lipid composition and organisation with maturation

A detailed study of the surface membrane lipid composition of the newly transformed and adult schistosome has not yet been undertaken. Some information concerning the nature of the schistosomulum surface membrane lipids which can be radiolabelled in situ has been provided by Rumjanek and

McLaren (1981) but studies relating to lipid composition in the adult worm have been confined to analysis of total as opposed to surface membrane lipid composition (Smith and Brooks, 1969; Meyer et al, 1970; Cesari and Marchiani, 1978). A comparison between the two stages of development is therefore not possible. In spite of this, it is known that the surface membrane of the adult worm has a greater cholesterol:protein ratio than that of the newly transformed parasite (Kusel et al, 1981). In addition, the fact that schistosomes rely on their host for provision of free fatty acids and sterols (Meyer et al, 1970); the finding that lung stage schistosomula have a different surface membrane lipid composition from newly transformed parasites; and the knowledge that the surface membrane lipid composition of schistosomula maintained in vitro is influenced by the origin of serum present in the culture medium (Rumjanek and McLaren, 1981) suggests that it is unlikely that the newly transformed and mature parasites should have a similar surface membrane lipid composition.

Evidence suggesting that the schistosomulum and adult worm differ with respect to the organisation of their surface membrane lipids has been obtained by Kusel et al, (1981). These workers have shown that amphotericin B(a polyene antibiotic), and vitamin A alcohol are independently able to damage the outer membrane of the schistosomulum surface as measured by ^{51}Cr release but are ineffective against the adult worm. Since amphotericin B is known to interact with cholesterol (Norman et al, 1972) it was considered that its inability to damage the adult surface membrane might be a

consequence of a decrease in membrane cholesterol content, with maturation. This hypothesis was proved to be incorrect however as the cholesterol content of the surface membrane was found to have increased with maturation. It has been suggested by Kinsky et al, (1966) that amphotericin B can be prevented from interacting with cholesterol in membranes if the number of phospholipid molecules in association with the sterol is increased. Kusel et al, (1981) have therefore proposed that the insensitivity of the adult worm to amphotericin B might be a consequence of an increased packing of phospholipid molecules within the membrane. Likewise, it was suggested that such a change might reduce the ability of vitamin A alcohol to penetrate the membrane.

Alternative evidence supporting the existence of a change in surface membrane lipid organisation or composition has been obtained using the fluorescence recovery after photobleaching technique. This technique allows an assessment of the relative mobilities of molecules within membranes (Axelrod et al, 1976). Johnson et al (1982), using 1,1'-dioctadeyl-3,3,3',3'-tetramethyl indocarbocyanine iodide a fluorescent lipid probe which is able to insert itself into the parasite surface membrane, have obtained results which indicate that there is a greater degree of lipid immobility in the adult surface membrane than in that of the schistosomulum.

1.4.5 Differences in the exposed surface carbohydrates of schistosomula and adult worms

The exposed surface carbohydrate composition of schistosomula and adult worms has been investigated using lectins by Simpson and co-workers (Simpson and Smithers, 1980; Simpson et al, 1983). This work has shown that although both stages of the parasite bind concanavalin A (specific for several sugars but likely to indicate the presence of internal α-D-mannosyl residues), wheat germ agglutinin (specific for N-acetyl-D-glucosamine), Ricinus communis agglutinin (major specificity is for terminal D-galactose) and peanut agglutinin (specific for D-galactose conjugated to N-acetyl-D-galactosamine) only the newly transformed schistosomulum is able to bind the fucose-binding protein from L. tetragonolobus and only the adult worm is able to bind soyabean agglutinin (after neuraminidase treatment). In addition sialic acid, as determined by comparing the binding of peanut agglutinin and soyabean agglutin to the parasite surface before and after neuraminidase treatment, could only be detected at the surface of the adult worm. The nature of the exposed carbohydrates at the schistosome surface would therefore appear to undergo a change as the parasite matures. Simpson et al (1983) have shown that this change is already evident by the time that the parasites are in the lungs. It should be mentioned however that although many of the above results have been confirmed by other workers (Stein and Lumsden, 1973; Bennett and Seed, 1977; Murrell et al, 1978a; Torpier and Capron, 1980) Murrell et al, (1978a) were unable to bind soyabean agglu-

tinin to lung stage schistosomula following neuraminidase treatment. In addition, Torpier and Capron (1980) could not promote interaction between newly transformed schistosomula and wheat germ agglutinin.

1.4.6 Changes in surface antigenicity associated with maturation

1.4.6.1 Introduction

Migration of newly transformed schistosomula from the skin to the lungs of an infected host is concomitant with a change in the nature of their surface antigens from solely parasite specific to molecules which share specificities with the host (McLaren et al, 1975). These cross-reacting antigens are also extensively distributed at the surface of the adult worm. The adult worm therefore expresses surface antigens which are either absent from, or only in, the process of being expressed at the surface of skin-stage schistosomula. The origin and nature of these antigens has been the subject of much investigation.

1.4.6.2 Origin of host-like antigens

Sharing of antigenic determinants between parasite and host was originally shown, using gel diffusion techniques by Damian (1964,1967) who considered the phenomenon to have arisen during the course of evolution as a method of reducing the schistosomes foreign appearance such that better survival was permitted. The host-like materials were therefore considered to be of parasite origin and could thus be thought

of in terms of a form of molecular mimicry. Capron et al (1968) suggested that the ability of the schistosome to survive in a range of hosts might be due to its possession of a set of genes coding for antigens relevant to different species, the gene required in a particular host being switched on by some form of induction.

An alternative explanation for the origin of host-like antigens was put forward by Smithers et al (1969). These workers considered that the host-like determinants were indeed of host origin and were acquired by the parasite surface membrane during the course of schistosome maturation. This hypothesis was put forward on the strength of in vivo observations which showed that adult schistosomes grown in mice ("mouse" worms) were rapidly killed when transferred to the hepatic portal system of monkeys immunised against mouse erythrocytes, but not of normal monkeys. This killing was entirely specific:parasites grown in species other than the mouse survived following transfer showing that only "mouse" worms expressed mouse erythrocyte antigens.

It is now generally accepted that this latter theory is correct and evidence in support of this statement is given in section 1.4.8.

1.4.6.3 Nature of acquired host antigens

The first clue, as to the nature of the host antigens present on the schistosome surface was produced by Clegg et al (1971). These workers established using similar techniques to Smithers et al (1969) that schistosomula cultured in vitro

for 15 days in the presence of human serum and red cells were able to acquire antigens which were universally common to human erythrocytes and also antigens which were blood group specific. Blood group specific antigen uptake was confirmed by Dean (1974) who showed that schistosomula cultured in the presence of human erythrocytes or alcohol extracts of erythrocyte membranes displayed the relevant blood group A or B antigen at their surfaces. Dean and Sell (1972) had previously shown that a Forssman-like antigen is acquired by schistosomes grown in mice and since Forssman antigen and alcohol-soluble A and B substances are known to be glycolipid in nature (Makita et al, 1966; Koscielak, 1963) it was suggested that adsorption of host molecules might be dependent on their possessing a sphingolipid structure (Dean, 1974). Further work by Goldring et al (1976) confirmed that A, B, and also H antigens could be acquired by schistosomula in their glycolipid form and in addition showed that several glycoprotein antigens - M, N, S, rhesus, and Duffy, were not adsorbe It was thus suggested that host antigen uptake, in vitro at least, was restricted to glycolipid molecules. This idea was strengthened by the finding that erythrocytes whose surface carbohydrates had been radiolabelled were shown to transfer glycolipids but not glycoproteins to the surfaces of schistosomula cultured in their presence (Goldring et al, 1977a).

Recently however, Sher et al (1978) have produced evidence demonstrating that glycoproteins can also be acquired by developing schistosomula. Using appropriate antisera and

the indirect fluorescent antibody method, these workers showed that schistosomula recovered from the lungs of mice or cultured in the presence of mouse lymphoid cells possess surface alloantigens which are gene products of the MHC, namely K and Ia antigens. Similarly McLaren and Smithers (see McLaren, 1980) have found that schistosomula cultured in the presence of human lymphoblasts, tonsil cells and platelets are able to acquire human histocompatibility antigens. That histocompatibility antigens are also present at the surface of the adult worm has recently been demonstrated using "mouse worms" by Gitter et al (1982).

Another type of glycoprotein molecule found at the adult schistosome surface is host immunoglobulin. This has been detected in the form of IgG at the surface of worms removed from infected baboons (Kemp et al, 1976) and in the forms of IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and IgA at the surface of "mouse worms" (Kemp et al, 1978). Not all of the immunoglobulin detected is directed against parasite antigens as molecules with additional specificities have been found (Kemp et al, 1977).

1.4.6.4 Host-like antigens synthesised by the parasite

It is now generally accepted that all the antigens mentioned above are truly of host origin and are not synthesized by the parasite. This is supported by :

i) The transfer of radiolabelled glycolipids from erythrocytes to schistosomes has been demonstrated (Goldring et al, 1977a) and in addition, dead schistosomula have been shown to acquire host antigens which are glycolipid in nature (Sell and Dean, 1972; Dean, 1974).

ii) Schistosomula have been shown to possess alloantigens of identical specificity to their host and to be able to change to the specificity of a new host following transfer (Sher et al, 1978). To achieve this by an intrinsic mechanism, the parasite would have to be able to differentiate host antigens and also produce an exact copy of the one which it requires. No evidence has been obtained to support the existence of such a phenomenon.

iii) Kemp et al (1977) have shown that examination of worms removed from infected mice which had been immunized with various antigens reveals the presence of antibody specific for these antigens at the parasite surface.

In spite of these results, however, evidence has been obtained which indicates that the parasite can synthesize some molecules which cross-react with mammalian antigens. Thus Damian et al (1973) have detected the presence of an antigen sharing specificity with mouse α2-macroglobulin on the surface of schistosomes grown in mice and also on monkeys, and Bout et al (1974) have shown that schistosomes derived from hamsters are able to synthesize a protein which cross-reacts with hamster liver proteins.

1.4.6.5 Mechanisms of acquisition of host antigens

The mechanisms by which the various host antigens bind to the schistosome surface have yet to be fully elucidated. Immunoglobulin is considered to bind via an Fc receptor (Kemp et al, 1977, 1980) but Torpier et al (1979) were unable to demonstrate such a receptor at the adult schistosome surface. The finding that the β2-microglobulin side chain of H-2K antigens is not exposed at the schistosomulum surface has led Sher (1979) to suggest that only the heavy chain of the molecule is acquired by the parasite or alternatively that the whole molecule is inserted in such a way as to make the β2-microglobulin component undetectable. Previously Sher (1978) had suggested that the schistosomulum surface might possess receptors for certain carbohydrate moieties of histocompatibility antigens and in addition, glycolipids. Clegg (1972) has also suggested that a specific receptor might exist for the sugar component of glycolipid molecules but in addition has put forward an alternative hypothesis, namely that the hydrophobic ceramide region of the molecule might simply become inserted into the outer lipid bilayer of the parasite membrane. Whichever of these two hypotheses is correct,remains to be established, but it is certain that the nature of the association between the glycolipid and the membrane is intimate as these antigens are not removed by prolonged washing of worms in saline (Clegg et al, 1970) and can still be detected on "mouse" worms three days after transfer to normal monkeys (Smithers et al, 1969).

The expression of host antigens at the parasite surface is clearly dependent on the nature of the surface membrane outer bilayer. Several findings indicate that the outer bilayer differs in several ways from the inner bilayer. These include :-

i) the inner membrane is readily fixed using a conventional glutaraldehyde-osmium method, whereas the outer membrane requires additional fixation with uranyl acetate (Hockley and McLaren, 1973). This finding indicates that the two membranes have different lipid compositions.

ii)astudy in which the affinity of both bilayers for various stains was compared indicated that the outer bilayer contained more lipid, possibly in the form of phospholipid than the inner bilayer (Wilson and Barnes, 1974b).

iii) The majority of IMP in the outer bilayer are found in the outermost leaflet; the reverse is true for the inner bilayer (Hockley et al, 1975).

The distribution of particles in the outer bilayer of the adult schistosome is unusual for a biological membrane and this finding has therefore led to the suggestion that the two trilaminate membranes are arranged with their external leaflets apposed (Hockley et al, 1975). Cesari and Marchiani (1978) have suggested that such an arrangement may provide schistosomes with a mechanism for acquiring antigens from the host as the internal leaflet of the RBC surface membrane, by virtue of its phospholipid composition, is considered a more accommodating environment for proteins than the external

one. With respect to this idea Cesari and co-workers (Cesari 1976; Cesari and Marchiani, 1978; Cesari and Polanco, 1980) have produced evidence which suggests that the phospholipid composition of the adult surface membrane outer bilayer may enable it to acquire lysine-containing molecules. This may therefore represent an alternative method by which the worm may acquire glycoproteins.

1.5 Evasion of the immune response

1.5.1 The host antigen hypothesis

The major issue of contention in relation to the ability of adult schistosomes to evade a self-induced host immune response concerns the role played by acquired host antigens. In other words, of the various developmental changes described in section 1.4, is the acquisition of molecules from the parasitised host the crucial factor which permits survival of the parasite ? The reasoning behind this idea is simple and attractive : the schistosomulum changes the nature of its surface antigens from parasite to host during migration to the lungs (McLaren *et al*, 1975) and at the same time becomes refractory to antibody dependent immune mechanisms *in vitro* (Clegg and Smithers, 1972; Ramalho-Pinto *et al*, 1978). One explanation for this result is that host antigens mask or disguise exposed parasite antigens such that antibody is no longer able to bind to the parasite surface (Smithers *et al*, 1969). This theory is also consistent with the hypothesis that antibody may only be effective *in vivo* during the first few hours or perhaps days of a challenge infection (section 1.4.1). Thus it could be argued that the adult schistosome

is insensitive to antibody-dependent host immunity by virtue of the fact that the antigens which stimulated this immunity are not expressed at the surface of the mature worm. This latter statement is in fact not strictly correct however as Goldring *et al* (1977b) and McLaren *et al* (1978) have independently shown that although lung stage schistosomula are unable to bind immune serum, older worms do show a little binding. This finding, therefore, suggests that the host-like disguise of the adult worm is incomplete. Goldring *et al* (1977b) have stated that this does not necessarily invalidate the host antigen hypothesis however as anti-schistosome antibody binding may be minimised to such an extent that the required quantity or pattern needed to bind cells or fix complement is not obtained.

1.5.2 Testing the host antigen hypothesis

The major strategy which has been adopted to investigate the"host antigen" hypothesis has been to determine if abrogation of schistosomulum sensitivity to *in vitro* antibody-mediated cytotoxicity is dependent on exposure to host components. Unfortunately, the experimental evidence accumulated to date does not provide a clear answer to this question. Thus, although some workers (Dessein *et al*, 1981) have demonstrated that mechanically transformed schistosomula cultured in chemically defined media become progressively resistant to immunity *in vitro*, no such protection has been reported by other workers (McLaren and Incani, 1982). In addition, although Tavares *et al* (1978b) have reported that a certain amount of protection can be acquired by schistosomula,

maintained in the absence of rabbit serum, it was also found that much more rapid and effective protection could be obtained in its presence. Similarly Dessein *et al* (1981) have demonstrated that the presence of human serum in the culture medium increases the rate at which schistosomula prepared by skin penetration become refractory to immunity *in vitro*. Penetration of skin itself would appear to confer a certain degree of protection (McLaren and Incani, 1982) and in addition, skin schistosomula become more rapidly protected against antibody mediated immunity than their mechanically transformed counterparts when cultured in medium lacking host macromolecules (Dessein *et al*, 1981).

1.5.3 Evidence for the existence of a role for host molecules independent of antigen masking

1.5.3.1 Introduction

The results described above can perhaps be summarised by saying that whether or not exposure to host components is a prerequisite for development of protection, it would certainly seem to enhance the procedure. There is some indication however that the role played by host molecules may not necessarily be related to masking of parasite antigens. Thus although a correlation has been demonstrated between loss of susceptibility to *in vitro* cytotoxicity and a reduction in binding of anti-parasite antibody following exposure of parasites to host molecules (Dessein *et al*, 1981; McLaren and Incani, 1982), it has also been shown that

schistosomula which have become evasive to antibody mediated damage following culture in medium containing serum are able to bind as much antibody as vulnerable parasites (Tavares et al, 1980; Levi-Schaffer et al, 1982). In addition, Dessein et al (1981) have shown that reduced antibody binding and reduced parasite death are both a consequence of pre-culturing schistosomula in chemically defined medium.

1.5.3.2 Role of host lipid

If the ability of host tissue or serum to promote or accelerate the development of evasion of immunity is not related to host antigen transfer, then upon what does it depend ? One possible answer is that it depends on lipid transfer : incubation of newly transformed schistosomula in medium containing foetal calf serum or human serum leads to changes in the parasite surface membrane lipid composition and at the same time promotes the development of protection against immunity in vitro (Rumjanek and McLaren (1981)). The nature of the change in both lipid composition and susceptibility to immunity is dependent on the type of serum employed. Thus both sera remove mono- and di- glycerides from the schistosomulum surface and also confer on the parasite the ability to evade antibody-dependent, complement mediated killing : in addition however, human serum transfers triglycerides and cholesterol to the parasite and at the same time renders it insensitive to antibody-dependent, eosinophil mediated killing. It could be argued therefore that developing

schistosomula become resistant to antibody-mediated immunity *in vivo* by virtue of changes in the nature and/or organisation of their surface membrane lipid phase. As discussed in section 1.4.4, such changes are almost certain to take place, and previous research has shown that the properties of the lipid phase of plasma membranes can influence their ability to express antigens (Borocher and Shinitsky, 1976) or be damaged by complement (Schlager *et al*, 1978).

1.5.3.3 Ability of host molecules to trigger intrinsic reorganisation of the parasite surface membrane

The serum-dependent protection observed in the work of Tavares *et al* 1978b,1980) was shown to be due to a macromolecule of molecular weight 7-19S. This unidentified molecule was shown to increase the turnover rate of most of the surface proteins and glycoproteins and was therefore considered to stimulate some form of developmental change which was dependent on parasite metabolism. (Dean (1977), on observing that various drugs which inhibit synthetic or secretory processes greatly increased the susceptibility of skin schistosomula to antibody dependent immunity *in vitro*, had previously concluded that the ability of skin schistosomula to evade immunity was dependent on metabolic processes). The idea of an intrinsic developmental change contributing to the acquisition of protection against host immunity has since been put forward by several groups and it has been suggested that in addition to serum, this change may be triggered by host skin (McLaren and Incani, 1982) and also by

concanavalin A (Pijkeren et al, 1981). McLaren and Incani (1982) have also suggested that Iscove's medium may possess this property, as this particular medium was employed by Dessein and co-workers (1981) in their study which demonstrated that schistosomula can become insensitive to host immunity when cultured in medium free of host molecules. McLaren and Incani (1982) have also therefore concluded that their own unsuccessful attempt to promote protection in the absence of skin or serum was due to the use of an alternative, probably inferior, medium (Eagles medium).

The idea of an intrinsic structural change at the schistosome surface contributing to the ability of the parasite to evade the immune response has also been investigated using a different approach by Moser et al (1980). These workers attached trinitrophenyl (TNP) to the surface of 3hr skin and 5-day lung schistosomula and then examined the survival rate of both following subjection to anti-TNP dependent immunity in vivo and in vitro. The results obtained from these experiments clearly indicated that whereas the skin-stage schistosomulum was susceptible to immunity both in vivo and in vitro, the lung-stage parasite, in spite of binding as much antibody as the younger form, was not. It was thus concluded that lung-stage schistosomula undergo some form of intrinsic modification at their surface which enables them to become resistant to an antibody-dependent immune response.

1.5.4 Differences between the adult worm and the lung stage worm and the possible role of membrane turnover

It has been reported that the 5-day lung stage worm is insusceptible to an *in vitro* killing mechanism which is dependent for its activity on anti-host RBC antibody and to which adult worms are vulnerable (McLaren and Terry, 1982). The surface membrane of the adult worm may therefore have properties which differ from those of the lung-stage worm and it has been suggested by McLaren and Terry (1982) that whereas the lung stage worm may have several mechanisms of defence, host antigens may constitute the major form of defence employed by the adult worm. Since the host antigen disguise of the adult worm is not complete however (Goldring *et al*, 1977; McLaren *et al*, 1978) it is reasonable to assume that some anti-parasite antibody may bind to the worm *in vivo*. As mentioned earlier (1.5.1) it has been suggested that this may not harm the worm in that binding may not be of the correct quantity or pattern to allow complement fixation and/or cell binding (Goldring *et al*, 1977b)and in fact antibody binding might even protect the parasite by acting in the manner of classical blocking antibody (see McLaren, 1980). Studies performed *in vitro* however suggest that the adult worm is able to cast off areas of membrane damaged by complement following binding of anti-host RBC antibody (Perez and Terry, 1973) and also shed surface antigens such as heterospecific host immunoglobulins which have been bound by a ligand (Kemp *et al*, 1980). Parasites may therefore employ a similar mechanism to remove any anti-parasite antibody which adheres to their

surfaces in vivo and this may therefore perhaps represent a second but minor form of evasion of the immune response.

1.6 Aims

The two membranes which enclose the adult schistosome are similar in structure to plasma membranes of mammalian cells in that they both appear to consist of a lipid bilayer into which a number of proteins have been inserted. Although a few preliminary studies have been undertaken, little is yet known of the organisation of these proteins with respect to other membrane components or cytoskeletal structures, but it has been established that at least some of them are antigenic and that these antigens are very poorly expressed at the parasite surface as determined by both radiolabelling techniques and the indirect fluorescent antibody method. Whether this latter phenomenon is due to some intrinsic property of membrane organisation; to the effect of host lipids on membrane organisation; or to masking by host antigens, is uncertain. The fact that the adult worm can be killed by anti-RBC antibody both in vitro and in vivo however raises the possibility that regardless, of the mechanism which brings it about, the low expression of parasite antigen at the adult surface enables the adult worm to evade antibody-dependent immunity. It is reasonable to postulate therefore, that increasing the expression of parasite antigens at the surface membrane, might lower the resistance of the worm to host immunity. In this respect, one hypothesis put forward to explain the observation that adult schistosomes treated with potassium antimony

tartrate are killed in the immune mouse is that the drug promotes an increased binding of antibody to the parasite surface (Doenhoff and Bain, 1979).

This latter hypothesis suggests that it may be possible to develop drugs which alter the expression of membrane parasite antigens. The major aim of this investigation was therefore to determine the properties such drugs might need in order to perform this function. It was proposed to do this by subjecting the adult worm to culture <u>in vitro</u> with a number of reagents which would alter some property of the surface membrane and then measuring the effect of the various treatments on parasite antigen expression. By using treatments which modify the membrane in a number of different ways, for example, by altering either the composition or organisation of either proteins or lipids, it should be possible to determine the type of drug which may be of use in promoting increased expression of parasite antigens <u>in vivo</u>.

In order to measure the effect of the various reagents on antigen expression, it was proposed to develop an indirect radiolabelled antibody method. Such a technique would provide a quantitative measurement of antigen expression and would therefore be more accurate than the qualitative or semi-quantitative methods which have been employed in previous studies on surface antigens of adult schistosomes.

It was also proposed to use this assay to determine if the level of expression of parasite antigens at the adult surface was related to the level of expression of host

antigens by also measuring the latter, following experimental manipulation of the worms. Such information should again be of use in designing drugs to promote increased parasite antigen expression. In addition, this form of experimentation should constitute a new approach to determining the validity of the much studied, but perhaps inadequately tested, host antigen hypothesis.

Finally, Thomas and Andrews (1977) have shown that praziquantel, a recently developed schistosomicidal drug whose mode of action is unknown is able to increase the susceptibility of parasitic cestodes to proteases. This result suggests that the drug may expose previously hidden protein molecules at the cestode surface. It was therefore decided to determine if praziquantel is able to increase the expression of parasite antigens at the schistosome surface in vitro. The existence of such an action in vivo may contribute to the elimination of the worm in praziquantel treated animals.

CHAPTER 2

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Biological materials

2.1.1.1 The parasite

The life cycle of a Puerto Rican strain of Schistosoma mansoni is maintained in the Department of Biochemistry, University of Glasgow. The parasite and the intermediate host, Biomphalaria glabrata, were originally obtained from the National Institute for Medical Research, Mill Hill, London. Some snails were also obtained from the Department of Biological Science, University of York.

2.1.1.2 Animals

Mice : balb/c, CBA and BIO A mice were obtained from the departmental animal house or from the Anatomy Department, University of Glasgow.

Hamsters : Syrian Golden hamsters were obtained from Wrights of Essex, England by the departmental animal house.

Rabbits : New Zealand white rabbits were obtained from the departmental animal house.

2.1.2 Reagents

All reagents were obtained from the British Drug House Ltd., Poole, Dorset, with the following exceptions :

Dimethyl Sulfoxide (DMSO); 2.5-diphenyloxazole (PPO); 2-mercaptoethanol; sodium citrate; Tween reagents:

Koch Laboratories Ltd., Colnbrook, England.

Freunds complete adjuvant; Freunds incomplete adjuvant; trypsin (for preparation of miracidia); Difco Laboratories Ltd., Colnbrook, Bucks.

Chloroform; formalin (40% (w/v) formaldehyde plus 12% (v/v) methanol); methanol; Sagatal : May and Baker Ltd., Dagenham, England.

Agarose; bovine serum albumin (BSA); chloramine T; 2, 4, 6 collidine; Coomassie Brilliant Blue R250; human serum albumin (HSA); NP 40; pepstatin; phenyl methyl sulphonyl fluoride (PMSF); phospholipase A_2 (Naja naja); retinol; Triton X-100; Tris (Hydroxymethyl) aminomethane (Trizma Base); trypsin (type XII, from bovine pancreas): Sigma Chemical Co., Poole, Dorset.

Sephadex G-25: Pharmacia (Gt. Britain) Ltd., Prince Regent Road, Hounslow, Middlesex.

Praziquantel : Bayer, Leverkusen, West Germany.

Sodium deoxycholate : Merck, Damstadt, West Germany.

Glutaraldehyde; osmium tetroxide : Taab Laboratories, Reading, Berks.

Ammonium sulphate : Fisons Scientific Apparatus, Loughborough, Leics.

Repelcote : Hopkins and Williams, Chadwell Heath, Essex.

Carrier free (^{125}I)-NaI; (^{51}Cr) - sodium chromate : Radiochemical Centre, Amersham, England.

Staphylococcal δ toxin : a kind gift from Dr. T.H. Birkbeck, Department of Microbiology, University of Glasgow.

Wheat germ agglutinin : a kind gift from Dr. G. Lindsay, Department of Biochemistry, University of Glasgow.

Penicillin; streptomycin; heparin : Vestric and Co. Ltd., Glasgow.

2.1.3 Photographic materials

Kodak-FX-40-X-Ray Liquid Fixer ; Kodak-DX-80-X-Ray Developer; Kodak Ektachrome 160 film (EPT 135); Kodak Electron Microscope film 4489; Kodak-X-O-mat Royal Film: Kodak (U.K.) Ltd., London.

2.1.4 Plasticware and Glassware

LP2 and LP3 tubes : Luckham Ltd., Beckenham, Kent.

Microscope slides and cover slips : Chance Proper Ltd., Warley, England.

Petri dishes (various sizes): Sterilin, Teddington, Middlesex.

Sterile containers, tubes and pipettes were provided by the Tissue Culture Unit, Biochemistry Department, University of Glasgow.

2.1.5 Standard Solutions

(1) Eagles medium

Sterile reagents to prepare Eagles medium were obtained from the Tissue Culture Unit, Department of Biochemistry, University of Glasgow. Formulation can be obtained in Flow Laboratories Manual. Penicillin and streptomycin were added at concentration of 10^4 u/l and 100mg/l respectively. Heparin was added at a concentration of 200µl/l. Newborn calf serum (Gibco-Bio-Cult, Paisley) was added at a concentration of 10% (v/v) when required.

(ii) Phosphate buffered saline, pH 7.2 or 7.4 (PBS)

Solution A : 500mM-$Na_2H\ PO_4/NaH_2\ PO_4$, pH 7.2 or 7.4

Solution B : 140mM-NaCl

PBS consists of 10% by vol Solution A and 90% by vol Solution B.

(iii) Alsevers solution

Alsevers solution was prepared by adding 20.5g dextrose, 4.2g NaCl, and 8.0g sodium citrate to 1l distilled H_2O. The solution was sterilised by membrane filtration using a 0.22µm Millipore filter.

(iv) Sorenson's buffer

Solution A : 50mM - $KH_2\ PO_4$

Solution B : 60mM - $Na_2\ HPO_4$

Sorenson's buffer consists of 5% by vol Solution A and 95% by vol solution B.

(v) Borate-saline

Solution A : borate buffer pH 8.4 containing (g/l)

6.2g boric acid ; 9.5g sodium

tetraborate; 4.4g NaCl.

Solution B : 140mM NaCl

Borate-saline contains 5% by vol Solution A and 95% by vol Solution B.

(vi) Citrate-saline

Citrate-saline is prepared by adding 17g NaCl and 30g sodium citrate to 2l of distilled H_2O.

(vii) Dulbecco's PBS

Solution A : 10g NaCl; 0.25g KCl; 1.44g Na_2HPO_4; 0.25g KH_2PO_4; 1l distilled H_2O

Solution B : 1g $CaCl_2.2H_2O$; 1l distilled H_2O

Dulbecco's PBS consists of 87.5% by vol Solution A and 12.5% by vol Solution B.

(viii) Glucose-PBS

Solution A : 1M-Na_2HPO_4, pH 7.6

Solution B : 140mM NaCl

Glucose - PBS consists of 1% by vol Solution A and 99% by vol Solution B plus dextrose added at a concentration of 10g/l.

2.2 Maintenance of the parasite

2.2.1 Preparation of miracidia

Miracidia were hatched from eggs obtained from the guts of infected mice according to the following procedure :

guts were dissected from mice infected 8 weeks previously, washed with 15mM -NaCl and then homogenised in 50ml Sorenson's buffer (2.1.5). The homogenate was transferred to a conical flask containing a further 50ml buffer, trypsin was added at a concentration of 1mg/gut, and the suspension was incubated at 37°C for 2h with gentle shaking. After trypsin digestion the homogenate was strained through 2 layers of muslin cloth and the filtrate was washed and centrifuged (800g for 10 min) twice. The sediment of packed eggs was recovered and added to a conical flask containing 1500ml/6 guts of aquarium water (copper free). The eggs were then allowed to hatch under a light source at room temperature.

2.2.2 Infection of snails

A stock of snails was maintained in plastic aquaria and fed regularly on dried lettuce. Snails were exposed to one (for preparation of clones) or a batch of 10-15 freshly hatched miracidia. Multi-welled trays, 140 x 100x 25mm (Linbro Scientific Co. Inc., Hampden, London), were used as snail containers for this purpose when dealing with a single miracidium, and 4 x 2cm glass bottles when using a batch. An aliquot of water containing the miracidium (-a) was added to snails individually placed in a well or a bottle and the snails were then left at 25°C for 24h. Following this, snails infected with a single miracidium were transferred individually into universal bottles (R and J. Wood, Paisley) of 20ml capacity and capped with muslin cloth to allow aeration; snails infected with multiple miracidia were pooled and transferred to a plastic aquarium. All snails were kept in the dark for 6 weeks at 25°C before being subjected to a bright light source in order to induce shedding of cercariae.

2.2.3 Infection of animals

2.2.3.1 Mice (Smithers and Terry, 1965)

Mice were anaesthetized using 0.9ml/100gm body weight of 10% (v/v) Sagatal in distilled water/ethanol (9:1 by vol). The anaesthetized mice were shaved to remove abdominal hair and placed face upward on polystyrene trays. A metal ring was then carefully placed on each abdomen. 100-200µl H_2O containing a fixed number of cercariae (50-200) was added to each ring. Mice were then left undisturbed for 10-15 min to allow cercarial penetration of skin.

2.2.3.2 Hamsters

Hamsters were infected by subcutaneous injection of approx. 1,000 cercariae (in 250µl H_2O)/animal.

2.2.4 Perfusion of animals for recovery of adult worms (Smithers and Terry, 1965).

6-8 week infected animals were killed by giving them an injection of sagatal (1ml/100gm body weight) intraperitoneally. The animals were dissected to expose the abdominal and thoracic cavities and then held in place by two thick rubber bands against a vertical perspex stand such that both cavities faced outward. An incision was made in the portal vein and then warm EMS or citrate-saline (2.1.5) was pumped into the heart using a 50ml disposable syringe (Plastipak - B - D, Becton, Dickinson and Co., Ireland). The flow of fluid through the circulation leads to worms being washed out through the incision in the portal vein. The worms were collected in a glass bowl, washed in EMS or EM + CS and employed in various experiments as described elsewhere.

2.2.5 Preparation of schistosomula by mechanical agitation (Colley and Wikel, 1974).

Cercariae freshly shed from snails were transferred to a universal bottle and concentrated on ice. Water was removed until approximately 1ml remained and then 9ml of EMS preincubated at 37°C was added. The cercariae were sucked up and down using a 10ml syringe (Plastipak - B-D, Becton, Dickinson and Co., Ireland) 5 or 6 times in order to separate the bodies (i.e. mechanically transformed schistosomula) from the tails. The mixture was then transferred to a 10ml test tube and centrifuged (100g for 5s) to sediment the schistosomula (the tails remain in the supernatant). Schistosomula were then washed (x 3) before being employed in the indirect fluorescent antibody method (2.4) or for immunisation (2.3.2).

2.3 Antisera

2.3.1 Anti-mouse RBC serum

2.3.1.1 Raising of antiserum

Venous blood was removed by cardiac puncture from freshly killed (by exposure to chloroform) Balb/c mice and added to a volume of Alsever's solution (2.1.5) such that the ratio of Alsevers solution to blood was greater than 3:1. The RBC were removed by centrifugation (300g for 5 min), washed (x 3) in glucose-PBS (2.1.5) and then 1ml (packed) added to an equal volume of Freunds complete adjuvant. This mixture was sonicated before being injected subcutaneously at several sites on the back of a rabbit. A booster inoculum consisting of 1ml packed RBC sonicated in the presence of 1ml Freunds Incomplete adjuvant was given 6 weeks later and at additional times when necessary.

2.3.1.2 Collection of antiserum

The antiserum was collected by bleeding the rabbit from the ear vein ten days after boosting. Approximately 20ml of blood was collected at each bleeding. Blood was allowed to clot at room temperature for 1h and then at $4^{o}C$ overnight. The serum was removed by pasteur pipette and then centrifuged (400g for 10 min) to remove any contaminating RBC. Serum samples were then aliquoted and stored at $-20^{o}C$.

2.3.1.3 Activity of antiserum

The antiserum was shown to be active by its ability to agglutinate mouse RBC. The haemagglutination assay was performed as follows :

(i) serum from the first bleed from the immunized rabbit, and normal rabbit serum (2.3.6) were decomplemented by heating for 30 min at 56°C;

(ii) doubling dilutions of both sera were prepared in PBS, pH 7.2, and 100µl aliquots added to the wells of a microtitre plate ;

(iii) 5ml of a 5% (v/v) solution of packed washed RBC (2.3.1.1) in PBS, pH 7.2, was added to each well and the plate was gently shaken before being incubated at 37°C;

(iv) after 1h plates were examined for evidence of agglutination. Anti-mouse RBC serum was found to have a titre of 512 : normal rabbit serum was without effect.

Binding of the antibody with respect to mechanically transformed and mature schistosomes was determined by using the indirect fluorescent antibody method (2.4). It was found that the antibody had no affinity for the surface of the juvenile parasite but bound to the surface of the adult male worm. The ability to interact with the adult worm was lost if the serum was first adsorbed with Balb/c RBC. Adsorption was performed as follows :

(i) 0.5ml aliquots of washed packed RBC (2.3.1.1) were added to 0.5ml aliquots of antiserum in LP3 tubes which were then rotated gently for 30 min at room temperature;

(ii) tubes were centrifuged (500g for 20 min) and the serum removed;

(iii) the serum was added to a further 0.5ml of RBC and the procedure repeated.

2.3.2 Anti-schistosomulum serum

2.3.2.1 Raising and collection of antiserum

Anti-schistosomulum serum was raised and collected using the techniques described in Section 2.3.1. Primary immunization and boosting employed 3,000 mechanically transformed schistosomula in 1ml of EMS.

2.3.2.2 Activity of antiserum

The antiserum was shown to be active by its affinity for the surface of newly transformed schistosomula as measured by the indirect fluorescent antibody method (2.4.2).

2.3.3 Anti-mouse immunoglobulin serum

Antisera raised in rabbits against mouse IgG 2b, IgM and IgA myelomas were a kind gift from Dr. D. Kipp, National Institute for Medical Research, Mill Hill, London. Each antiserum was found by the Ouchterlony double diffusion method to react with the myeloma with which it was raised but to have no affinity for the other two immunoglobulins. The myelomas were a kind gift from Dr. W. Cushley, Department of Biochemistry, University of Glasgow.

The Ouchterlony double diffusion method was performed as follows:

(i) Ouchterloney plates were prepared by (a) dissolving 1g agarose (Sigma type II) in 100ml borate-saline (2.1.5) and boiling for 5 min; (b) allowing the agarose to cool; and then finally (c) pouring 2-3ml of it on to a microscope slide. When the agarose had set, rosettes of wells were prepared using a punch.

(ii) Each myeloma was placed in the central well of a rosette and each antiserum was added to wells on the outside of the three rosettes. A volume of 2-3µl was employed when administering both myelomas and antisera. Following loading of the wells, the slides were incubated in a humid sandwich box for 24h at $4^{o}C$ to allow formation of precipitin lines.

2.3.4 Anti-MHC antibody

Monoclonal antibodies against the K and I-A regions of the mouse MHC were obtained from Dr. D. Kipp (National Institute for Medical Research, Mill Hill, London) as ascites fluid. The antibody directed against the K region has affinity for the k, p, q and r haplotypes; the antibody directed against the I-A region has affinity for the k, r, f and s haplotypes. The ascites fluid was centrifuged (400g for 5 min) to remove peritoneal cells. An IgG fraction of the two antibody preparations was then prepared by ammonium sulphate precipitation as follows :

(i) 2ml of saturated ammonium sulphate (cold) was added slowly, with constant stirring over a period of several min to 2ml of ascites fluid placed on ice. The prepar-

ation was left on ice for 45 min.

(ii) The preparation was centrifuged at 5,000g for 10 min and the supernatant was removed. The pellet was dissolved in a small volume of 20mM Tris-HCl; 40mM NaCl, pH 7.8.

(iii) The dissolved pellet was dialysed against 50-100 volumes of 20mM-Tris-HCl; 20mM-NaCl, pH 7.8 in a measuring cylinder by hanging the dialysis bag as high as possible without stirring ($(NH_4)_2SO_2$ dialysing out streams to the bottom of the tube). The dialysis buffer was changed 3 times in 24h.

(iv) The sample was transferred to a centrifuge tube and spun for 10 min at 15,000g to remove precipitated denatured proteins. The supernatant was removed, aliquoted and stored at -20°C.

2.3.5 Rabbit anti-human serum albumin (HSA):- raised in rabbits as in 2.3.1 by Dr. J.R. Kusel.

2.3.6 Second antisera

The following second antisera were supplied as indicated :

Goat anti-rabbit IgG (for coprecipitation)	Scottish Antibody Production Unit, Law Hospital, Carluke.
Goat anti-rabbit IgG-FITC	Institut Pasteur, Paris
Goat anti-mouse IgG Fc-FITC	Cappel Laboratories, Cochranville, U.S.A.
Goat anti-rabbit IgG (purified by affinity chromatography)	Cappel Laboratories, Cochranville, U.S.A.

2.3.6.1 Iodination of purified goat anti-rabbit IgG (Hunter and Greenwood, 1962).

All reagents for iodination were prepared in 50mM-$Na\ H_2\ PO_4/Na_2\ H\ PO_4$ buffer pH 7.2. To 20µg (in 20µl) of IgG were added in order : 20µl of 500mM - NaH_2PO_4/Na_2HPO_4 buffer, pH 7.2; 200µCi (in 20µl) ^{125}I (NaI); 20µg (in 20µl) of chloramine T; 25µg (in 20µl) of sodium metabisulphite; and 400µl of 0.1% (w/v) BSA. The solution was immediately vortexed and then left for 1 min following addition of chloramine T, and also following addition of sodium metabisulphite. Vortexing was also carried out on completion of the protocol.

Labelled antibody was separated from free ^{125}I by passing the sample through a small (12 x 1cm) column of Sephadex G-25. 0.1% (w/v) BSA in PBS pH 7.2 was used for elution and the column was equilibrated with this solution before use. The column was also "saturated" with protein before use in order to minimize the adsorption of the very low concentration of labelled protein which is present. Saturation was accomplished by passing 0.5ml of a 100mg/ml solution of BSA in PBS pH 7.2 through the column.

The labelled antibody is eluted from the column in a volume of 2-3ml. This was divided into 100µl aliquots and stored at $-20^{o}C$.

2.3.7 Normal rabbit and normal mouse serum

Normal rabbit serum (NRS) and normal mouse serum (NMS) were either obtained from the Scottish Antibody Production

Unit, Law Hospital, Carluke or by bleeding animals (2.3.1.2) from the departmental animal house.

2.4 Indirect fluorescent antibody method (IFAM)

2.4.1 Adult worms

Worms (4/tube) were incubated in 100µl of the antibody being tested for 30 min in an LP3 tube and then washed (x 3) in EM + CS. 100µl of an FITC-labelled antibody, specific for the first, was added and a further 30 min incubation followed. Worms were again washed, immobilised in carbachol (2mg/ml in EMS), washed, mounted on slides, and examined using a Leitz Ortholux II microscope equipped with a Ploempak epi-illuminator and a GG475 filter.

The assay was carried out at various temperatures. All reagents, washing fluids, and also the worms were incubated at the appropriate temperature for at least 30 min before use in the assay.

All first antisera were titrated in the first assay in which they were employed. In subsequent experiments anti-mouse RBC serum was employed at a 1:80 dilution; anti-mouse IgG and anti-H-$2K^k$ at a 1:40 dilution; and all other first antisera at a 1:20 dilution. Antisera were diluted in EMS + 10% NRS or EMS + 10% NMS and were heated at 56°C for 30 min before use. FITC-labelled second antibodies were employed at a 1:25 dilution with the exception that goat-anti rabbit-FITC was used at a 1:100 dilution when anti-mouse RBC serum was employed as first antibody.

Intensity of fluorescence was measured using a + system (- to ++++ on an arbitrary scale).

Photographs were taken using Kodak Ektochrome 160 film (EPT 135).

2.4.2 Schistosomula

100µl aliquots of the antibodies under test were added to centrifuge tubes containing approximately 200 schistosomula in 200µl EMS. The tubes were incubated for 30 min at 37°C and then the schistosomula were washed in EMS. The washing fluid was removed as supernatant following centrifugation (100g for 5s). This washing procedure was repeated twice and following the last centrifugation approx. 200µl of EMS was left in each tube. 100µl of FITC labelled anti-IgG was then added and the incubation and washing steps were repeated. Schistosomula were then prepared for examination by fluorescence microscopy as described in 2.4.1.

Anti-mouse RBC serum and anti-schistosomulum serum were both employed at a 1:10 dilution. Second antibody was employed at a 1:100 dilution. All three antisera were diluted in EMS.

2.5 Indirect radiolabelled antibody method (IRAM)

(see Fig. 7)

2.5.1 Assay protocol

Worms (4/tube) were washed (x 3) in EM + CS either after removal from mice or after treatment with a membrane-active

reagent and then incubated with 50µl of specific antibody or NRS for 30 min at 37°C in an LP3 tube. Following incubation, worms were washed (x 3) in EM + CS and transferred to a fresh LP3 tube by pasteur pipette. All residual washing medium was removed and 50µl of ^{125}I labelled antibody having specificity for the first antibody was added. A second incubation of 30 min at 37°C was carried out before worms were again washed (x 2) in EM + CS and transferred to a Beckman Biogamma counting vial. A final wash in EM + CS was undertaken, all residual washing medium was removed and worms were then counted for radioactivity in an LKB gamma counter. Anti-mouse RBC serum and anti-schistosomulum serum were employed at a 1:50 and a 1:10 dilution respectively. ^{125}I-goat anti rabbit IgG was employed at a concentration of 200-300ng/50µl. First antisera were prepared in EM + 10% NRS; second antibody in EM + CS. First antisera were heated for 30 min at 56°C before use.

During the course of the development of the assay a number of parameters were varied including the washing conditions; the assay volume, temperature and incubation time; the number of worms/tube; and the antibody concentrations. The conditions for experiments in which assay temperature was varied were identical to those employed in the IFAM (2.4).

Exposure of worms to membrane active reagents was normally carried out in petri dishes. The details of the incubation times employed are described in the results section. Worms subjected to 24h culture periods were

maintained under sterile conditions in an atmosphere of 5% CO_2/95% air (by vol.).

2.5.2 Calculation and expression of results

All samples were investigated in triplicate. The average value obtained for binding of radiolabelled antibody in the absence of specific antibody (i.e. following incubation in NRS) was subtracted from each value obtained for binding in the presence of specific antibody. The mean value and standard deviation (s.d.) attributed to specific antibody was then determined. Results are thus expressed as mean specific antibody bound/four worms (in c.p.m.) ± s.d. Statistical analysis was undertaken using the Students t-test.

2.6 Preparation of membrane-active reagents and purified lipoproteins for use in the IRAM

2.6.1 Formalin, glutaraldehyde and methanol

All three reagents were prepared by dilution in PBS, pH 7.2.

2.6.2 Trypsin

Trypsin was prepared in EMS immediately before use. Trypsin powder was stored at -20°C.

2.6.3 Retinol

A stock solution of retinol in ethanol (100mg/ml) was stored at -20°C under nitrogen in the dark. Retinol solutions for use in the investigation were prepared from this stock solution in EMS immediately before use.

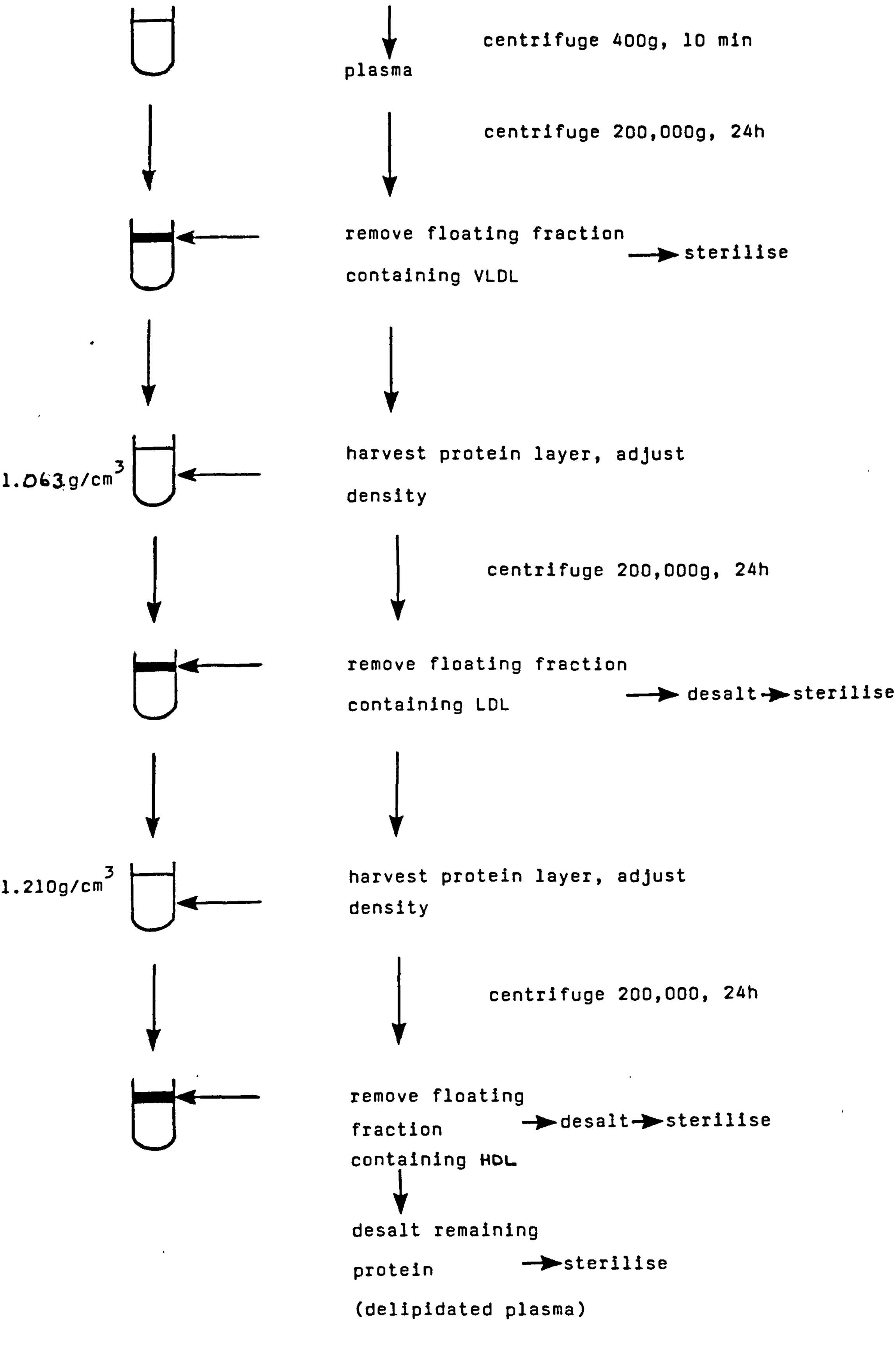
2.6.4 Tween reagents

Tween reagents were dissolved in EMS by constant stirring for 5-10 min at room temperature. The solutions were prepared immediately before use.

2.6.5 Lipoproteins (Fig 2)

20ml of freshly obtained human venous blood was added to 1ml of 10% sodium citrate. The plasma was isolated by centrifugation (400g for 10 min) in an MSE bench centrifuge and then centrifuged (200,000g for 24h at $4^{o}C$) in a Beckman 250-5B ultra-centrifuge to isolate very low density lipoproteins (VLDL). This lipoprotein class is present in the turbid floating fraction. The orange-coloured protein layer in the lower one-third of the tube was then harvested with a pasteur pipette and its density adjusted to 1.063 g/cm^3 by dilution with 0.8x its own volume of 1.1320 g/cm^3 solution (3.398M - NaCl; 0.1mM - Na_2 EDTA). This was centrifuged (200,000g for 24h at $4^{o}C$) as before. The yellow floating fraction containing low density lipoproteins (LDL) was removed and desalted by passage through a column packed with Sephadex G-25. PBS, pH 7.2 was employed as elution buffer. The orange infranate fraction was harvested with a pasteur pipette and its density was readjusted to 1.210 g/cm^3 by dilution with 1.12x its own volume of 1.3224 g/cm^3 solution (0.202M - NaCl; 4.599M - NaBr; 0.1mM - Na_2 EDTA). This was again centrifuged (200,000g for 24h at $4^{o}C$) as before. The floating fraction containing high density lipoproteins (HDL) and the yellow 1.210g/cm^3 infranate were each harvested and then desalted as described for LDL. All fractions were sterilised by membrane filtration using a Millipore

Fig. 2. Isolation of lipoproteins



filter with a 0.2μm pore size. They were then stored at $4^{o}C$ and had a shelf life of two weeks. The density of the 1.132g/cm^3 and 1.3224g/cm^3 solutions was measured using a digital density meter (Department of Chemistry, University of Glasgow).

For use in the IRAM, lipoproteins and delipidated plasma (1.210g/cm^3 infranate) were restored to approximately their original serum concentration with EMS, and then diluted 1:10, again in EMS.

2.6.6 Praziquantel

Praziquantel was prepared immediately before use by first dissolving it in ethanol (2mg praziquantel/ml ethanol) and then diluting in EMS to give a final concentration of 10μg/ml. A solution of 0.5% ethanol/EMS (to act as a control) was also prepared.

2.6.7 Staphylococcal δ toxin

δ toxin was dissolved in EMS and then stored in aliquots at $-20^{o}C$.

2.6.8 Phospholipase A_2 (Naja naja)

Phospholipase A_2 was dissolved in Dulbecco's PBS (2.1.5) immediately before use.

2.7 Preparation and iodination of membrane sample

2.7.1 Preparation

Approximately 700 adult worms, freshly obtained from Balb/c mice, were washed (x 3) in EMS and then frozen in 1ml PBS pH 7.4 containing 100µM-PMSF and 1µM-pepstatin (PPP). The PBS was then thawed, vortexed to remove loosened membranes, and transferred to ice. The loosened membranes were washed with 11ml PPP and the washing fluid containing isolated membranes and soluble proteins was then mixed thoroughly before being divided into four equal aliquots. Each aliquot was centrifuged (200,000g for 2h at 4^{o}C) on a Beckman L5-L6 centrifuge. The supernatants were then removed and the membrane pellets were solubilised for 15 min at 37^{o}C with 100µl of either :

(a) 1% (v/v) Triton X-100; (b) 1% (v/v) NP40; (c) 1% (w/v) sodium deoxycholate; (d) 1% (v/v) Triton X-100; 9M-urea. All detergents were prepared in 50mM-NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4, containing 100µM-PMSF and 1µM-pepstatin. The solubilised membranes were transferred to Eppendorf tubes and then centrifuged (10,000g for 2 min). The supernatants were recovered, transferred in 100µl aliquots to fresh Eppendorf tubes, and then stored at -20^{o}C.

PMSF was dissolved in DMSO and pepstatin in 50% ethanol. 20µl of 100mM-PMSF and 20µl of 1mM-pepstatin were added to 20ml of the various solutions at 4^{o}C whilst stirring. Stirring was then continued for a further 30 min.

2.7.2 Iodination (Hunter and Greenwood, 1962)

All reagents for iodination were prepared in 50mM-sodium phosphate buffer, pH 7.4. Membrane samples were thawed and centrifuged (10,000g for 2 min) to remove insoluble material. The supernatants were transferred to LP3 tubes and to each was added in order : 25µl 500mM - sodium phosphate buffer pH 7.4; 500µCi (in 25µl) ^{125}I (NaI); 40µg (in 20µl) chloramine T ; 50µg (in 20µl) sodium metabisulphite; and 50µg BSA in 300µl of a solution containing 1% of the detergent in which the membrane preparation was initially solubilised (2.7.1). 1% (v/v) Triton X-100 was employed when dealing with the sample initially solubilised using 1% (v/v) Triton X-100; 9M-urea. The solutions were immediately vortexed and then left for 1 min following addition of chloramine T, and also following addition of sodium metabisulphite. Vortexing was also carried out on completion of the protocol.

All four preparations were next dialysed twice (for 2h and then overnight) against PBS pH 7.4 containing the relevant detergent at a concentration of 0.25% (Triton X-100 was again employed when dealing with the sample initially solubilised with 1% (v/v) Triton X-100; 9M-urea). Samples were removed from dialysis tubing, transferred to Eppendorf tubes, and then made up to 500µl with PPP. Samples were stored at -20°C.

2.8 Co-precipitation assay

2.8.1 Anti-schistosomulum serum

The technique employed was a modification of the method of Kusel *et al* (1975). 25µl of iodinated membrane was made up to 100µl in an Eppendorf tube with detergent solutions as follows :

(a) membrane initially solubilised with 1% (v/v) Triton X-100 (2.7.1) was added to 75µl of 0.25% (v/v) Triton X-100 in PBS pH 7.4;

(b) membrane initially solubilised with 1% (v/v) Triton X-100; 9M-urea (2.7.1) was prepared as in (a);

(c) membrane initially solubilised with 1% (w/v) sodium deoxycholate (2.7.1) was added to 75µl of 0.25% (w/v) sodium deoxycholate in PBS pH 7.4.

The samples were centrifuged (10,000g for 2 min) and the supernatants retained and transferred to fresh Eppendorf tubes. The following were then added rapidly in sequence with vortexing : 10µl (5mg/ml) of human serum albumin (HSA); 40µl of a 1:2 dilution of rabbit anti-HSA; and 50µl of 0.5% of the relevant detergent in PBS pH 7.4. Eppendorfs were then incubated for 30 min at 37^{o}C and then centrifuged as before. During the incubation period a precipitate forms between HSA and anti-HSA and this helps remove. "sticky" iodinated proteins which bind to antibody non-specifically. The use of HSA and anti-HSB therefore represents a clearance step. The supernatants were retained following centrifugation, and each divided into two equal aliquots during transfer to LP2 tubes. 10µl of either anti-schistosomulum serum or NRS were added to each tube and the samples were

incubated for 1h at 37°C. To carry out co-precipitation, 100µl of a 1:4 dilution of goat anti-rabbit IgG containing 0.25% of the relevant detergent was added to each tube, before samples were subjected to a final incubation consisting of 1h at 37°C followed by 1h at 4°C. Samples were then centrifuged (1000g for 20min), and the pellets recovered and washed twice - once with 0.25% of the relevant detergent in PBS, pH 7.4, and once in PBS. LP2 tubes containing the pellets were inserted in counting vials and counted for radioactivity using an LKB gamma counter. All samples were examined in duplicate. Following counting, samples were stored at -20°C.

2.8.2 Anti-mouse RBC serum

Co-precipitation was performed using anti-mouse RBC serum as first antiserum as described in 2.8.1. The membrane material employed was initially solubilised by 1% (v/v) Triton X-100 or 1% (v/v) Triton X-100; 9M-urea (2.7.1).

2.8.3 Determination of precipitating antibody dilutions

2.8.3.1 Anti-HSA

Doubling dilutions of anti-HSA were added to 100µl PBS, pH 7.4 and 10µl (5mg/ml) HSA in LP3 tubes. The tubes were incubated for 30 min at 37°C and then examined visually for evidence of precipitation. The dilution of antibody producing the largest precipitate (1:2) was selected for use in the assay.

2.8.3.2 Anti-rabbit IgG

The co-precipitation assay was performed in the absence of membrane (replaced by PBS pH 7.4) using doubling dilutions of second antibody. Visual examination of the precipitates obtained following the final incubation step revealed that dilutions up to 1:8 had produced similar precipitation and that the nature of the first antiserum had not influenced the results.

2.9 SDS Polyacrylamide Gel Electrophoresis (Laemmli, 1970)

2.9.1 Stock solutions

(i) 1.5M-Tris/HCl buffer, pH 8.8

(ii) 0.5M-Tris/HCl buffer, pH 6.8

(iii) Acrylamide-bisacrylamide solution : 30g acrylamide plus 0.8g bisacrylamide was dissolved in, and made up to 100ml with distilled water. The solution was stored at $4^{o}C$ and used within one month.

(iv) 10% (w/v) SDS solution : this solution was stored at room temperature.

(v) Running buffer : 3g Tris base, 14.4g glycine and 1g SDS was dissolved in 1l distilled water.

2.9.2 Preparation of glassware

Glass plates for slab gels (65 x 80 and 65 x 65mm) and gel tubes (internal diameter = 10cm) were washed with dilute nitric acid, oven dried, and then siliconised by soaking for 30 min in Repelcote solution. After siliconising, the plates and tubes were thoroughly washed with distilled water and then oven dried.

2.9.3 Preparation of slab gels

2.9.3.1 Gel casting mould

Two plastic strips (80 x 3 x 1 mm) were cemented along the long sides on the surface of a 65 x 80mm glass plate using vacuum grease. A second glass plate, 65 x 65mm, was then cemented on to the glass strips and the whole sandwich secured by two paper clips.

2.9.3.2 Sealing gel (0.85% (w/v)).

The bottom of the gel casting mould was sealed by immersing it in a gel solution containing 2.5ml acrylamide - bisacrylamide solution; 7.5ml 1.5M-Tris/HCl buffer pH 8.8; 0.25ml TEMED and 0.2ml 10% (w/v) ammonium persulphate (freshly prepared). The solution was degassed at a vacuum pump before addition of TEMED and 10% ammonium persulphate.

2.9.3.3 Separating gel (10% (w/v))

A solution was prepared containing 10ml acrylamide - bisacrylamide solution; 7.5ml 1.5M-Tris HCl buffer, pH 8.8; 12.05ml distilled water; 0.3ml 10% SDS; 0.15ml 10% (w/v) ammonium persulphate (freshly prepared) and 10µl TEMED. After the addition of the acrylamide, water and Tris HCl buffer the solution was degassed at a vacuum pump before addition of the other components. The gel solution was poured between the glass plates of the gel mould, overlayed with distilled water to ensure that the surface of the gel was flat, and then allowed to polymerise for 30 min. The layer of water was then removed and the stacking gel poured.

2.9.3.4 Stacking gel (3% (w/v))

A solution was prepared containing : 1.5ml acrylamide - bisacrylamide; 3.75ml 0.5M Tris/HCl buffer, pH 6.8; 9.45ml distilled water; 0.15ml 10% SDS; 0.15ml 10% (w/v) ammonium persulphate (freshly prepared) and 10µl TEMED. Degassing was undertaken as described for the separating gel. The gel solution was poured on top of the separating gel and left for 30-60 min to polymerise around a well template with 7 sample application wells. On completion of polymerisation the template was removed.

2.9.4 Preparation of tube gels

2.9.4.1 Separating gel (10 and 15% (w/v))

The composition and preparation of 10% (w/v) separating gel is described in Section 2.8.3.3. 15% (w/v) gel was prepared in the same manner and has the following composition: 15ml acrylamide - bisacrylamide solution; 7.5ml 1.5M - Tris/HCl buffer pH 8.8; 7.05ml distilled water; 0.3ml 10% SDS; 0.15ml 10% (w/v) ammonium persulphate (freshly prepared) and 10µl TEMED. Gel solutions were poured into gel tubes, overlayed with water and allowed to polymerise for 30 min. The layer of water was then removed and the stacking gel poured.

2.9.4.2 Stacking gel (3% (w/v))

The composition and preparation of stacking gel is described in 2.8.3.2. The gel mixture was poured on top of the separating gel, overlayed with water and allowed to set for 30-60 min.

2.9.5 Sample preparation and loading

The sample buffer was prepared as follows :

10ml Tris/HCl buffer, pH 6.8; 10ml 10% SDS; 10ml glycerol; 68ml distilled water; 2ml 0.5% Bromophenol blue.

2.9.5.1 Isolated membrane preparation

Isolated membrane proteins were resolved using slab gels. Frozen samples were thawed, vortexed and then centrifuged (10,000g for 2 min) to remove any aggregated material. 10µl samples were then denatured in an equal volume of sample buffer containing 1% (v/v) mercaptoethanol by heating for 2 min at 100°C. Once the electrophoresis apparatus had been completely assembled and the running buffer added, the samples were loaded into wells using a micropipette.

2.9.5.2 Coprecipitated membrane antigens

Coprecipitated membrane antigens were resolved using tube gels. Precipitates containing antigen (2.8) were thawed, dissolved in 20µl of sample buffer containing 1% (v/v) mercaptoethanol and then denatured and loaded on to the top of gel columns as described in Section 2.9.5.1.

2.9.6 Electrophoresis conditions and sample running

The samples were electrophoresed at 6 mA per tube and 15-20 mA per slab using a constant current at room temperature until the marker dye had almost reached the bottom of the gels. The gels were then removed from the tubes or slabs and fixed and stained for protein (to detect molecular weight markers 2.9.7) as described in Section 2.9.8.

2.9.7 Molecular weight calibration of SDS PAGE

Molecular weight estimations of polypeptides resolved by SDS-PAGE were determined by calibrating gels with a set of molecular weight standards (Bio-Rad Labs. Ltd. Caxton Way, Watford, Herts). These were :

phosphorylase B, mol. wt : 92,000; BSA, mol. wt. : 66,000; ovalbumin, mol. wt : 45,000 ; carbonic anhydrase, mol. wt : 31,000 ; soyabean trypsin inhibitor, mol. wt : 21,500 ; lysozyme, mol. wt : 14,500.

2.9.8 Fixing and staining

Slab and tube gels were fixed and stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 in methanol/acetic acid/ H_2O (50:10:40 by vol) for 30 min at 60°C. The gels were then destained with methanol/acetic acid/H_2O (10:10:80 by vol) at 60°C. Following destaining gels were analysed for radioactive proteins, by fluorography in the case of slab gels (2.10) and by counting of gel slices in the case of tube gels (2.11).

2.10 Fluorography (Bonner and Laskey, 1974)

Slab gels were given 3 successive washes, each of 30 min, in 200ml DMSO, with gentle shaking. This was followed by immersion of gels in 100ml of 20% (w/v) PPO in DMSO for 45 min. Gels were next washed twice in H_2O, each wash lasting 30 min, before being dried onto Whatman 3MM filter paper under vacuum. Finally gels were placed in contact with Kodak-X-O-mat-Royal film and exposed at -70°C for 18-30h.

2.11 Gel-slicing technique

Tube gels were frozen by immersion in solid CO_2 in methanol and sliced transversely into 1mm sections. Each slice was then placed in a counting vial and measured for radioactivity in an LKB gamma counter.

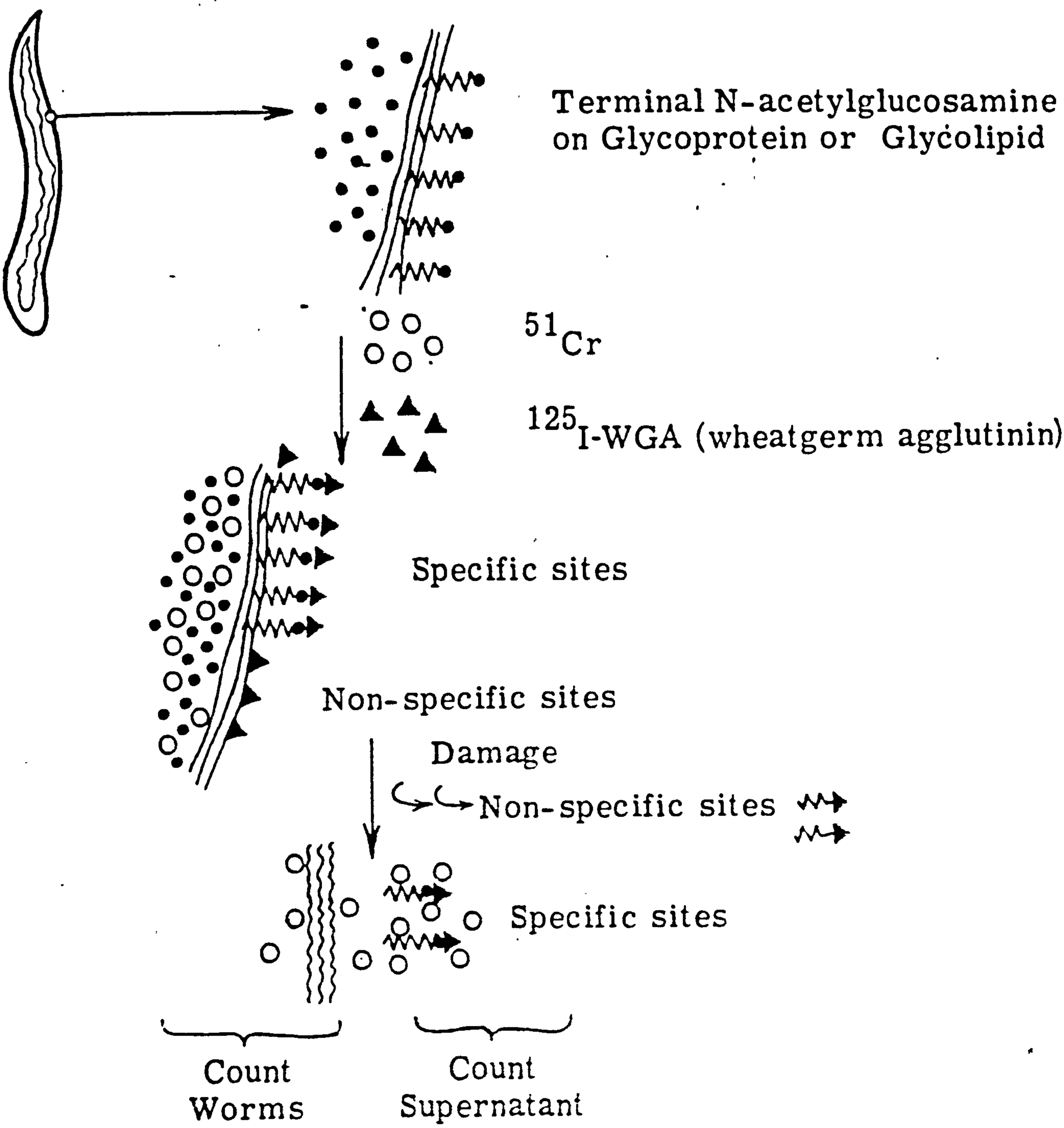
Gels were sliced by gently pressing them into an apparatus consisting of a continuous row of blades, spaced 1mm apart.

2.12 Use of the release of ^{51}Cr and ^{125}I-labelled wheat-germ agglutinin (^{125}I-WGA) from labelled parasites as an indicator of membrane damage (Kusel et al, 1981;1982) (Fig. 3)

2.12.1 Principle of method

Incubation of adult schistosomes in the presence of ^{51}Cr (sodium chromate) leads to uptake of the isotope into the cytoplasm of the worm. Treatment of worms with reagents which increase membrane permeability will however cause the isotope to be released. Incubation of schistosomes with ^{125}I-WGA results in binding of the lectin partly specifically and partly non-specifically to N-acetyl glucosamine residues on surface glycoproteins or glycolipids. If such molecules are released following treatment of worms with membrane-active reagents, then ^{125}I can be detected in the incubation medium. It thus follows that release of ^{51}Cr can be taken as an indication of a permeability change and release of ^{125}I-WGA as a measure of surface macromolecule release.

Fig.3 The $^{51}Cr/^{125}I$-WGA release assay



$$\text{Damage} = \frac{\text{Counts in Supernatant}}{\text{Counts in Supernatant} + \text{Worms}} \times 100\%$$

2.12.2 Iodination of WGA (Hunter and Greenwood, 1962)

All reagents for iodination were prepared in 50mM-sodium phosphate buffer, pH 7.2. To 400µg (in 100µl) of WGA were added in order : 40µl 500mM-sodium phosphate buffer pH 7.2; 200µCi (in 20µl) ^{125}I (NaI); 20µg (in 20µl) chloramine T; 25µg (in 20µl) of sodium metabisulphite; and 300µl 0.1% (w/v) BSA. The solution was immediately vortexed and left for 1 min following addition of chloramine T and also following addition of sodium metabisulphite. Vortexing was also carried out on completion of the protocol.

Labelled lectin was separated from free ^{125}I by dialysing twice (for 2h and then overnight) against PBS, pH 7.2. The WGA was then recovered from dialysis tubing, aliquoted, and stored at -20°C.

2.12.3 Assay protocol

100-150 adult worms (male unless otherwise stated) were incubated in 1.0-1.5ml EMS containing 100µl ^{51}Cr (1.0 mCi/ml as sodium chromate in 0.9% saline) and 10µl ^{125}I-WGA (0.8mg/ml) for 1h at 37°C, washed (x 3) in EMS, and then added to fresh EMS in a petri dish. Groups of 4 worms were then placed in specially prepared incubation containers consisting of 500µl Oxford pipette tips which had plastic netting fused to a cut surface 0.5cm from the delivery end. The incubation containers were placed within counting vials containing 500µl of EMS or the reagent under test and incubated for 1h at 37°C. At the completion of the incubation period, the incubation containers were lifted above the fluid level in the counting vials with

a pair of tweezers and the worms in each container were then rinsed with 0.5ml EMS. The washing fluids are thus added to the incubation medium in the counting vials. The incubation containers were transferred to fresh vials and the radioactivity associated with the worms and the incubation supernatant measured in an LKB gamma counter. Correction was made for spill over of ^{51}Cr counts into the ^{125}I channel.

2.12.4 Calculation and expression of results

The radioactivity (in c.p.m.) released from the parasite into the culture medium was expressed as the percentage of the total radioactivity in the system (i.e. worms plus medium) and expressed as the mean of triplicate determinations ± standard deviation. Statistical analysis was undertaken using the Students t-test.

2.13 Electron microscopy (Hockley and McLaren, 1973)

Worms were washed (x 3) in EMS and then exposed to :

(i) 2% (w/v) glutaraldehyde in 50mM-sodium cacodylate buffer pH 7.4, containing 2mM-calcium acetate for 1.5h at $4^{\circ}C$ followed by washing (x 3) in isotonic buffer (Lewis and Shute, 1966)

(ii) 1% (w/v) osmium tetroxide in 100mM-s-collidine buffer, pH 7.4 for 1.5h at $4^{\circ}C$ and then washing (x 3) in distilled H_2O;

(iii) 0.5% (w/v) aqueous solution of uranyl acetate pH 5, containing 45mg/ml sucrose for 1h at $4^{\circ}C$ with a final wash in distilled H_2O.

The worms were dehydrated in ethanol and embedded in Araldite. Sections were cut using a diamond knife and stained with uranyl acetate and lead citrate. Examination was undertaken using a Phillips EM 300 electron microscope. Photographs were taken using Kodak Electron Microscope film 4489.

CHAPTER 3

The development of an assay to measure changes in surface antigen expression

3.1 Introduction

In order to measure changes in antigen expression at the surface membrane of adult Schistosoma mansoni it is necessary to employ a technique which will provide quantitative data. Studies on surface antigens of the adult worm to date have provided qualitative or at best semi-quantitative information. Such studies invariably involve the use of an indirect antibody technique employing for example, an FITC labelled second antibody as an indicator system (the indirect fluorescent antibody method, Goldring et al 1977b). The indirect fluorescent antibody method (IFAM) can be employed to provide quantitative data if used in conjunction with a spectrophotofluorimeter and indeed this system has been used to measure antibody binding to schistosomula (Tavares et al, 1980; Samuelson et al, 1980). An alternative approach however, would be to label the second antibody with an isotope such as ^{125}I, a molecule whose quantitation is known to be rapid and reliable. Indirect antibody assays employing an ^{125}I-labelled second antibody have previously been employed to measure antigen expression at the cell surface (for review, see Williams, 1977) and also at the schistosomulum surface (Levi-Schaffer et al, 1982). It was thus decided to develop such an assay to measure changes in antigen expression at the adult schistosome surface.

3.2 Selection of an antibody to measure host antigen expression.

The ability of a number of antisera of different specificities to interact with the adult worm surface was determined by using the IFAM. The results obtained when the experiment was performed at 37°C are shown in Table 2.

Anti-mouse RBC antibody was found to bind extensively over the entire surface of both male (Fig 4a,4b) and female worms. The fluorescence associated with worms incubated with anti-IgG was in general of a patchy appearance (Fig 4c) and was lower in intensity than that obtained with anti-mouse RBC serum. Binding of anti-IgA and anti-IgM to both male and female worms was extremely variable in each of four separate experiments. In spite of this, some indication of differences between sexes was obtained. The binding of antibodies directed against histocompatibility antigens was also variable but in general was weak or absent.

To determine if the low and variable binding of anti-IgA, anti-IgM and anti-MHC antibody was due to shedding of antigen/ antibody complexes (Kemp et al, 1980) the experiment was repeated at both 37°C and 4°C (Table 3). Comparison of the results obtained at the two different temperatures revealed that lowering the temperature had not influenced the result. Interestingly however, low temperature appeared to have an effect on the binding of anti-mouse RBC antibody as although it was still extensive it appeared to have lessened in intensity.

Worms were incubated for 30 minutes at 37°C with the first antibody and then washed (x 3) with EM + CS. A further incubation of 30 minutes at 37°C in the presence of the second antibody then followed before worms were washed, immobilised using carbochol (2mg/ml) and examined for intensity of fluorescence (++++, +++; ++; +; - representing intensity of fluorescence on an arbitrary scale), using a Leitz Ortholux II microscope equipped with a Ploempak epi-illuminator and a GG475 filter. Each antiserum was examined in the assay on at least three separate occasions.

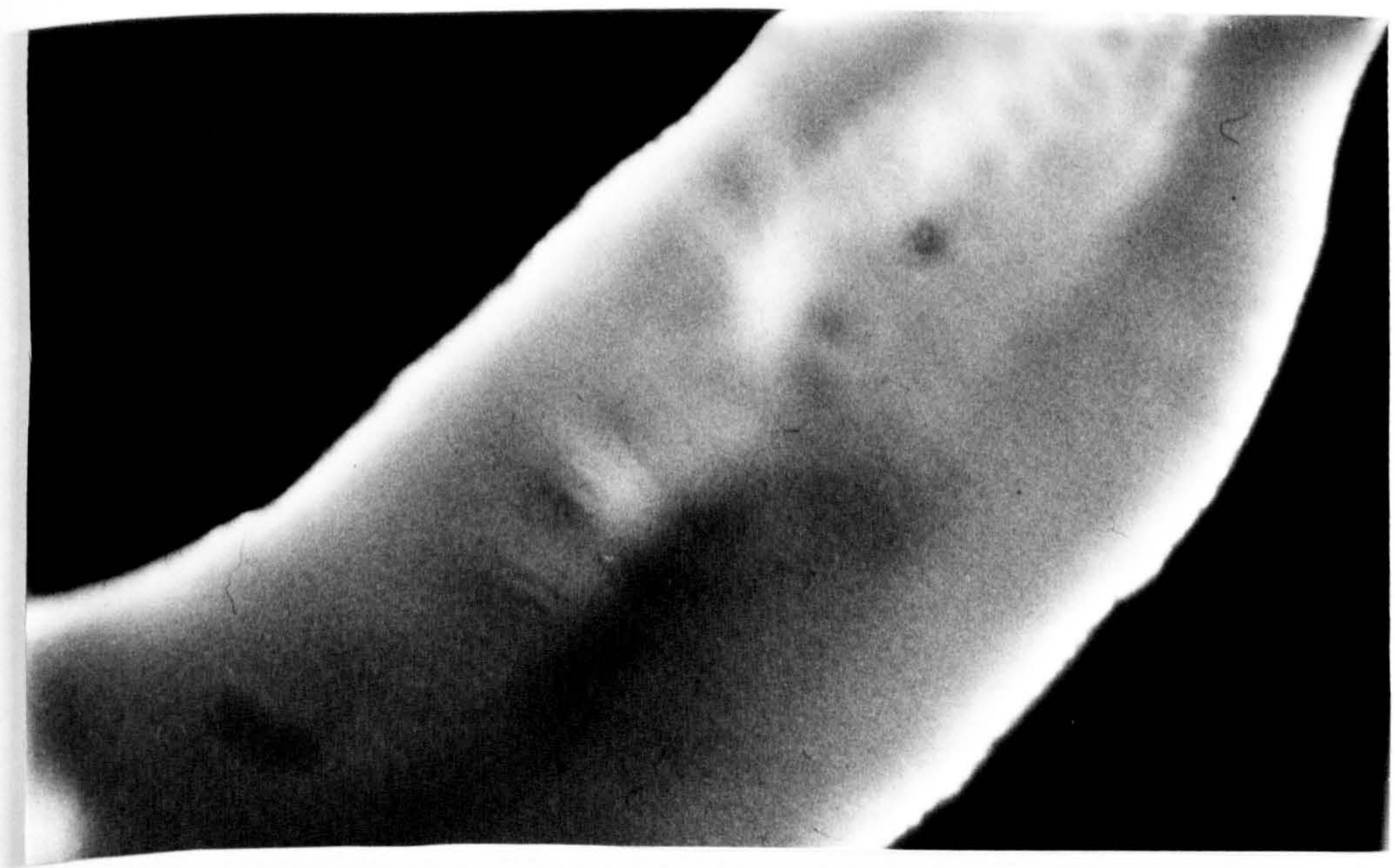
* Variation in intensity of fluorescence of individual worms within the one experiment.

** the result in which intensity of fluorescence was ++ was obtained in only one experiment. Female worms were not examined in this particular experiment.

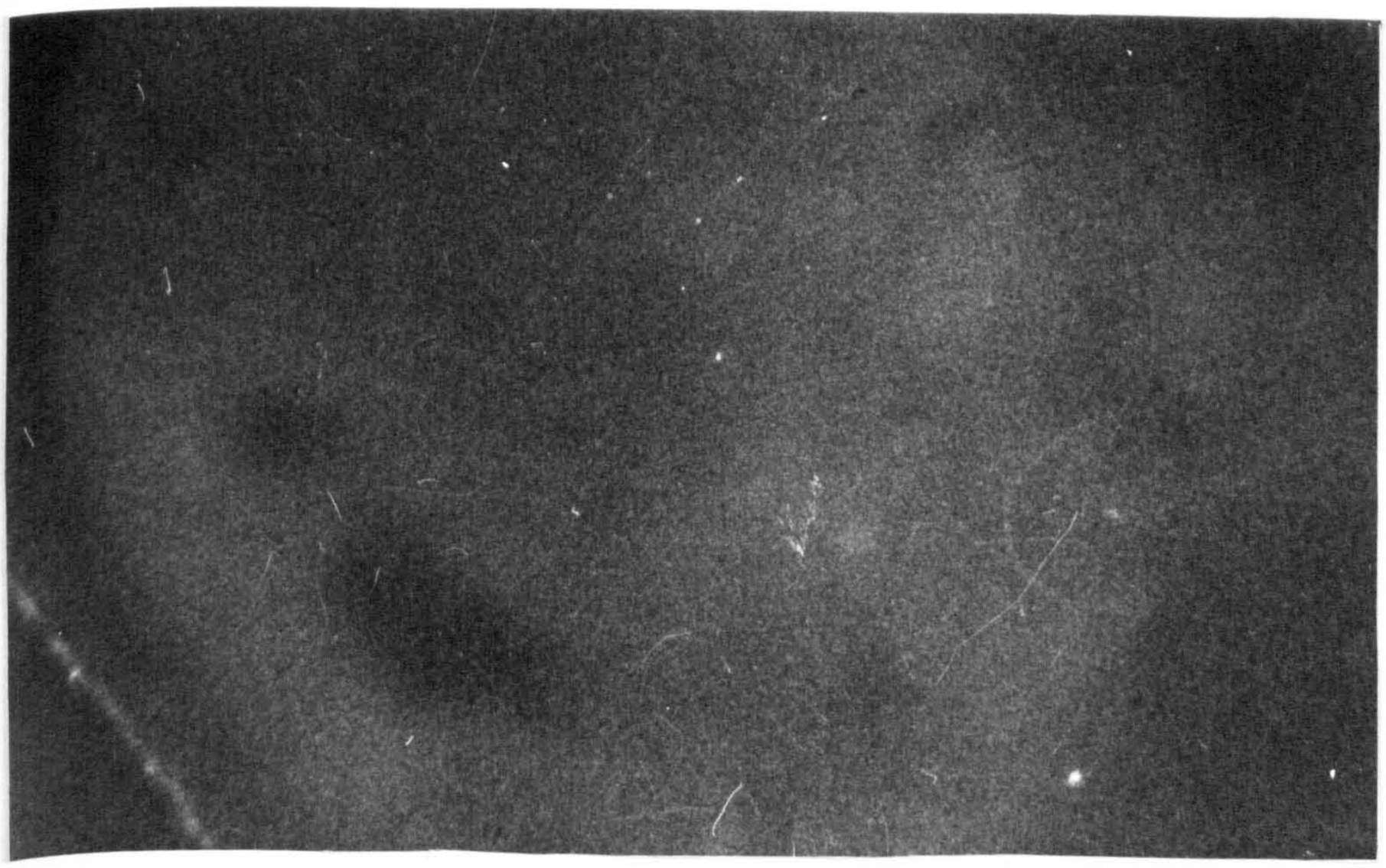
Table 2 Intensity of binding of FITC labelled second antibody to adult worms after incubation with a number of first antisera

Strain of mouse providing worms	Sex of worms	First antibody	Second FITC labelled antibody	Intensity of fluorescence
Balb/c	M + F	anti-balb/c RBC	anti-rabbit IgG	++++
Balb/c	M + F	anti-mouse IgG	anti-rabbit IgG	++
Balb/c	M	anti-mouse IgM	anti-rabbit IgG	++, +, - *
Balb/c	F	anti-mouse IgM	anti-rabbit IgG	+, - *
Balb/c	M	anti-mouse IgA	anti-rabbit IgG	++, +, - *
Balb/c	F	anti-mouse IgA	anti-rabbit IgG	+, - *
Balb/c	M + F	normal rabbit serum	anti-rabbit IgG	-
CBA ($H\text{-}2^K$)	M	anti-H-$2K^K$	anti-mouse IgG Fc	++, +, - * **
CBA ($H\text{-}2^K$)	F	anti-H-$2K^K$	anti-mouse IgG Fc	+. - *
CBA ($H\text{-}2^K$)	M + F	anti-H-$2IA^K$	anti-mouse IgG Fc	+, -
CBA ($H\text{-}2^K$)	M + F	normal mouse serum	anti-mouse IgG Fc	-
Balb/c ($H\text{-}2^d$)	M + F	anti H-$2K^K$	anti-mouse IgG Fc	-
Balb/c ($H\text{-}2^d$)	M + F	anti-H-$2IA^K$	anti-mouse IgG Fc	-
BIO/A ($H\text{-}2^a$)	M + F	anti-H-$2K^K$	anti-mouse IgG Fc	-
BIO/A ($H\text{-}2^a$)	M + F	anti-H-$2IA^K$	anti-mouse IgG Fc	-

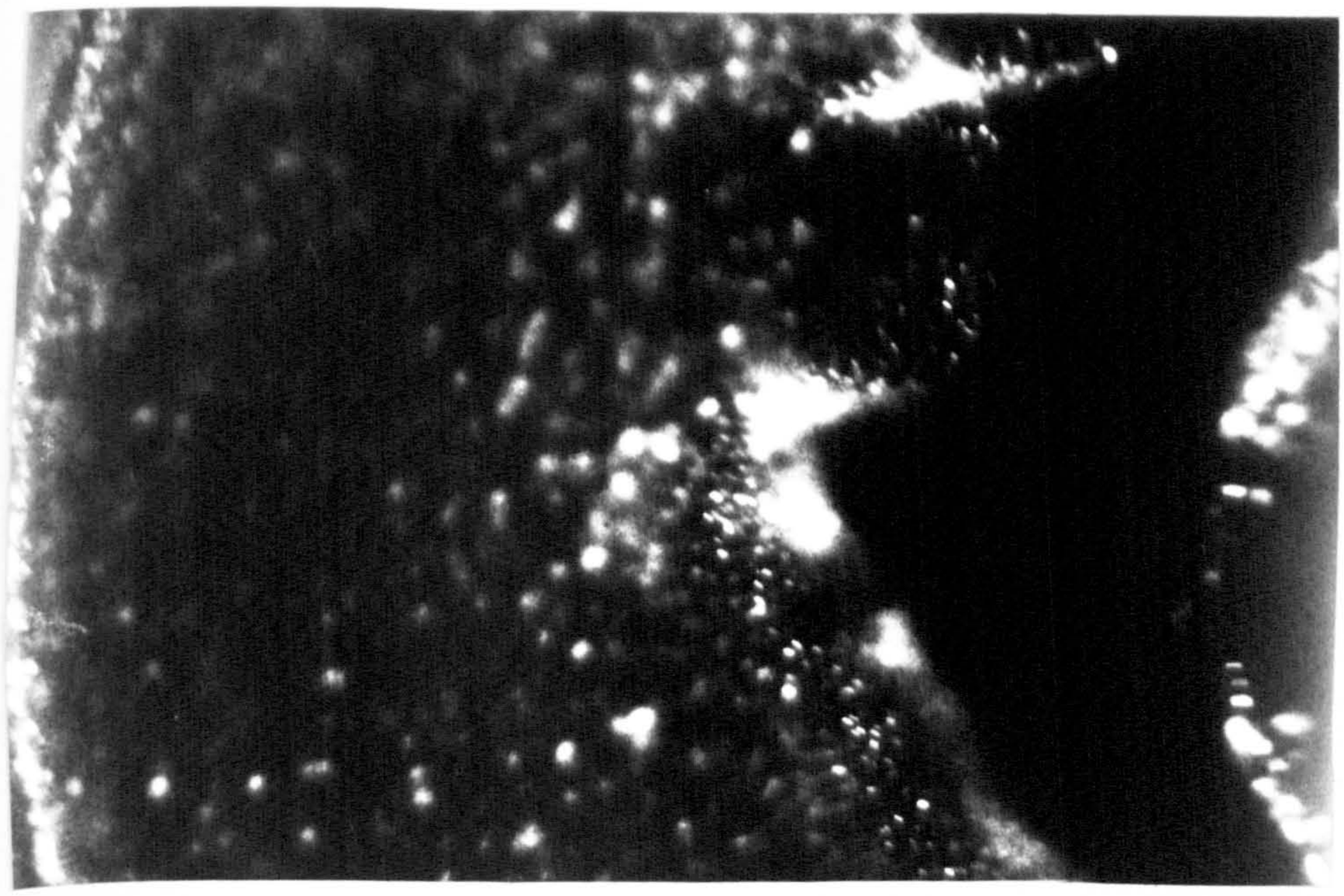
Figure 4 Incubation of adult schistosomes with antibodies directed against host antigens



a) Adult male schistosome (x 125) incubated with anti-mouse RBC serum.



b) Adult male schistosome (x 312.5) incubated with normal rabbit serum.



c) Adult male schistosome (x 125) incubated with anti-mouse IgG.

All worms were examined with the appropriate first antiserum for 30 minutes at 37°C and then washed (x 3) in EM + CS. To detect binding of the first antibody, worms were exposed to a second incubation in the presence of FITC-labelled anti-rabbit IgG. Following this step, worms were again washed (x 3) in EM + CS, immobilised using carbachol, and then examined by fluorescence microscopy.

Worms were incubated for 30 minutes with the first antibody and then washed (x 3) with EM + CS. A further incubation of 30 minutes in the presence of the second antibody then followed before worms were again washed, immobilised using carbachol (2mg/ml), and examined for intensity of fluorescence (- to ++++ representing intensity of fluorescence on an arbirtrary scale) using a Leitz Ortholux II microscope equipped with a Ploempak epi-illuminator and a GG475 filter. Worms were maintained at either $4^{o}C$ or $37^{o}C$ as stated above during the course of the entire experiment. The experiment was performed on three separate occasions.

* Variation in intensity of fluorescence of individual worms within the one experiment.

Table 3 Influence of incubation temperature on binding of a number of first antisera to adult male worms as measured by the indirect fluorescent antibody method

Strain of mouse providing worms	First antibody	Second FITC-labelled antibody	Incubation Temp. (^{o}C)	Intensity of fluorescence
Balb/c	anti-balb/c RBC	anti-rabbit IgG	37	++++
Balb/c	anti-balb/c RBC	anti-rabbit IgG	4	+++
Balb/c	anti-mouse IgG	anti-rabbit IgG	37	++
Balb/c	anti-mouse IgG	anti-rabbit IgG	4	++
Balb/c	anti-mouse IgM	anti-rabbit IgG	37	++, +, - *
Balb/c	anti-mouse IgM	anti-rabbit IgG	4	++, +, - *
Balb/c	anti-mouse IgA	anti-rabbit IgG	37	++, +, - *
Balb/c	anti-mouse IgA	anti-rabbit IgG	4	++, +, - *
Balb/c	normal rabbit serum	anti-rabbit IgG	37	-
Balb/c	normal rabbit serum	anti-rabbit IgG	4	-
CBA ($H\text{-}2^{K}$)	anti $H\text{-}2K^{K}$	anti-mouse IgG Fc	37	+, - *
CBA ($H\text{-}2^{K}$)	anti $H\text{-}2K^{K}$	anti-mouse IgG Fc	4	+, - *
CBA ($H\text{-}2^{K}$)	normal mouse serum	anti-mouse IgG Fc	37	-
CBA ($H\text{-}2^{K}$)	normal mouse serum	anti-mouse IgG Fc	4	-
Balb/c ($H\text{-}2^{d}$)	anti-$H\text{-}2K^{K}$	anti-mouse IgG Fc	37	-
Balb/c ($H\text{-}2^{d}$)	anti-$H\text{-}2K^{K}$	anti-mouse IgG Fc	4	-
BIO/A ($H\text{-}2^{a}$)	anti-$H\text{-}2K^{K}$	anti-mouse IgG Fc	37	-
BIO/A ($H\text{-}2^{a}$)	anti-$H\text{-}2K^{K}$	anti-mouse IgG Fc	4	-

The results described above indicated that the distribution of antigens having specificities shared with mouse red blood cell antigens, unlike that of other antigens examined, appeared to be even and extensive and in addition could be detected in a reproducible manner. For these reasons, in subsequent experiments in which host antigen expression is quantitated by an indirect radiolabelled antibody method (IRAM) the antiserum raised against mouse erythrocyte antigens was employed as first antibody.

3.3 Selection of an antibody to measure parasite antigen expression

In order that the same labelled second antibody could be used to measure expression of both host and parasite antigens, it was decided to prepare an anti-parasite antibody in rabbits. It would thus be possible to quantitate both antigen types with an iodinated preparation of anti-rabbit IgG. To prevent contamination by host components rabbits were not exposed to adult worm material but were immunised with schistosomula, newly transformed by mechanical agitation (Colley and Wikel, 1974). Since schistosomula and adult worms are known to share membrane antigens (Kusel et al, 1975; Snary et al, 1980; Shah and Ramasamy, 1982) it was considered that an anti-schistosomulum serum which had affinity for the surface of the juvenile parasite (Methods 2.3.22) should also recognise antigens in the surface membrane of the adult worm.

3.4 Specificity of anti-schistosomulum serum

Membrane antigens of adult parasite origin are likely to

constitute a heterogeneous population of molecules only a portion of which may react with any particular antiserum. It was thus decided to determine which particular antigens were recognised by the anti-schistosomulum serum which was to be employed in this investigation.

3.4.1 Preparation of an isolated ^{125}I-labelled adult schistosome membrane fraction

The method adopted to investigate antiserum specificity was a modification of the co-precipitation assay of Kusel *et al* (1975) (Methods 2.8). This method involves labelling membrane proteins with ^{125}I. Since previous attempts by other workers to label membrane proteins at the adult worm surface have produced variable and frequently unsuccessful results (see Hayunga *et al*, 1979a; Snary *et al*, 1980; Cesari *et al*, 1981) it was decided to attempt to iodinate an isolated, solubilised membrane fraction prepared by the freeze-thaw method of Kusel (1972).

A number of different treatments were employed at the membrane solubilisation step. An indication of the extent of solubilisation produced by a particular treatment is given by the amount of ^{125}I incorporated into the solubilised membrane fraction during the iodination reaction. Thus it can be seen that the extent of solubilisation is influenced by the reagent(s) employed (Table 4).

Table 4 Influence of method of solubilisation on incorporation of ^{125}I into solubilised adult schistosome membrane fraction

Solubilisation Reagent(s)	c.p.m. ^{125}I incorporated/5µl solubilised membrane material
1% Triton X-100	360,124
1% NP 40	194,032
1% Triton X-100/9M urea	698,822
1% Sodium Deoxycholate	608,551

The isolated membrane material from 175 worms was solubilised in 100µl of each reagent(s) for 15 minutes at 37°C Each solubilised preparation was then iodinated using 500µCi of ^{125}I by the method of Hunter and Greenwood (1962). On completion of the iodination step the final volume of each solubilised preparation was 500µl. 5µl samples of each preparation were counted for radioactivity in a Beckman Biogamma counter. The values given are the average of two samples.

3.4.2 SDS-PAGE analysis of schistosome membrane fraction

3.4.2.1 Influence of solubilisation method on incorporation of ^{125}I into membrane polypeptides

The pattern of polypeptides obtained from the material released from membrane pellets solubilised by three different methods is shown in Figure 5 . An equal volume (10µl) of all three samples was added to the gel. 1% Triton X-100 generally appears to solubilise the lower molecular weight components as they are selectively labelled when this non-ionic detergent is employed. If the detergent is used in combination with 9M urea however a number of polypeptides of higher molecular weight are also labelled. The anionic bile salt sodium deoxycholate used at a concentration of 1% produces a pattern similar to that obtained with the Triton X-100/urea combination. The pattern obtained with 1% NP40 (not shown) which consisted of only a few low molecular weight bands was similar to, but less intense than, the Triton X-100 pattern.

3.4.2.2 Molecular weights of membrane polypeptides

Polypeptides of the following molecular weights were labelled : > 100,000, 95,000, 78,000, 66,000, 62,000, 53,000, 45,000, 43,000, 34,000-28,000, 23,000, 20,000, 18,000 and < 14,000 (Fig 5). This pattern of bands was found to be reproducible although it should be noted that i) the intensity of some bands varied between different preparations and ii) it was sometimes possible to determine additional minor bands

Figure 5 <u>10% (w/v) SDS Polyacrylamide Gel Fluorograph of membrane polypeptides solubilised by three different techniques.</u>

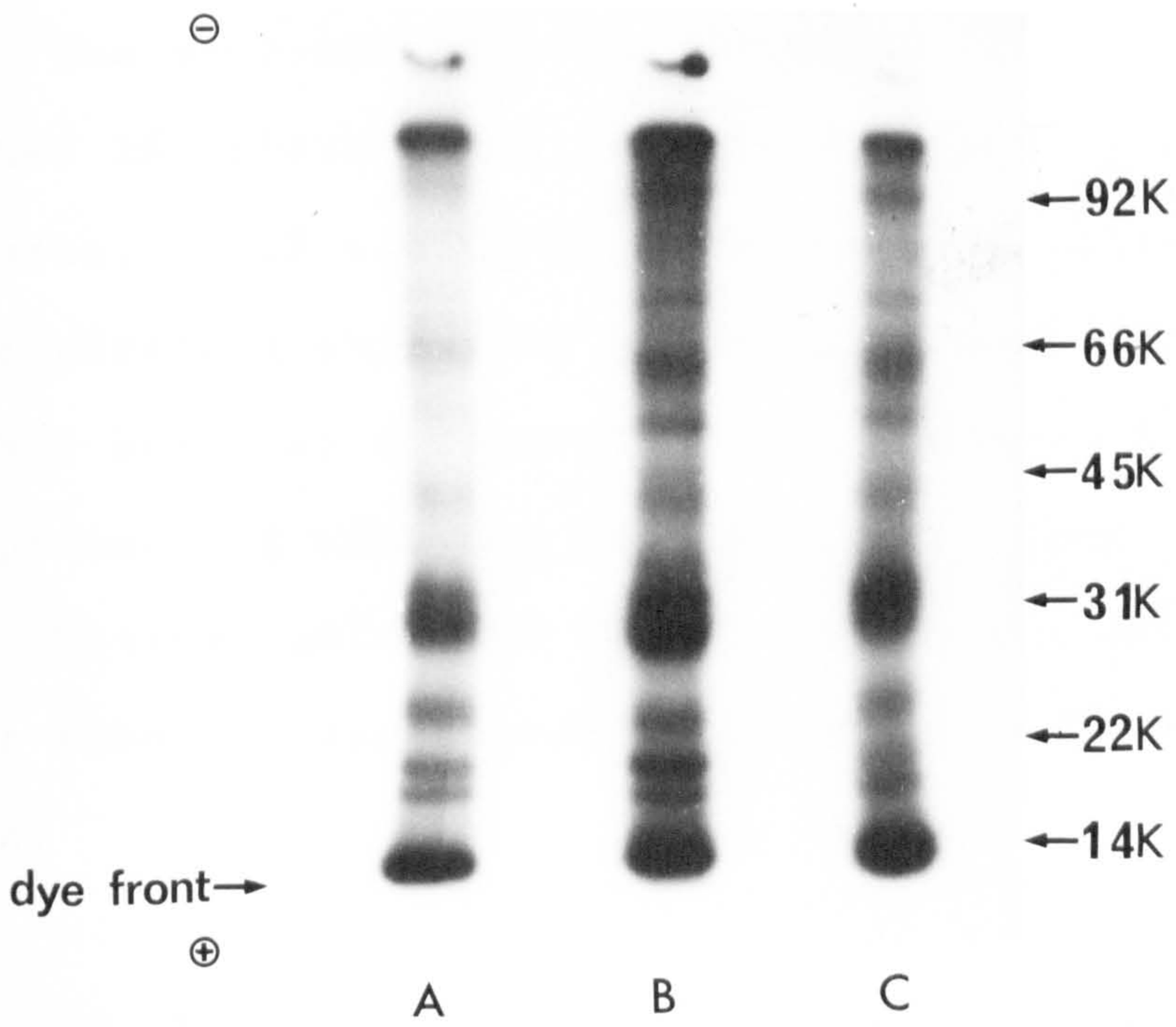
The isolated membrane material from 175 worms was solubilised in 100µl of each reagent(s) for 15 minutes at 37°C. Each solubilised preparation was then iodinated using 500µCi of ^{125}I by the method of Hunter and Greenwood (1962). On completion of the iodination step the final volume of each solubilised preparation was 500µl. 10µl of each iodinated preparation was subjected to SDS-PAGE and visualised by fluorography.

Lane A - Solubilisation with 1% Triton X-100

Lane B - Solubilisation with 1% Triton X-100 in combination with 9M urea

Lane C - Solubilisation with 1% sodium deoxycholate

Running positions of Coomassie Blue stained Bio-Rad molecular weight markers are shown at the right hand side of the gel.


⊖
92K
66K
45K
31K
22K
14K
dye front
⊕
A
B
C

with some preparations.

A comparison of the membrane protein composition with that obtained by some other workers is shown in Table 5 . It can be seen that molecules of identical or similar molecular weight to most of the proteins resolved in this investigation have been described by other workers.

3.4.3 Co-precipitation of isolated membrane fraction

3.4.3.1 Selection of material for analysis by SDS-PAGE

The membrane material obtained after solubilisation by either 1% Triton X-100, 1% Triton X-100 in combination with 9M urea, or 1% sodium deoxycholate was subjected to co-precipitation employing anti-schistosomulum serum and normal rabbit serum as a control. The greatest number of counts (representing membrane material) precipitated specifically was obtained using the Triton X-100/urea preparation and this material was therefore selected for analysis by SDS-PAGE.

3.4.3.2 Nature of polypeptides precipitated by anti-schistosomulum serum

The amount of membrane material precipitated was often found to be low relative to the amount of starting material. This was mainly due to removal of aggregated and "sticky" material (Kusel et al, 1975) in addition to the presence in the sample of labelled material which did not bind antibody. Precipitates were thus examined by the gel slicing method

Table 5 A comparison of the adult schistosome surface membrane protein composition as determined by various investigators employing SDS-PAGE

Molecular Weight (x 10^{-3})			
(A)	(B)	(C)	(D)
Present thesis	Ruppel (1978)	Hayunga et al (1979_a)	El-adhami (1980)
> 100	120	100	112
95			94
78	78	78	74
66		68	70
62		60	60
53	55		54
45	44	43	46
43	39	36	36
28-34	30	30	34
23	25	26	28
20	20	21	
18			
< 14			

Methods used for detecting surface membrane proteins :

A - iodination of isolated membrane fraction

B - surface labelling with ^{125}I (lactoperoxidase method)

C - surface labelling with ^{125}I (use of Bolton-Hunter reagent)

D - Coomassie Blue staining of isolated membrane fraction.

rather than by fluoragraphy as the former technique allows more rapid and sensitive assessment of samples having a low number of counts.

As shown in Fig 6 , the antiserum would appear to have affinity for the majority of polypeptides in the membrane fraction (c.f. Fig 5) and should therefore permit the detection of an increase in antigen expression due to increased exposure of almost any of the antigens. Such an antiserum is ideal for this particular investigation, as current knowledge concerning the organisation of the surface membrane does not allow one to predict which particular antigens are likely to be exposed following experimental manipulation.

3.5 Information on the nature of the RBC antigen acquired by schistosomes

3.5.1 Co-precipitation of isolated membrane fraction with anti-mouse RBC serum

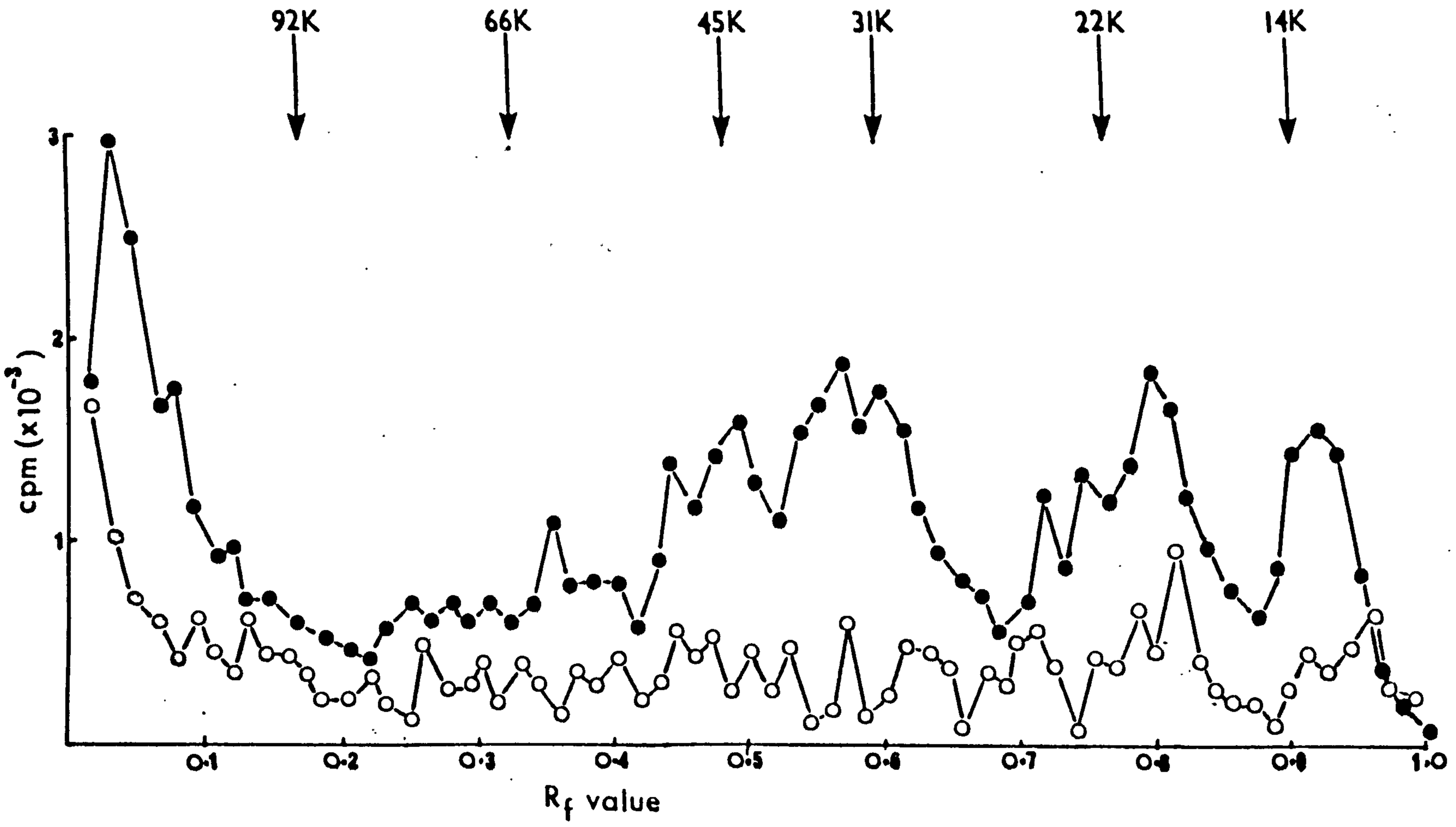
The co-precipitation technique was also employed to determine if any of the labelled membrane antigens would bind anti-mouse RBC antibody. Results however proved to be inconsistent. On occasion certain polypeptides, in particular one having a molecular weight of 11,000 (resolved by 15% (w/v) SDS-PAGE) were successfully precipitated but it was found to be impossible to precipitate any particular polypeptide in a reproducible manner. This result may therefore be consistent with the RBC antigen at the worm surface being a glycolipid which is reversibly attached to one or more membrane proteins.

Figure 6 <u>10% (w/v) SDS polyacrylamide gel electrophoresis of coprecipitated iodinated membrane polypeptides</u>

25µl of iodinated adult membrane preparation was subjected to coprecipitation using anti-schistosomulum serum as first antibody or NRS as a control (2.8). Coprecipitated polypeptides were then subjected to SDS-PAGE and the distance migrated by each polypeptide determined by slicing the gel and counting each portion for radioactivity in an LKB gamma counter (2.11). The distances migrated by Bio-Rad molecular weight standards are shown by the use of arrows.

●- anti-schistosomulum serum

o- normal rabbit serum


92K
66K
45K
31K
22K
14K
cpm (x10⁻³)
3
2
1
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Rf value

3.5.2 Sharing of RBC antigen by different mouse strains and the hamster

The ability of worms grown a) in a number of mouse strains of different H-2 specificity (Balb'lc, CBA, and BIO A) and b) in hamsters, to bind anti-mouse RBC antibody was investigated using the IFAM. All worms examined were shown to bind the antibody and apparently to the same extent, at least as determined by this subjective form of measurement.

The fact that the different mouse strains share the antigen indicates that it is not a histocompatibility antigen or at least that a major host antigen is present which is not a histocompatibility antigen. This result is perhaps not surprising in view of the earlier result obtained using anti-H-2 antibodies (3.2). The finding that hamster worms also bind the antibody indicates that the antigen is not species specific but that it is common to both hamsters and mice. The possibility that the antigen is a cross-reacting calf serum component acquired by both mouse and hamster worms prior to initiation of the assay was ruled out by using rabbit serum as a substitute for calf serum during the perfusion step.

3.6 Selection of second antibody

As discussed in section 3.3, it was proposed to use an anti-rabbit IgG as second antibody in the assay. To prevent

incorporation of ^{125}I into other serum components including other antibodies it was decided to employ a highly purified form of antibody. Such a step means that iodination can be performed using much lower amounts of ^{125}I and thus reduces the amount of ^{125}I to which the worm is exposed. In addition the field of RIA has shown that labelled impure antibody preparations give more problems with non-specific binding than do pure preparations (see Parker, 1976). The antibody preparation employed was commercially purified and was obtained from Cappell Laboratories, U.S.A.

3.7 Influence of assay protocol on specific and non-specific binding

The influence of a number of factors on specific and non-specific binding was investigated in order to establish an optimum assay protocol. This work employed anti-mouse RBC serum (1:30 dilution) as first antibody (the anti-schistosomulum serum is unsuitable for this purpose as it shows little affinity for the unaltered adult worm surface). In addition, the use of second antibody was restricted to small amounts in order to preserve supplies of this valuable reagent. A sample

of between 0.2 and 0.4 µg having a specific activity of 3-6 µCi/µg was usually added in the presence of EM + CS to each tube.

3.7.1 Influence of worm transfer to new test tubes during the course of experiment

It was initially planned to perform the assay in a similar manner to the indirect fluorescent antibody method but to use gamma counting tubes (LKB Instruments, London) rather than LP3 tubes in order to prevent the need for transfer of worms at the end of the experiment. However, it was soon found that a transfer of worms was necessary due to adhesion of labelled antibody to the side of the counting tube resulting in the recording of incorrect results for non-specific binding. The tendency of radiolabelled antibody or indeed most radiolabelled proteins to adhere to plastic surfaces is well known. Some workers (Shumak et al, 1973; Dorvall et al, 1975) have attempted to overcome this problem by pre-coating test tubes with unlabelled protein. This approach was investigated by coating tubes with 1% (w/v) BSA before use but it was found that adhesion of labelled antibody was still extensive.

The effect of transferring worms to a new tube before exposure to second antibody was also investigated and was

found to increase the value attained for specific binding of antibody. The reason for this increase is uncertain but it is possible that if first antibody binds to the side of the test tube, it may compete with worm-bound first antibody for interaction with second antibody molecules. Transfer of worms to a new test tube before addition of second antibody would automatically obviate this problem. The alternative approach of precoating tubes with 1% BSA to prevent the supposed binding of first antibody was found to be unsuccessful in promoting an increase in specific antibody binding. Thus it was decided that in all future experiments a double worm transfer would be undertaken. A typical experiment showing the value of the double transfer is shown in Table 6 .

The final protocol with the number of washes performed at each step is shown in Fig 7 . The number of washes required after the second antibody incubation was determined by calculating the ratio of total counts bound to non-specific counts bound after each wash : when this no longer increases, sufficient washing has taken place.

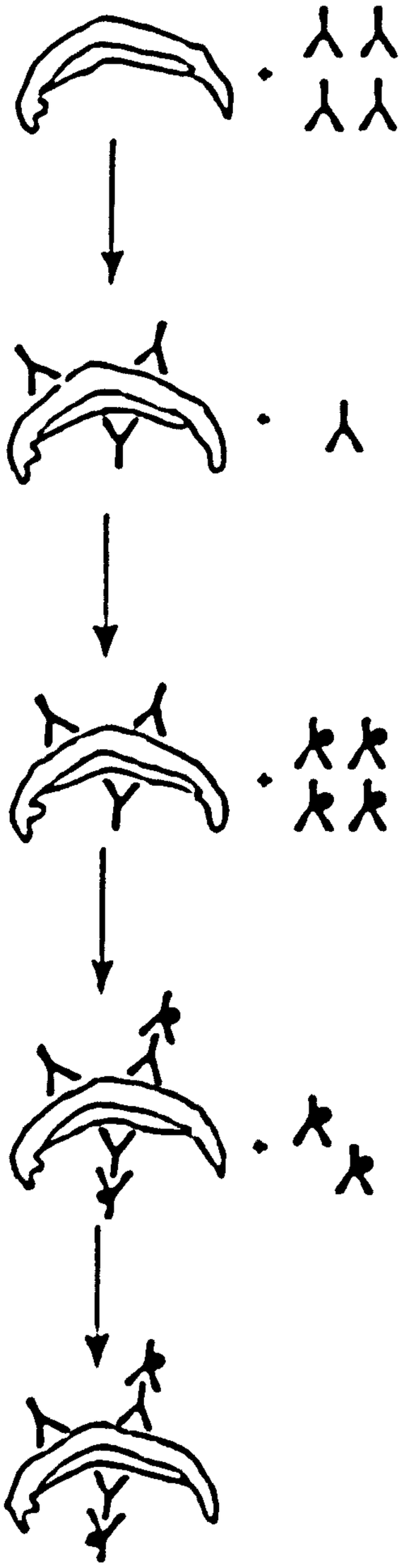
3.7.2 Influence of calf serum in washing fluid

As can be seen from Fig 7 the medium employed for washing and transfer of worms contains calf serum. This serum is included for two reasons : first of all, worms assayed in the absence of serum were on occasion found to show a significant reduction in binding of specific antibody (Table 7); and secondly, the use of medium lacking in serum appears to

Three experimental procedures were compared. Procedure 1 :- worms were incubated with first antibody (100µl) for 30 min at 37°C in a gamma counting tube, washed (x 3) in EM + CS, and then incubated with second antibody (100µl) for 30 min at 37°C. After further washing in EM + CS (x 3), worms were counted for radioactivity in a Beckmán Biogamma counter. Worms were maintained in a single counting tube during the course of this procedure. Procedure 2 :- worms were incubated with first antibody in an LP3 tube for 30 min at 37°C washed (x 3) in EM + CS and then incubated with second antibody for 30 min at 37°C. After two washes in EM + CS, worms were transferred to a gamma counting tube, washed once with EM + CS and counted for radioactivity. Procedure 3 :- worms were incubated with first antibody in an LP3 tube for 30 min at 37°C, washed (x 3) in EM + CS and then transferred to a fresh LP3 tube. A second incubation for 30 min at 37°C in the presence of second antibody then followed before worms were washed (x 2) in EM + CS and then transferred to a gamma counting tube. Worms were then given a final wash in EM + CS before counting for radioactivity.

Table 6 Influence of worm transfer on the results obtained for specific and non-specific binding in the indirect radio-labelled antibody assay.

Experimental Procedure	1st serum	Total antibody bound/ four worms (c.p.m.)
1. No worm transfer	anti-mouse RBC	3,909 ± 545
	normal rabbit	1,805 ± 901
	normal rabbit (no worms)	1,399 ± 676
2. Transfer after second incubation	anti-mouse RBC	2,685 ± 801
	normal rabbit	417 ± 319
3. Transfer after both incubations	anti-mouse RBC	6,344 ± 1,201
	normal rabbit	312 ± 242



(4) male worms + first antibody;

incubate 30 min/37°C in LP3 tube

Wash (x 3) in EM + CS; transfer

to new LP3 tube (by Pasteur pipette)

Add second labelled antibody;

incubate 30 min/37°C

Wash (x 2) in EM + CS; transfer

to gamma counting vial

Wash (x 1) in EM + CS; count for

radioactivity

- 1st antibody

- 2nd antibody (radiolabelled)

Fig. 7. Protocol for indirect radiolabelled antibody method (IRAM)

The IRAM was performed using either EM or EM + CS as washing medium as described above. The results given for worms assayed in the presence of anti-mouse RBC antibody are expressed as specific antibody bound (second antibody bound following incubation of worms in anti mouse-RBC serum- second antibody bound following incubation of worms in NRS).

Table 7 Comparison of the effects of EMS and EM + CS as washing media on specific and non-specific antibody binding in the IRAM

Experiment Number	First serum	Washing medium	Specific antibody bound/four worms (c.p.m.)	Statistical analysis
1	anti-mouse RBC	EM	6232 ± 749	P > 0.05
	anti-mouse RBC	EM + CS	7061 ± 1880	
	NRS	EM	195 ± 25	P > 0.05
	NRS	EM + CS	148 ± 30	
2	anti-mouse RBC	EM	6188 ± 244	P < 0.05
	anti-mouse RBC	EM + CS	8670 ± 1314	
	NRS	EM	316 ± 58	P < 0.025
	NRS	EM + CS	140 ± 31	
3	anti-mouse RBC	EM	7413 ± 1139	P < 0.001
	anti-mouse RBC	EM + CS	14462 ± 503	
	NRS	EM	310 ± 41	P < 0.05
	NRS	EM ± CS	233 ± 25	
4	anti-mouse RBC	EM	6081 ± 846	P > 0.05
	anti-mouse RBC	EM ± CS	5876 ± 925	
	NRS	EM	131 ± 38	P > 0.05
	NRS	EM & CS	101 ± 26	

increase non-specific binding (Table 7). The former result is not due to loss of RBC antigens induced by contact with serum-free medium as worms can be incubated in EMS for 30 minutes before introduction to the assay with no detectable significant change in antigen expression (section 4.3.1.2). One possible explanation is that the result is due to tegument damage caused by the tendency of worms transferred in serum-free medium to adhere to the inside of the pasteur pipette used for transfer. Examination of such worms by the light microscope reveals evidence of tegument flattening and shedding.

3.7.3 Influence of temperature on antibody binding

The results obtained with the IFAM suggested that less anti-mouse RBC antibody bound at $4^{\circ}C$ than at $37^{\circ}C$. Confirmation of this was obtained using the IRAM (Table 8) and all further experiments were therefore performed at $37^{\circ}C$.

3.7.4 Influence of incubation time on binding of antibody

An experiment was performed in which the influence of incubation time on antibody binding was examined. This experiment (Table 9) clearly showed that maximum binding had been attained when both incubation times were fixed at 30 minutes ; the times used in all experiments up to this stage. These incubation times were therefore maintained for use in all further experiments. The fact that increasing both incubation times to 120 minutes did not alter the result suggests that antigen/antibody complexes are not shed during the course of an experiment with 30 minute incubation times.

Table 8 Influence of incubation temperature on binding of anti-mouse RBC antibody in the IRAM

Experiment	Incubation Temperature (^{o}C)	Specific antibody bound four worms (c.p.m.)	Statistical analysis
1	37	8215 ± 2488	
	22	7344 ± 608	P> 0.05
	4	3425 ± 975	P> 0.05
2	37	10338 ± 1027	
	22	7095 ± 812	P<0.05
	4	2892 ± 560	P<0.001

An IRAM was performed in which the temperature of the incubation and washing media were varied as described above. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum. The results obtained when NRS was employed were independent of assay temperature.

Table 9 Influence of incubation time on results obtained for specific binding of anti-mouse RBC antibody in IRAM

First incubation time (min)	Section incubation time (min)	Specific antibody bound/ four worms (c.p.m.)
30	30	8593 ± 828
60	60	8236 ± 1013
120	120	9168 ± 127

IRAM was performed with varying incubation times as described above. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum. The results obtained when NRS was employed were independent of assay incubation time.

3.7.5 Influence of assay volume on antibody binding

At constant second antibody concentration results obtained were found to be independent of the volume of reagent employed. Thus in order to preserve antibody the volume of second antibody administered to each tube in all further experiments was fixed at 50µl rather than 100µl, as had been used up till now.

3.8 Quantitation of differences in antibody binding and antigen expression

3.8.1 Selection of methodology

If an immunoassay is employed in which both first and second antibody are present in excess then the total number of antibody molecules required to saturate a particular cell surface antigen can be determined. In addition, by measuring the number of molecules of antibody which are bound it follows that the number of molecules of antigen can be estimated (Williams, 1977). By using such a system it is thus possible to compare antibody binding or antigen expression at two different cell surfaces (Morris and Williams, 1975). The main problem relating to the use of this type of assay to study RBC antigens at the adult worm surface concerns the amount of first and second antibody which would be required for saturation. Thus using first antiserum undiluted does not lead to saturation of all membrane antigen receptors yet still requires the use of > 64 µg/tube of second antibody in order for the labelled reagent to be present in excess

(Fig 8 and Table 10). This means that even if a first antiserum was produced with enough activity to saturate all binding sites the amount of second antibody required would render the assay impracticable. This is because a) the use of such large amounts of second antibody would be too expensive, and b) the specific activity of the antibody preparation would have to be very low if exposure of worms and in addition, the researcher, to ^{125}I was to be kept minimal. The use of such a preparation would almost certainly have a deleterious effect on assay sensitivity (Parker, 1976). To overcome these problems, therefore, it was decided to develop an assay in which limiting amounts of antibody were employed.

3.8.2 Attempts to measure antigen expression using limiting amounts of antibody

In reagent excess assays, the amount of antibody bound is proportional to the amount of antigen present in the system. It was considered uncertain as to whether such a relationship would exist in an assay utilising limiting amounts of antibody. If this type of relationship did not exist, then any detectable difference in antibody binding, although indicative of a change in antigen expression, might not necessarily allow quantitation of this change.

The possibility of using limiting amounts of antibody to quantitate antigen in reagent excess sandwich type RIAs was discussed and considered feasible by Ekins(1978) and indeed Rodbard et al (1978) have performed two site immunoradiometric assays using limiting amounts of labelled antibody. Conversely,

Figure 8 Titration of second antibody when first antibody is employed undiluted in the IRAM

IRAM was performed using various second antibody concentrations, as described. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum. Each sample was examined in duplicate. The specific activity of the second antibody was calculated to be 0.016μCi/μg.

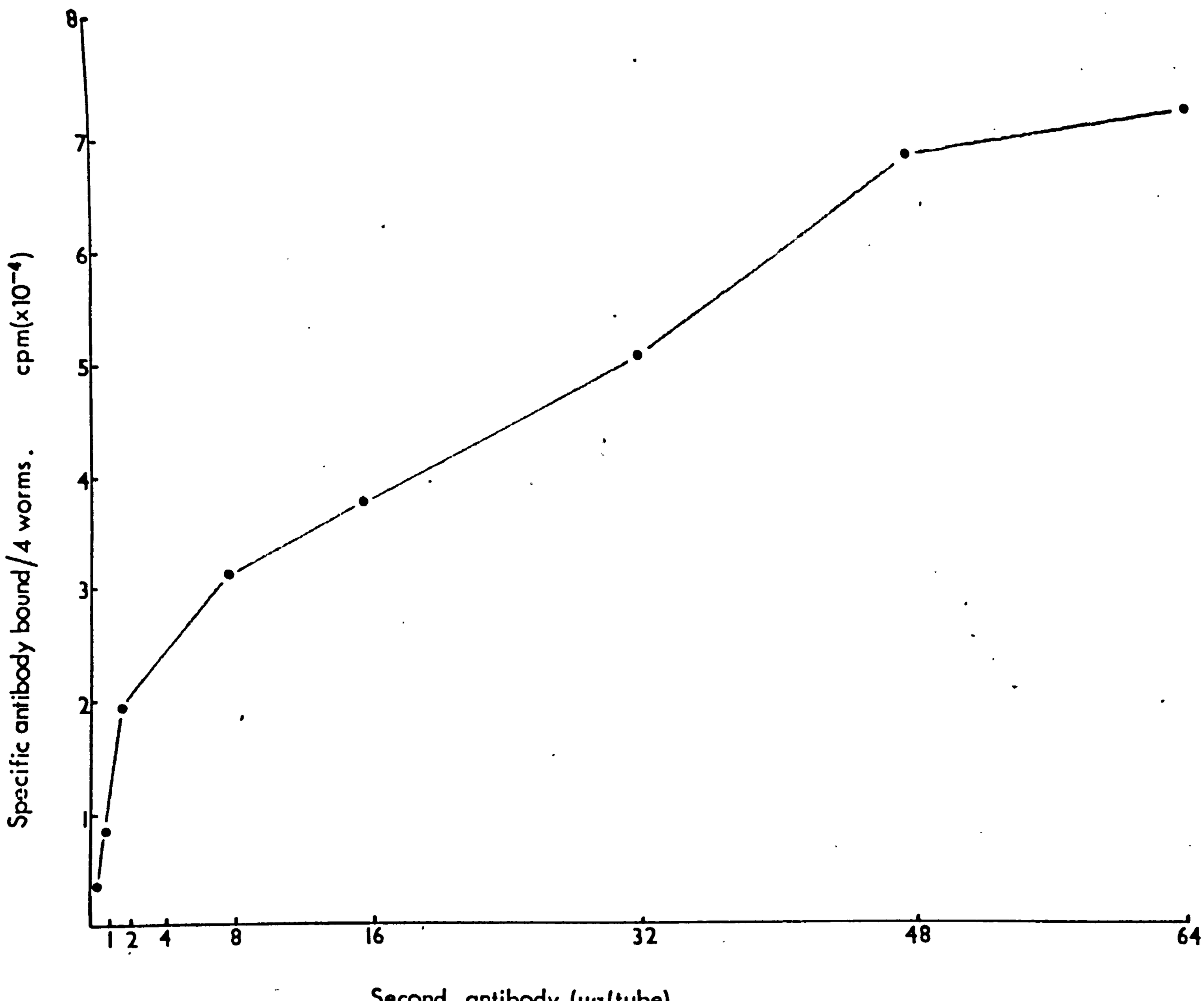

Specific antibody bound/4 worms. cpm(x10⁻⁴)
8
7
6
5
4
3
2
1
1 2 4 8 16 32 48 64
Second antibody (µg/tube)

Table 10 Titration of first antibody when second antibody is employed in excess in the IRAM

Anti-mouse RBC serum dilution	Specific antibody bound/ four worms (c.p.m.)
neat	61,934
1:2	46,675
1:4	31,435

IRAM was performed with the first antibody being varied as described above. Second antibody was employed at a concentration of 150µg/ml. This value was calculated by Scratchard analysis to be sufficient for second antibody to be present in excess. The specific activity of the preparation was calculated to be 0.016 µCi/µg. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum. Each sample was examined in duplicate.

Batchelor, Shumak, and Watts (1973) whilst employing a direct immunoassay with limiting amounts of labelled antibody were unable to demonstrate a linear relationship between density of cell surface antigen and binding of antibody. Hutchison and Ziegler (1972) were however able to use limiting amounts of labelled second antibody to quantitate the amount of first antibody bound to the surface of E. coli, as a linear relationship between concentration of first antibody and labelled second antibody was established. This latter result suggested the possibility that in addition to quantitating antigens by limiting amounts of first antibody it might be possible to quantitate first antibody bound to antigen using limiting amounts of second antibody. It was therefore decided to investigate this idea initially.

3.8.2.1 Quantitation of bound first antibody

Fig 9 shows the influence of changing the first antibody concentration on the binding of second antibody. Clearly, under these conditions in which the concentration of first antibody does not approach saturation, a linear relationship exists between first antibody concentration and second antibody bound when the latter is present at a concentration of 20µg /50 µl (the flattening effect observed when the first antibody dilution is > 1:80, may be a consequence of the very low specific activity of this labelled antibody (0.01 µCi/µg)). This second antibody concentration is likely to provide sufficient molecules to saturate or almost saturate all binding sites when first antibody is employed at a 1:10

Figure 9 Influence of second antibody concentration on quantitation of specific antibody binding.

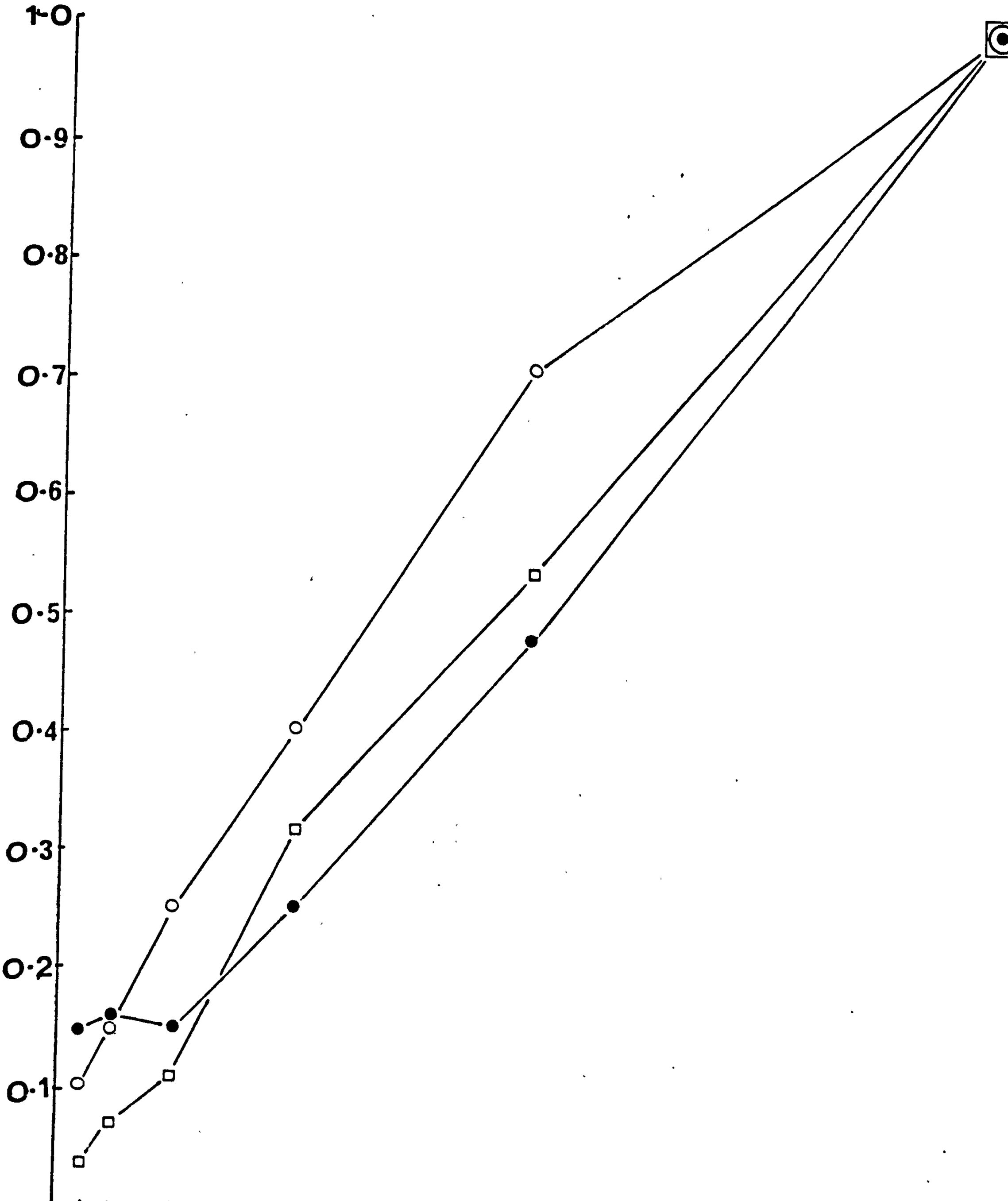
IRAM was performed using varying amounts of first and second antibody. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum. Each point is the mean of two samples and the data were normalized by setting the binding achieved at a 1:10 first antibody dilution equal to one.

●- second antibody concn. = 20µg/50µl
specific activity = 0.01µCi/µg

□- second antibody concn. = 2µg/50µl
specific activity = 0.08µCi/µg

○- second antibody concn. = 200ng/50µl
specific activity = 4.2µCi/µg

The 20µg/50µl and 2µg/50µl antibody solutions were prepared by diluting labelled antibody obtained by the iodination procedure (2. 3. 6. 1) with unlabelled antibody.


1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Normalised specific antibody binding
320
160
80
40
20
10
Reciprocal dilution of anti-mouse RBC antiserum

dilution (see legend to Table 10). It is also clear from Fig 9 however that a linear relationship is achieved when using much lower and limiting quantities of second antibody. Indeed such a relationship is evident when a second antibody concentration of 200ng/50µl is employed providing the maximum first antibody concentration in use is < 1:20 dilution. This result therefore shows that the amount of first antibody bound can be quantitated by limiting amounts of second antibody and in addition, suggests that the assay can be developed to suit the amount of available second antibody by manipulating the concentration of first antibody.

3.8.2.2 Quantitation of antigen

To determine if limiting amounts of first antibody could be used to quantitate antigens, the binding of antibody to different numbers of worms was investigated. The reasoning behind this experiment was that if the amount of antibody bound was indicative of the amount of antigen present then one would expect that reducing the number of worms would lead to a proportional reduction in the total amount of antibody bound. Table 11 shows that this in fact happens. This result therefore suggested that limiting amounts of first antibody could be utilised to monitor the influence of membrane active reagents on surface antigen expression.

Confirmation of this suggestion was achieved by performing a further experiment employing worms exposed to glutaraldehyde (this reagent is employed in later work to investigate antigen expression as described in detail in

Table 11 Influence of worm number on specific binding of anti-mouse RBC antibody

Anti-mouse RBC serum dilution	Number of worms per tube	Normalised specific antibody bound
1:30	4	1.00
1:30	3	0.70
1:30	2	0.44
1:30	1	0.26
1:120	4	1.00
1:120	3	0.69
1:120	2	0.56
1:120	1	0.25

An IRAM was performed in which the number of worms added to each tube was varied. Each result is the mean of two samples. Results were normalized to allow rapid assessment. This was done by setting the value obtained for specific antibody binding in the presence of four worms equal to one. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum. Second antibody was employed at a concn. of 200ng/50µl.

section 4.3.2). If limiting amounts of first antibody are able to quantitate changes in antigen expression then it can be argued that the results obtained should be independent of first antibody concentration. As shown in Table 12, changing the antibody concentration does not alter the result obtained when the effect of 0.01% glutaraldehyde on host antigen expression is being measured.

The ability to quantitate differences in parasite antigen expression is also dependent on forming linear relationships between first antibody bound and antigen, and between second and first antibody bound. The amount of each antibody required to maintain such relationships however will clearly depend on the extent of the increase in antigen expression which arises following treatment of worms with any particular reagent. If insufficient antibody levels are employed then it is likely that linearity and therefore quantitation will be lost.

The levels of antibody selected to measure parasite antigen expression were a 1:10 dilution of first antibody and a second antibody concentration of 200-300ng/50µl. These concentrations were chosen for the following reasons : adult worms incubated with a 1:10 dilution of anti-schistosomulum serum will in general bind less than 10% of the amount of second antibody bound by worms pre-incubated in a 1:20 dilution of anti-mouse RBC serum. A 1:20 dilution rests at the top of the linear part of the curve obtained when anti-mouse RBC serum is titrated by second antibody employed at a concentration of 200ng/50µl (Fig. 9).

It thus follows that this second antibody concentration should provide adequate antibody to quantitate increases in anti-

Table 12 Influence of first antibody concentration on ability of IRAM to detect changes in host antigen expression induced by 0.01% glutaraldehyde

Anti-mouse RBC serum dilution	Incubation medium	Specific antibody bound/four worms (c.p.m.)	Percentage binding of control
1:25	EMS	9125 ± 129	
1:25	0.01% glutaraldehyde	4912 " 712	53.83
1:50	EMS	6771 ± 467	
1:50	0.01% glutaraldehyde	3378 ± 438	49.88
1:100	EMS	4588 ± 92	
1:100	0.01% glutaraldehyde	2432 ± 248	53.00

Worms were precultured for 15 min at 37°C in either EMS or 0.01% glutaraldehyde/PBS, pH 7.2 and then following washing, were assayed for binding of anti-mouse RBC antibody by the IRAM. The washing procedure for worms subjected to glutaraldehyde treatment is described in the legend to Fig. 15.

Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum.

schistosomulum antibody binding represented by up to, at least, a 10 fold increase in binding of second antibody. This was considered adequate with which to begin experimentation as it was unknown as to what extent parasite antigen expression was likely to be increased following treatment of worms.

The heterogenous nature of schistosome antigens must be considered when attempting to measure parasite antigen expression, as it is possible that different reagents may increase the expression of different antigens. A first antibody dilution which can be employed to quantitate antigen expression following treatment of worms with one reagent may therefore be unsuitable when using an alternative reagent. Thus although a 1:10 dilution of first antibody is sufficient to quantitate the change in parasite antigen expression induced by 0.01% glutaraldehyde (i.e. increasing the first antibody concentration does not lead to an increase in the measured change in antigen expression (Table 13)), it is possible that results obtained with some other reagents may be an underestimate.

3.8.2.3 Relative nature of antigen quantitation

An assay using limiting amounts of antibody can thus be used to compare antigen expression in control and experimental worms in a quantitative manner. Such an assay cannot quantitate the exact number of molecules of antigen but can only measure antigen expression in relative terms. This

Table 13 Influence of first antibody concentration on ability of IRAM to detect changes in parasite antigen expression induced by 0.01% glutaraldehyde

Anti-schistosomulum serum dilution	Incubation medium	Specific antibody bound/four worms (c.p.m.)	Percentage binding of control
1:2.5	EMS	5925 ± 691	
1:2.5	0.01% glutaraldehyde	25711 ± 2100	417
1:5	EMS	6061 ± 1128	
1:5	0.01% glutaraldehyde	26242 ± 6409	433
1:10	EMS	5656 ± 929	
1:10	0.01% glutaraldehyde	22787 ± 1343	403
1:20	EMS	4051 ± 1940	
1:20	0.01% glutaraldehyde	18164 ± 3753	448

Worms were precultured as described in the legend to Fig. 15 and then assayed for binding of anti-schistosomulum serum by the IRAM. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-schistosomulum serum. Second antibody was employed at a concn. of 700ng/50ml.

however is sufficient to give an indication of the extent of reduction or increase in antigen expression following treatment with a particular reagent.

3.9 Quantitation of antigen expression at the surface of the female worm

The work described in this thesis is concerned with measuring changes in antigen expression at the surface of the adult male schistosome only. The assay can however be used to measure antigen expression at the surface of the female worm. As shown in Figure 10 female worms appear to bind less anti-mouse RBC antibody but more anti-parasite antibody than their male counterparts. In neither case however is the result significant (at $p < 0.05$).

The differences in antibody binding shown by male and female worms dictated that they could not be used arbitrarily in the assay. In addition, it was considered possible that their outer membranes, perhaps as a consequence of differences in composition and/or organisation might have different susceptibilities to some of the membrane active reagents under investigation. Some evidence in support of this idea is shown in Tables 14 and 15. Two reagents initially considered for use in the investigation were the δ toxin of Staphylococcus aureus, and phospholipase A_2 from cobra venom (Naja naja). These reagents were rejected however as results from a $^{51}Cr/^{125}I$-WGA release assay suggested that they probably induced extensive membrane damage. It can be seen in Tables 14 and 15

Fig. 10 Binding of anti-mouse RBC antibody and anti-schistosomulum antibody by male and female worms

Male and female worms were assayed for antigen expression by the IRAM. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum or anti-schistosomulum serum. Anti-schistosomulum serum was employed at a 1:10 dilution; anti-mouse RBC serum at a 1:50 dilution. Second antibody was present at a concentration of 200ng/50µl.

A. First antiserum : anti-mouse RBC serum

B. First antiserum : anti-schistosomulum serum

M : male worms

F : female worms

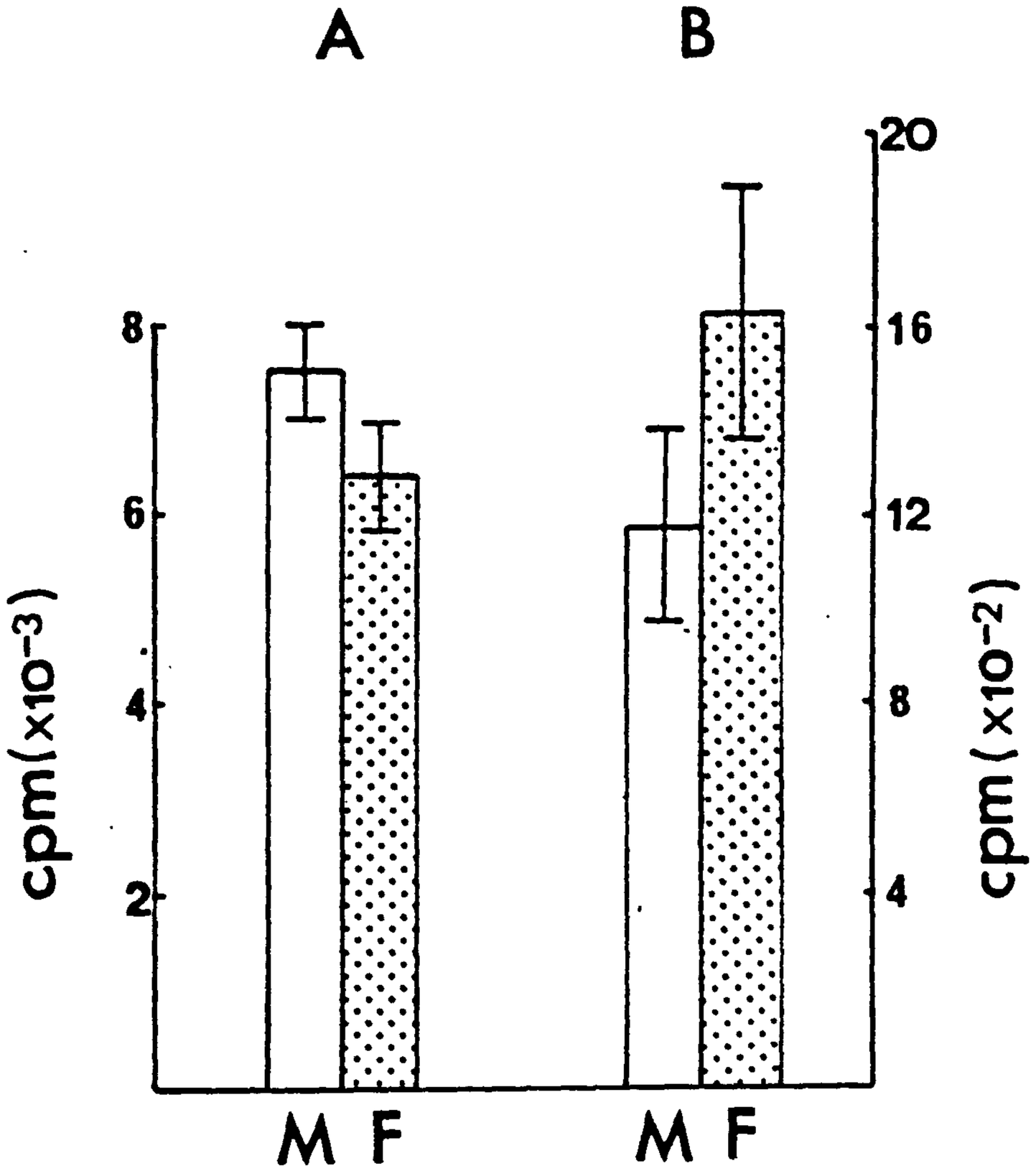

A
B
8
6
4
2
cpm(x10-3)
20
16
12
8
4
cpm(x10-2)
M F
M F

Table 14 The effect of Staphylococcal delta-toxin on adult male and female schistosomes as measured by ^{51}Cr and ^{125}I-WGA release

Incubation medium	Worm Sex	% ^{51}Cr release		% ^{125}I-WGA release	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
EMS	M+F *	10.5±2.1	8.7±1.3	12.44±4.1	9.2±1.6
10 Hu delta-toxin	M	51.5±4.9	63.8±3.8	35.3±17.2	52.6±6.4
10 Hu delta-toxin	F	10.4±7.1**	50.4±4.2**	18.4±8.2	31.4±6.6**
5 Hu delta-toxin	M	28.5±9.2	42.7±7.7	12.8±3.4	18.3±3.0
5 Hu delta-toxin	F	10.4±8.4	34.3±2.6	13.6±9.8	16.6±2.2

Worms were labelled with ^{51}Cr and ^{125}I-WGA, washed in EMS and then incubated for 1hr at 37°C in the appropriate concentration of delta toxin in EMS. The release of the two labels during the incubation period was then measured.

The values expressed are the percentage of total counts in the incubation. Each sample was examined in triplicate.

* A preliminary experiment revealed no difference in extent of release of either isotope from male and female worms. Either sex was thus employed indiscriminately for the purpose of a control measurement.

** Value significantly different from that obtained when male worms were assayed ($P<0.025$).

Table 15 The effect of Phospholipase A_2 on adult male and female schistosomes as measured by ^{51}Cr and ^{125}I-WGA release

Incubation medium	Worm sex	% ^{51}Cr release		% ^{125}I-WGA release	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
EMS	M+F *	24.8±3.3	5.5±1.0	10.2±1.8	6.5±0.3
Phospholipase A_2 5ou/ml	M	64.0±12.5	63.8±1.4	53.8±5.5	44.5±5.1
Phospholipase A_2 5ou/ml	F	72.9±4.6	61.5±15.7	53.3±4.0	42.5±4.0
Phospholipase A_2 10u/ml	M	39.0±2.3	39.2±6.5	15.9±7.3	13.4±3.9
Phospholipase A_2 10u/ml	F	63.7±10.1**	47.7±9.2	43.6±0.9**	29.7±4.7**

Worms were labelled with ^{51}Cr and ^{125}I-WGA, washed in EMS and then incubated for 1hr at 37°C in the appropriate concentration of phospholipase A_2 in Dulbecco's PBS. The release of the two labels during the incubation period was then measured. Controls with Dulbecco's PBS were not significantly different from the EMS control and have been omitted.

* A preliminary experiment revealed no different in extent of release of either isotope from male and female worms. Either sex was thus employed indiscriminately for the purpose of a control measurement.

** Value significantly different from that obtained when male worms were employed ($P<0.025$).

that the extent of the damage is influenced by the sex of the worm. Male worms appear to be more susceptible to the detergent like properties (Arbuthnott, 1976) of δ toxin (Table 14). Females on the other hand appear to possess a membrane which is more sensitive to the phospholipase (Table 15).

3.10 Use of clones of schistosomes derived from a single miracidium

The use of clones of male schistosomes derived from a single miracidium offered a number of advantages over the use of males derived from a mixed population. These were :

i) all worms recovered from an infected animal could be used in the assay;

ii) the need to tease apart male and female worms, a procedure which is time consuming, but more importantly may cause tegument damage, is avoided;

iii) the possibility of accidentally assaying worm pairs as opposed to individual worms is avoided.

Clones were therefore used in the investigation whenever possible. It should be mentioned however that the use of female clones presents problems as their small size (due to underdevelopment in the absence of males) makes them difficult to work with in this type of assay.

The possibility that individual clones possess quantitative differences in host antigen expression or quantitative or qualitative differences in parasite antigen expression is an exciting one which, if confirmed, would no doubt have

great influence on the nature of immunological strategems designed to combat the disease. With regard to my own work it could be argued that if differences do exist then results obtained from a particular experiment might be influenced by the individual antigenic properties of the clone employed. For this reason, it was decided to use the IRAM to compare antibody binding to different clones. Results (Fig 11) indicated that the three clones which provided the majority of worm material in this investigation bound both the anti-mouse RBC antibody and the anti-schistosomulum antibody to the same extent.

Fig. 11 Binding of anti-mouse RBC antibody and anti-schistosomulum antibody to worms derived from three individual clones of cercariae.

Worms derived from the three clones of cercariae which provided the majority of worms for this investigation were compared for binding of anti-mouse RBC antibody and anti-schistosomulum antibody using the IRAM. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum or anti-schistosomulum serum. Anti-schistosomulum serum was employed at a 1:10 dilution and anti-mouse RBC serum at a 1:50 dilution. Second antibody was present at a concentration of 200ng/50µl.

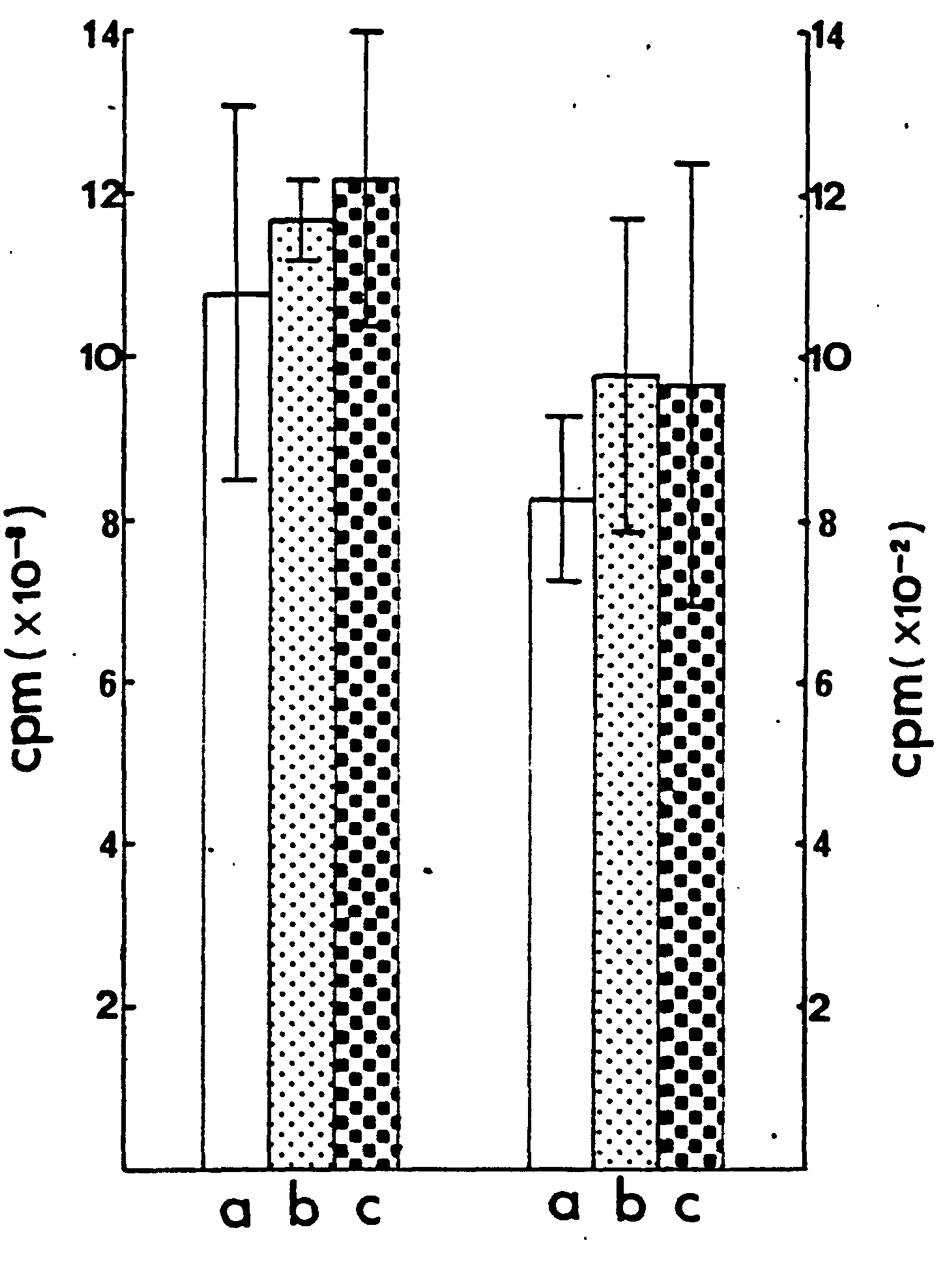
A. First antibody : anti-mouse RBC serum

B. First antibody : anti-schistosomulum serum

a : clone a

b : clone b

c : clone c


A
B
14
12
10
8
6
4
2
cpm (x10⁻¹)
cpm (x10⁻²)
a b c
a b c

CHAPTER 4

The measurement of expression of parasite and host antigens following incubation of adult schistosomes with a variety of membrane-active reagents and culture conditions

4.1 Introduction

The objectives of this work were two fold :

(a) to investigate the ability of a variety of membrane - active reagents and culture conditions to promote increased expression of surface membrane parasite antigens ;

(b) to investigate the hypothesis that host antigens mask or disguise parasite antigens, by determining if increased expression of parasite antigen is related to decreased expression of host antigen.

In order to meet these objectives it is necessary to preserve the integrity of the parasite surface membrane. Thus, before selection for use in the investigation, reagents considered to be potentially capable of altering antigen expression were initially examined for their effect on the surface membrane as measured by transmission electron microscopy and/or a $^{51}Cr/^{125}I$-wheat germ agglutinin (WGA) release assay. Reagents considered to be causing gross membrane damage were usually discarded. Examples of such reagents include the δ-toxin of Staphylococcus aureus and phospholipase A_2 from cobra venom (Naja naja). The effect of these reagents on the surface membrane as measured by $^{51}Cr/^{125}I$-WGA release is shown in Table 14 (δ toxin) and Table 15 (phospholipase A_2).

The reagents employed in this work were selected for their ability to alter either surface membrane protein composition or organisation; or surface membrane lipid composition or organisation. By using reagents with different

properties it was hoped to gain information concerning the properties which drugs might require in order to promote increased expression of parasite antigens *in vivo*.

4.2 Quantities of antibody employed in investigation

Second antibody was employed at a concentration of 200-300ng/50µl when measuring expression of both host and parasite antigens. The specific activity of the preparation was usually 3-5µCi/µg. Anti-mouse RBC serum and anti-schistosomulum serum were employed at a 1:50 and a 1:10 dilution respectively.

4.3 Influence of formalin and glutaraldehyde on antigen expression

4.3.1 Formalin

4.3.1.1 Introduction

Formaldehyde or formalin (37-40% formaldehyde in aqueous solution containing 10-12% methanol) are reagents which have been used as fixatives for cells in the study of cell surface antigens (see for example Chen (1977)). Fixation is carried out to preserve membrane structure and/or prevent antibody induced turnover or relocation of antigen. The value of a fixative is not only determined by its ability to perform these functions however, but is also measured by its effect on the antigenicity of the molecules under study. If these molecules are protein or glycoprotein in nature, it is likely that formaldehyde will combine directly with them during the

fixation process due to its potential to interact with a number of amino acid side chains (French and Edsall, 1945). Such a reaction may have no effect on the ability of antibody to bind to antigen in which case the use of formaldehyde as a fixative would be justified. Alternatively, however, it is possible that interaction between formaldehyde and antigen may interfere with antigen-antibody binding. In this respect Collis and Ritzi(1980) have reported a reduction in antibody binding to the cell surface located mouse mammary tumour cell virus antigen gp 52 following fixation of cells with 1% formaldehyde.

A lowering of antibody binding could arise as a consequence of either destruction of the antigen receptor, or of its displacement within the membrane due to interaction between antigen and formaldehyde resulting in the formation of intra- or inter-molecular methylene bridges (Fraenkel-Conrat and Olcott, 1948; Fraenkel-Conrat and Mecham, 1949). In this respect Matre and Tander (1982) have suggested that abolition of Fc receptor activity of placental cell membranes following treatment with formalin is due to masking of the receptors as a consequence of the formation of intra- and inter-molecular crosslinks.

If formaldehyde induced changes in cell surface architecture can result in a lowering of the exposure of certain cell surface antigens then it is reasonable to expect that they may also be able to cause exposure of antigenic sites which are not normally able to interact with antibody.

Recent findings reported by two groups of workers are in agreement with this idea. Deppert and co-workers (Deppert and Pates,1979a; Deppert and Pates, 1979b; Deppert, Hanke and Henning, 1980) have reported that fixation of simian virus 40-transformed monolayers with formalin or formaldehyde results in exposure of SV40 T-antigen-related molecules. These molecules could not be detected at the surface of unfixed cells. It was thus concluded that the SV40 proteins at the cell surface were normally masked by other cell surface components and were therefore unable to react with antibody until exposed as a consequence of formaldehyde fixation. Also, Collis and Ritzi (1980) have found that fixation of mouse mammary tumour virus infected cells with 1% formaldehyde leads to an increase in expression of the viral cell surface antigens p.27 and gp 36. To explain these results the authors suggested that fixation with formaldehyde produced an alteration in the cell surface which exposed additional antigenic determinants.

If formaldehyde is able to increase the expression of certain antigens at the cell surface then it is possible that it may also be able to increase the expression of parasite antigens at the schistosome surface. This idea is in fact consistent with the results of Gitter et al (1982) who reported that adult worms treated with 10% formalin bind more antibody from infected mouse serum than do living worms as measured by immunofluroescence. It was thus decided that formalin should be selected as one of the reagents whose effects on antigen expression would be measured by the indirect radiolabelled antibody method.

4.3.1.2 Influence of 10% (w/v) formalin on host and parasite antigen expression

Initially formalin fixation was performed in a manner similar to that utilised by Gitter et al (1982) (see Kemp et al, 1977) in order that a comparison of results could be made. This involved incubating worms with 10% formalin in PBS for one hour at 37°C and then washing periodically with PBS during a further two hour period. The results of an experiment in which the effects of 10% formalin on both host and parasite antigen expression was measured is shown in Fig. 12. Clearly formalin fixation leads to an increase in expression of parasite antigens and in addition, a decrease in expression of host antigens. The same effect can in fact be obtained using much shorter incubation periods (Fig. 14) and therefore in all further experiments the incubation period was reduced to 15 min and the washing period to 30 min.

In the above two experiments, the living worms employed as a control were removed from infected mice immediately before use in the IRAM. It could be argued however that control worms should be incubated in EM + CS or PBS during the period in which experimental worms are subjected to formalin fixation. Incubation of worms in EM + CS (Or EMS) for 45 min does not alter the expression of host or patasite antigens (Fig 13). Incubation in PBS appears to have no effect on host antigen expression but was usually found to induce a slight increase (usually less than 30%) in parasite antigen expression. As a consequence of this latter result, it was therefore decided that controls in all further experiments

Fig. 12 Influence of 10% formalin on expression of host and parasite antigens at the adult schistosome surface

Adult schistosomes were removed from the mesenteric veins of infected mice, washed in EMS (x 3) and then incubated in 10% formalin/PBS for 1h at 37°C. Following the fixation step, worms were washed in PBS at 15 min intervals during a two hour period and were then finally washed in EM + CS (x 3). Formalin fixed worms and living worms were then measured for antigen expression by the IRAM. Living worms employed in the assay were removed from infected mice and washed in EM + CS (x 3) immediately before use.

Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum or anti-schistosomulum serum.

A. First antibody : anti-mouse RBC serum

B. First antibody : anti-schistosomulum serum

a: incubation in EMS

b: incubation in 10% formalin/PBS

Statistical analysis

A : avb - $P<0.001$

B : avb - $P<0.05$

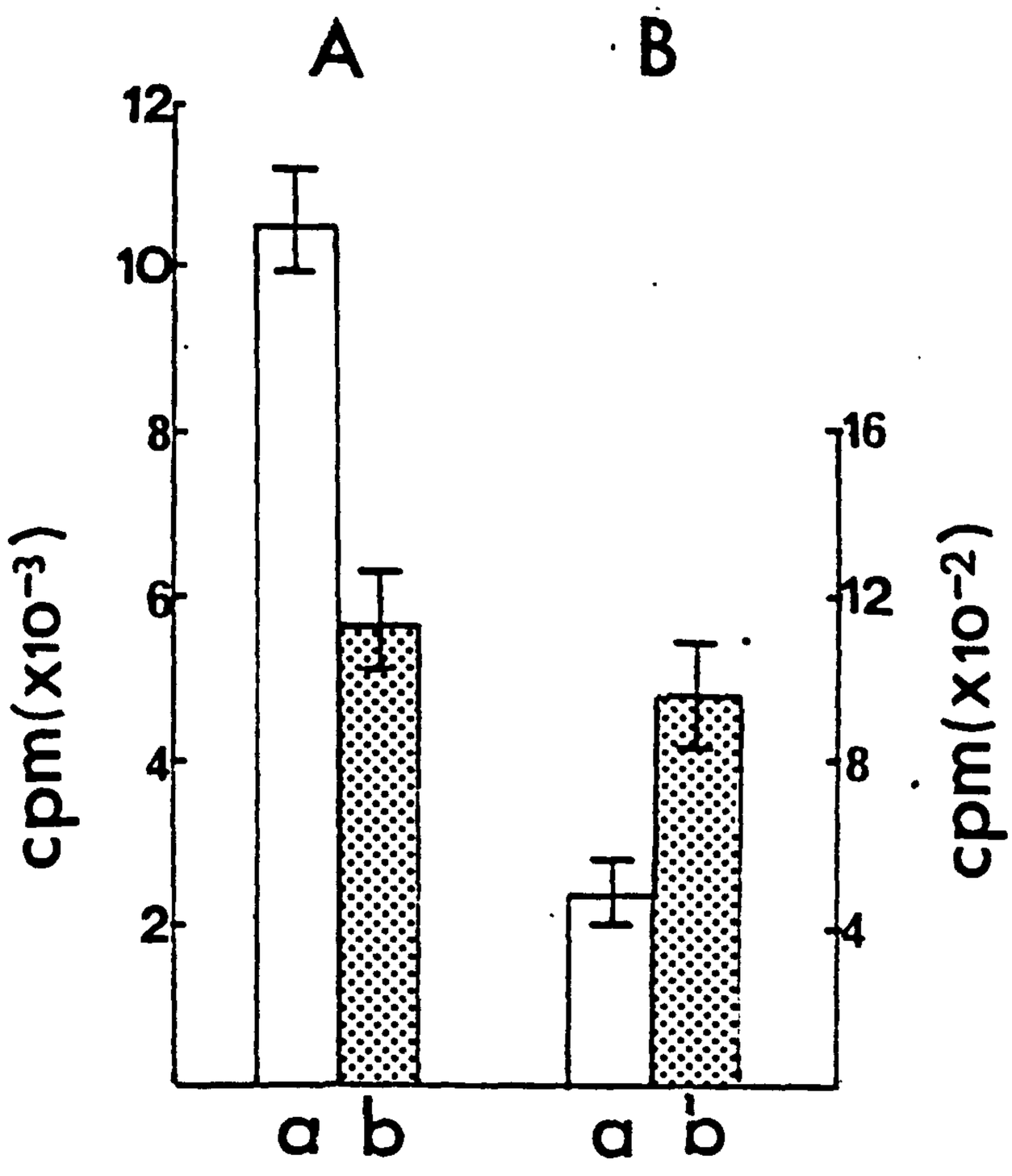

A
B
12
10
8
6
4
2
cpm(X10⁻³)
16
12
8
4
cpm(X10⁻²)
a b
a b

Fig. 13 Influence of 45 min culture in EM + CS or EMS on antigen expression at the adult schistosome surface

Adult worms were measured for antigen expression by the IRAM either (a) immediately following perfusion, or following 45 min culture in (b) EM + CS, or (c) EMS. The experiment was performed such that all worms were assayed simultaneously.

Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-schistosomulum serum or anti-mouse RBC serum.

A. First antibody : anti-mouse RBC serum

B. First antibody : anti-schistosomulum serum.

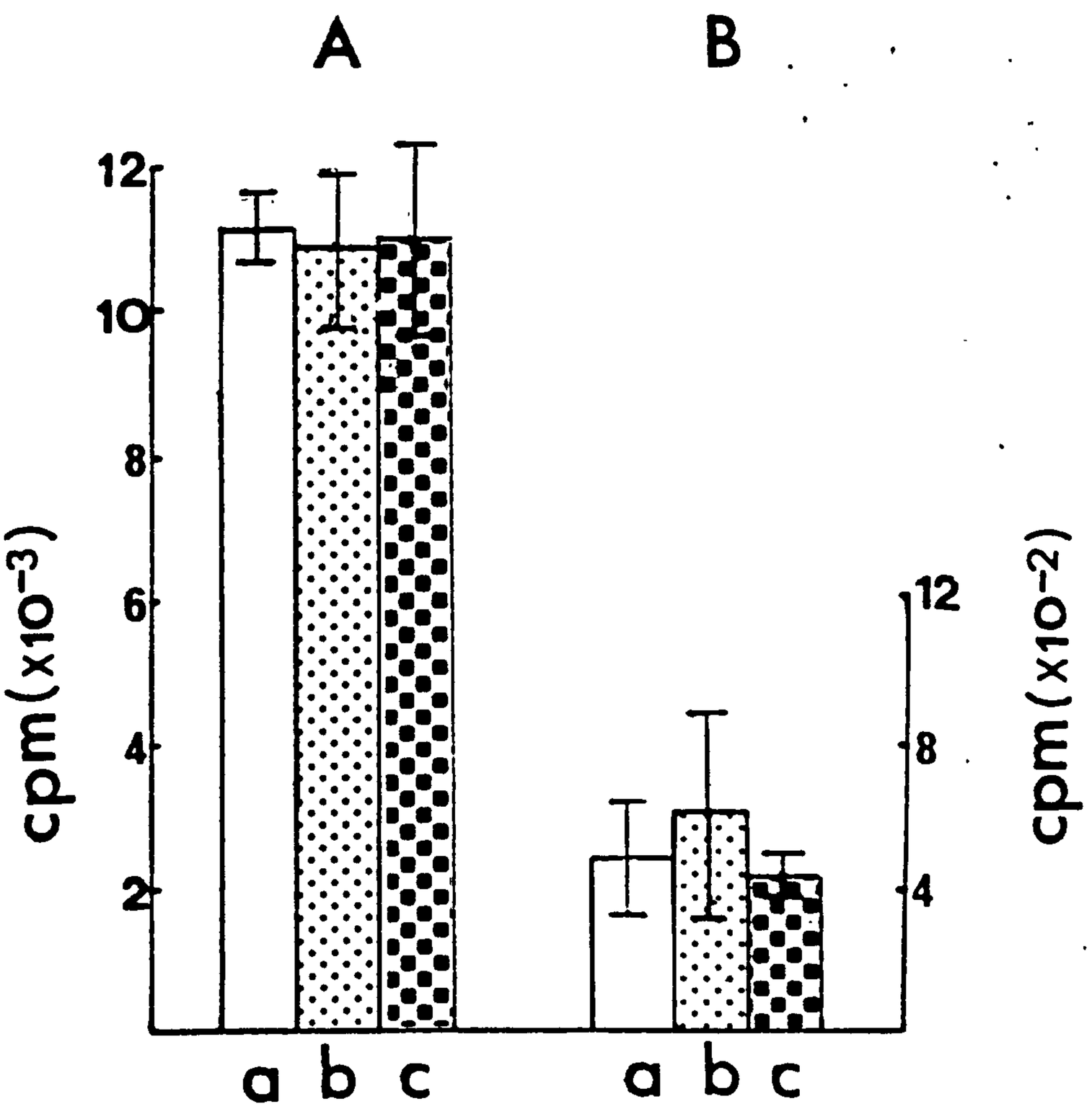

A
B
12
10
8
6
4
2
cpm(x10⁻³)
12
8
4
cpm(x10⁻²)
a b c
a b c

Fig. 14 Influence of different concentrations of formalin on antigen expression at the adult surface membrane

Adult worms were removed from infected mice, washed (x 3) in EMS and then either incubated for 45 min at 37°C in either EM + CS or PBS or subjected to fixation with one of a number of concentrations of formalin. Fixation involved incubating worms in formalin/PBS for 15 min at 37°C and then washing in PBS (x 6) during a 30 min period. Both living and formalin fixed worms were then washed (x 3) in EM + CS before being measured for antigen expression by the IRAM.

A. First antibody : anti-mouse RBC serum

B. First antibody : anti-schistosomulum serum

C. First antibody : NRS (control)

a : incubation in EM + CS

b : incubation in PBS

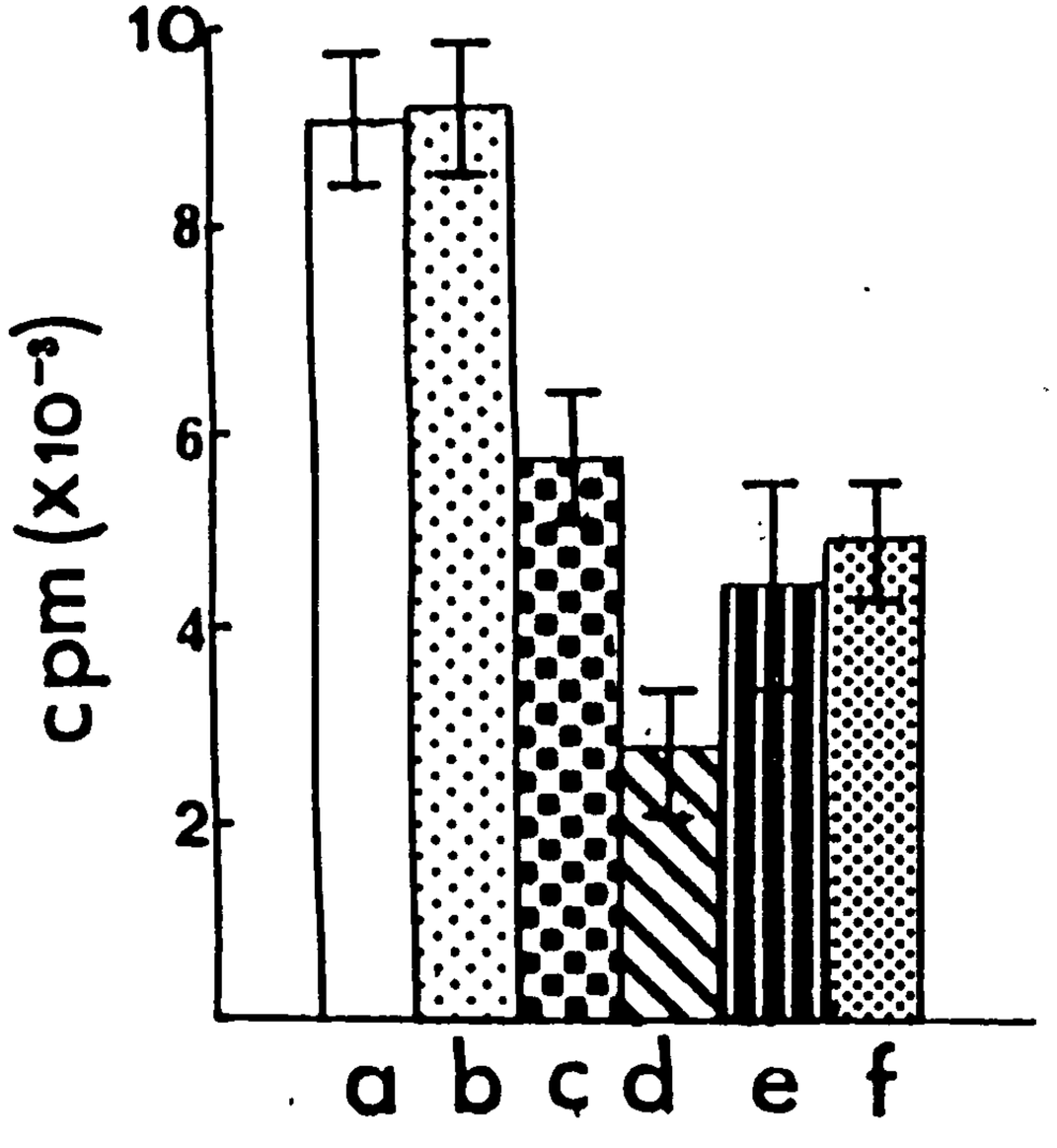
c : incubation in 0.01% formalin

d : incubation in 0.1% formalin

e : incubation in 1% formalin

f : incubation in 10% formalin

The values given in A and B are for specific counts bound (second antibody bound following incubation of worms in either anti-mouse RBC serum or anti-schistosomulum serum - second antibody bound following incubation in NRS).


A
B
C
cpm (X10⁻³)
cpm (X10⁻³)
cpm (X10⁻²)
10
8
6
4
2
4
3
2
1
8
4
a b c d e f
a b c d e f
a b c d e f

employing reagents prepared in PBS would consist of worms incubated in EM + CS and also worms incubated in PBS.

4.3.1.3 Influence of formalin concentration on antigen expression

The results of an experiment in which the effect of different concentrations of formalin on antigen expression was measured are shown in Fig 14. A concentration of 0.1% (w/v) appears to induce the maximum alteration in expression of both host and parasite antigens. The pattern of results was found to be reproducible although the extent of the change in antigen expression varied between experiments. This can be seen with respect to 0.1% formalin in Table 16.

Formalin fixation also tends to increase the amount of labelled antibody bound non-specifically to worms (Fig 14). The extent of the increase varied between different experiments but it was usually less than twice the value obtained with living worms. In general, the effect was greatest when lower concentrations (0.01 or 0.1%) were being employed.

4.3.1.4 Influence of 0.025% (v/v) methanol on antigen expression

0.1% (w/v) formalin, the concentration of the reagent which is most effective at altering antigen expression contains 0.025% (v/v) methanol. An experiment was therefore performed to determine if 0.025% methanol (in EMS) was able to alter antigen expression. No effect was found however and the result has therefore not been shown.

Adult worms were removed from infected mice, washed (x 3) in EMS and then either a) incubated for 45 min at 37°C in either EM + CS or PBS or b) subjected to fixation with formalin. Fixation involved incubating worms in formalin/PBS for 15 min at 37°C and then washing in PBS (x 6) during a 30 min period. Both living and formalin fixed worms were then washed (x 3) in EMS before being measured for antigen expression by the IRAM. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum or anti-schistosomulum serum.

Table 16 Influence of 0.1% (w/v) formalin on expression of host and parasite antigens at the adult schistosome surface

Experiment Number	First anti serum	Incubation medium	Specific antibody bound/4 worms (c.p.m.)	% control value	Statistical analysis
1	anti-mouse RBC	EM + CS	9180±776		
	anti-mouse RBC	0.1% formalin	2720±723	29	P< 0.001
2	anti-mouse RBC	EM + CS	8775±1334		
	anti-mouse RBC	0.1% formalin	2946± 226	34	P< 0.005
3	anti-mouse RBC	EM + CS	7101± 832		
	anti-mouse RBC	0.1% formalin	3121± 376	44	P< 0.0025
1	anti-schisto-somulum	EM + CS	1326± 163		
	anti-schisto-somulum	0.1% formalin	3202±1581	241	P> 0.05
2	anti-schisto-somulum	EM + CS	1232± 600		
	anti-schisto-somulum	0.1% formalin	3729± 254	302	P< 0.00025
3	anti-schisto-somulum	EM + CS	1168± 515		
	anti-schisto-somulum	0.1% formalin	2837± 210	243	P< 0.01
1	anti-schisto-somulum	PBS	1713± 140		
	anti-schisto-somulum	0.1% formalin	3202±1581	187	P> 0.05
2	anti-schisto-somulum	PBS	901± 350		
	anti-schisto-somulum	0.1% formalin	3729± 254	413	P< 0.0005
3	anti-schisto-somulum	PBS	1250± 360		
	anti-schisto-somulum	0.1% formalin	2837± 210	227	P< 0.005

4.3.2 Action of glutaraldehyde on antigen expression

4.3.2.1 Introduction

Glutaraldehyde, like formaldehyde, is a reagent which is commonly employed as a fixative in morphological studies (see for example Wisse et al (1974). Like formaldehyde it is able to form crosslinks between and within proteins (Bowes and Carter, 1968; Hopwood, 1969; Steck, 1972) by virtue of being able to interact with a number of amino acid side chains (Habeeb and Hiramoto, 1968; Avraemas and Ternynck, 1969). It could thus be argued that glutaraldehyde might be expected to alter antigen expression at the adult schistosome membrane in a manner similar to formaldehyde. Reports in the literature however indicate that glutaraldehyde and formaldehyde differ with respect to certain properties : glutaraldehyde has been shown to a) be more effective at inhibiting the catalatic activity of crystalline beef liver catalase (Herzog and Fahimi, 1974), b) be less effective at preserving the antigenicity of BSA (Habeeb, 1969), c) be a more effective crosslinker of proteins (Habeeb, 1969; Hopwood, 1969), d) unlike formaldehyde, increase the surface negative charge of human erythrocytes by 10% at pH 7 (Vasser et al, 1972). Such differences in activity suggest that it is possible that glutaraldehyde and formaldehyde might in fact have a dissimilar effect on surface antigen expression. This possibility was investigated.

4.3.2.2 Influence of glutaraldehyde on antigen expression

The influence of glutaraldehyde on antigen expression is shown in Fig 15. Host antigen expression was found to be reduced following incubation with all three concentrations of glutaraldehyde but the maximum effect was obtained using a 0.01% (w/v) concentration. Parasite antigen expression was increased by both 0.01 and 0.1% glutaraldehyde, the former concentration being more effective. Parasite antigen expression was not increased however when glutaraldehyde was employed at a concentration of 1% and indeed was slightly reduced. Although this decrease was not statistically significant (at $P < 0.05$), it was found to be reproducible. The pattern of results as a whole was also reproducible although the extent of the change varied between experiments (with respect to 0.01% compare Fig 15 with Tables 12 and 13).

Glutaraldehyde, like formalin, tended to increase the non-specific binding of radiolabelled antibody to worms (Fig 15). The extent of the increase was similar to that induced by formalin fixation and the maximum effect was usually obtained when glutaraldehyde was employed at a concentration of 0.01%.

4.3.3 Influence of formalin and glutaraldehyde on membrane permeability as measured by ^{51}Cr release

Table 17 clearly shows that incubation of schistosomes in formalin or glutaraldehyde leads to a concentration-dependent increase in the loss of the cytoplasmic label ^{51}Cr during the course of the IRAM. This result raises the

Table 17 The effect of pre-treatment with formalin or glutaraldehyde on the release of ^{51}Cr from adult schistosomes during the incubation period of the IRAM

Incubation	% ^{51}Cr release.
EMS	13.57 ± 3.84
PBS	38.83 ± 4.40
1% (w/v) formalin	42.89 ± 3.84
0.1% formalin	48.82 ± 2.42
0.01% formalin	50.67 ± 4.84
1% (w/v) glutaraldehyde	34.36 ± 4.65
0.1% flutaraldehyde	39.05 ± 4.96
0.01% glutaraldehyde	48.29 ± 4.17

Worms were labelled with ^{51}CR, washed in EMS, and then incubated for 15 min at 37°C in either EMS, PBS, formalin or glutaraldehyde. Worms were then removed from these reagents and washed with 0.5mls PBS before being incubated for 30 min at 37°C in 0.25mls PBS or in the case of worms previously immersed in EMS, a further 0.25mls EMS. All supernatants and washings were kept for counting of radioactivity. The incubation conditions of the IRAM were then mimicked by adding a 0.25ml aliquot containing appropriate materials such that all worms were immersed in EM + CS and allowing incubation to proceed for a further 90 min. Finally, worms were removed from the incubation media and washed with 0.5mls PBS before counting for radioactivity. The supernatants and washings were again kept and added to earlier material before also being counted.

Fig. 15 Influence of glutaraldehyde on antigen expression at the adult surface membrane

Adult worms were removed from infected mice, washed (x 3) in EMS and then either incubated for 45 min at 37°C in either EM + CS or PBS or subjected to fixation with glutaraldehyde. Fixation involved incubating worms in glutaraldehyde/PBS for 15 min at 37°C and then washing in PBS (x 6) during a 30 min period. Both living and glutaraldehyde fixed worms were then washed (x 3) in EM + CS before being measured for antigen expression by the IRAM.

A. First antibody : anti-mouse RBC serum

B. First antibody : anti-schistosomulum serum

C. First antibody : NRS (control)

a : incubation in EM + CS

b : incubation in PBS

c : incubation in 0.01% glutaraldehyde

d : incubation in 0.1% glutaraldehyde

e : incubation in 1% glutaraldehyde

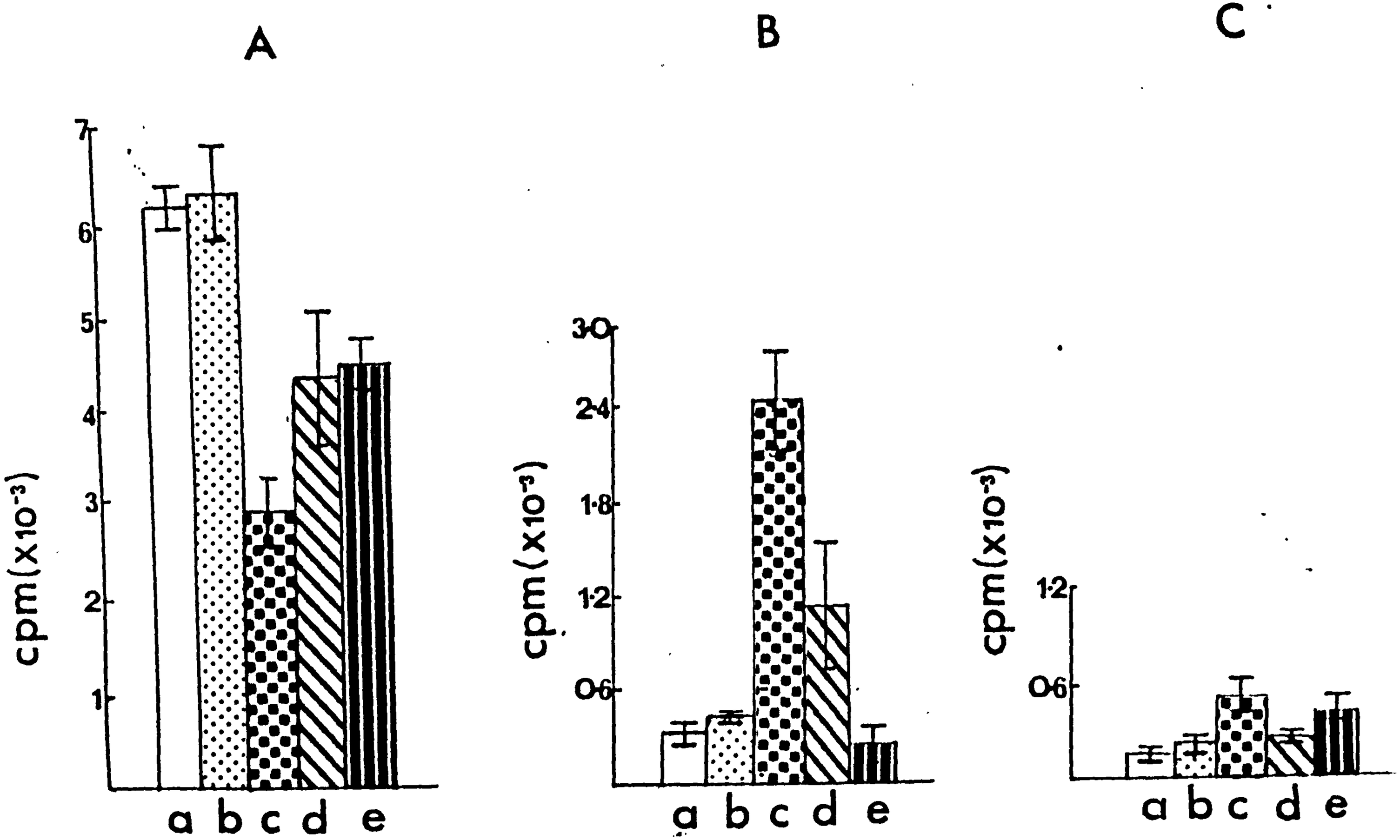
The values given in A and B are for specific antibody bound (second antibody bound following incubation in either anti-mouse RBC serum or anti-schistosomulum serum - second antibody bound following incubation in NRS).

Statistical analysis.

A : avc - $P < 0.0005$; avd - $P < 0.05$; ave - $P < 0.0025$

B : avc - $P < 0.0025$; avd - $P < 0.05$; ave - $P > 0.05$

bvc - $P < 0.0025$; bvd - $P > 0.05$; bve - $P > 0.05$


A
B
C
cpm(X10⁻³)
7
6
5
4
3
2
1
30
2·4
1·8
1·2
0·6
1·2
0·6
a b c d e
a b c d e
a b c d e

possibility that treatment of worms with aldehydes may in addition to allowing binding of anti-parasite antibody to exposed membrane antigens, also enable some antibody to pass through the membrane and bind to internal antigens. This is considered unlikely however for the following reason :- Incubation of worms in PBS results in a loss of three times as much ^{51}Cr as is lost following incubation of worms in EMS. PBS treatment however is only accompanied by a slight and inconsistent increase in binding of anti-parasite antibody as compared to, for example, that obtained when 0.1% (w/v) glutaraldehyde is employed (Fig 15). Since 0.1% glutaraldehyde is equal to PBS in its ability to promote release of ^{51}Cr it must be assumed that the increase in antigen expression associated with the aldehyde is due largely, if not wholly, to binding of antibody to the worm surface. This result does not rule out the possibility however that antibody may enter the tegumental cytoplasm when the increase in ^{51}Cr release is greater. Even in such a situation however ^{51}Cr release still does not correlate with antibody binding, as 0.01% (w/v) and 0.1% formalin have a similar effect on ^{51}Cr release but differ in their ability to increase expression of parasite antigens (Fig.14).

4.4 Action of trypsin on surface antigen expression

4.4.1 Introduction

A number of workers have shown that surface antigens of schistosomula are susceptible to the action of the proteolytic enzyme trypsin (Dissous *et al*, 1981; Taylor *et al*, 1981;

Gazzinelli et al, 1982). Since schistosomula and adult worms are known to share surface antigens (Kusel et al, 1975; Snary et al, 1980) it is possible that trypsin may also interact with antigens at the adult surface membrane. In support of this idea is the finding that trypsin has been shown to remove binding sites for concanavalin A from both schistosomula (Gazzinelli et al, 1982) and adult worms (Bennett and Seed, 1977). It is therefore possible that interaction between trypsin and surface membrane antigens could lead to changes in surface antigen expression. Arguments in support of this hypothesis are as follows :

1) Trypsin has as its site of action peptide bonds to which lysine or arginine have contributed the carboxyl group. The ε-amino group of lysine can be attacked by both formaldehyde (French and Edsall, 1945; Fraenkel-Conrat and Olcott, 1948) and glutaraldehyde (Habeeb and Hiramoto, 1968; Avraemas and Terrynck, 1969; Peters and Richards, 1977), reagents which have been shown to change antigen expression. It is therefore possible that trypsin may have a site of action on certain membrane polypeptides in close proximity to that of the aldehydes and may therefore be able to mimic their effects on antigen expression. In relation to this idea trypsin (Schochetman et al., 1978) and formaldehyde (Collis and Ritzi, 1980) have both been shown to expose viral antigens at the surface of mouse mammary tumour virus infected cells and Collis and Ritzi have in fact suggested that the two reagents may act in a similar manner.

ii) Treatment of schistosomula with trypsin renders them more sensitive to the action of lethal antibody plus complement (Gazzinelli et al, 1982). One possible explanation for this result is that trypsin exposes certain normally inaccessible antigenic sites such that antibody binding and in turn, complement fixation, are increased at the schistosomulum surface. If this hypothesis is true then treatment of adult schistosomes with trypsin may also increase the expression of certain parasite antigens.

iii) Kodani (1962) has shown that human amniotic cells of blood group O or A will express blood group B if exposed to the glycolipid form of this antigen in culture. Similarly, the A substance can be acquired by O or B cells. This effect is lost however when cells are exposed to trypsin, presumably as a consequence of loss of a protein receptor. It is possible that uptake of glycolipid antigens by schistosomes is also dependent on a protein receptor and that this receptor is trypsin sensitive. Treatment of adult worms with trypsin may therefore lead to a reduction in expression of host antigens.

In order to confirm or refute these possibilities trypsin treated and control worms were compared for host and parasite antigen expression.

4.4.2 The influence of trypsin on the adult surface membrane as measured by release of ^{51}Cr and ^{125}I-WGA

The influence of trypsin (400µg/ml for 1h) on release of ^{51}Cr and ^{125}I-WGA from adult schistosomes is shown in Table 18.

Table 18 The effect of trypsin on the adult surface membrane as measured by ^{51}Cr and ^{125}I-WGA release

Incubation	% ^{51}Cr release	% ^{125}I-WGA release
EMS	9.57 ± 4.94	7.18 ± 1.05
Trypsin (0.4mg/ml)	13.08 ± 1.96	12.76 ± 0.23

Worms were labelled with ^{51}Cr and ^{125}I-WGA, washed in EMS and then incubated for 1h at 37°C in either EMS or trypsin in EMS. The release of both isotopes during the incubation period was then measured.

The values expressed are the percentage of total counts in the incubation. Each sample was examined in triplicate. Trypsin was prepared in EMS immediately before use.

Although the enzyme has increased the release of both labels the increase in both cases is slight when compared to, for example, the results obtained following treatment of worms with staphylococcal delta toxin (Table 14). Trypsin treatment as measured by this method was therefore considered to be not excessively damaging to the worms.

4.4.3 The influence of trypsin on the adult surface membrane as determined by transmission electron microscopy

Trypsin treatment (400µg/ml for 30 min) was found to preserve the parasite outer membrane as determined by transmission electron microscopy (Fig 16) although loosening of membrane was observed in some of the surface pits (cf. Fig 19). Loosened membrane for the most part was trilaminate in nature suggesting that trypsin treatment may split the outer bilayer from the inner bilayer and in addition, was removed in the form of a continuous unbroken structure. Examination of some sections of trypsin treated worms also revealed the presence of abnormal structures consisting of concentric whorls of membrane in the parasite tegument (Fig 17).

4.4.4 The influence of trypsin on parasite antigen expression

Figure 18 clearly shows that treatment of schistosomes with trypsin results in an increase in parasite antigen expression. The effect is concentration dependent, increasing in extent with increasing concentration of enzyme. As with formalin and glutaraldehyde the pattern of results was found to be reproducible. The inter-assay variation

Figure 16 Transmission electron micrograph showing the surface membrane and surface pits of the dorsal tegument of the adult worm following exposure to trypsin

Adult worms were removed from infected mice, washed (x 3) in EMS and then incubated in EMS containing trypsin (400µg/ml) for 30 min at 37°C. Following incubation worms were washed (x 3) in EMS and then prepared for electron microscopy.

T = tegument; SM = surface membrane; SP = surface pits;
LTM = loosened trilaminate membrane; S = spine

Magnification = x 132,750

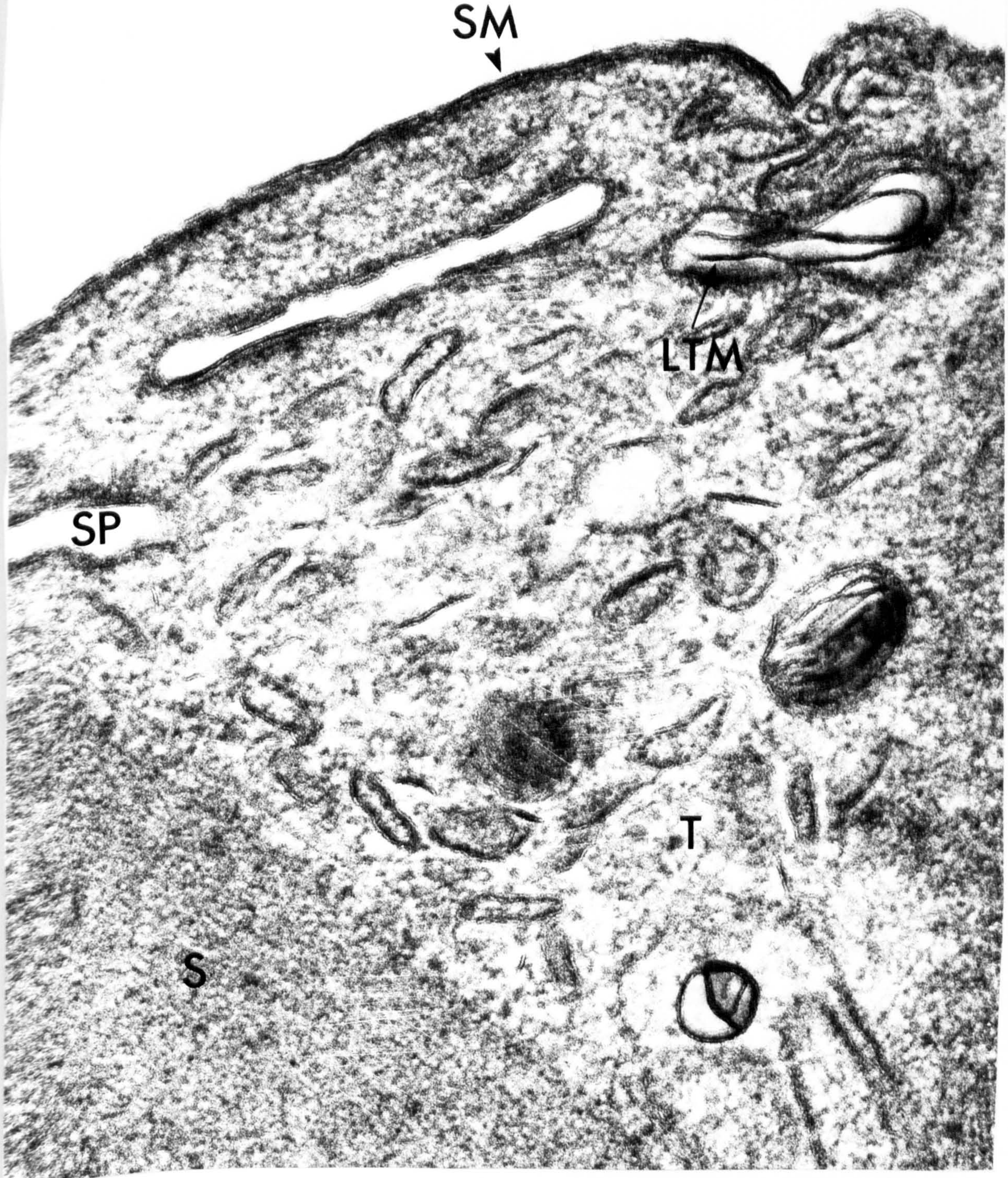

SM
LTM
SP
T
S

Figure 17 Transmission electron micrograph showing the dorsal tegument of the adult worm following exposure to trypsin

Adult worms were removed from infected mice, washed (x 3) in EMS and then incubated in EMS containing trypsin (400µg/ml) for 30 min at 37°C. Following incubation worms were washed (x 3) in EMS and prepared for electron microscopy.

T = tegument; SM = surface membrane; C.W. = abnormal structure consisting of concentric whorls of membrane.

Magnification = x 81,000

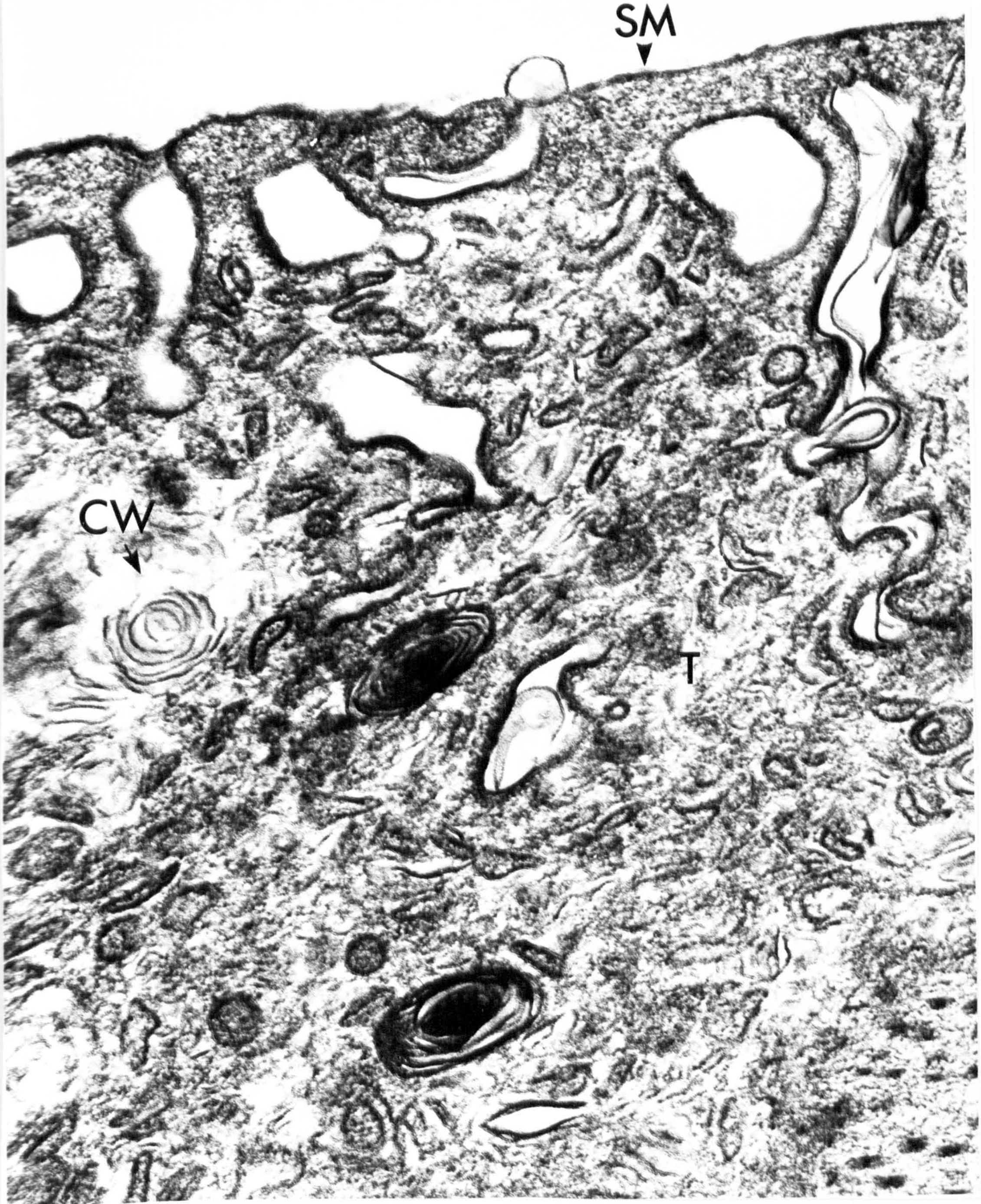

SM
CW
T

Figure 18 Influence of trypsin on parasite antigen expression at the adult schistosome surface membrane

Adult worms were removed from infected mice, washed (x 3) in EMS and then incubated in various concentrations of trypsin for 20 min at 37°C. Following incubation, worms were washed (x 3) in EM + CS and then measured for parasite antigen expression by the IRAM.

Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-schistosomulum serum. Trypsin treatment did not alter non-specific binding.

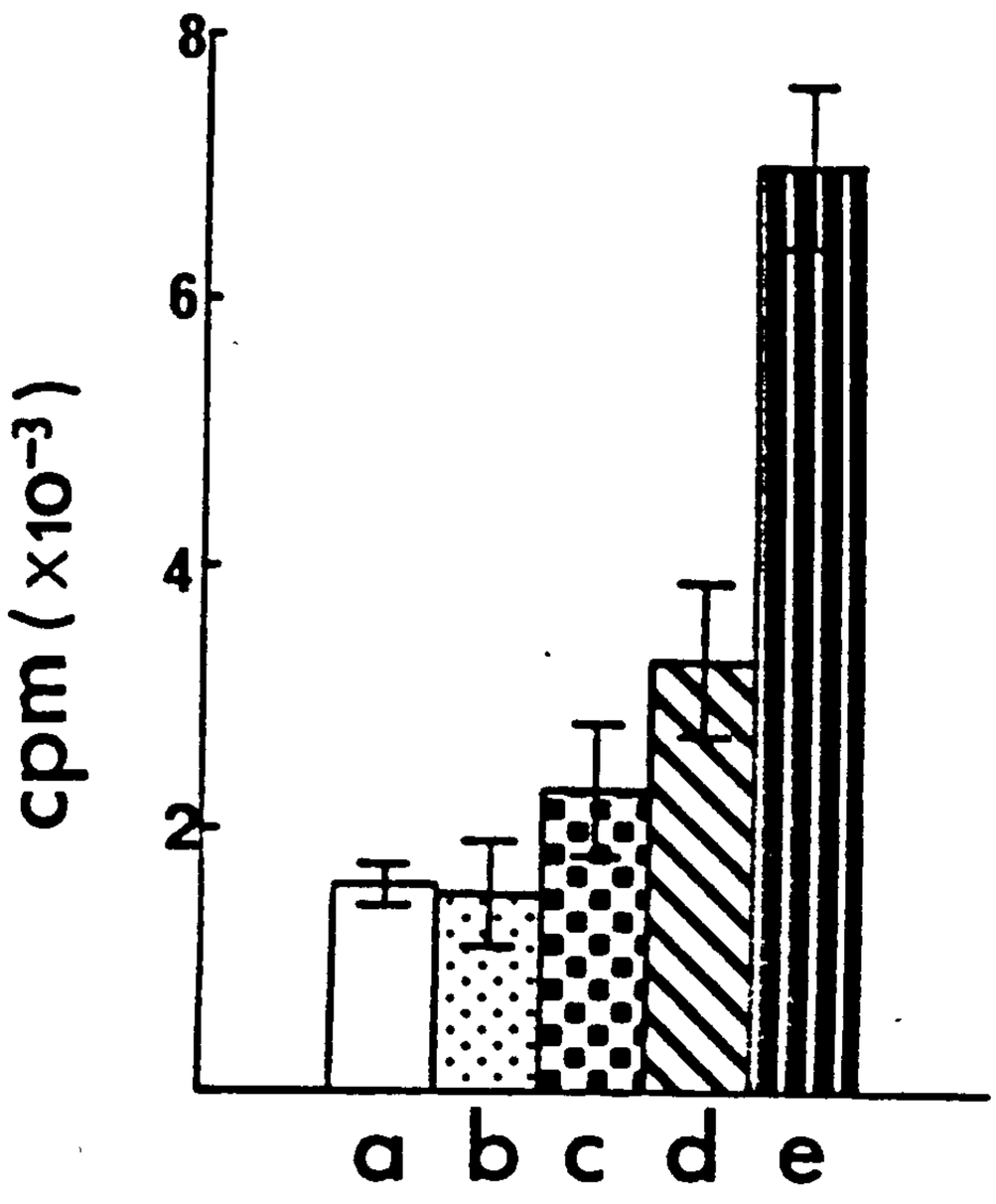
a - incubation in EMS

b - incubation in 50µg/ml trypsin

c - incubation in 100µg/ml trypsin

d - incubation in 200µg/ml trypsin

e - incubation in 400µg/ml trypsin


8
6
4
2
cpm (x10⁻³)
a b c d e

associated with worms treated with 400µg/ml of the enzyme is shown in Table19 .

4.4.5 The influence of trypsin on host antigen expression

Attempts to measure the effect of trypsin on host antigen expression were unsuccessful due to a change in affinity of the second antibody for the first, following binding of the latter to trypsin treated schistosomes (Table 20). This effect was not observed when measuring host antigen expression at the surface of glutaraldehyde or formalin fixed worms and may be due to trypsin, unlike the aldehydes interacting with the surface membrane in such a way as to alter the affinity of RBC antigens for first antibody. Such a situation could perhaps arise if trypsin treatment a) chemically altered the antigenic site or b) resulted in relocation of the antigen in relation to other membrane components.

4.5 The influence of retinol (vitamin A alcohol) on surface antigen expression

Incubation of certain cells and bacterial protoplasts in the presence of excess retinol leads to alterations in their plasma membranes (for review see Dingle and Lucy, 1965). In the case of the red blood cell, retinol used at a concentration of 10µg/ml is able to penetrate and expand the membrane such that loss of haemaglobin and cell lysis soon follow. Retinol (1mg/ml) is also able to interact with the adult schistosome surface membrane as shown by spectrofluorimetry and fluorescence microscopy (Kusel et al, 1982) but apparently does not damage

Table 19 Influence of Trypsin (400µg/ml) on expression of parasite antigens at the adult schistosome surface

Experiment Number	Incubation	Specific antibody bound/worms (c.p.m.)	% control value	Statistical analysis
1	EMS	627 ± 132		
	trypsin/EMS	2192 ± 412	350	P< 0.005
2	EMS	914 ± 203		
	trypsin/EMS	1961 ± 214	215	P< 0.025
3	EMS	1799 ± 147		
	trypsin/EMS	7000 ± 557	389	P< 0.00025

Adult worms were removed from infected mice, washed (x 3) in EMS, and then incubated in either EMS or EMS containing 400µg/ml trypsin for 20 min at 37°C. Following incubation worms were washed (x 3) in EM + CS and then measured for parasite antigen expression by the IRAM. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-schistosomulum serum.

Table 20 The influence of second antibody concentration on the change in host antigen expression detected following treatment of worms with trypsin

Incubation medium	conc. 2nd antibody (µg/ml)	specific antibody bound/4 worms * (c.p.m.)
EMS	0.2	1.00
trypsin (400µg/ml)	0.2	0.44
EMS	0.6	1.00
trypsin (400µg/ml)	0.6	0.55
EMS	8.0	1.00
trypsin (400µg/ml)	8.0	1.57

Adult worms were removed from infected mice, washed (x 3) in EMS and then incubated in trypsin/EMS or EMS for 30 min at 37°C. Following incubation worms were washed in EM + CS and then measured for host antigen by the IRAM. The concentration of second antibody employed in the IRAM was varied as indicated. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum.

* Results were normalized to allow rapid assessment.

it as measured by release of ^{51}Cr and ^{125}I-WGA (Table 21) or transmission electron microscopy (Fig 19). This ability of retinol to interact with, but not damage, the schistosome outer membrane permits the effect of the reagent on membrane antigen expression to be measured. It was thus decided to determine if retinol, a different type of reagent from trypsin, formaldehyde and glutaraldehyde in that it is able to bind strongly to the hydrophobic regions of membrane lipids (Lucy and Dingle, 1964) was able to alter the expression of host and/ or parasite antigens. In relation to this idea Borochov and Shinitsky (1976) have shown that reorganisation of lipids in the cell membrane can alter the expression of surface proteins.

Figure 20 clearly shows that retinol used at a concentration of 1mg/ml does not alter expression of either host or parasite antigens. This result was reproducible.

4.6 The influence of Tween detergents on surface antigen expression

Non-ionic detergents of the polyoxyethelene sorbitol series (Tween series (Fig 21)) vary in their ability to disintegrate HVJ virions (Hosaka, 1968), extract rhodopsin from an isolated photoreceptor fraction of bovine retinal tissue (Zorn and Futterman, 1973) and improve trypsin banding of human chromosomes.(Neumann et al, 1980). As shown in Table 22 they also vary in their ability to damage the adult schistosome outer membrane as measured by release of ^{51}Cr and ^{125}I-WGA. Tween 40 and Tween 80 appear to have little effect on the leakage of the labels : Tween 20 conversely is able to promote their release.

Table 21 The effect of retinol on the adult surface membrane as measured by ^{51}Cr and ^{125}-WGA release

Incubation medium	% ^{51}Cr release	% ^{125}I-WGA release
EMS	8.46 ± 1.96	8.07 ± 2.87
Retinol (1mg/ml)	11.51 ± 2.15	7.58 ± 1.26

Worms were labelled with ^{51}Cr and ^{125}I-WGA, washed in EMS and then incubated for 1hr at 37°C in either EMS or retinol in EMS. The release of both isotopes during the incubation period was then measured.

The values expressed are the percentage of total counts in the incubation. Each sample was examined in triplicate. Retinol, from a stock solution in ethanol, was added to EMS immediately before use.

Figure 19 Transmission electron micrograph showing the dorsal tegument of the adult worm following exposure to retinol

Adult worms were removed from infected mice, washed (x 3) in EMS and then incubated for 15 min at 37°C in EMS containing retinol (1mg/ml). Following incubation worms were washed (x 3) in EMS and then prepared for electron microscopy. Examination of the tegument following retinol treatment revealed that it did not differ from the tegument of untreated worms.

T = tegument; SM = surface membrane; SP = surface pits; MF = muscle fibres.

Magnification = 61,000

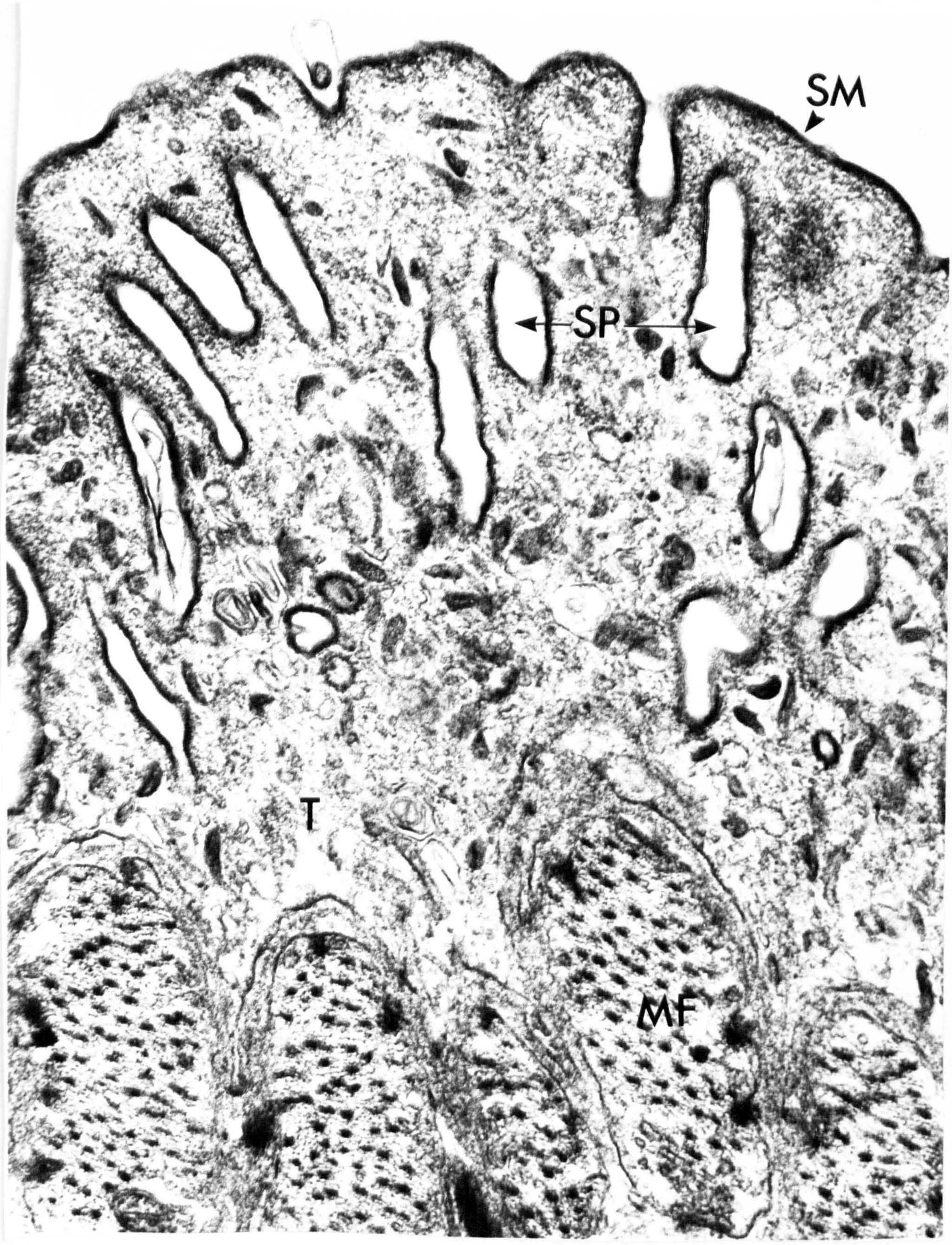

SM
SP
T
MF

Fig. 20 Influence of Retinol on expression of host and parasite antigens at the adult schistosome surface

Adult schistosomes were removed from the mesenteric veins of infected mice, washed in EMS (x 3) and then incubated for 15 min at 37°C in either retinol (1mg/ml in EMS) or EMS. After washing in EM + CS (x 3) worms were then measured for antigen expressions by the IRAM.

Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum or anti-schistosomulum serum. The use of retinol did not alter non-specific binding.

A. First antibody : anti-mouse RBC serum

B. First antibody : anti-schistosomulum serum

a. incubation in EMS

b. incubation in retinol/EMS

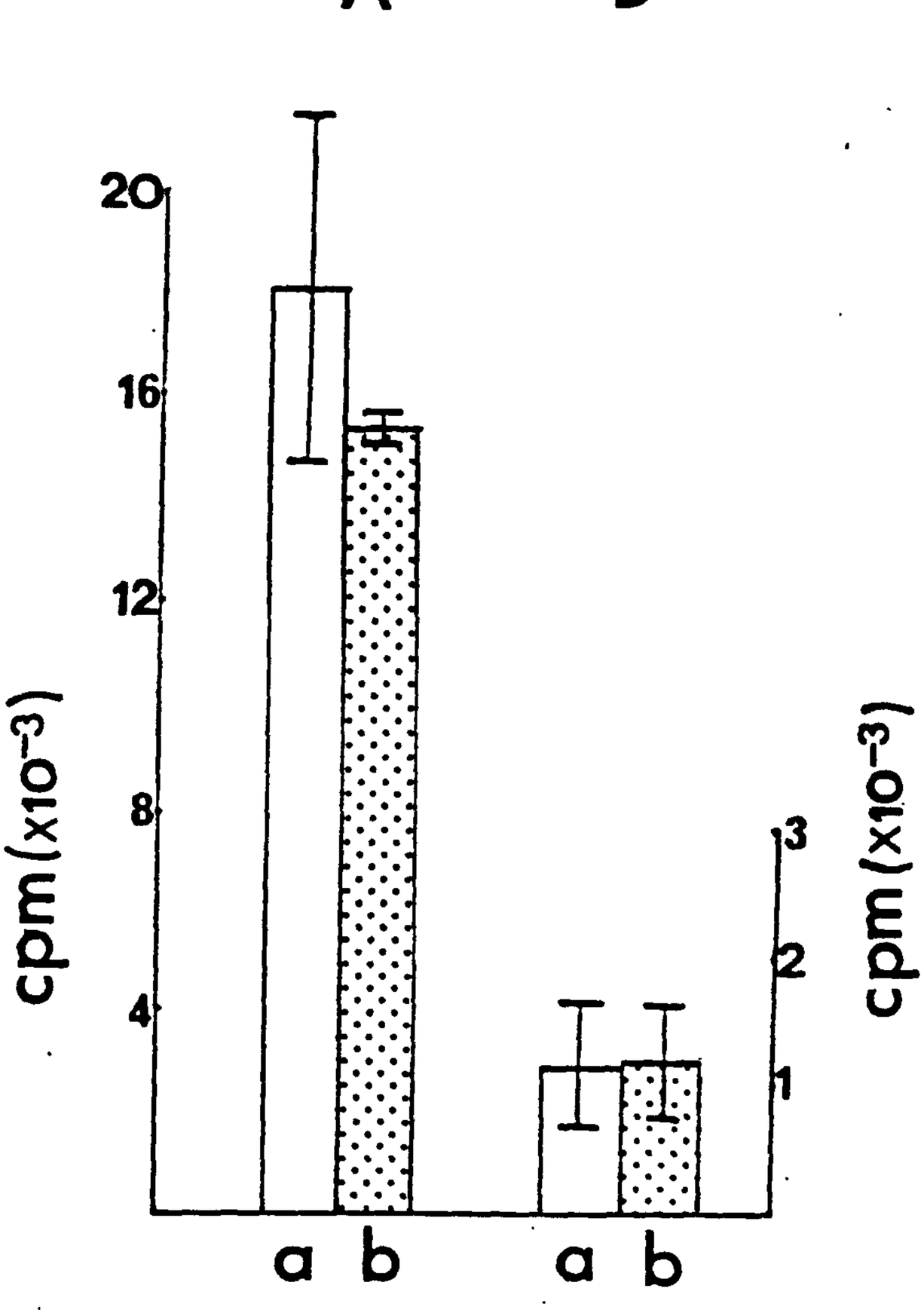
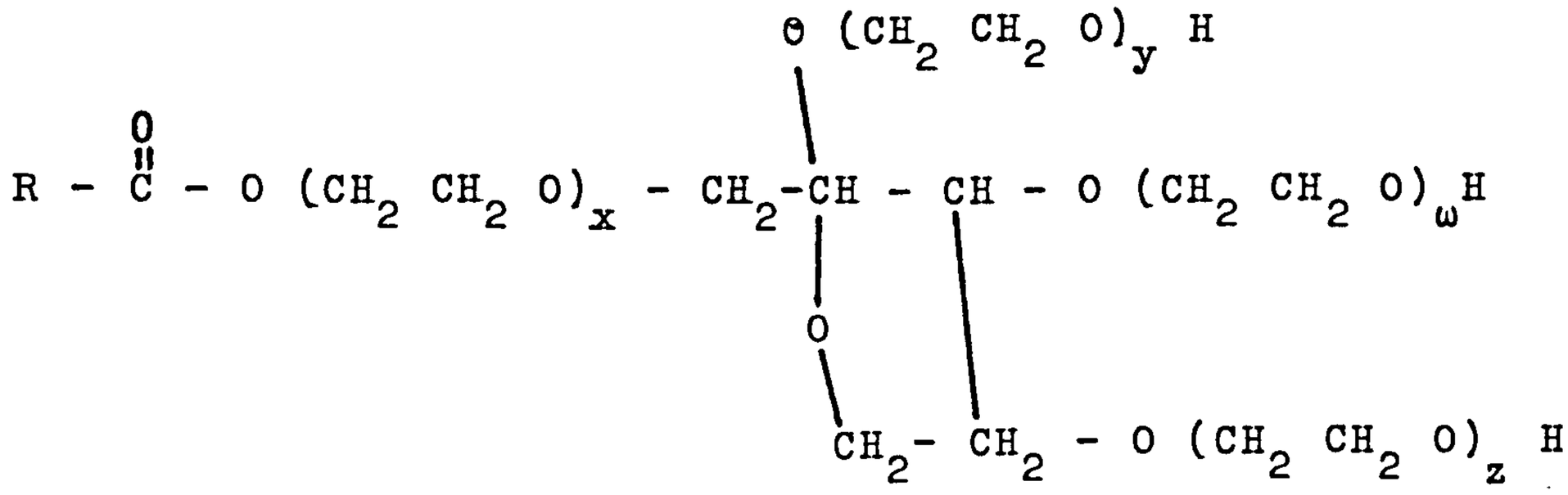

A
B
20
16
12
8
4
cpm (x10⁻³)
3
2
1
cpm (x10⁻³)
a
b
a
b

Figure 21 Structure of Tween detergents



$x + y + z + \omega = 20$

R = mono-laurate : Tween 20

mono-palimitate: Tween 40

mono-oleate : Tween 80

Table 22 The effect of Tween reagents on the adult surface membrane as measured by ^{51}Cr and ^{125}I-WGA release

Incubation medium	% ^{51}Cr release	% ^{125}I-WGA release
EMS	16.89 ± 3.17	11.09 ± 3.11
2.5% Tween 20	27.04± 1.98*	24.36 ± 1.94*
2.5% Tween 40	16.41 ± 2.44	14.12 ± 2.24
2.5% Tween 80	17.56 ± 4.60	12.80 ± 2.68

Worms were labelled with ^{51}Cr and ^{125}I-WGA, washed in EMS and then incubated for 1 hr at 37^{o}C in the appropriate reagent. The release of the two labels during the incubation period was then measured.

The values expressed are the percentage of total counts in the incubation. Each sample was examined in triplicate. Tween reagents were prepared in EMS.

* Values significantly different from control ($P<0.05$).

Since Tween 40 and Tween 80 are able to extract lipid from (J.R. Kusel, unpublished results), but do not damage the surface membrane, their influence on membrane antigen expression can be readily determined. However, the results obtained when the effect of retinol on surface antigen expression was measured suggests that amphiphiles which are able to insert themselves into the membrane without "damaging" it may have little influence on antigen expression. For this reason in addition to determining the effect of Tween 40 and Tween 80 on antigen expression it was decided to also measure the effect of Tween 20.

Figure 22 clearly shows that incubation of worms in the presence of Tween 40 or Tween 80 does not alter surface antigen expression.Conversely, Tween 20 was found to significantly increase parasite antigen and significantly decrease host antigen expression. These results were found to be reproducible.

4.7 Influence of various culture conditions on surface antigen expression

4.7.1 Introduction

Shaw and Erasmus (1977) using a culture medium containing human serum have shown that adult schistosomes can be maintained *in vitro* for a period of several days without any effect on the morphology of the parasite tegument. Overnight culture of schistosomes in EM + CS produces a similar result (Fig 23) with the exception that some vacuolation can be detected at the

Fig. 22 Influence of Tween reagents on expression of host and parasite antigens at the adult schistosome surface.

Adult schistosomes were removed from the mesenteric veins of infected mice, washed in EMS (x 3) and then incubated in the appropriate Tween reagent for 15 min at 37°C. After washing in EM + CS (x 3) worms were then measured for antigen expression by the IRAM.

Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum or anti-schistosomulum serum. Treatment of worms with Tween reagents did not alter their ability to bind antibody in a non-specific manner.

A. First antibody : anti-mouse RBC serum

B. First antibody : anti-schistosomulum serum

a : incubation in EMS

b : incubation in 2.5% (v/v) Tween 20/EMS

c : incubation in 2.5% (v/v) Tween 40/EMS

d : incubation in 2.5% (v/v) Tween 80/EMS

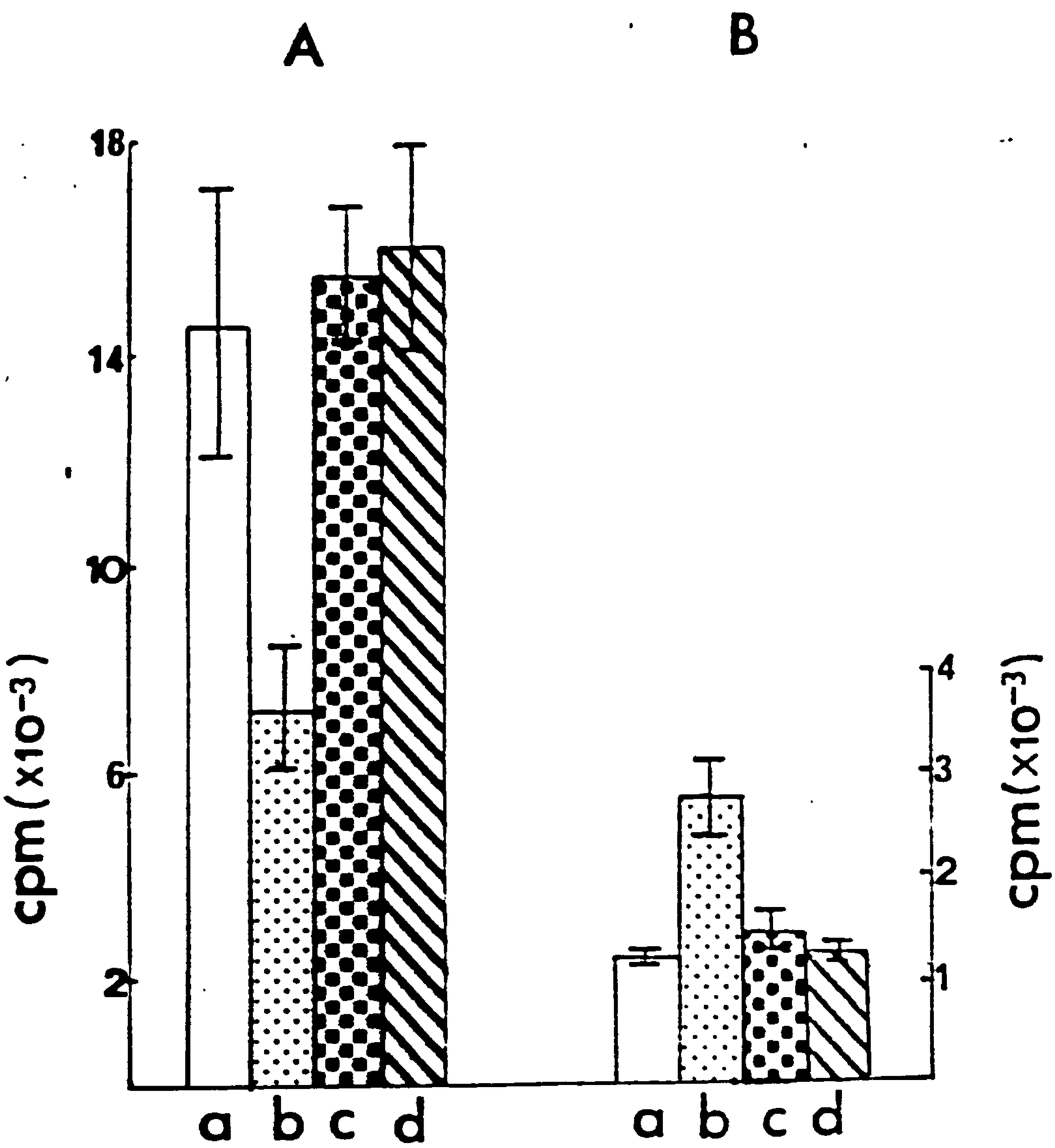
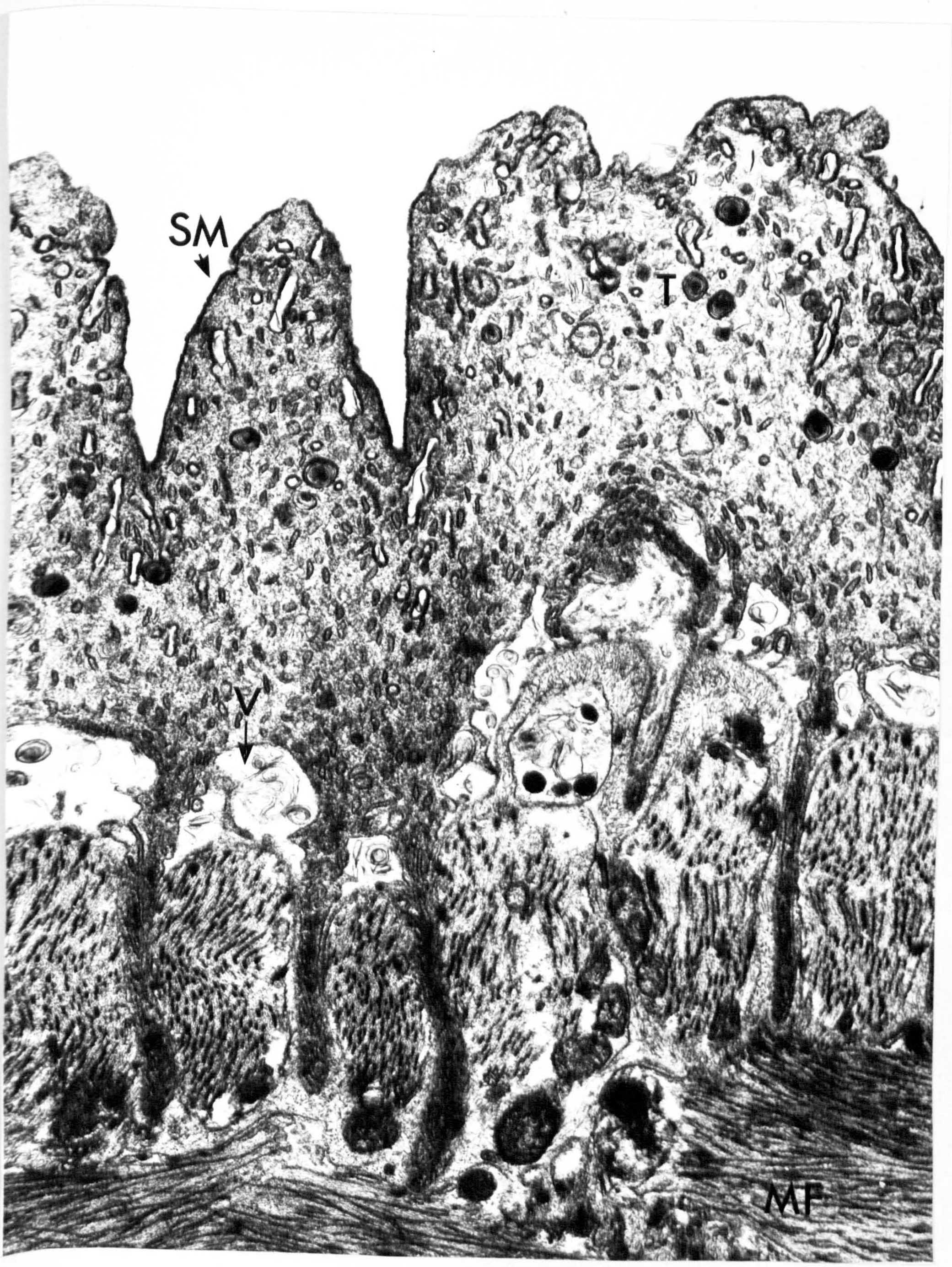

A
B
18
14
10
6
2
cpm(x10⁻³)
4
3
2
1
cpm(x10⁻³)
a b c d
a b c d

Figure 23 Transmission electron micrograph showing the dorsal tegument of the adult worm following 24 hours culture in EM + CS.

Adult worms were removed from infected mice, washed (x 3) in EMS and then cultured for 24 hours at 37°C in EM + CS. Following culture, worms were washed in EMS (x 3) and then prepared for electron microscopy.

T = tegument; SM = surface membrane ; V = vacuolation; MF = muscle fibres.

Magnification = 22,050


SM
T
V
MF

tegumental basement membrane. Although these two results indicate that *in vitro* culture has not disrupted the parasite outer membrane it is likely that it has changed its properties. This idea is supported by the findings that a) incubation of schistosomula in medium containing foetal calf serum leads to changes in surface membrane lipid composition (Rumjanek and McLaren, 1981) and b) incubation in medium containing human serum leads to changes in membrane lipid composition (Rumjanek and McLaren, 1981) and in addition surface antigen expression (Rumjanek, personal communication). It is thus tempting to speculate that similar changes may take place in adult worms subjected to culture. Certainly adult worms are able to absorb and utilise as membrane components, exogenous lipid present in culture media (Rumjanek and Simpson, 1981).

In order to investigate if parasite antigen expression and in addition host antigen expression at the adult worm surface can be altered by *in vitro* culture, worms incubated under a variety of conditions were subjected to the IRAM. The idea of culture media being able to alter host antigen expression has been suggested previously by Cesari and Polanco (1980).

4.7.2 The influence of various culture conditions on expression of host and parasite antigens

Figure 24 shows that overnight culture of worms in EM + CS or EM + NHP does not alter the expression of host antigens. Both culture conditions are, however, able to promote an increase in expression of parasite antigens, and to a similar

Fig. 24 Influence of various culture conditions on expression of host and parasite antigens at the adult schistosome surface.

Adult worms were removed from the mesenteric veins of infected mice, washed in EMS (x 3) and then incubated for 24h at 37°C in the appropriate culture medium. After washing in EM + CS (x 3) worms were then measured for antigen expression by the IRAM.

Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum or anti-schistosomulum serum. All culture media employed were found to have no effect on non-specific binding.

A. First antibody : anti-mouse RBC serum

B. First antibody : anti-schistosomulum serum

a : freshly perfused worms

b : culture in EM + CS

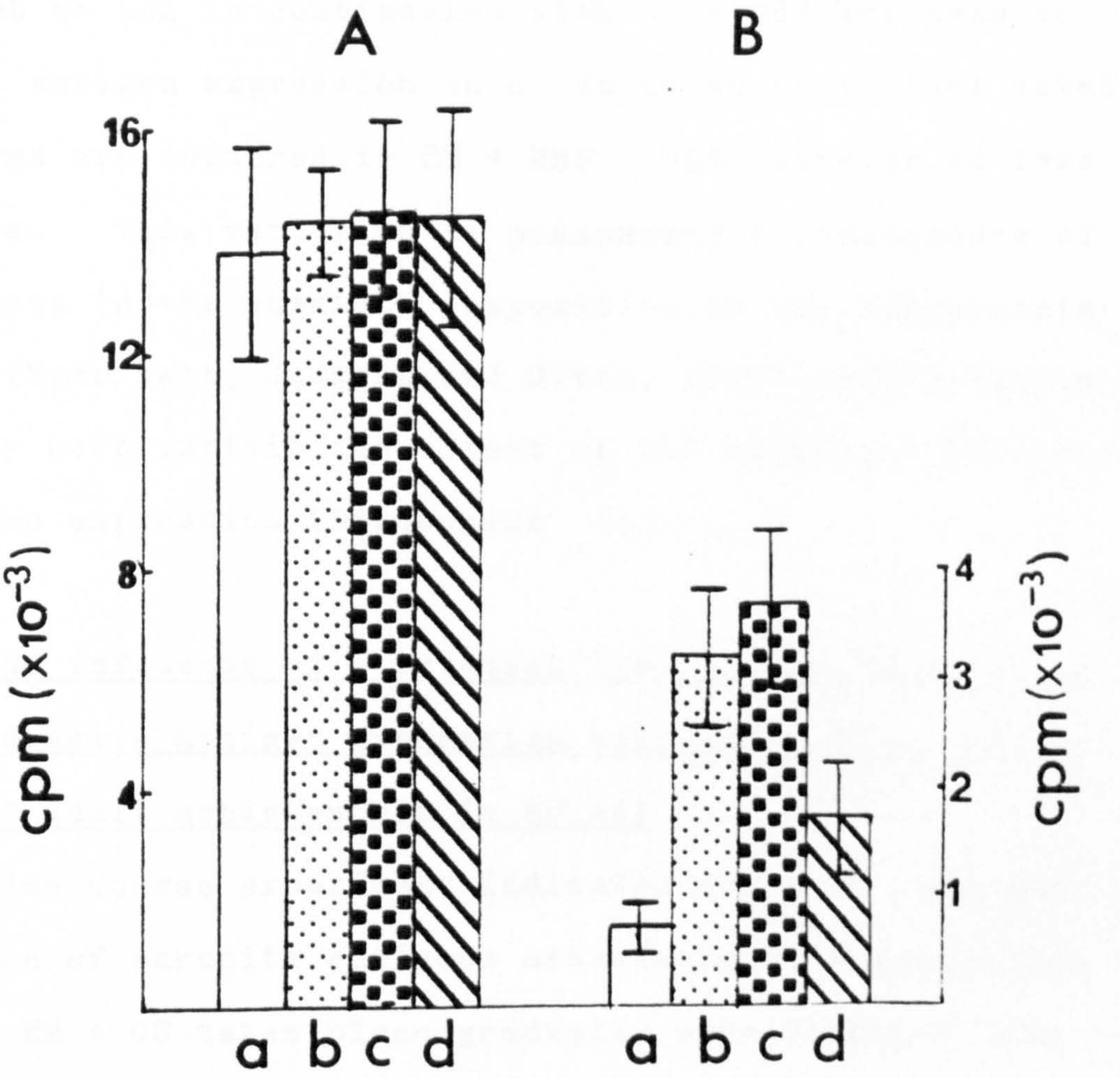
c : culture in EM + NHP

d : culture in EM + DHP

Statistical analysis

B : avb - $P < 0.025$; avc - $P < 0.0025$; avd - $P < 0.05$;

cvd - $P < 0.025$.


A
B
16
12
8
4
4
3
2
1
cpm (×10⁻³)
cpm (×10⁻³)
a b c d
a b c d

extent. A role for plasma lipoproteins in this effect is suggested by the reduction in the value for parasite antigen expression obtained when NHP is replaced by DHP in the culture medium. Confirmation of this hypothesis was obtained from two experiments in which purified lipoproteins were added to the medium containing DHP (Fig 25).

The increase in parasite antigen expression associated with addition of lipoproteins to the culture medium varies according to the class of lipoprotein employed (Fig 25). Thus, HDL or LDL in combination with EM + DHP are able to increase antigen expression to a value equal to that obtained when worms are cultured in EM + NHP : VLDL however is less effective. This variation is presumably a consequence of differences in the chemical composition of the lipoprotein classes (Morrisett, Jackson and Gotte, 1977) consideration of which may help explain the effect of the different lipoproteins on antigen expression (Discussion 6.3).

4.7.3 <u>The influence of incubation time on the increase in parasite antigen expression associated with incubation of adult schistosomes in EM +CS</u>

A time course experiment indicated that the increase in expression of parasite antigens associated with incubation of worms in EM + CS takes place gradually over 24 hours (Fig 26).

Fig. 25 Influence of different lipoprotein classes on expression of parasite antigens at the adult schistosome surface

Adult worms were recovered from the mesenteric veins of infected mice, washed in EMS (x 3) and then incubated for 24h at 37°C in the appropriate culture medium. After washing in EM + CS (x 3) worms were then measured for parasite antigen expression by the IRAM.

Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-schistosomulum serum. All culture media employed were found to have no effect on non-specific binding.

a: freshly perfused worms

b: culture in EM + CS

c: culture in EM + NHP

d: culture in EM + DHP

e: culture in EM + DHP + HDL

f: culture in EM + DHP + LDL

g: culture in EM + DHP + VLDL

Statistical analysis

Experiment one : avb - $P<0.01$; avc - $P<0.005$; avd - $P<0.05$;
dve - $P>0.05$; dvf - $P<0.05$; dvg - $P<0.025$;
evg - $P>0.05$; fvg - $P>0.05$

Experiment two : avb - $P<0.0005$; avc - $P<0.005$; avd - $P<0.025$;
dve - $P<0.01$; dvf - $P>0.05$; dvg - $P>0.05$
evg - $P>0.05$; fvg - $P>0.05$.

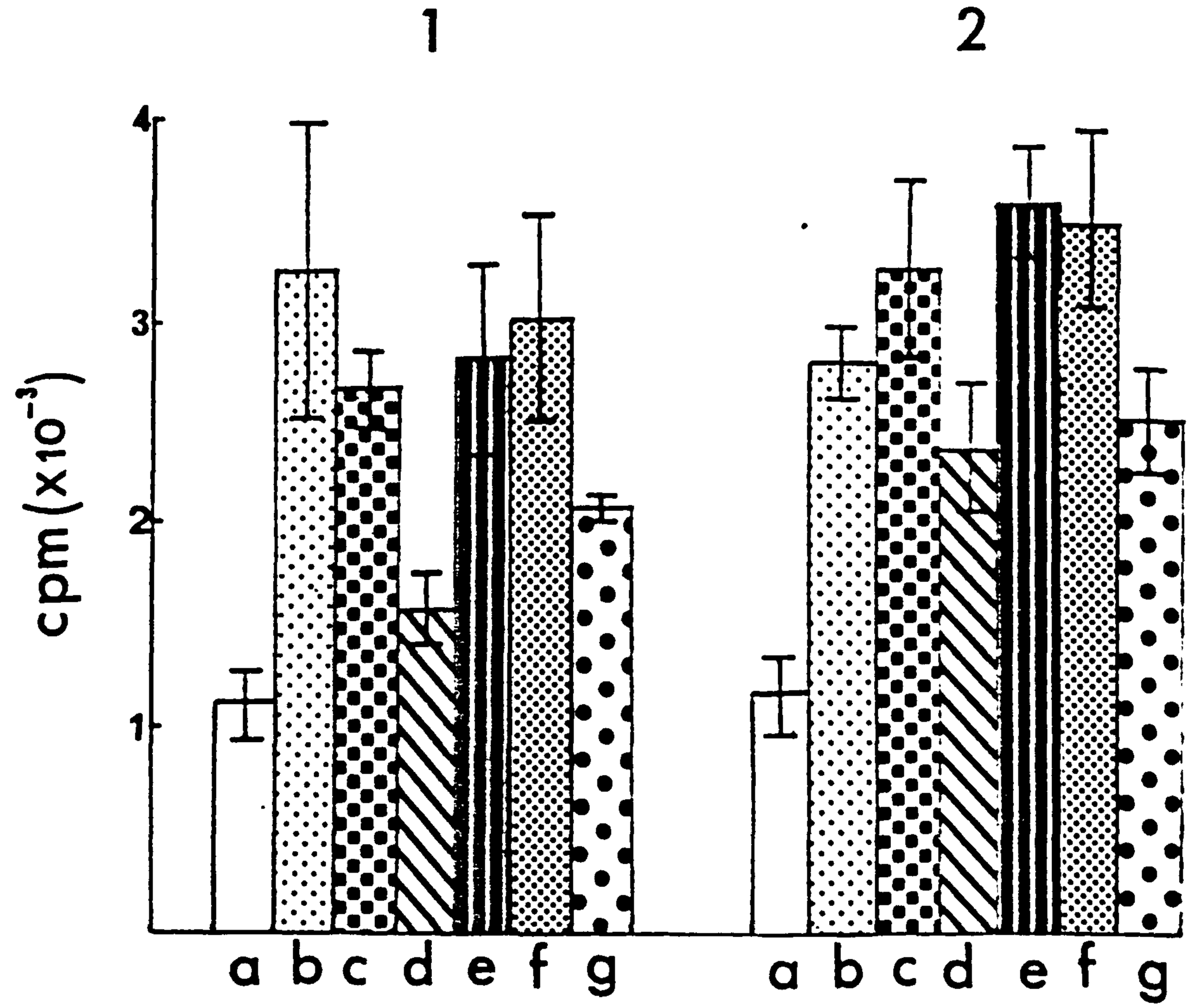
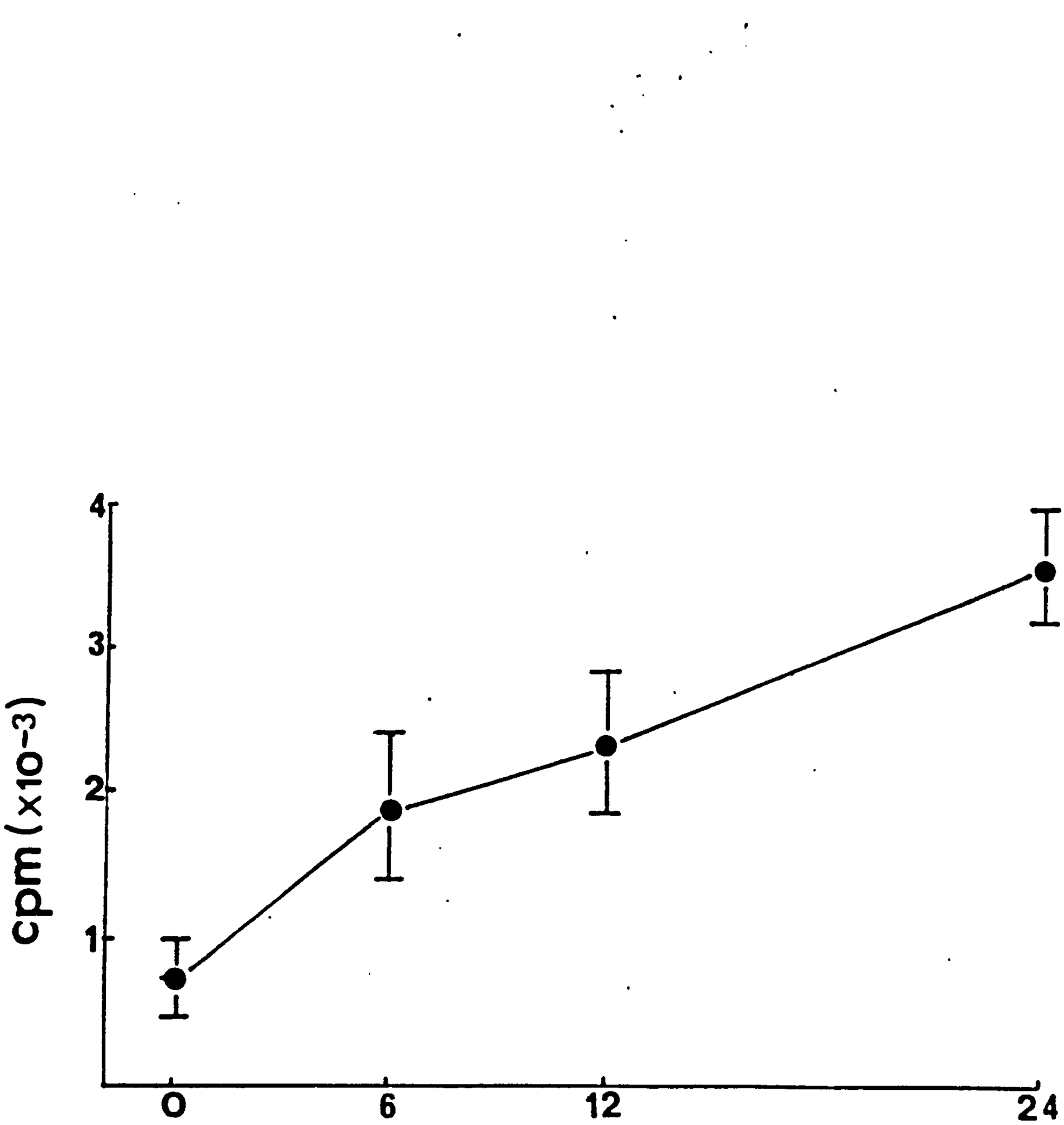

1
2
4
3
2
1
cpm (x10⁻³)
a b c d e f g
a b c d e f g

Fig. 26 Influence of incubation time on the ability of EM + CS to promote an increase in expression of parasite antigens

Adult worms were recovered from the mesenteric veins of infected mice, washed in EMS (x 3) and then incubated for various time intervals in EM + CS. After washing in EM + CS (x 3) worms were then measured for parasite antigen expression by the IRAM. The experiment was performed such that all worms were assayed simultaneously. Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-schistosomulum serum.


4
3
2
1
cpm (x10-3)
0
6
12
24
Incubation time (h.)

CHAPTER 5

The influence of praziquantel on surface antigen expression

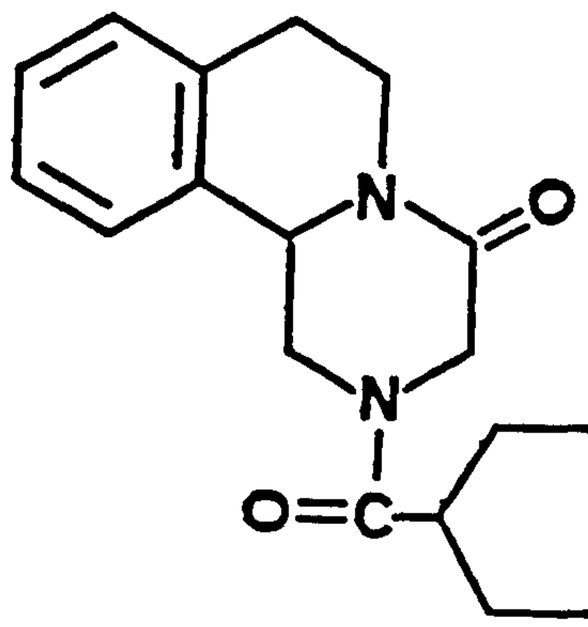
5. The influence of praziquantel on surface antigen expression

5.1 Introduction

Praziquantel (Fig. 27) is a recently developed broad-spectrum drug which has been shown to be effective against S. mansoni in mice, Mastomys, hamsters and Cebus monkeys (Gonnert and Andrews, 1977; Pellegrino et al, 1977; Webbe and James, 1977). It is thus hoped that praziquantel will provide a safe effective chemotherapeutic treatment for schistosomiasis in man. In this respect, the first clinical trial in Brazil has found the drug to be highly active but to have low toxicity and generally mild side effects (Katz et al., 1979).

The mechanism by which praziquantel is able to exert its effect on schistosomes is at present unknown. In vitro studies have indicated that praziquantel causes spastic paralysis of the male schistosome musculature and it has been suggested that this effect is due to an influx of Ca^{2+} into the worm (Pax et al, 1978; Coles, 1979). The reason for the change in permeability of the surface membrane to Ca^{2+} following drug treatment has yet to be elucidated. Praziquantel is not thought to act as an ionophore (Chubb et al, 1978; Coles, 1979). Fetterer et al (1980a) have concluded that since the drug also alters Na^{+} and K^{+} levels in the worm, it is possible that the increase in permeability to Ca^{2+} might be a non-specific effect which is independent of the mechanism which regulates Ca^{2+} transport.

Fig. 27 Structure of praziquantel



$C_{19}H_{24}N_2O_2$

Coles (1979) has suggested that praziquantel might alter membrane permeability to Ca^{2+}, by opening pores in the membrane through which Ca^{2+} can flow, either directly or as a consequence of an effect on Na^{+}. Fetterer *et al* (1980b) have suggested that the action of praziquantel on the movement of Na^{+} and K^{+} might be due to an effect on the parasite's Na^{+} K^{+} pump. Likewise, Terada *et al* (1982) have put forward the idea that praziquantel may have an effect on Na^{+} K^{+} ATPase activity in schistosomes. The schistosome Na^{+} K^{+} pump ATPase is located in the basal membrane of the parasite tegument (Podesta and McDiarmid, 1982).

Becker *et al* (1980) have suggested that the main factor which eventually leads to the death of schistosomes subjected to praziquantel is disruption of the parasite tegument. Morphological studies undertaken by these workers have demonstrated that praziquantel causes vacuolation and degeneration of the tegument, the magnitude of this damage being dependent on the exposure time. Extensive vacuolation and breakdown of organisation are evident after 60 min but the surface membrane appears unchanged, at least as measured by intensity of staining using Thierys PAS silver proteinate reaction for carbohydrates.

Treatment of schistosomes *in vivo* with amascanate (Voge and Bueding, 1980) or 1:7 bis (p-aminophenoxy) heptane (Standen, 1963) leads to binding of host leucocytes to the parasite surface. When considering the mechanism of action of praziquantel it is thus perhaps pertinent to ask the question as to whether the drug facilitates an immune response-

dependent rejection of the parasite. One way in which it might do this would be to increase the expression of parasite antigens. Such a mechanism has been put forward to explain the mode of action of other anti-helminth drugs, such as diethylcarbamazine (Gibson et al, 1976; Piessens and Beldekas, 1979) but as far as can be ascertained has not been attributed to praziquantel. Praziquantel may however expose previously hidden proteins at the surface of cestodes as treatment of animals infected with these parasites results in the worm being attacked by host proteases (Thomas and Andrews, 1977).

It was thus decided to investigate if praziquantel was able to expose additional parasite antigens at the schistosome surface. Worms were only exposed to the drug for 3-4 min thus probably ensuring that the surface membrane would not be shed during the course of the assay (Becker et al, 1980).

5.2 The influence of praziquantel on host and parasite antigen expression

Becker et al,(1980)employed praziquantel in the range from 1 to 100µg/ml and found the effect of the drug on schistosomes to be independent of concentration. An arbitrary drug concentration of 10µg/ml was thus selected for this investigation. Incubation of schistosomes in the presence of this concentration of praziquantel was found to lead to their almost immediate contraction and immobilization.

The results of an experiment in which the effect of praziquantel on surface antigen expression was measured is shown in Fig 28. Praziquantel increased parasite antigen expression

Fig. 28 Influence of praziquantel on expression of host and parasite antigens at the adult schistosome surface

Adult schistosomes were removed from the mesenteric veins of infected mice, washed in EMS (x 3) and then incubated in the presence of praziquantel (10 µg/ml EMS) for 2-3 min at 37°C. After washing in EM + CS (x 3) the worms were measured for antigen expression by the IRAM.

A. First antiserum : anti-mouse RBC serum

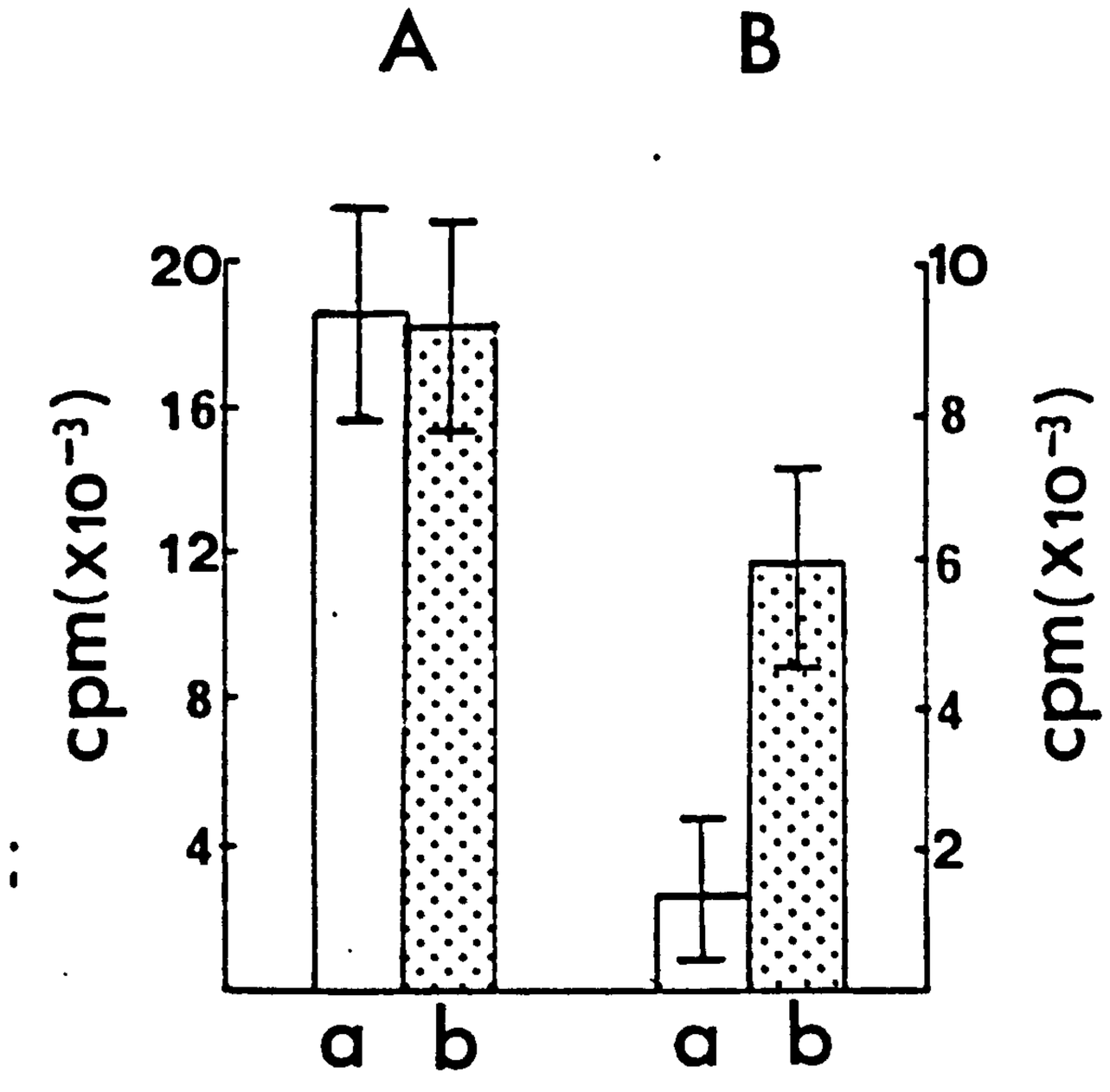
B. First antiserum : anti-schistosomulum serum

a: incubation in EMS

b: incubation in praziquantel/EMS

Specific antibody binding was determined by subtracting the results obtained when worms were incubated in NRS from the results obtained when worms were incubated in anti-mouse RBC serum or anti-schistosomulum serum. Praziquantel treatment did not alter non-specific binding.

Praziquantel was solubilised in ethanol before addition to EMS. The concentration of ethanol present in the final solution was 0.5% (v/v). Incubation of worms in 0.5% (v/v)ethanolin EMS does not alter antigen expression and results of this control experiment have therefore not been shown.


A
B
20
16
12
8
4
cpm(X10⁻³)
10
8
6
4
2
cpm(X10⁻³)
a
b
a
b

to give a value equal to 405% ($P<0.025$) of the control value but apparently had no effect on expression of host antigens. This pattern of results was repeated in two additional experiments in which the values for parasite antigen expression as compared to the controls were 472% ($P<0.05$) and 288% ($P>0.05$) respectively.

5.3 Influence of praziquantel on release of ^{51}Cr

Although praziquantel is able to alter the permeability of the schistosome surface membrane to Na^+, K^+ and Ca^{2+} (Pax et al, 1978) as shown in Table 23, it apparently has little effect on permeability to ^{51}Cr.

5.4 Influence of praziquantel on release of ^{125}I-WGA

The results of four experiments in which the influence of praziquantel on release of ^{125}I-WGA was measured is shown in Table 24. The massive increase in release of the label following praziquantel treatment, recorded in experiment one, suggested that a correlation might exist between ^{125}I-WGA release and expression of parasite antigens. This result could not be repeated however in subsequent experiments even when the time of exposure to the drug (experiment 3) or the time allowed for release of the label (experiment 4) was increased. Praziquantel treatment in fact lowered the release of ^{125}I-WGA in experiments 2, 3 and 4 but in no case was this statistically significant.

Table 23 . . The influence of praziquantel on the adult surface membrane as measured by ^{51}Cr release

Incubation medium	% ^{51}Cr release
EMS	2.33 ± 1.04
Praziquantel	3.01 ± 1.74

Worms were labelled with ^{51}Cr, washed in EMS and then incubated for 4 min in praziquantel (10µg/ml) of EMS. The release of the label during the incubation period was then measured.

The values expressed are the percentage of total counts in the incubation. Each sample was examined in triplicate.

Praziquantel was solubilised in ethanol before addition to EMS.

Table 24 The influence of praziquantel on the adult surface membrane as measured by ^{125}I-WGA release

Experiment Number	Incubation medium	% ^{125}I-WGA release
1	EMS	9.23 ± 2.52
"	praziquantel	54.90 ± 6.44*
2	EMS	7.75 ± 1.43
"	praziquantel	3.25 ± 2.79
3	EMS	7.79 ± 4.20
"	praziquantel	3.97 ± 3.65
4	EMS	25.52 ± 6.54
"	praziquantel	17.59 ± 3.32

Worms were labelled with ^{125}I-WGA, washed in EMS and then incubated in praziquantel or EMS the following time periods : experiments 1 and 2 - 4 min; experiments 3 and 4 - 15 min. The release of the label during the incubation period was then measured in experiments 1, 2, and 3. Release of the label in experiment 4 was measured after worms had been maintained in the presence of EM + CS for 1 hr following the praziquantel incubation.

The values expressed are the percentage of total counts in the incubation. Each sample was examined in triplicate.

Praziquantel was solubilised in ethanol before addition to EMS. Ethanol was thus present in the praziquantel/EMS solution at a concentration of 0.5%. Ethanol employed at this concentration has no effect on release of ^{125}I-WGA (Kusel et al, 1982).

* Value significantly different from control value ($P<0.0005$).

CHAPTER 6

DISCUSSION

6.1 The indirect radiolabelled antibody method

The development of the indirect radiolabelled antibody method enabled changes in antigen expression at the surface of the adult schistosome to be measured in a quantitative manner. The use of limiting amounts of antibody prevents the assay from being employed to measure the total number of molecules of antigen present at the worm surface but it can be used to measure the change in antigen expression as a percentage of the control value. Such information clearly provides a more accurate measurement of altered surface antigenicity than can be obtained by using subjective forms of measurement such as visual assessment of FITC-labelled antibody binding (Goldring et al, 1977b;Gitter et al, 1982).

6.2 The influence of reagents which interact with membrane protein on the expression of parasite antigens

When employed at certain concentrations, formalin (Fig.14), glutaraldehyde (Fig.15) and trypsin (Fig.18) are clearly able to increase the expression of parasite antigens at the surface of the adult worm. It is possible that these increases may arise as a consequence of :

(a) direct interaction with parasite membrane antigens resulting in exposure of new determinants;

(b) interaction with host proteins resulting in exposure of parasite epitopes;

(c) in the case of formalin and glutaraldehyde, interaction with the $-\ddot{N}H_2$ group of membrane lipids such as

phosphatidyl serine or phosphatidyl ethanolamine leading to changes in lipid-protein interactions (Komarowska et al, 1982) which in turn may result in altered antigenicity (Borochov and Shinitsky, 1976).

With respect to hypothesis (b), host proteins such as immunoglobulin and histocompatibility antigens have been detected at the surface of the adult schistosome by other workers (Kemp et al, 1977; 1978; Gitter et al, 1982) although their distribution as determined by the present investigation is not extensive (Tables 2 and 3). Nevertheless, it is possible that some parasite antigens may be masked by host proteins and may therefore be exposed when the host molecules are removed or have their conformations altered. Concerning hypothesis (c) Shiga et al. (1977) have reported that treatment of human erythrocytes with glutaraldehyde (≥ 0.05%) results in increased immobility of membrane lipids. Jost et al. (1973) however, have found that 2% glutaraldehyde does not alter the mobility of phospholipid components of lobster nerves or liposomes. In addition, Komarowski et al. (1982) has concluded that interaction of glutaraldehyde with the surface of horse RBC is predominately due to binding to membrane proteins. Glutaraldehyde would therefore appear to vary in its reactivity for the lipid phase of different membranes and this is presumably due to differences in membrane composition and organisation (Shiga et al, 1977). In attempting to predict whether or not glutaraldehyde will react with the lipid portion of the schistosome outer membrane, it is pertinent to note that Cesari and Marchiani (1978) have suggested that

phosphatidyl serine and phosphatidyl ethanolamine may be exposed at the parasite surface. However, the finding that trypsin has a similar effect to formalin and glutaraldehyde on antigen expression (see later) suggests that the aldehydes may mediate their effects through reactivity with membrane protein components. Thus, if binding of aldehydes to membrane phospholipid takes place it may have little or no effect on antigen expression.

In relation to hypothesis (a) it is likely that formaldehyde, glutaraldehyde and trypsin will each interact directly with parasite membrane antigens. The aldehydes will combine readily with exposed terminal-NH_2 groups and also with a number of amino acid side chains (French and Edsall, 1945; Habeeb and Hiramoto, 1968; Avraemas and Ternynck,1969); trypsin has been shown to cleave a number of antigens at the surface of the schistosomulum (Dissous et al, 1981; Taylor et al, 1981; Gazzinelli et al, 1982) and it is known that the immature and mature forms of the trematode share a similar membrane antigen composition (Kusel et al, 1975; Snary et al, 1980; Shah and Ramasamy, 1982). It is likely that interaction of membrane antigens with the aldehydes will cause conformational changes which may result in exposure of previously hidden antigenic determinants, and trypsin may act by cleaving regions of membrane proteins such that underlying antigenic sites are exposed, or become exposed as a consequence of a change in conformation of the remaining protein segments. Support for the idea that trypsin increases parasite antigen expression by direct interaction with parasite membrane antigens is given

by the finding that mechanically transformed schistosomula which have been exposed to trypsin are more sensitive than untreated controls to the action of lethal antibody plus complement (Gazzinelli et al, 1982).

The nature of the antigens which act as putative targets for trypsin and the two aldehydes have yet to be established. Several studies indicate that the majority of proteins which are exposed at the surface of the schistosomuhm are sensitive to the action of trypsin (Dissous et al, 1981; Taylor et al, 1981; Gazzinelli et al, 1982). A number of different antigens may therefore be cleaved by trypsin at the surface of the adult worm although it is also possible that certain membrane proteins may no longer be accessible to the enzyme as a consequence of the reduction in parasite antigen expression which takes place with maturation (Goldring et al, 1977b ; McLaren et al, 1978). In addition, there is some indication from the results obtained using transmission electron microscopy (Fig.16) that anti-parasite antibody may be able to bind to membrane protein at the surface of the inner bilayer due to loosening of regions of outer bilayer at the surface pits. This putative binding may be relatively slight however as a freeze fracture study suggests that the majority of membrane proteins are located in the outer leaflet of the outer bilayer (McLaren et al, 1978). Moreover, not all surface pits examined displayed signs of membrane loosening.

Glutaraldehyde and formaldehyde may have similar sites of action to trypsin on certain membrane proteins as both aldehydes

are able to interact with the ε-amino group of lysine (French and Edsall, 1945; Fraenkel-Conrat and Olcott, 1948; Habeeb and Hiramoto, 1968; Avraemas and Ternynck, 1969) and trypsin breaks peptide bonds to which lysine or arginine have donated a carboxyl group. In relation to this idea Collis and Ritzi (1980) have suggested that trypsin and formaldehyde may act in a similar manner in exposing viral antigens at the surface of mouse mammary tumour virus infected cells. Thus, although the aldehydes may combine with a number of different amino acid side chains (French and Edsall, 1945; Habeeb and Hiramoto, 1968;Avraemas and Ternynck, 1969), their ability to attack the ε-amino group of lysine may enable them to expose antigenic sites which are also exposed by trypsin. This idea could be tested by performing an IRAM using a number of monoclonal antibodies such that the expression of individual epitopes could be measured following treatment of worms with a particular reagent. It should be possible to test the hypothesis that all three reagents interact with the same molecules by :

(a) comparing the SDS-PAGE patterns of the surface membrane proteins before and after treatment with trypsin; and

(b) incubating the worms with radioactive aldehydes, isolating the surface (Kusel, 1972), and then detecting labelled proteins by SDS-PAGE in combination with autoradiography or fluorography.

The effect of trypsin on parasite antigen expression can be increased by raising the concentration of the enzyme to which the worm is exposed (Fig.18). Formaldehyde and glutar-

aldehyde conversely were each found to be most effective at a concentration of 0.1% (w/v) and 0.01% (w/v) respectively (Fig. 14and Fig.15). Increasing the concentration of aldehydes above these levels reduced the amount of parasite antigen which could be detected at the surface of the worm in both cases. One explanation for this result is that higher concentrations of the aldehydes, in addition to exposing antigenic sites, may also combine with some of them such that their reactivity for antibody is lost. In relation to this idea Boron et al, (1977) have shown that the antigenic reactivity of Ia antigens at the surface of lymphoid cells can be destroyed during glutaraldehyde fixation of cells if the concentration of the aldehyde employed is greater than 0.024% (w/v). In addition, the antigenicity of various proteins, including rabbit secretory IgA, α-chymotrypsinogen, trypsinogen,and DNase can be reduced by exposure to glutaraldehyde and the extent of this effect is proportional to the concentration of aldehyde employed (Kraehenbuhl and Jamieson, 1974). There is no proof however that these results are due to destruction of antigenic sites. An alternative hypothesis involves consideration of the fact that the ability of glutaraldehyde to cross-link proteins increases as its concentration is raised (Hopwood, 1969; Cheung and Nimmi, 1982). It could thus be argued that treatment of proteins with high concentrations of glutaraldehyde will lead to the formation of inter-molecular cross-links which may prevent antibody from coming into contact with certain antigenic sites (Hopwood, 1969). 1% (w/v) glutaraldehyde may therefore reduce parasite

antigen expression at the surface of the adult schistosome by virtue of the fact that it extensively cross-links membrane proteins. Conversely lower concentrations (0.1 and 0.01%) may induce the binding of more anti-parasite antibody because they are able to interact with, and expose antigenic sites on membrane proteins, but are less able to link the proteins together. According to this hypothesis a 0.01% concentration of glutaraldehyde might be considered to be more effective at increasing parasite antigen expression than a 0.1% concentration and this was found to be the case. Also, if this hypothesis is valid then the inability of 1% glutaraldehyde in contrast to 1% (w/v) and 10% formalin to increase parasite antigen expression can be attributed to the fact that glutaraldehyde is a more effective cross-linker of proteins than formaldehyde (Habeeb, 1969; Hopwood, 1969). The fact that 0.01% formalin is less able than 0.1% to increase expression of antigens may indicate the existence of a simple concentration effect.

A third hypothesis to explain the results obtained using different aldehyde concentrations involves consideration of the effect which changes in charge may have on surface antigenicity. Interaction of aldehydes with membrane proteins will cause the proteins to become more negatively charged. Changes in charge are likely to cause alterations in conformation which may, in turn, lead to changes in surface antigenicity. 0.1% formalin and 0.01% glutaraldehyde may therefore be the concentrations of the respective aldehydes which are most effective at increasing parasite antigen expression because of the

effect which they have on the charge of the antigens. Higher concentrations of either aldehyde will clearly have a greater effect on charge but this may result in conformational changes which expose lower levels of parasite antigens.

Kraehenbuhl and Jamieson (1974) have reported that treatment of pancreatic exocrine cells with 4% formaldehyde enables antibody molecules to diffuse through the plasma membrane, but this effect is not observed when 0.5% glutaraldehyde is employed. The authors concluded that glutaraldehyde fixed cells were unable to take up antibody as a consequence of the superior cross-linking properties of this fixative enabling it to form an impenetrable barrier at the cell surface. It is therefore possible that schistosomes subjected to fixation with formalin and low concentrations of glutaraldehyde, may take up antibody into the tegument. This hypothesis may explain the finding that the concentrationsof formalin and glutaraldehyde which are most effective at increasing parasite antigen expression are also the most effective at increasing non-specific binding of labelled antibody (Fig. 14and15). In addition, increasing the concentration of formalin or glutaraldehyde above the levels at which they are most effective at increasing parasite antigen expression results in a concomitant decrease in release of ^{51}Cr from the tegument of labelled worms (Table 17). In spite of these results however the following findings and observations suggest that the parasite antigens being detected following exposure of worms to the aldehydes are membrane components :

(a) the detected increases in parasite antigen expression appear to be accompanied by a related decrease in host antigen expression suggesting that the two species may be in association or at least in close proximity to each other (Fig.14 and 15);

(b) a relationship does not exist between the extent of parasite antigen expression and membrane permeability as measured by ^{51}Cr release (see section 4.3.3);

(c) formalin and glutaraldehyde may expose the same antigenic determinants which are unmasked by trypsin and the proteolytic enzyme does not cause the schistosome to release ^{51}Cr (Table 18);

(d) the anti-parasite antibody binding to formalin fixed worms observed by Gitter et al. (1982) is clearly surface located as fluorescent Staphylococcus aureus was employed as the indicator system;

(e) Murrell et al. (1978a) have reported that fixation with 1% formalin will prevent the binding of soya bean agglutinin and Ulex europaeus agglutinin I, to internal components of dead or damaged schistosomula. In this situation therefore, treatment of parasites with aldehyde inhibits internal entry of antibody.

It should be stated that the relevance of the latter result to the effects of formalin fixation on the adult worm is uncertain as the parasite surface membrane is known to undergo a number of changes as the worm matures (1.4). Nevertheless, it is possible that the permeability of both membranes may be affected to a similar extent following formalin fixation.

It should be possible to determine if parasite antigens detected following treatment of worms with formalin or glutaraldehyde are located at the schistosome surface by using fluorescent Staph. aureus as an indicator system as demonstrated by Gitter et al., (1982).

In addition, by altering the nature of the label from FITC to ^{125}I, the technique can readily be made quantitative. Although this technique cannot determine whether antibody has penetrated the membrane, it can provide an indication of the extent of any change in antigen expression at the parasite surface. The possibility that antibody may have penetrated the membrane can be determined by establishing if formalin or glutaraldehyde fixed worms are able to bind FITC-labelled anti-actin antibodies (Deppert et al, 1980).

Solutions of formalin and glutaraldehyde which were employed in the investigation were diluted in PBS pH 7.2. It was thus decided to determine if exposure of worms to this solution produced any change in surface antigen expression. The results indicated that parasite antigen expression was in general increased following incubation but that the increase was usually slight (4.3.1.2). This result is of interest as Simpson et al. (1981) have demonstrated that incubation of adult worms in PBS, pH 7.4 leads to shedding of substantial amounts of the parasite tegument. This is shown by the finding that 37% of ^{125}I-WGA which had been bound to the schistosome surface was released within 5 min of exposure to the saline. This result was not obtained in this investigation however, as incubation of ^{125}I-WGA labelled worms in PBS, pH 7.2 for 15 min resulted in a release of label equal to 5% of the total (result not shown). A similar release was observed when worms were incubated in EMS (result not shown). In addition, incubation of worms in PBS does not affect host antigen expression. A similar result is suggested by the finding that mouse worms subjected to prolonged incubation in Hanks balanced salts solution are able to survive in normal monkeys but succumb to immunity in monkeys which have been immunized against mouse RBC (Clegg et al., 1970).

No evidence therefore exists to suggest that tegumental outer membrane components are being shed following incubation of worms in saline in this particular investigation. A similar conclusion was arrived at by Shah and Ramasamy (1982) following transmission electron microscopic examination of worms exposed to PBS, pH7.2. The reason for the difference in results obtained by Simpson et al. (1981) is uncertain. One possibility is that the result is due to genetic differences between the parasites employed in the various experiments. An alternative explanation is that the effect observed by Simpson and co-workers (1981) is pH dependent as these workers employed PBS, pH 7.4, whereas the work of Shah and Ramasamy (1982) and the experiments described in this thesis used PBS, pH 7.2. A preliminary experiment has indicated however that the ability of PBS to increase parasite antigen expression is not influenced by the pH of the saline, at least in the range from pH 6-8.

Although there is no evidence in this investigation that surface membrane components are being shed following incubation of worms in PBS, there is some indication of a permeability change as measured by ^{51}Cr release (Table 17). Some leakage of tegumental components may therefore take place. This permeability change may also help explain the small increase in binding of anti-parasite antibody which takes place as the more permeable nature of the membrane may allow better access of anti-body to membrane antigens.

6.3 The influence of (a) reagents which interact with the membrane lipid phase or (b) various culture media on expression of parasite antigens

Incubation of adult schistosomes for 15 min in EMS containing retinol at a concentration of lmg/ml did not increase the expression of parasite antigens at the worm surface (Fig. 20). This result is not a consequence of an inability of retinol to penetrate the membrane as the reagent can be detected in the membrane systems of the worm following incubation, by fluorescence microscopy (Kusel et al., 1982). It must be concluded therefore that although retinol is likely to alter membrane lipid organisation (Dingle and Lucy, 1965) this does not result in rearrangement of membrane proteins such that they are accessible to antibody. Studies on the RBC have shown that in addition to interacting with membrane lipids retinol is also able to bind to membrane proteins (Dingle and Lucy, 1965). Thus some of the retinol which enters the schistosome membrane may associate with protein antigens. If such interactions do take place however, it must be concluded that as with lipid-retinol interactions, they do not result in increased antigen expression.

Like retinol, Tween 40 and Tween 80 possess lipophilic properties and it has been shown that both detergents remove ^{3}H-cholesterol and ^{14}C-palmitate from the surface membrane (unpublished observations). Results obtained in this investigation indicate that Tween 40 and Tween 80 do not increase the expression of parasite antigens at the worm surface (Fig. 22). It was concluded therefore that cholesterol and phospholipid can be removed from the parasite surface membrane without exposing parasite antigens. However, the amount of lipid removed from the membrane is only in the region of 1% and it is possible that any increase in membrane parasite antigen expression induced by

this loss would not be detected by the IRAM.

Parasite antigen expression can be increased if worms are exposed to Tween 20 (Fig. 22). This detergent is able to extract similar levels of lipid from the parasite surface as are removed following incubation of worms with Tween 40 or Tween 80 (unpublished observations) but appears to be much more disruptive as measured by $^{51}Cr/^{125}I$-WGA release (Table 22). It could be argued therefore that in order to increase expression of parasite antigens, lipophilic reagents must promote a certain, perhaps crucial, amount of disruption to the lipid phase of the surface membrane. Tween 40, Tween 80 and in addition retinol (Table 21) may not increase antigen expression because they are unable to attain this level. Since the only difference between Tween 20 and the other two Tween detergents is in the nature of the fatty acid moiety (Fig. 21), it must be concluded that this part of the molecule is responsible for the disruptive effects of Tween 20.

Since schistosomes are dependent upon their host for provision of fatty acids and cholesterol (Meyer et al., 1970) it must be assumed that the composition of the lipid phase of the parasite surface membrane is sensitive to changes in the lipid composition of the worms environment. This is clearly demonstrated in the case of the schistosomulum by the finding that incubation of worms in mammalian serum leads to changes in surface membrane lipid composition, the nature of which depend on the species of mammal from which blood was obtained (Rumjanek and McLaren, 1981). It was thus considered that incubation of adult worms in mammalian serum was likely to result in changes in lipid composition and it was therefore decided to determine if cultured worms displayed an altered surface antigenicity. Worms were therefore exposed to either 10% (v/v) newborn calf serum/EMS or normal human

plasma/EMS for 24h at 37°C whereupon it was found that the expression of parasite antigens had increased. That the increased expression of parasite antigens is due,at least in part, to the lipid fraction of plasma, is shown by the reduction in parasite antigen expression associated with culture of worms in delipidated rather than whole plasma (Fig.24). In addition, it was shown that addition of lipoproteins (the vehicle employed by mammals to transport lipid in the bloodstream) to delipidated plasma raised its ability to promote increased parasite antigen expression (Fig.25). When high density lipoproteins (HDL) and low density lipoproteins (LDL) were employed, parasite antigen expression was raised to a level equal to that obtained when worms are cultured in whole plasma; very low density lipoproteins (VLDL) conversely were found to be less effective. Although not statistically significant(at $P < 0.05$) this difference was found to be reproducible (Fig.25).

The differences in ability to increase parasite antigen expression demonstrated by the lipoproteins are of interest as they may indicate the mechanism behind the effect of serum or plasma on antigen expression. The major lipid component of VLDL is triglyceride whereas HDL and LDL carry a greater proportion of cholesterol and phospholipid (Jackson et al, 1976). It is tempting to speculate therefore that HDL and LDL are more effective at increasing antigen expression as a consequence of containing more cholesterol and/or phospholipid than VLDL. HDL and LDL may transfer lipid to, or remove lipid from, cells (Jackson et al., 1976). The results obtained when parasite antigen expression was measured following treatment of worms with Tween 40 or Tween 80 suggest that cholesterol and phospholipid can be removed from the schistosome membrane without exposing parasite antigens. HDL and

LDL may therefore increase parasite antigen expression by increasing the concentration of cholesterol and/or phospholipid in the membrane. In this respect, Borochov and Shinitzky (1976) have shown that increasing the cholesterol content of the RBC leads to a greater exposure of proteins at the cell surface.

In order for this hypothesis to be valid it is necessary to show that lipoproteins are able to interact with the schistosome surface. Preliminary studies have indicated that binding does take place but there is some conflict as to the conditions required. Thus Rumjanek (personal communication) has demonstrated that purified human LDL will bind to the surface of mechanically transformed schistosomula but this is dependent on worms being pre-incubated in the presence of human serum. Conversely Campbell and Kusel (unpublished observations) have obtained results which suggest that binding may be inhibited by delipidated plasma. Clearly further work is required to resolve this particular problem but it appears likely that interaction with host lipoproteins may constitute the mechanism by which the schistosome acquires lipid molecules. Attempts by Rumjanek (personal communication) to demonstrate albumin mediated lipid exchange have proved unsuccessful.

The finding that incubation of worms in delipidated plasma results in an increase in parasite antigen expression suggests that either :
(a) some non-lipid component of plasma can alter antigenicity;
or (b) that the effect is due to some lipid component which was not removed during the delipidation process (fatty acid bound to albumin or perhaps lipid-containing cell membrane debris ?).

The idea of albumin transferring fatty acids to schistosomes is inconsistent with the results of Rumjanek (personal communication)

mentioned earlier although Rumjanek's work employed schistosomula and the present work involved the use of adult worms. It may be that the schistosome develops the ability to acquire fatty acid from albumin as the properties of its surface membrane alter (McLaren, 1980) but this has yet to be investigated.

It may be possible to test hypothesis (a) by incubating worms overnight in serum-free medium. Although there is evidence to show that incubation of worms in such media can result in tegument damage (Simpson et al., 1981) a preliminary experiment performed during this investigation has shown that overnight incubation of worms in EMS does not reduce host antigen expression. Nevertheless, EMS may alter the permeability of the membrane in a manner similar to PBS, pH 7.2 (Table 17).It is possible that EMS + delipidated plasma may also produce this effect and this may in turn alter surface antigenicity. This suggestion therefore constitutes a third hypothesis to explain the increase in parasite antigen expression induced by delipidated plasma.

The nature of the antigen or antigens exposed at the worm surface following culture have yet to be determined. Lipoproteins may promote the expression of a different type of antigen from delipidated plasma so it is possible that incubation of worms in whole plasma may result in increased expression of more than one antigen. Whether or not this is true, it is possible that the increase in parasite antigen expression induced by serum or plasma involves a different set of molecules from the antigens exposed by formalin, glutaraldehyde and trypsin, as unlike these reagents, serum or plasma did not cause a decrease in host antigen expression. Work by Rumjanek (personal communication) has shown that incubation of mechanically transformed schistosomula in the presence of human serum results in the exposure of two parasite antigens

each having a molecular weight of approx. 45,000. The expression of these proteins is dependent on a dialysable serum factor and cannot be induced by purified lipoprotein (LDL). It is therefore possible that the increased parasite antigen expression associated with incubating worms in delipidated human plasma is due to such a factor and the antigens involved may therefore have a molecular weight of approx. 45,000. Examination of the protein composition of the iodinated isolated membrane fraction reveals the presence of two polypeptides of molecular weight 43,000 and 45,000 (Fig. 5) both of which are antigenic (Fig. 6). It should be mentioned however that Rumjanek was only able to detect the parasite proteins when human serum was employed whereas the results obtained in this investigation show that parasite antigen expression can be increased if worms are incubated in newborn calf serum. Although Rumjanek did not employ this particular serum in his work, he did investigate foetal calf serum and five mammalian sera, other than human. In addition, in Rumjanek's study, parasite antigen was detected following a 30 minute incubation period. The increased expression of parasite antigen detected in this study conversely, was not evident unless longer incubation times were employed (Fig.13 and Fig.26). This difference in results may however represent a difference in sensitivity between the surface labelling technique employed by Rumjanek, and the IRAM used in the present study.

The finding that schistosomes subjected to culture have an altered surface antigenicity has important implications in relation to the use of such worms for experimental purposes. Clearly, cultured worms do not possess an entirely normal surface membrane and therefore it is possible that they may act abnormally in experiments in which aspects of

the parasite surface are under investigation. If such experiments are undertaken using cultured worms, it must be concluded that the results should be treated with caution.

6.4 Nature of the RBC antigen at the schistosome surface and the relationship between RBC and parasite antigens.

In comparison to other host molecules located at the schistosome surface membrane antigens sharing specificities with the RBC were found to be evenly and extensively distributed over the entire surface of the parasite (Table 2). Strong binding of anti-RBC antibody has been demonstrated previously by other works (for example, Goldring et al., 1977b). The relatively low binding of anti-immunoblobulin antibodies is of interest in that it could not be increased by incubating worms at 4°C (Table 3). This result is different from the findings of Kemp et al. (1980) who showed that binding of antibody to the worm surface at 37°C induces shedding of antigen-antibody complexes but that this can be inhibited if the experiment is performed at 4°C. It is possible that in the present investigation shedding was taking place but at a much slower rate (shedding had taken place within 20 min when worms were incubated at 37°C in Kemp's work). This idea would explain the variability observed when expression of immunoglobulin was being measured but it is also possible that individual worms may simply vary in the amount of immunoglobulin expressed at their surfaces. The presence or absence of shedding could be investigated by fixing worms using, for example, formalin or glutaraldehyde, but the results of the present investigation have shown that such a step alters the surface antigenicity of the worm. Also of interest is the finding that low temperature may alter surface antigen expression as witnessed by the

reduction in RBC antigen expression following incubation of worms at $4^{o}C$ (Table 3). It has been demonstrated by Fetterer et al. (1981) that incubation of adult schistosomes in medium at low temperature results in an increase in muscle tension and also an increase in Na^{+} concentration and a decrease in K^{+} concentration within the worm. In addition, Kusel et al. (1982) have shown that adult schistosomes are less able to take up retinol from retinol/Tween solutions if the incubation medium temperature is lowered to $4^{o}C$. These results clearly demonstrate that the surface membrane of worms incubated at $4^{o}C$ possesses different properties from that of worms incubated at $37^{o}C$. This should therefore be taken into account when planning studies on antigen expression in which the use of low temperature is envisaged.

Previous attempts to detect host proteins in the schistosome membrane by precipitation techniques employing antibody to host serum antigens have been unsuccessful (Hayunga et al., 1979b; Shah and Ramasamy, 1982). In addition, the finding by Snary et al. (1980) that very little protein can be detected at the adult schistosome surface by lactoperoxidase catalysed iodination led these authors to conclude that most adsorbed host antigen is not protein in nature. This observation is consistent with the findings of the present investigation in that host immunoglobulin and histocompatibility antigen are sparsely distributed at the worm surface. An attempt to unequivocally demonstrate the presence of a RBC protein antigen in the present work was also found to be unsuccessful. In two out of four experiments, a polypeptide having a molecular weight of 11,000 was precipitated using

rabbit anti-mouse RBC serum. However in the first of these experiments a polypeptide of identical molecular weight was also precipitated using normal rabbit serum, although the quantity of antigen detected was lower. In addition a polypeptide of lower molecular weight was exclusively precipitated using normal rabbit serum in the second experiment. It is difficult to draw any conclusions from these experiments but they clearly cannot be taken as evidence of the existence of host RBC proteins in the adult surface membrane. It is of interest however that a schistosome proteolipid with a molecular weight of 11,000 has recently been demonstrated by Rumjanek and Curiel (1983). This molecule is considered to be a surface membrane component, of parasite origin, but its functional significance has yet to be established. Although there is no evidence to support this idea one possible role might be to act as a receptor for host glycolipid. The 11,000 molecular weight component precipitated by anti-mouse RBC antibody in this investigation may therefore represent a molecule which is reversibly attached to RBC glycolipid. The variation associated with the results of the coprecipitation assay may thus be a consequence of the samples employed for coprecipitation differing in the extent of binding between the two molecules. The reason for the existence of such variation is not readily apparent however.

The finding that anti-mouse RBC antibody was able to adhere to worms grown in hamsters was unexpected as Torpier et al (1979b) have shown that incubation of parasites with anti-mouse serum leads to changes in the IMP density of the surface

membrane of mouse, but not hamster worms. This result suggests that the antigen being recognised in the present study may be a RBC component which is not present in serum and which is shared between mice and hamster. Evidence in support of schistosomes acquiring a purely cellular antigen is given by the finding that a proportion of mouse worms (19-56 days in age) transferred to the mesenteric veins of hamsters preimmunized against mouse RBC are rejected, whereas worms transferred to animals immunized with mouse serum show normal survival levels (Cioli, 1976). This result however suggests that the mouse and the hamster worm may acquire a different RBC antigen from their respective hosts although it has been suggested by Cioli (1976) that the finding that the immunized hamster, in contrast to the immunized rhesus monkey (Smithers et al., 1969) does not reject all mouse worms, may indicate the existence of a partial homology between the two antigens. In addition, although other workers have reported that transfer of mouse worms to hamsters immunized against mouse RBC results in parasite destruction (Cioli and Neis, 1972; Boyer et al., 1977), it has been demonstrated by Boyer and Ketchum (1976) that hamster worms transferred to mice immunized with hamster RBC show a normal survival rate.

A further indication as to the nature of the RBC antigen is given by the finding that unlike mouse RBC, hamster RBC are not agglutinated by anti-mouse RBC serum and in addition do not appear to adsorb the antibody activity which binds to the parasite surface (preliminary observation).

Cioli (1976) has reported that significant levels of mouse worm rejection are obtained in immunized hamsters with relatively low titres of anti-mouse RBC agglutinating anti-bodies in their circulation. On the strength of these results it was suggested that the antibodies involved in worm rejection and the antibodies involved in haemagglutination may constitute two molecules of different specificity. This idea is certainly in agreement with the observations of the present investigation. The inability of hamster RBC to adsorb the antibody molecules which bind to the surface of the adult worm suggests that the target for these antibodies is either not exposed or not accessible at the cell surface. How then does one explain the observation that mouse RBC are able to adsorb this antibody ? One possible explanation concerns the ability of the anti-mouse RBC serum to lyse mouse RBC but not hamster cells. During the adsorption experiments sera were not decomplemented and lysis of mouse RBC was clearly demonstrated by release of haemaglobin. Lysis of the cells may enable the antibody activity which binds to the adult schistosome to be adsorbed either as a consequence of exposure, or increased accessibility, of membrane antigen. This theory could be tested by adsorbing using decomplemented serum. An alternative possibility relates to the observation that hamster RBC show little tendency as compared to mouse RBC to agglutination by acid phospholipids of the schistosome membrane (Cesari, 1976; Cesari and Marchiani, 1978). It has been suggested by

Cesari and Polanco (1980) that acid phospholipids may enable schistosomes to acquire host proteins by a mechanism dependent on interaction between the negatively charged phospholipid molecule and the ε-amino group of lysine molecules. If the host antigen being recognised in this investigation is the same RBC component to which acid phospholipids bind then it would be expected that hamster RBC would have little affinity for the anti-host antigen antibody. It is also worth commenting at this point that a lysine - phospholipid interaction would be sensitive to the action of glutaraldehyde, formalin and trypsin, reagents which have been shown to alter expression of host antigens (Fig. 14, Fig.15 , and Table 20).

If this latter hypothesis is valid then the RBC antigen detected at the worm surface must be, at least in part, protein, in nature. No evidence in support of this idea has been obtained from coprecipitation experiments in this or other investigations (Hayunga et al., 1979b; Shah and Ramasamy, 1982). It appears likely therefore that the ability of formalin, glutaraldehyde and trypsin to alter host antigen expression may be a consequence of their interaction with parasite membrane antigens. If this hypothesis is valid then it is likely that RBC antigens and parasite antigens are present in the schistosome surface membrane in close association. This situation is of course entirely consistent with the commonly discussed idea that host antigens may act to mask or disguise membrane parasite antigens.

Additional support for the idea that host and parasite antigens are in association is provided by the findings that:

(a) the concentrations of formalin and glutaraldehyde which were most effective at increasing parasite antigen expression were most effective at decreasing host antigen expression;

(b) the effect of trypsin on host antigen expression was only observed when employing concentrations which were able to increase parasite antigen expression (results not shown).

These observations do not of course provide conclusive evidence and it might be argued that host and parasite antigens are both subject to the greatest change in expression by a distinct concentration of each of the three reagents because these particular concentrations are most able to disrupt the surface membrane. This idea is supported by the membrane permeability levels (for ^{51}Cr) of glutaraldehyde fixed worms but does not correlate with the results obtained when using formalin fixed worms (Table 17). In addition, PBS, pH 7.2 has a similar effect to 0.1% glutaraldehyde on membrane permeability and yet unlike the aldehyde has little effect on antigen expression. Also, and perhaps most importantly, trypsin does not disrupt the parasite surface membrane as measured by ^{51}Cr-release (Table 18) and yet has a similar effect to the two aldehydes on parasite antigen expression and probably also on host antigen expression. It appears likely therefore that gross membrane disruption does not account for the observed changes in antigen expression and that the two antigen species are likely to be

in association or at least in close proximity to each other within the membrane.

An approach to determining the number of host molecules in possible association with parasite antigens is to compare the total number of both molecules which can be detected before and after treatment of worms. This unfortunately is not possible in the case of RBC antigens as the antiserum lacked sufficient activity to saturate all binding sites (Table 10). Nevertheless, it is possible to get a crude estimate of the minimum number of sites. This is done as follows : the amount of second antibody required to saturate all first antibody bound when first antiserum is employed neat was found by Scratchard analysis to be approx. 150µg/four worms (legend to Table 10). The amount of this antibody specifically bound by the worms is equal to approx. 0.7%. It can thus be calculated that each worm possesses at least 10^{12} RBC antigens at its surface. Using the same method of calculation, a preliminary experiment indicates that there may be approx. 1.2×10^{9} parasite antigen molecules exposed at the surface of the normal worm. These calculations make a number of assumptions including :

(i) that all second antibody molecules possess antigen binding activity ;

(ii) that the binding of one molecule of second antibody represents the expression of one antigenic site ;

(iii) that each antigenic site represents one antigen ;

(iv) and that there is no steric hindrance which inhibits antibody binding at the worm surface.

If the effect of 0.01% glutaraldehyde on antigen expression is now examined (Fig. 15) it can be seen that the increased expression of approx. 6×10^9 parasite antigens is accompanied by a loss of at least 4.5×10^{11} host antigen molecules. Thus, although the calculated figures for antigen expression at the surface of the normal worm represent a crude estimate and the value for parasite antigen expression requires confirmation it appears that something in the region of at least 100RBC antigens are lost for every parasite antigen whose expression is increased when worms are subjected to fixation with 0.01% glutaraldehyde. The RBC antigen being studied in this investigation may be an identical or similar molecule to the Forssman-like antigen at the surface of mouse worms described by Dean and Sell (1972). In support of this idea is the finding that the RBC antigen located at the mouse-worm surface in the present investigation is also found in the hamster, a species reported to be Forssman-positive (Buchbinder, 1935). The Forssman antigen is known to exist in tissues as a glycolipid-protein complex (Raffel, 1961) and this supports the idea that RBC antigens may exist in some form of association with certain proteins in the adult surface membrane. If this is true, then the high RBC antigen: membrane protein ratio suggested by the findings discussed above may indicate that either :

(a) each membrane protein antigen associates with a large number of RBC antigens ;

or (b) that not all RBC antigens mask epitopes of parasite proteins.

Alternatively, it is possible that all glycolipid molecules may simply insert themselves into the lipid bilayer as apposed to forming more intimate associations with protein molecules. Molecules bound to the surface membrane in this way may also mask antigenic sites if they are in close proximity to them (Clegg,.1972) and may also therefore be displaced by reagents which interact with membrane proteins.

The inability of the IRAM to quantitate changes in host antigen expression following treatment of adult schistosomes with trypsin was assumed to indicate the existence of a change in RBC antigen expression concomitant with a change in some aspect of the association between the antigen and its specific antibody. Trypsin treatment may alter the distribution and accessibility of the RBC antigen such that its affinity for first antibody is altered. This in turn may lead to changes in the affinity of the second antibody for the first. The effect observed when dealing with trypsin treated worms was not encountered when dealing with worms subjected to measurement of host antigen expression following fixation with formalin or glutaraldehyde. In addition no indication of differences in antibody affinity was obtained when dealing with measurement of parasite antigens.

6.5 Validity of the host antigen hypothesis

The host antigen hypothesis states that adult schistosomes are able to survive in the bloodstream of a susceptible mammalian host because surface antigens which act as targets

for antibody-dependent immunity are masked or disguised by host molecules (Smithers et al., 1969). In attempting to determine the validity of this hypothesis it is necessary to establish :

(a) whether RBC antigens and parasite antigens are in some form of association, or in close proximity to each other, within the membrane ;

(b) whether a reduction in expression of host antigens at the schistosome surface is accompanied by a related increase in expression of parasite antigens;

(c) whether exposure of parasite antigens at the surface of the worm would lead to antibody-dependent destruction of the worm in vivo.

As regards (a), although direct proof is lacking, a considerable amount of circumstantial evidence, discussed in detail in section 6.4, suggests that the two antigens may associate or be adjacent to each other within the membrane. Moving on to (b), the results of the present investigation show that the concentration of formalin or glutaraldehyde which is most effective at increasing parasite antigen expression is most effective at reducing host antigen expression. This result is therefore consistent with the idea that the amount of parasite antigen which can be detected at the surface of the adult worm is related to the amount of host antigen which is present. In relation to (c), it should be possible to measure the survival rate of worms with increased parasite antigen expression in vivo by using for example, trypsin treated worms. A preliminary in vitro experiment has

indicated that trypsin treated schistosomes are more adhesive for mouse peritoneal macrophages in the presence of antibody, than untreated worms. Parasite survival was not investigated but it is of interest to note that some recent studies by Bout et al. (1981) and Moser and Sher (1981) have indicated that schistosomulum killing by host cells in vitro is enhanced by increasing the effector cell to target ratio. On the basis of their observations, Moser and Sher (1981) concluded that the ability of different granulocyte populations to kill the juvenile parasite in the presence of antibody may be strongly dependent on their ability to adhere in sufficient numbers to the worm surface. It is therefore possible that increased expression of parasite antigens at the surface of the adult worm in vivo would enable a sufficient number of host cells to adhere such that the worm suffered immune damage and then elimination.

Taken together these observations must be considered as being entirely consistent with the host antigen hypothesis. They do not unequivocally confirm the validity of the hypothesis but it may be possible to do this by, as mentioned earlier, measuring the survival rate in an immune host of worms whose parasite antigen expression has been increased by removal or displacement of host antigens (trypsin treated ?). Such an experiment would therefore constitute a priority in any continuation of this work.

6.6 The influence of praziquantel on antigen expression

The ability of praziquantel (10µg/ml) to increase the

expression of parasite antigens (Fig. 28) raises the possibility that worms may suffer rejection from drug treated animals either wholly or partly as a consequence of increased susceptibility to antibody dependent immunity. As far as can be ascertained, this is the first report which suggests that praziquantel may act in this way although it has been suggested that other anti-helminth drugs may employ such a mechanism (Gibson et al, (1976); Doenhoff and Bain (1979)). Praziquantel has however been reported as being able to increase the susceptibility of parasitic cestodes to host proteases so it appears likely that the drug is also able to expose antigens at the surface of this type of worm (Thomas and Andrews, 1977).

Worms were exposed to praziquantel for only 3-4 min in an attempt to prevent the surface membrane and tegumental cytoplasm components from being shed (Becker et al., 1980). The finding that praziquantel does not alter host antigen expression strongly suggests that this attempt was successful. In addition, praziquantel did not promote increased release of ^{51}Cr (Table 23) or with the exception of one experiment, ^{125}I-WGA from labelled worms (Table 24). It is strongly suggested therefore that the increase in binding of anti-parasite antibody induced by praziquantel is due to increased expression of parasite antigens at the schistosome surface membrane.

What then is the mechanism by which praziquantel is able to expose additional antigenic determinants at the surface of the adult worm ? The finding that praziquantel does not effect host antigen expression suggests that praziquantel

may increase parasite antigen expression in a manner different from that of trypsin, glutaraldehyde and formalin. The drug has lipophilic properties (Fig. 27) and is clearly able to penetrate the surface membrane of the parasite with extreme rapidity. Penetration is associated with changes in the permeability of the membrane to Na^+, K^+ and Ca^{2+} (Pax et al., 1978) and it has been suggested by Coles (1979) that praziquantel may open pores in the membrane. The formation of pores is indicative of an alteration in organisation of the surface membrane and this may account for the increase in expression of parasite antigens which is detectable at the surface of praziquantel treated schistosomes.

The nature of the antigenic sites which are exposed by praziquantel may differ from sites exposed by aldehydes or trypsin. This idea is supported by the finding that unlike the other three reagents praziquantel does not have any measurable effect on expression of RBC antigens. It could be argued that praziquantel may therefore expose schistosome antigenic determinants which are not associated with, or not in close proximity to, RBC determinants. However, in view of the fact that praziquantel has little disruptive effect on the surface membrane as measured by ^{51}Cr and ^{125}I-WGA release it is perhaps equally likely that the parasite antigenic determinants are exposed due to alterations in membrane structure which are of an insufficient level to interfere with host antigen expression.

6.7 Conclusions and implications

The major aim of the present investigation was to elucidate a strategy for increasing the expression of parasite antigens at the surface of the adult worm without causing membrane disruption. The experimental approach employed to achieve this aim was to subject the worm to reagents which interacted with either surface antigens or the lipid phase of the surface membrane and then measure antigen expression by an indirect radiolabelled antibody method. In this way, it was hoped to establish the properties required by drugs to promote antibody-dependent rejection of the worm *in vivo*.

The results of this investigation show that it is possible to expose parasite epitopes at the schistosome surface *in vitro* by exposing the worm to reagents which interact with membrane proteins. It is apparent however that such a strategy could not be employed to increase expression of parasite antigens *in vivo* unless a reagent could be produced which reacted exclusively with a parasite membrane protein. The level of specificity required is clearly characteristic of the antibody molecule and the possibility of using a reagent of limited specificity (a proteolytic enzyme ?) in combination with anti-schistosome antibody is perhaps worthy of consideration and investigation. This idea is perhaps rather ambitious but the possibility of carrying drugs to their desired targets by using antibody is currently under a great deal of investigation in the field of tumour immunology (Moolten *et al.*, 1976; Thorpe, *et al.*, 1981).

A more attractive approach to increasing parasite antigen expression in that it is perhaps likely to be less detrimental to the host, is to incubate the worm with lipophilic reagents which are able to penetrate the membrane but at the same time preserve its integrity. The reasoning behind this approach is that by inserting themselves into the lipid phase of the membrane, the reagents may cause membrane reorganisation and this may result in exposure of antigens. No evidence of such an effect was observed when employing retinol, Tween 40 or Tween 80. It was found however that praziquantel was able to increase expression of parasite antigens and this drug has a lipophilic moiety which enables it to readily penetrate the parasite surface membrane. This result therefore raises the intriguing possibility that the type of reagent which is most suitable for increasing expression of parasite antigens may already be in use to treat schistosomiasis. If the in vitro result can be confirmed by showing that treatment of infected animals results in exposure of parasite determinants at the schistosome surface membrane then the need to develop drugs which increase parasite antigen expression is clearly already satisfied. Determination of the nature of the interaction between praziquantel and the surface membrane would however be of value in designing alternative drugs should such development be required. The nature of the antigen exposed by praziquantel must also be the subject of further investigation because if praziquantel treated animals reject worms by an immunological mechanism then this antigen must clearly act as a susceptible target for the host immune response. Purification of this antigen may thus provide suitable material

for immunization studies.

If praziquantel is able to increase the exposure of parasite antigens *in vivo*, in a manner similar to that which is observed *in vitro*, then it is clear that parasite antigen expression can be increased in the absence of any effect on the expression of host antigens. Removal of host antigens may therefore not be a prerequisite for the antibody-dependent rejection of adult schistosomes *in vivo*. This is an important idea in that it dismisses the need to overcome the considerable obstacle of the densely packed coating of host molecules at the worm surface (Table 2 and Fig. 4). It is important to realise however that it does not disprove the host antigen hypothesis as the parasite epitopes exposed may : (a) be uncovered due to a change in organisation of host antigens which is not detected by the IRAM ; or (b) constitute a population of molecules which perhaps because they are normally deeply embedded in the surface membrane, do not require masking by host molecules.

It is possible that newly exposed antigens may vary both with respect to each other and in relation to antigens detectable at the surface of the normal worm, in their ability to promote antibody dependent complement fixation or cell binding. Such variation might arise as a consequence of :

(a) differences in the rate of ease with which antigens are shed following immune recognition;

(b) differences in the distribution of the antigens (Goldring *et al.*, 1977b);

(c) differences in the subclass of antibody by which the antigens are bound (Shah and Ramasamy, 1982).

Elimination of the worm *in vivo* may therefore depend on exposing certain distinct antigenic determinants at the worm surface (for example the epitope(s) exposed by praziquantel ?). If the nature of these putative epitopes can be established then it would be possible to investigate mechanisms of increasing their expression *in vitro* by developing assays which employ mono-specific antibodies. The present investigation in combination with the recent production of anti-schistosome monoclonal antibodies (Smith *et al*., 1982; Taylor and Butterworth, 1982) has paved the way for the development of such assays.

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