1983

Surface Membrane Biology Of Schistosoma Mansoni

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SURFACE MEMBRANE BIOLOGY OF *SCHISTOSOMA MANSONI*

by

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Submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Ontario

July, 1983

ABSTRACT

The apical and basal membranes of the surface syncytium of Schistosoma mansoni were separated and found to exhibit the predictable, polarised ATPase distributions of other transporting epithelial layers. A multilamellar body fraction was prepared from adult worms and the major phospholipid classes determined. Phosphatidylcholine constituted the major phospholipid class. An apparent projection core was described in schistosome multilamellar bodies, which showed structural analogy to multilamellar bodies from lung.

Methods were devised for sequentially stripping the outer (OB) and inner (IB) bilayers from the surface apical membrane complex of adult worm pairs. Tritiated Concanavalin A and [^{125}I] diazotised iodosulfanilic acid were found to be useful markers for the OB, while alkaline phosphatase and a Mg^{2+} dependent, Na^{+} ATPase were described from the IB. Removal of the OB did not result in cytosolic leakage from the syncytium, while removal of the IB resulted in the loss of high molecular weight soluble proteins. Differences in phospholipid composition between the OB, IB and multilamellar bodies were found to be quantitative rather than qualitative. Preliminary evidence suggested that major phospholipid classes of the OB and IB exhibited heterogeneous turnover or post-synthetic modulation.

Radiolabelled glycerol was incorporated into the lipids of adult worms and had a turnover half-time (t1/2) of 13.0 hours. Loss of glycerol from the aqueous phase exhibited biphasic kinetics. Glycerol was found not to be ideal for measurements of turnover rates of OB and

IB lipids. ^{14}C Palmitate was incorporated into the lipid phase of adult worms and was lost with t1/2 = 11.5 hours. The IB lipids showed biphasic kinetics with t1/2 = 11.5 hours. The IB lipids showed biphasic kinetics with t1/2 = 12 minutes and 17.3 hours. Results of the turnover studies emphasised the ability of S. mansoni to rapidly renew its surface and to modulate surface phospholipids.

A negatively charged, sialic acid - containing glycocalyx was discovered on the schistosome surface and was found to show regional and sexual heterogeneity. The presence of this surface coat on lung stage and adult parasites, combined with the lack of a coat on very young schistosomula, may help to explain the phenomenon of concomitant immunity.

To Chet

for unwavering

support

ACKNOWLEDGEMENTS

I am indebted to my supervisor, Dr. Ron Podesta, for his encouragement and for the many stimulating ideas and opportunities afforded to me whilst a student in his laboratory.

I should also like to thank the members of my advisory committee, Drs. Dean Befus, Stan Caveney and John Wiebe for their useful criticisms and suggestions. Gratitude is extended to my fellow students, Lyn Dean, Bruce Young, Afzal Siddiqui, Mano Edwards and to Dr. Saidur Rahman for their cooperation, discussion and friendship during this work. Helpful advice from Mr. Rick Harris is appreciated.

This research was supported by grants from The Natural Sciences and Engineering Research Council of Canada and UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases. Postgraduate scholarships from The University of Western Ontario and The Province of Ontario are gratefully acknowledged.

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CHAPTER 1

GENERAL INTRODUCTION AND THESIS OBJECTIVES

1.1 LITERATURE REVIEW

1.1.1 Introduction

Schistosomiasis, is not widely recognised in the western world as one of the major world health problems of developing nations (Knopf, 1982). Recent estimates suggest that this disease affects the lives of over 300 million people (Capron et al., 1980a). Furthermore, manipulation of water resources for new land use schemes is currently enlarging the intermediate host habitats and prevalence (Phillips and Colley, 1978). The main aetiological agents of human schistosomiasis are three species of the genus Schistosoma (Platyhelminthes: Trematoda). Schistosoma haematobium is distributed throughout much of Africa and the Middle East, Schistosoma japonicum is restricted to East Asia, whilst Schistosoma mansoni occurs in Africa, South and Central America, the Middle East and the Caribbean (Mahmoud, 1982). Global research efforts have been directed primarily at S. mansoni, which is also the subject of this thesis.

1.1.2 The Life Cycle of Human Schistosomes

Schistosomes, unlike most trematodes, are dioecious - the slender cylindrical female being held in the gynecophoric canal of the male. The canal results from folding and overlapping of the ventral surface (Figure 1.1). The sexually mature adults of S. mansoni and S. japonicum are 10 - 20 mm long and occupy the inferior and superior

Figure 1.1 Scanning electron micrograph showing an adult worm pair of S. mansoni. The thin cylindrical female (f) is held in the gynecophoric canal (g) of the male parasite. The dorsal surface of the male (d) is clearly visible, whilst the ventral surface forms the inside of the gynecophoric canal. Both male and female parasites possess a ventral sucker for attachment. The ventral sucker of the male is visible (v).


f
d

mesenteric veins respectively, whilst S. haematobium resides in the vesical plexus of the urinary bladder. Adult worm pairs of S. mansoni live for up to 20 years producing an average of 300 unembryonated eggs per worm pair per day in the small venules supplying the intestinal walls (Mahmoud, 1982). Within six days the eggs embryonate and lytic enzymes present in their secretions aid the passage of the singly spined eggs through the host tissue and into the lumen of the intestine, or bladder for S. haematobium (Bogitsch and Wikel, 1974). Many eggs however, become trapped in the intestinal wall, or are disseminated in the bloodstream to the liver, lungs or other organs (Warren, 1976). Embryonated eggs which are successfully voided to the exterior in faeces or urine hatch in freshwater to release a small ciliated larva - a miracidium, which is only viable for 24 hours, within which time it must locate and penetrate the molluscan intermediate host. Within the snail tissues and hepatopancreas there is asexual reproduction of sporocyst stages, leading to the production of thousands of cercariae which escape from the snail 4-5 weeks after initial infection. The free living cercariae actively swim, utilising their bifurcate tails, but die within 48 hours if a suitable mammalian host is not located. Upon contact with a host the cercariae penetrate the skin with the aid of enzymatic secretions (Stirewalt, 1974). During penetration through the skin the cercariae lose their tails and become transformed to juvenile forms called schistosomula, which migrate via the venous system to the pulmonary capillaries. The juveniles then enter the systemic circulation to eventually become lodged in the hepatic portal system (Miller and Wilson 1978; 1980). Five to six weeks post-infection the female worms commence oviposition.

1.1.3 Pathology of Schistosomiasis

Repeated exposures to penetrating cercariae may involve hypersensitivity reactions, causing papular dermatitis while severe inflammation can result in the lungs from migrating schistosomula. However, the egg is the main factor responsible for symptoms of the disease (Phillips and Colley, 1978). Acute schistosomiasis, manifest as katayama fever, usually occurs in visitors to endemic areas who pick up large single infections. The onset of katayama fever, involving fever, myalgia, lymphadenopathy, hepatosplenomegaly and eosinophilia; usually coincides with the start of oviposition and is thought to be a form of serum sickness, resulting from the massive onslaught of antigenic material (Mahmoud, 1982). Chronic schistosomiasis is the more common and sometimes asymptomatic form of the disease. Again the majority of the pathology can be attributed to the eggs, most of which are not voided but become embolised in the liver (Warren, 1976). Maturing eggs, trapped in the tissues, secrete antigenic and lytic material, attracting lymphocytes, macrophages, eosinophils, neutrophils and plasma cells. The resulting lesions or granulomas become epithelioid, then fibroblastic and eventually collagen containing fibrotic plaques (Phillips and Colley, 1978). Fibrotic tissue may occupy an area around the egg 100 times the volume of the egg (Warren, 1972), causing obstruction of portal circulation and leading to portal hypertension with subsequent development of hepatomegaly, liver disfunction, congestive splenomegaly and portal systemic collateral circulation, such as oesophageal varices. (Cha,

1978; Dunn et al., 1979). Granuloma formation in the small intestine affects gut physiology and nutrient absorption (Vengesa and Leese, 1976; Tiboldi, 1979; Vengesa and Leese 1979).

A cell-mediated delayed hypersensitivity reaction has been implicated in granuloma formation - the response being anamnestic, correlated with delayed hypersensitivity skin tests and is greatly reduced in T-cell or eosinophil deprived animals (Phillips et al., 1977a; Byram et al., 1979; Epstein et al., 1979; Mahmoud, 1979; 1982). Mahmoud suggested that reduction of the granuloma would lessen pathology (Mahmoud et al., 1975a), but suppression of the granuloma resulted in liquefactive necrosis of liver tissue and increased susceptibility (Mahmoud, 1982). Granulomas have a protective function in sequestering the damaging products of the egg (Phillips and Colley, 1978). In long-term infections spontaneous modulation of granuloma formation occurs, probably mediated by suppressor T-cells (Chen and Dean, 1977; Naggar and Colley, 1982), increased B-cell stimulation and antibodies (Boros et al., 1975; Colley et al., 1977; Chensue and Boros, 1979). In addition to egg secretions adult worms release soluble antigens and particulate antigens in the form of shed surface components (Rotmans et al., 1981; Simpson et al., 1981a; Samuelson and Caulfield, 1982). The resulting circulating immune complexes, as well as modulating the immune response, are directly pathogenic. Schistosome specific and non specific IgG, IgM and C_3 have been eluted from granular deposits on the glomerular basement membrane of damaged kidneys (Bout et al., 1977; Jones et al., 1977; Danno et al., 1979; Digeon et al., 1979).

Anaemia is a frequent correlate of schistosomiasis and although each worm consumes only 0.88 ul of blood per day, rectal blood loss may reach 30 ml per day (Mahmoud, 1982). The spectrum of disease caused by this helminth is diverse, ranging from a total lack of symptoms to an acute fever or chronically debilitating disease and even death.

1.1.4 Control of Schistosomiasis

Diagnosis of schistosomiasis is often confirmed by the Kato stool smear technique, which detects eggs in the faeces (Peters et al., 1980). The number of eggs per gram of faeces however is not a good indication of the disease state, as in chronic infections most of the eggs are trapped internally by fibrotic reactions (Phillips and Colley, 1978). Current immunodiagnostic methods suffer from a lack of specificity, compounded by the frequency of heterospecific infections (Knopf, 1982). Perhaps the recent development of monoclonal antibodies against unique schistosome determinants and the purification of stage specific antigens may allow accurate immunological characterisation of the disease state of the patient (Nash et al., 1981; Strand et al., 1982).

There are three major approaches to the control of schistosomiasis sanitation, use of molluscicides and the prophylactic and therapeutic treatment of definitive hosts (Knopf, 1982). Oxamniquine and Praziquantel are two of the drugs of choice for S. mansoni infections (Mahmoud, 1982). These drugs, although effective, may not reverse liver damage and also leave the host susceptible to reinfection. Currently a major research thrust is towards the

development of a protective vaccine. To date some resistance to reinfection in experimental animals has been obtained with irradiated cercariae and cercarial antigen, unisexual infections, bovine schistosomes, extracts of Fasciola hepatica and Toxoplasma gondii (Phillips and Colley, 1978; Taylor and Bickle, 1980; Hillyer and Serrano, 1982; Horowitz et al., 1982). The search for a vaccine more practically suited to large scale production and application will be aided by the recent production of monoclonal antibodies to schistosome determinants (Dissous et al., 1982; Strand et al., 1982; Taylor and Butterworth, 1982; Dresden et al., 1983). These monoclonal antibodies have induced resistance in passive transfer experiments (Zodda and Phillips, 1982) and in mediating eosinophil dependent cytotoxicity in vitro (Grzych et al., 1982). Purification of those antigens relevant to this immunity is now underway (Zodda and Phillips, 1982).

1.1.5 An Introduction to the Schistosome Surface: Structure and Function

The surface of S. mansoni has evolved to accommodate a drastic change from a fresh water habitat to the internal milieu of the mammalian host. Once inside the host this versatile surface serves as an interface between the worm and the hostile effectors of the provoked immune system, whilst mediating crucial biochemical, physiological and nutritive functions. Although originally referred to as a cuticle (Morris and Threadgold, 1968) the schistosome surface is very different from the inert impermeable cuticle of parasitic nematodes (Lee and Atkinson, 1976). Many studies have attempted to

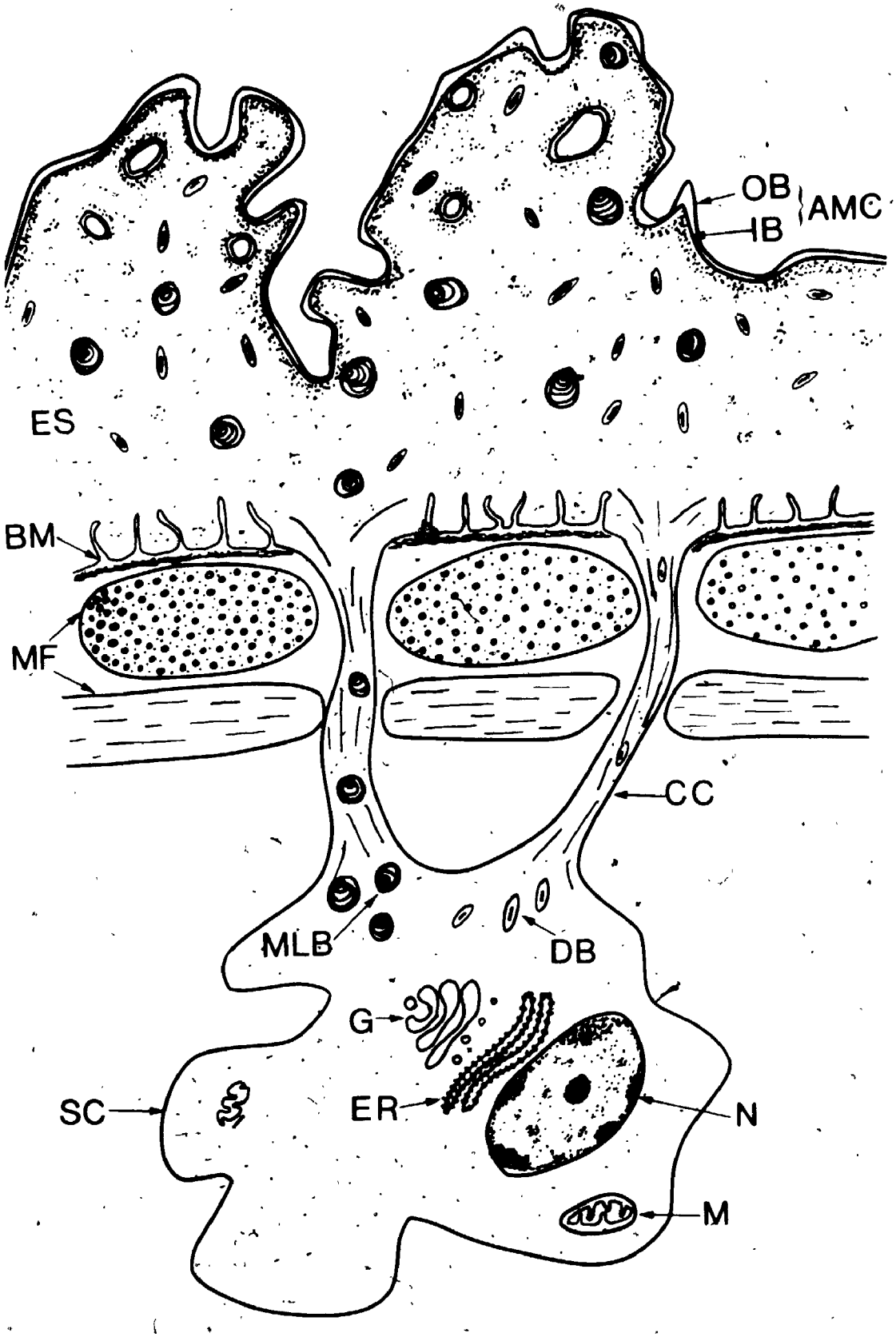
Figure 1.2 Electron micrograph showing the surface of S. mansoni. The surface layer, or tegument, consists of a syncytial epithelium, bounded on the outside by an apical membrane complex (A) and on the inside by a basal membrane (B). Osmiophillic multilamellar bodies (ML), the presumed precursors of the apical membrane complex, are visible in the tegument. The basal membrane rests on a basal lamina and underlying muscle layers (M), which are traversed by internuncial processes (I). These link the syncytium to the underlying nucleated (N) cell bodies.


A

characterise the structure and function of the schistosome surface and to account for its insusceptibility to host immune factors, but many basic questions remain unanswered and the role of some simple structures is still a matter of dispute.

Cercariae, schistosomula and adult S. mansoni are totally invested by a continuous, anucleate layer of cytoplasm 0.5 - 5.0 μm in depth (McLaren, 1980; Figure 1.2). The possession of this syncytial epithelium or tegument is considered a primary adaptation to parasitism (Podesta, 1982a), because a similar arrangement is found in other parasitic Platyhelminthes, whereas in contrast free living flatworms have a cellular epithelium (Matricon-Gondran, 1980; Podesta, 1982a). The inward-facing aspect of the epithelium is bounded by an infolded trilaminate basal plasma membrane, attached by hemidesmosomes to the underlying basal lamina, interstitial material, circular and longitudinal muscles. Cytoplasmic connections or internuncial processes form tortuous channels through this material to unite the epithelium with the sunken cell bodies, situated in the parenchyma underlying the muscle layers (Figure 1.2). The cytoplasm of the anucleate epithelium is therefore continuous with that of the nucleated cell bodies (Morris and Threadgold, 1968; Silk et al., 1969; Reissig, 1970; Hockley, 1973). Figure 1.3 illustrates the basic features of the schistosome surface. As many as 50 microtubule lined channels may enter each cell body (Wilson and Barnes, 1974a). Based on the presence of nuclei, endoplasmic reticulum and golgi (Morris and Threadgold, 1968; Hockley, 1973; McLaren, 1980), on positive histochemical staining for RNA and mitochondrial enzymes (Wheater and Wilson, 1976) and on autoradiography of [^{3}H] leucine incorporation (Wilson and Barnes, 1979) the cell bodies have been implicated as

Figure 1.3 Diagram illustrating the basic organisation of the adult schistosome surface. The surface epithelial syncytium (ES) is bounded by an apical membrane complex (AMC), composed of an inner (IB) and outer bilayer (OB). The inner side of the epithelium is limited by an infolded basal membrane. The subtegumental cells (SC) are the major sites of synthesis and contain nuclei (N), endoplasmic reticulum (ER) and golgi (G). The two main inclusion bodies of the syncytium : discoid bodies (DB) and multilamellar bodies (MLB) are thought to be synthesised in the subtegumental cells and pass up the microtubule - lined cytoplasmic connections (CC), which traverse the muscle fibres (MF).


OB
AMC
IB
ES
BM
MF
CC
MLB
DB
G
SC
ER
N
M

major sites of biosynthesis of precursor material for the surface epithelium. The two major inclusion bodies of the tegument - the discoid and multilamellar bodies - are thought to be synthesised in the golgi complex of the cell bodies and transported via the cytoplasmic connections to the tegument.

In 1973 the use of uranyl acetate as a post-fixative revealed an unusual feature of S. mansoni - the presence of a heptalaminate membrane complex (Hockley and McLaren, 1973), which is now known to consist of two closely apposed trilaminate bilayers (Hockley et al., 1975; Torpier, 1977). This aspect of the schistosome surface appears to be an adaptation to the sanguineous environment, as it has been found only in blood flukes and not in those inhabiting the intestine or other sites (McLaren and Hockley, 1977). The free swimming cercaria has a trilaminate outer membrane, invested with a thick mucopolysaccharide - containing glycocalyx. By three hours post-penetration the structural and physiological change to a schistosomulum with a double outer bilayer is complete (Hockley, 1973). More recent work however suggests that development of the outer bilayer may be delayed for up to 48 hours after transformation (Butterworth et al., 1982). Surface microvilli have been observed 30-60 minutes after penetration and these may effect shedding of the original cercarial membrane (McLaren and Hockley, 1976). The possible loss of cercarial plasma membrane and the addition of an extra bilayer pose the problem of the origins of new membrane. Multilamellar bodies are thought to fuse their bounding membranes with invaginations of the outward-facing membrane and to spread their lamellate contents onto the surface of the worm, forming a second lipidic layer (Hockley, 1973; Wilson and Barnes, 1974a; 1977). Although this theory does not

explain the incorporation of externally applied horseradish peroxidase into the multilamellar bodies (Smith and Von Lichtenberg, 1974), it does bear a striking similarity to another secretory system - that of surfactant production in type II pneumocytes of mammalian lung (Massaro and Massaro, 1976). It also provides a mechanism for what is thought to be a continual process of rapid membrane turnover (Kelligan et al., 1977; Wilson and Barnes, 1977; Samuelson and Caulfield, 1982; Podesta, 1982a). Histochemical studies suggest that the multilamellar bodies are composed primarily of lipid and possibly contain carbohydrate (Reissig, 1970; Wilson and Barnes, 1977). Similarly the adult surface reacts positively with stains for lipid and carbohydrate (Wheater and Wilson, 1976; McLaren, 1980). The surface, in addition to possessing exposed sugar groups, has a negative charge and binds cationised ferritin (Wilson and Barnes, 1977) and colloidal iron (Hockley, 1973; Wheater and Wilson, 1976). Despite this evidence to the contrary, the worms are considered not to have a glycocalyx (McLaren, 1980), indeed the membranous outer bilayer has been postulated to replace the ubiquitous glycocalyx of other cell types (Wilson and Barnes, 1974a). In most other trematodes the discoid bodies are thought to contribute to the surface membrane and although those of S. mansoni stain strongly for carbohydrate (Reissig, 1970; Hockley, 1973) they are considered to be precursors of tegument "ground substance" (Wilson and Barnes, 1974a) or of the spines (Hockley, 1973). A recent paper reports that the spines are composed of actin, based on their crystalline patterning, but no biochemical support was offered for this hypothesis (Cohen et al., 1982).

Freeze fracture studies of the schistosome surface membrane complex indicate that intramembranous particles are located mainly in the outermost leaflet of the outer bilayer, with some in the cytoplasmic leaflet of the inner bilayer. On the basis of this atypical arrangement researchers have suggested that the outermost membrane is "inside out" (Hockley et al., 1975) and that the intramembranous particles migrate from the inside of the inner bilayer to the exterior of the outer bilayer (Torpier et al., 1977). An effective understanding of the role of intramembranous particles in S. mansoni will have to await further research.

The schistosome surface, in addition to its role in immune evasion has long been recognised as an important site for the uptake of nutrients (Senft, 1959). Early uptake studies in this area have failed to take the caecum, an active absorbing surface, or unstirred layer into account (Isseroff et al., 1972; Asch and Read, 1975; Isseroff et al., 1976). More recently two methods have been described for circumventing these problems by compartmental analysis (Podesta and Dean, 1982a; 1982b) or triple isotope labelling (Cornford and Oldendorf, 1979). These studies have revealed the role of the tegument in the transport of amino acids and simple sugars. The surface is also involved in ion, water and volume regulation (Brodie and Podesta, 1981). Any theory of immune evasion involving the schistosome surface must therefore be consistent with its function as a transporting epithelium.

1.1.6 Host Immunity and the Surface of S. mansoni

The success of the primary infection and the degree of immunity engendered following an initial infection with S. mansoni varies for different hosts. Humans, baboons, hamsters, mice and gerbils are examples of permissive hosts, which permit the passage of viable ova, whilst rats, guinea pigs and foxes are non-permissive (Knopf, 1982). Rats and rhesus monkeys, after an initial infection with S. mansoni become immune to reinfection (Phillips et al., 1977b), whilst mice and hamsters exhibit only partial immunity. Human immunity has been less well demonstrated, but epidemiological studies suggest that people can slowly acquire at least partial resistance (Smithers and Terry, 1969a).

Schistosomes evoke a considerable humoral response from their host and both IgG and IgE have demonstrated cytotoxic activity in vitro, in the presence of complement or immune effector cells. Cytotoxicity in vitro or in vivo appears to be directed against the surface membranes of the flukes. Attempts to transfer immunity passively have been disappointing, although some success has been achieved with transfer of immune rat serum (Mangold and Knopf, 1981). In infected mice IgE may be involved in a protective response (Horowitz et al., 1982), IgE levels being correlated with worm expulsion in the rat (Capron et al., 1980b). In mice IgG2a, the second most potent anaphylactic immunoglobulin class, is the most effective specific mediator of immunologic injury (McLaren, 1980). The specific protective humoral response against schistosomes is directed primarily against surface membrane antigens (Sher et al., 1974; Murrell et al., 1977; Rotmans and Mooij, 1982) and currently

considerable research activity is directed at detecting and isolating the relevant antigens, most of which appear to be glycoproteins, (Strand et al., 1982) from the surface membranes. Hayunga et al. (1983) have isolated and partially characterised a major glycoprotein antigen from the tegument and monoclonal antibodies have recently been raised to tegument glycoproteins (Strand et al., 1982).

In 1977 adult S. mansoni were found to bind not only specific antibody but heterospecific antibody, suggesting the presence of an Fc receptor (Kemp et al., 1977). Schistosomula and adult worms have since been shown to possess Fc receptors which will bind IgG, immune complexes and ß-2 macroglobulin (Tarleton and Kemp, 1981). Proteases at the surface may subsequently cleave off the F(ab) portion (Auriault et al., 1981). Receptors for C_3 have also been detected (McGuinness and Kemp, 1981). Young schistosomula and their soluble products (Santoro et al., 1980) are known to activate complement both by alternate and classical pathways (Ouaissi et al., 1981). The earliest signs of damage are manifest as blebbing of the surface membrane (Ouaissi et al., 1980). By the time Schistosomula reach the lungs, they no longer activate complement by the alternative pathway (Santoro et al., 1979; Dias da Silva and Kazatchkine, 1980), which is therefore of prime importance in the non-specific killing of skin schistosomula in the naive host (Santoro et al., 1982). A protective role has also been proposed for the C_3 receptor in binding and subsequently sequestering or inactivating C3b, thus preventing completion of the complement cascade (Ouaissi et al., 1981). The beneficial effects of complement extend to C_3, as worm recoveries are lower from C_3 deficient mice compared to otherwise identical, normal mice (Ruppel et al., 1982).

Fc and C_3 groups on the schistosome surface have been shown to promote the adherence of a variety of immune effector cells. Much interest has been focussed on antibody-dependent, cell mediated mechanisms of schistosomular destruction and in particular on eosinophils, which are almost always associated with helminthic infections. Peripheral eosinophilia and tissue eosinophilopoiesis are characteristic of the schistosome-infected host (Salih et al., 1977; Byram et al., 1978; Knopf, 1979) and peak slightly during schistosomular migration and greatly at commencement of oviposition (Mahmoud, 1982). Development of a specific antieosinophil serum (Mahmoud et al., 1973) elucidated the role of the eosinophil in vivo, as exposure of immune mice to antieosinophil serum abbrogated their previous immunity (Mahmoud et al., 1975b). The degree of host resistance has been found to correlate with the peak of eosinophilia (Knopf and Cioli, 1980). Specific IgG2a and IgG1 have been shown to direct eosinophil mediated killing of schistosomula in vitro (McLaren, 1980). Immune damage was detected morphologically, by the ability of the schistosomula to mature and by [^{51}Cr] release assays, although there has been some failure to correlate morphological damage with [^{51}Cr] release (Butterworth et al., 1982). Eosinophil adherence may be more effectively stimulated by C_3 and the attendant eosinophil-chemotactic factors generated by complement activation (Ramalho-Pinto et al., 1978). Eosinophil activating factors are also released from stimulated mast cells and lymphocytes (Capron et al., 1980b; Mahmoud, 1982). Eosinophils adhere rapidly to opsonised schistosomula and degranulate, releasing major basic protein, cationic protein and peroxidases, which attack the surface (McLaren, 1980;

Kazura et al., 1981). Adherance and degranulation reactions may be independently mediated, as Concanavalin A will stimulate adherance without degranulation, whilst subsequent addition of a calcium ionophore stimulates degranulation (David et al., 1980). Damage, proceeding degranulation, is manifest by surface membrane blebbing and tegumental vacuolation, followed by destruction of surface integrity and removal of the tegument (Von Lichtenberg et al., 1977; McLaren, 1980). In chronic infections there is a modulation of the eosinophil response, mediated by a T-cell suppressor factor (Naggar and Colley, 1982). Some researchers have also found eosinophils from eosinophilic patients to be less effective in in vitro cytotoxicity assays, due either to their immaturity or to immune complex blocking of receptors (Butterworth et al., 1975; Butterworth et al., 1977; Kazura et al., 1981).

The efficacy of cell types, other than eosinophils, in schistosomular killing is controversial. In some systems neutrophils have proven totally ineffective (Butterworth et al., 1977), whilst Dean et al., (1974) found that, in the presence of complement and antibodies, neutrophils would attach to and damage schistosomula. These discrepancies in the literature however may result from the different specificities of sera used. Moser and Sher (1981) demonstrated neutrophils to be minimally effective against schistosomula in the presence of immune serum, whilst trinitrophenol (TNP) - coated schistosomula, in the presence of anti-TNP, were damaged more readily by neutrophils than eosinophils. The usual inefficiency of neutrophils in immune killing may also be related to their apparent ability to fuse their plasma membranes with the outer bilayer of the schistosomula, without stimulating degranulation

(Caulfield, et al., 1980). Additionally, mast cells have been shown to attach to schistosomula (Sher, 1976; Caulfield et al., 1981). Macrophages (Perez and Smithers, 1977; Mahmoud et al., 1979) and T-lymphocytes have been shown to damage schistosomula (Ellner et al., 1982). Antibody-dependent cell mediated damage to schistosomula in vitro is not affected by agents inhibiting DNA replication, protein synthesis or oxidative respiration, but is inhibited by cytochalasin B, inhibitors of glycolysis and other agents likely to induce alteration of cell surface membranes and interfere with cell-target interactions (David et al., 1977). In conclusion several mechanisms of schistosome destruction have been demonstrated in vitro, but their relevance in vivo is not fully understood.

1.1.7 Concomitant Immunity

A central problem in schistosomiasis research and one of the main hindrances to successful vaccine development is the type of immunity engendered. The term concomitant immunity has been borrowed from tumour immunology to describe the phenomenon whereby newly invading schistosomula are killed, concomitant with survival of the established population of adult worms from the primary infection. (Smithers and Terry, 1969a and 1969b). Loss of the adult worm population is frequently accompanied by a loss of immunity (McLaren, 1980). Several theories have evolved to explain concomitant immunity and these will be summarised briefly here. Historically, the most popular theory, the disguise hypothesis, accredits survival of the adult worms to an adsorbed layer of host antigen, which prevents the

worms from being recognised as non-self. Smithers and Terry (1968) suggested the existence of protective host antigens following transfer experiments, whereby worms grown in mice and transferred to the veins of monkeys survived, but not if the monkeys had previously been immunised against mouse red blood cells, rendering them "anti-mouse". These results have been duplicated with the mouse-monkey system (Erickson et al., 1973) but other workers who transferred rat worms to "anti-rat" hamsters or hamster worms to "anti-hamster" mice found no increased killing (Boyer and Ketchum, 1976; Coelho et al., 1976). The presence of host antigens on the worm surface has been amply demonstrated by several techniques including mixed agglutination, fluorescent antibody, damage in vitro from anti-host serum, antibody-enzyme bridge labelling, Ouchterlony test and fluorescein isothiocyanate (FITC) labelled Staphylococcus aureus. Host antigens detected on the surface include Forssman (Damian, 1967; Dean and Sell, 1972), mouse α-2-macroglobulin (Damian et al., 1973; Kemp et al., 1976), blood group determinants (Clegg, 1974, Dean 1974), major histocompatibility antigens (Gitter et al., 1982) and heterologous antibodies bound via the Fc portion (Kemp et al., 1977; Torpier et al., 1979). The antigens are thought to be acquired via hydrophobic interactions of their ceramide groups (Clegg, 1972), glycosyl transferases (Simpson et al., 1981b), Fc and C_3 receptors (Tarleton and Kemp, 1981) or possibly by neutrophil plasma membrane fusions (Caulfield et al., 1980). Many types of cells in culture will pick up host antigens (Clegg, 1974) and little significance is usually attached to this. Masking of schistosome antigens could also be effectively accomplished by the presence of worm-derived immunogens, exhibiting strong cross-reactivity with host components. An example

of this is the presence of a surface antigenic determinant that cross reacts with mouse 2-macroglobulin, which is even found in worms raised in monkeys (Damian, 1964; Damian et al., 1973). The significance of this mouse determinant on a parasite which is not primarily murine and does not protect naive mice from infection remains obscure.

Definitive proof of the disguise hypothesis has recently been deduced from the susceptibility of skin stage schistosomula to immune serum and eosinophils, coupled with the insusceptibility of lung stage and adult worms, which were damaged only with anti-host serum (McLaren and Terry, 1982). However this may be attributed, not to decreased antibody binding to masked worm immunogens, but to lessened susceptibility resulting from some intrinsic change in the surface of the developing schistosomula (Sher and Benno, 1982). As schistosomula mature over a six day postransformational period they decline in antigenicity and in susceptibility to lethal antibody dependent, complement or eosinophil mediated damage (Bickle and Ford, 1982). This holds true even if the host "disguise" is specifically bypassed by testing haptenated schistosomula with anti-hapten antibody (Sher and Moser, 1981; Levi-Schaffer et al., 1982). Older schistosomula no longer activate complement by the alternative pathway, they prevent prolonged attachment of eosinophils and resist damage by eosinophil degranulation products (Dessein et al., 1981; Bickle and Ford, 1982). Schistosomula develop impurity to immune effectors both in vivo and in vitro and in the presence or absence of host components. (Dean, 1977; Dessein et al., 1981; Levi-Schaffer and Smolarsky, 1981). These results are incompatible with the disguise hypothesis and point to an

unknown developmental change of the maturing schistosomular surface.

Alternative methods of parasite protection have been suggested, including immunosupression via inhibition of lymphocyte proliferation (Camus et al., 1977; Colley et al., 1977; Mogueira and Machado, 1978) or responsiveness (Colley et al., 1979), reduction in surface antigen expression (Butterworth et al., 1982), modulation of antibody titres with prolonged infection despite continued antigenic stimulation (Nash et al., 1978), induction of T-suppressor cells (Mota-Santos et al., 1977), suppression of haemagluttinin activity (Colley et al., 1979) and enhancing or blocking antibody binding (Sogandres - Bernal, 1976; McLaren, 1980).

The surface membranes of the tegument of S. mansoni are continually undergoing a dynamic turnover process and since the immune response of the host is directed against this surface, the ability of the worms to survive in an immunologically hostile environment may be related to their capacity to repair damaged membrane. Most researchers concur that the time constant for surface membrane renewal is between 2 and 10 hours (Wilson and Barnes, 1977; Dean and Podesta, 1982; Samuelson and Caulfield, 1982). There is evidence that some glycoproteins may have half lives as short as 15 minutes (Hayunga et al., 1982). Membranous material, shed by S. mansoni in vitro, precipitates immune serum and shows identity with freeze-thaw surface preparations (Murrell et al., 1974; Kusel et al., 1975a; Rotmans et al., 1981).

1.2 RATIONALE

The surface tegument of S. mansoni is known to be involved in

the transport of nutrients, ions and water (Senft, 1959; Isseroff et al., 1972; Cornford and Oldendorf, 1979; Brodie and Podesta, 1981) and may therefore be expected to share many features with other transporting epithelia. In addition the host immune response is directed at the worm surface (McLaren, 1980), which must therefore have evolved some protective features. The "disguise hypothesis", discussed previously, does not adequately account for the remarkable resistance of this parasite, especially as evidence now exists which suggests that resistance may develop in the absence of "disguising" host components (Dean, 1977; Dessein et al., 1981). A completely masking layer of adsorbed host material would be expected to alter and impede the vectorial transporting function of the surface. Furthermore, the rapid turnover times reported for the surface layers (Wilson and Barnes, 1977; Dean and Podesta, 1982; Samuelson and Caulfield, 1982) would appear to be incompatible with a passive disguise process.

Protection from immune effectors may well be afforded by the rapid synthesis, modulation and turnover of the apical membrane of the epithelial syncytium, allowing damaged membrane to be shed and replaced before the integrity of the cytoplasm becomes affected (Podesta, 1982a). The unusual structure of the platyhelminth tegument can be explained in terms of this hypothesis. The lack of cellular compartments in the tegument may be an adaptation which allows efficient coupling in the field of the syncytium, permitting rapid turnover and efficient repair of the surface. This hypothesis rests on the assumption that it is energetically more feasible to rapidly turn over the surface membrane of a syncytium than it is to

replace entire cells of a cellular epithelial layer (Podesta, 1982a).

Schistosomula progressively develop resistance to immune recognition and effectors over a period of several days (Dessein et al., 1981) suggesting that a developmental change occurs which involves the aquisition of the ability of the parasite to regulate the rate of surface renewal and in the structure of the schistosomular outer bilayer. These changes may render mature schistosomula relatively insusceptible to immune destruction and be partly responsible for the phenomenon of concomitant immunity. The signal transduction mechanisms responsible for directing membrane turnover may present susceptible targets for directed immunological attack and vaccine development.

1.3 OBJECTIVES

1. Dissection of the membranous components of the surface epithelial syncytium of S. mansoni, including preparation of fractions enriched in apical and basal membranes, outer and inner bilayers of the apical membrane complex and multilamellar bodies.

2. Quantitation of enzyme markers for these fractions.

3. Determination of the major phospholipid classes of the membranous fractions.

4. Measurement of the turnover rates of the outer and inner bilayer phospholipids from the apical membrane.

5. Investigation of surface alterations in developing schistosomula.

CHAPTER 2

SEQUENTIAL REMOVAL OF APICAL AND BASAL MEMBRANES FROM THE SURFACE EPITHELIAL SYNCYTIUM OF SCHISTOSOMA MANSONI

2.1 INTRODUCTION

The syncytial surface of parasitic platyhelminthes is thought to function in nutrient uptake as well as in synthesis, secretion and various sensory roles (Halton, 1982). In light of these proposed functions and the surface location of the tegument it is likely that the syncytium functions as a typical transporting epithelium. To extend this analogy it is useful to consider one definition of an epithelium suggested by MacKnight and co-workers (1980) - "A highly polarised structure composed of epithelial cells, which lie on variable amounts of supporting tissue and separates an external from an internal milieu". Two major difficulties are encountered in attempting to fit the schistosome surface into this definition. I refer here to the absence of epithelial cells per se and to the lack of information on functional polarisation. The unusual acellular surface, which presumably evolved from the cellular epithelium of free living platyhelminths, is thought to represent a specialised adaptation to parasitism (Podesta, 1982a). Polarization is a central feature of transporting epithelia and is a prerequisite for vectorial transport and regulation of the internal medium (Cereijido et al., 1980). In all epithelia examined to date therefore the apical and basal membranes are asymmetric both in morphological and functional aspects (MacKnight et al., 1980). This complies with the two membrane

model of Koefoed-Johnsen and Ussing (1958), which is central to most theories of epithelial transport. However the simple concept of two independent and dissimilar membrane barriers mediating vectorial transport is complicated by the existence of paracellular shunt pathways (Reuss, 1979; Podesta, 1982b). The relative importance of this third barrier in epithelial transport has been the subject of considerable investigation and the resistance of the shunt pathway has been found to be important in determining the transepithelial chemical and electrical potential gradients (Graf and Giebisch, 1979; Cereijido et al., 1980; Podesta, 1980). The schistosome surface provides a unique model for elucidating the importance of paracellular pathways to epithelial functioning, as it lacks morphologically detectable shunt pathways.

Although the schistosome surface has been implicated in a variety of transport processes (Cornford and Oldendorf, 1979; Bocash et al., 1981) there has been no definitive proof of polarised epithelial functioning. In Hymenolepis diminuta, however, the asymmetric transporting properties of the apical and basal membranes, which bound the surface syncytium, have been well demonstrated (Podesta, 1980). A central problem in this research area is posed by the acoelomate nature of the worms, which precludes the isolation of epithelial sheets and the concomitant exposure of the basal membrane to experimental manipulation. One solution to this problem is the comparison of tissue sections, where specific inhibitors gain access to the basal membrane, with entire organisms, where only the apical surface is exposed (Podesta, 1977; 1980). Another approach is to obtain separate fractions enriched in either apical or basal membranes

(Grinstein et al., 1980). Given the syncytial nature of the schistosome surface it should be feasible to sequentially remove the apical and basal membranes from intact worms. Membrane separation has been accomplished in other epithelial systems and ubiquitous marker enzymes characterised for both preparations (Armstrong et. al., 1979; Grinstein et al., 1980; MacKnight et. al., 1980). Similar enzyme distributions in S. mansoni would therefore be indicative of polarization and epithelial functioning. Varying amounts of the surface of S. mansoni have previously been removed by several workers (Kusel, 1972; Cordeiro and Gazzinelli, 1979; Oaks et. al., 1981; Simpson et. al., 1981a), but there has been no attempt to isolate apical and basal membranes or to demonstrate their functional dissimilarity - an essential prerequisite of transepithelial transport.

This chapter describes initial experiments designed to strip sequentially the apical and basal membranes from the intact worms, with the ultimate aim of locating marker enzymes and showing functional polarization of the surface syncytium.

2.2 MATERIALS AND METHODS

2.2.1 Maintenance of Schistosoma mansoni in the laboratory

Schistosoma mansoni (Puerto Rican strain) was maintained in our laboratory by routine passage between the molluscan intermediate host, Biomphalaria glabrata and Syrian hamsters, which served as the definitive host. The tropical snails, B. glabrata, were bred in our laboratory and supplemented by monthly shipments of infected snails from the Center for Tropical Diseases, Lowell, Massachusetts. They were housed either in large aerated aquaria or in flat horticultural

bedding trays, supplemented with a spoonful of mud, taken from deposits on the banks of the River Thames (London, Ontario) then sieved and autoclaved. Trays were cleaned twice weekly and refilled with dechlorinated tap water. Snails were fed Romaine lettuce as required. Optimal breeding conditions were found to include a room temperature of approximately 75°F and constant overhead fluorescent lighting. Eggs deposited on the walls of the aquaria were allowed to develop and hatch without interference. Styrofoam floats were provided for egg deposition in the trays and the floats, with eggs attached, were then transferred to the aquaria for hatching.

Groups of 50-100 young snails (3-5 weeks of age) were infected with S. mansoni by placing them in small petri dishes, containing 10 miracidia per snail in minimal volumes of dechlorinated tap water, for 3 hours. Infected snails were maintained in trays and examined for the presence of mother sporocyts 14 days later. Thirty days after infection the snails were classified as patent and kept under subdued light. Twice weekly patent snails were transferred to beakers and allowed to dry for 15 minutes before being left for 2 hours in small volumes of water under direct fluorescent light. Cercariae, larval schistosomes, emerge from the snails in response to the light and swim actively in the water. The cercarial suspension was decanted into test tubes on ice and a few minutes later the cercariae were pipetted from a concentrated layer on the surface into a small beaker. Aliquots of the suspension were transferred to depression slides and mixed with a drop of iodine in 50% ethanol. Cercariae were counted with the aid of a dissecting microscope, providing known concentrations of cercariae for hamster infections.

Syrian hamsters (Charles River Canada Ltd., Quebec or from stocks bred in the zoology animal care facilities, U.W.O.) were maintained with constant access to Purina rat chow and water. Young male hamsters (approximately 6 weeks old) were infected with S. mansoni by subcutaneous injection of 1,000 - 1,500 fresh cercariae in less than 1 ml of water. Adult S. mansoni were recovered from the hamsters 40 - 45 days post infection. Following cervical dislocation, the body cavity was exposed by ventral incision and part of the rib cage was removed to reveal the heart. The hepatic portal vein was cut close to the liver and worms perfused out with Krebs Ringer Phosphate (KRP) at pH 7.4, delivered to the left ventricle by a 23 gauge needle attached to a Manostat varistaltic pump, generating 10 lbs/inch^2 in perfusate pressure. Eluted worm pairs were either picked up directly with a flat spatula or were collected on fine plastic mesh. Gentle "milking" of the small mesenteric veins removed those worms not eluted by perfusion. Throughout the necropsy procedure eluted worms were maintained in KRP on ice and prior to experimental manipulation they were washed in several changes of fresh KRP and any blood clots removed.

Granulomatous livers were excised from the hamster carcasses and stored overnight in KRP at 4°C. The following day the livers were homogenised in KRP or 0.8% saline using a Virtis blender. The resulting homogenate was passed consequetively through 20 and 60 mesh/cm sieves to remove larger debris. Granulomas were repeatedly left to settle, the supernatant discarded and fresh saline added. Washed eggs were poured into a volumetric flask, covered in tin foil up to 2 inches from the top, which was then filled with dechlorinated tap water and set under a fluorescent light. Miracidia hatched from

Figure 2.1 Maintenance of the life cycle of S. mansoni in the laboratory.

1) Six week old male Syrian hamsters are infected by subcutaneous injection of 1,200 - 1,500 cercariae in dechlorinated tap water.

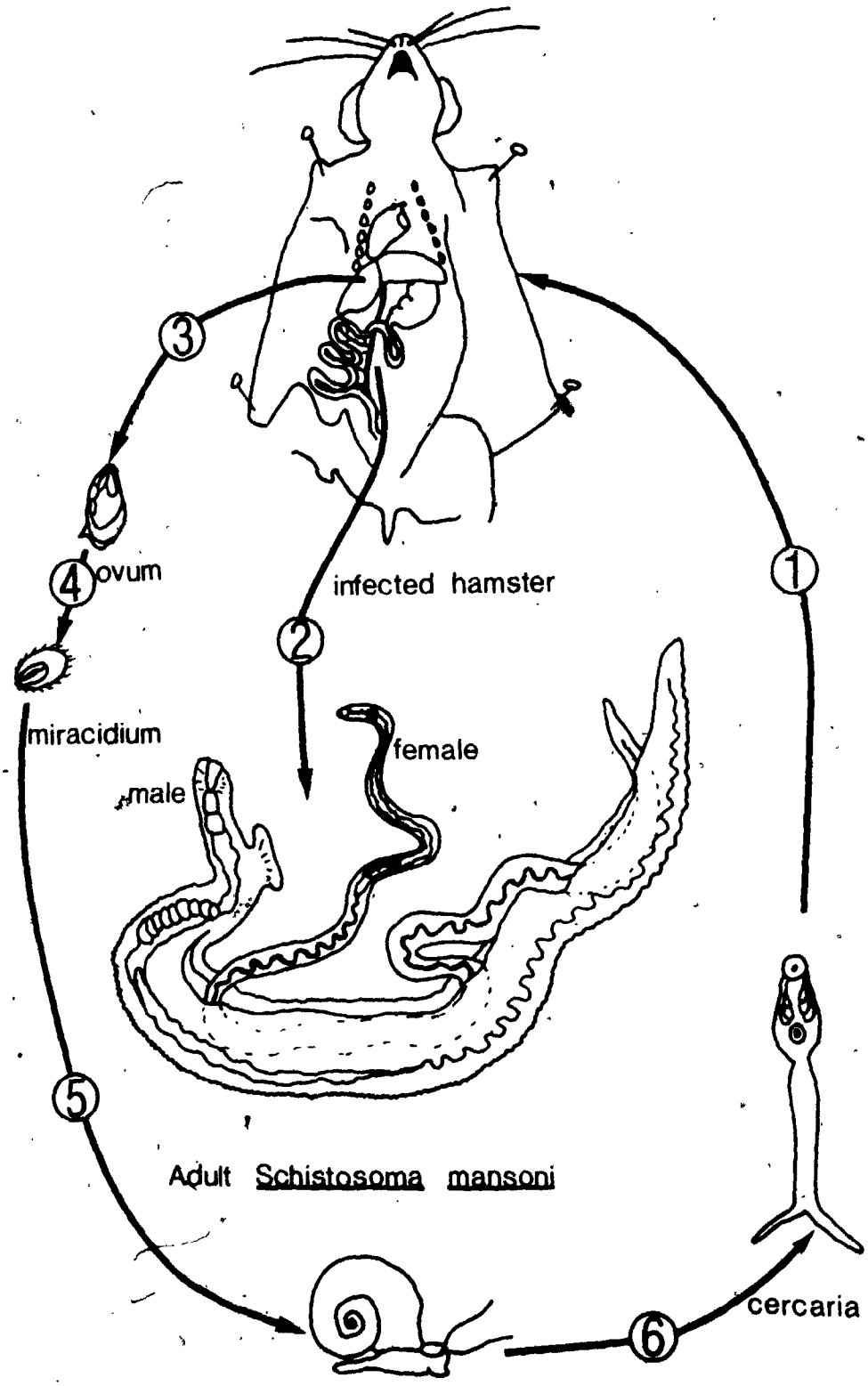
2) 40 - 45 days post-infection the hamsters are killed by cervical dislocation and adult worm pairs are perfused out of the cut ends of the hepatic portal vein.

3) Eggs are sedimented from homogenised livers and washed in Krebs Ringer Phosphate.

4) The ciliated larvae, miracidia, hatch from the eggs in response to reduced salinity.

5) Miracidia are concentrated in a light beam and allowed to penetrate the intermediate host - tropical snails Biomphalaria glabrata.

6) One month later exposure of infected snails to bright light stimulates cercariae to escape from their host and swim vigorously in the water. Cercariae are then concentrated for hamster infection.



Biomphalaria glabrata

the eggs and swam to the surface (by a positive phototaxis), from where they were pipetted into a small petri dish. Miracidia were concentrated with a narrow beam of light and counted under a dissection microscope. These miracidia were then available for the infection of a new batch of snails. The life cycle of S. mansoni in our laboratory is summarised in Figure 2.1.

2.2.2 Membrane removal

Adult worm pairs of S. mansoni were obtained from hamsters, infected 40-45 days previously, by cardiac perfusion with cold KRP (pH 7.4) as described above. After several rinses in ice cold KRP the worms were counted under a dissection microscope and any that were damaged or attached to blood clots were discarded. At least 500 washed worm pairs were incubated in membrane disruption fluid in a shaking water bath (2 oscillations/second) at 4°C, for 5 minutes. The membrane disruption fluid contained 0.5% saponin (Sigma Chemical Co.) and 3.0% $CaCl_2$ in 0.1 M Tris-sucrose buffer (pH 7.4), the complete fluid being 350 mOsM (Instrumentation Laboratories osmometer). Following incubation the worms were vortexed for 30 seconds at high speed and filtered through a fine plastic mesh. The fluid resulting from the first incubation was stored on ice and the worms placed in fresh membrane disruption fluid for a second incubation of 7 minutes. Worms from the second incubation were vortexed and filtered as before. After each digest several worms were removed and stored on ice for electron microscopy.

The suspensions from both incubations were spun at 500xg for 10 minutes to remove larger debris. Membranes from both fractions were pelleted at 20,000 x g for 1 hour at 4°C. For enzyme analysis the membrane pellets were resuspended in Tris buffer.

2.2.3 Electron microscopy

Untreated S. mansoni adults and samples of carcasses from the first and second incubations were processed for electron microscopy. Membrane pellets and worms were fixed for 3 hours at 4°C in 2.5% glutaraldehyde/0.05 M cacodylate buffer with 3% sucrose and 2.0μM calcium acetate. After several rinses in a solution containing 0.05 M cacodylate and 12% sucrose the fractions were postfixed at 4°C in 1.0% osmium tetroxide in 0.05 M cacodylate for 1.5 hours, washed in distilled water and left in 50% saturated aqueous uranyl acetate overnight. Specimens were washed repeatedly and dehydrated through an ethanol series to propylene oxide. They were then infiltrated in propylene oxide: resin mixtures and embedded in Araldite 6005.

Thin sections (600 - 1,000Å) were cut, with glass knives, on a Philips OMU 3 ultramicrotome. Grids were stained with uranyl acetate and Reynold's lead citrate and examined in a Philips 201 electron microscope.

2.2.4 Membrane Lipid Extraction

Prior to lipid extraction membrane pellets were usually washed once, resuspended in Tris-sucrose and either extracted immediately or frozen until required. Lipids were extracted following the method of Bligh and Dyer (1959). The membrane preparations, in Tris-sucrose, were added to 20 volumes of a 2:1 chloroform-methanol mixture and stirred for at least 30 minutes. After filtering through Whatman number one filter paper (previously washed in chloroform) the extract was shaken with 1/5th volume of de-oxygenated deionised distilled water (boiled and cooled in a stream of nitrogen). The suspension was left to settle in a separatory funnel and the lower layer drawn off,

whilst the top layer was washed in chloroform-methanol 2:1. The lower layer was decanted, combined with the previously removed layer and taken to dryness in a flash evaporator (Buchler Instruments). The extract was then resuspended in chloroform-methanol and either used immediately or stored at -20°C.

2.2.5 Thin Layer Chromatography of Membrane Phospholipids

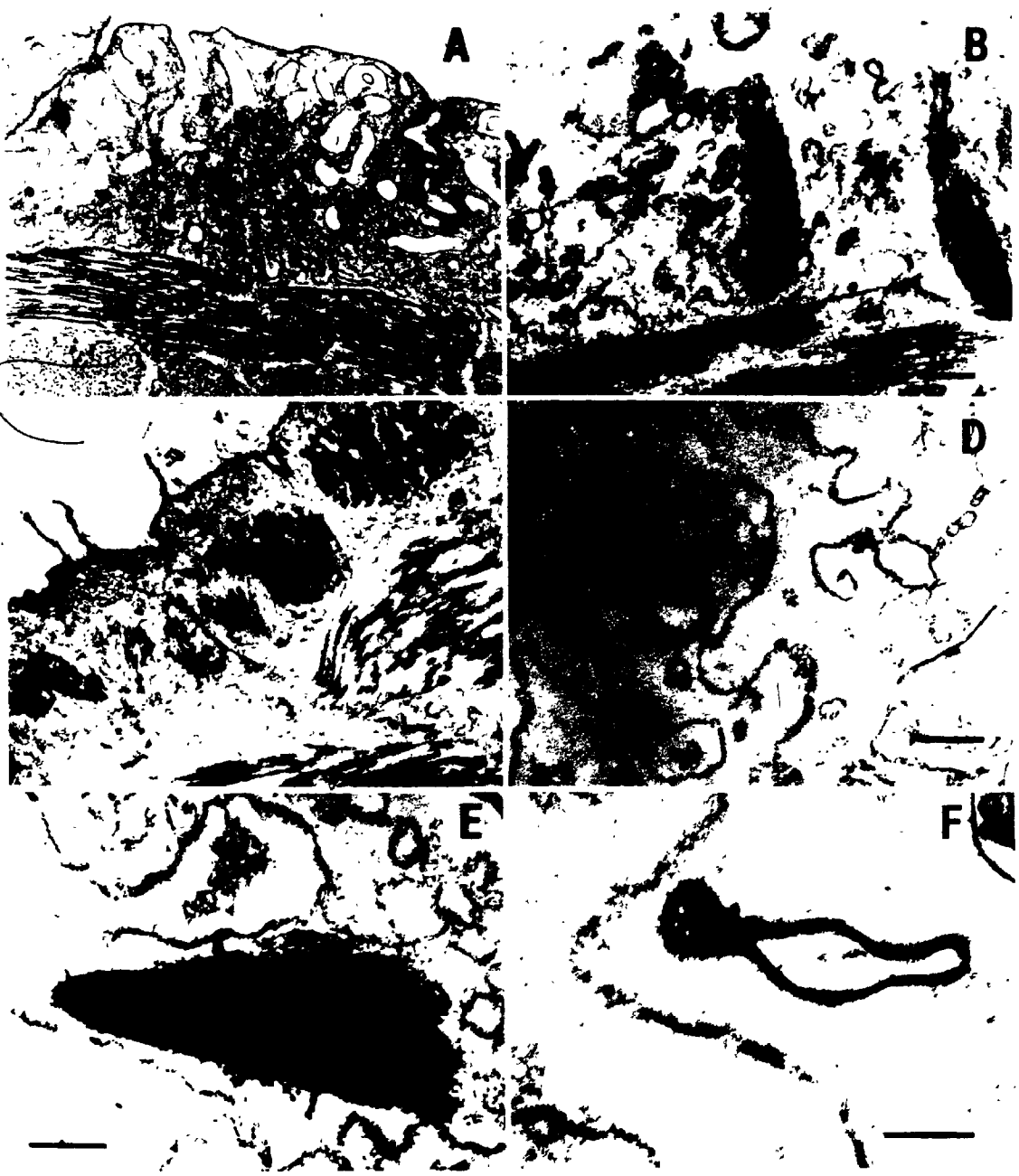
Phospholipid classes were separated by means of two-dimensional thin layer chromatography on silica gel G Rediplates (Fisher Scientific). Immediately before use the plates were activated at 110°C for one hour. Samples, in minimal volumes of chloroform-methanol were spotted on the lower left hand corner of the plate, whilst standards were spotted on the lower right. All applications were performed under a stream of nitrogen gas. A variety of solvent systems were tested and the following were found to yield optimal separation. The first dimension was run in chloroform-methanol - 28% ammonium hydroxide (130:70:10) and the second dimension in chloroform - acetone-methanol-acetic acid - water (100:40:20:20:10) or butanol-acetic acid - water (130:40:40). Between the first and second dimensions the plates were dried with nitrogen and a second set of standards applied. Spots were visualised with iodine vapor or by spraying with sulphuric acid, followed by charring on a hot plate. Major phospholipid classes were identified by comparison with the standards included on each plate. Ninhydrin spray was utilised for phosphatidylethanolamine identification.

2.3 RESULTS

Electron micrographs of the surface epithelial syncytium of S. mansoni prior to membrane removal and after removing the apical and basal membranes, with associated pelleted material, are shown in figure 2.2. Prior to membrane removal the syncytium is bounded by an apical and basal membrane and contains two membrane-bound inclusion bodies - the multilamellar and discoid bodies (Figure 2.2a). Following the first exposure to the saponin solution the apical membrane was removed, leaving intact much of the syncytial material, including the spines and the basal membrane (Figure 2.2b). The pellet derived from the first saponin exposure contained mostly membrane material (Figure 2.2d), which was characterised by having two bilayers (Figure 2.2f), a feature consistent with the apical membrane being a heptalaminate barrier. Following the second saponin incubation, the surface of S. mansoni was devoid of syncytial material and the pellet was characterised by the presence of spines and membrane material (Figure 2.2e). These results, when considered with enzyme data derived from these pellets, Appendix II, (Podesta and McDiarmid, 1982) are consistent with the epithelial nature of the syncytium and no further purification of the membrane material was attempted.

Phosphlolipids identified in the apical membrane included lysolecithin, sphingomyelin, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine and a spot co-migrating with bovine cerebrosides. Phosphatidylcholine and phosphatidylethanolamine appeared to be the most abundant, based on size and density of the spots. Lysolecithin, sphingomyelin, phosphatidylcholine and phosphatidylethanolamine were found in the preparation of basal membrane.

Figure 2.2 Electron micrographs of the surface epithelial syncytium of S. mansoni prior to and after sequential removal of the apical membrane complex and basal membranes. A, the normal surface prior to incubation in the membrane disruption fluid; B, surface after the first incubation in the membrane disruption fluid. The apical membrane and some syncytial components are no longer present. C, surface after the second incubation in the membrane disruption fluid. The entire syncytium has been removed, whilst the basal lamina remains intact. D and F, membrane pellets resulting from the first incubation; E, membrane pellet after the second incubation. A, B, C bar represents 1.0 μm; D, E, F bar represents 0.1 μm.


A
B
D
E
F

Following this cursory examination of phospholipid classes it was decided that further purification of membrane fractions, especially the apical membranes, would be desirable before further characterisation or quantitation was carried out.

2.4 DISCUSSION

The primary aim of this chapter - to obtain separate fractions enriched with apical and basal membranes, has been accomplished. The membrane removal procedure was adapted from Kusel (1972), who employed it to obtain one undefined tegumental fraction. In the present study exposure times were varied until morphological evidence indicated separate removal of apical and basal membranes. Following the procedure detailed previously, the first exposure to saponin was found to remove the apical membrane and approximately half of the tegumental material (Figure 2.2). The basal membrane and spines remained intact. Pelleted material resulting from this exposure consisted predominantly of bilayer material. As most of the membrane in this fraction was composed of double bilayers it must represent the apical membrane complex. Although intact multilamellar bodies were not observed in this fraction, such contamination is likely and would pass unnoticed if the multilamellar bodies were disrupted. We have evidence from other studies that disrupted multilamellar bodies can appear as double bilayer structures (McDiarmid et al., 1982). However, as multilamellar bodies are the presumed precursors of the apical membrane, such contamination should not alter the chemical properties of the fraction. Discoid bodies are the other possible membranous contaminant and few of these were observed in the pellet.

The second exposure to saponin removed the remainder of the syncytium, including the basal membrane. Following this procedure the general integrity of the surface was maintained, presumably by the fibrous basal lamina (Figure 2.2). The pellet resulting from the second exposure was more heterogeneous than that obtained from the first exposure. Despite contaminating spines, this fraction consisted primarily of membranous material. The double bilayer appearance, characteristic of the initial pellet, was not found in this fraction.

Purity of the membrane fractions was further quantitated by assaying for ATPase activity: The results of this work are presented in detail in Appendix II (Podesta and McDiarmid, 1982). ATPases are the most commonly used enzyme markers of epithelial membranes. In particular the oubain sensitive Na^+ - K^+ -activated, Mg^{2+} - dependent ATPase has been demonstrated to have a ubiquitous location on the basolateral membranes of transporting epithelia (Grinstein et al., 1980; MacKnight et al., 1980). Ion gradients created by the active extrusion of Na^+ are thought to account for the passive entry of Na^+ across the apical membrane down its concentration gradient (Cereijido et al., 1980), passive transport of non-electrolytes against their concentration gradient (Armstrong et al., 1979) and the transport of water across the epithelial layer (Diamond, 1978). The basal membrane preparation described in this study was found to contain the typical oubain-sensitive, Na^+ -K^+ - ATPase (Podesta and McDiarmid 1982). An ethacrynate-sensitive, Na^+ - Mg^{2+} - ATPase was found to be associated with the apical membrane and probably functions to augment the regulatory cell volume decreasing function of the Na^+ pump ATPase. Previous reports concerning ATPase activity in the schistosome tegument have not localised the activity

to particular membranes (Nechay et al., 1980). Ion dependencies in these studies are also conflicting and suggest that the activity is actually the result of more than one ATPase in the experimental fraction (Cesari et al., 1981).

Saponin was the detergent of choice for membrane removal in this study, as the surface membranes of S. mansoni are known to be susceptible to the solubilising effects of this detergent (Kusel, 1972). Futhermore saponin, at concentrations below the critical micellar concentration, has the ability to enhance ATPase activity. Saponin has even been employed to protect enzyme activity from the more severe effects of other detergents (Skou and Esmann, 1979). The activity of integral membrane enzymes is thought to be stimulated in the presence of saponin by the resulting increased permeability of membrane vesicles to ions and substrates of the catalytic site of the enzyme (Jørgensen, 1975; Hokin, 1981; Stekhoven and Bonting, 1981). Triton X-100 has been used to solubilise schistosome membranes prior to ATPase assays, however this detergent is known to decrease ATPase activity through its action on associated lipids (Hokin, 1981; Stekhoven and Bonting, 1981).

The initial phospholipid characterisation suggests that phosphatidylcholine is the major phospholipid of both apical and basal membranes. Whilst the differences between the two preparations may indicate a greater complexity of the apical membrane, it may simply reflect increased amounts of lipid material in the fraction. The phospholipids detected have all been found previously in whole worm homogenates (Meyer et al., 1970). Further phospholipid quantitation was postponed until fractions of higher purity could be obtained.

In conclusion, apical and basal membranes of the schistosome surface have been separated and their asymmetric properties demonstrated, providing evidence for a transporting epithelial function.

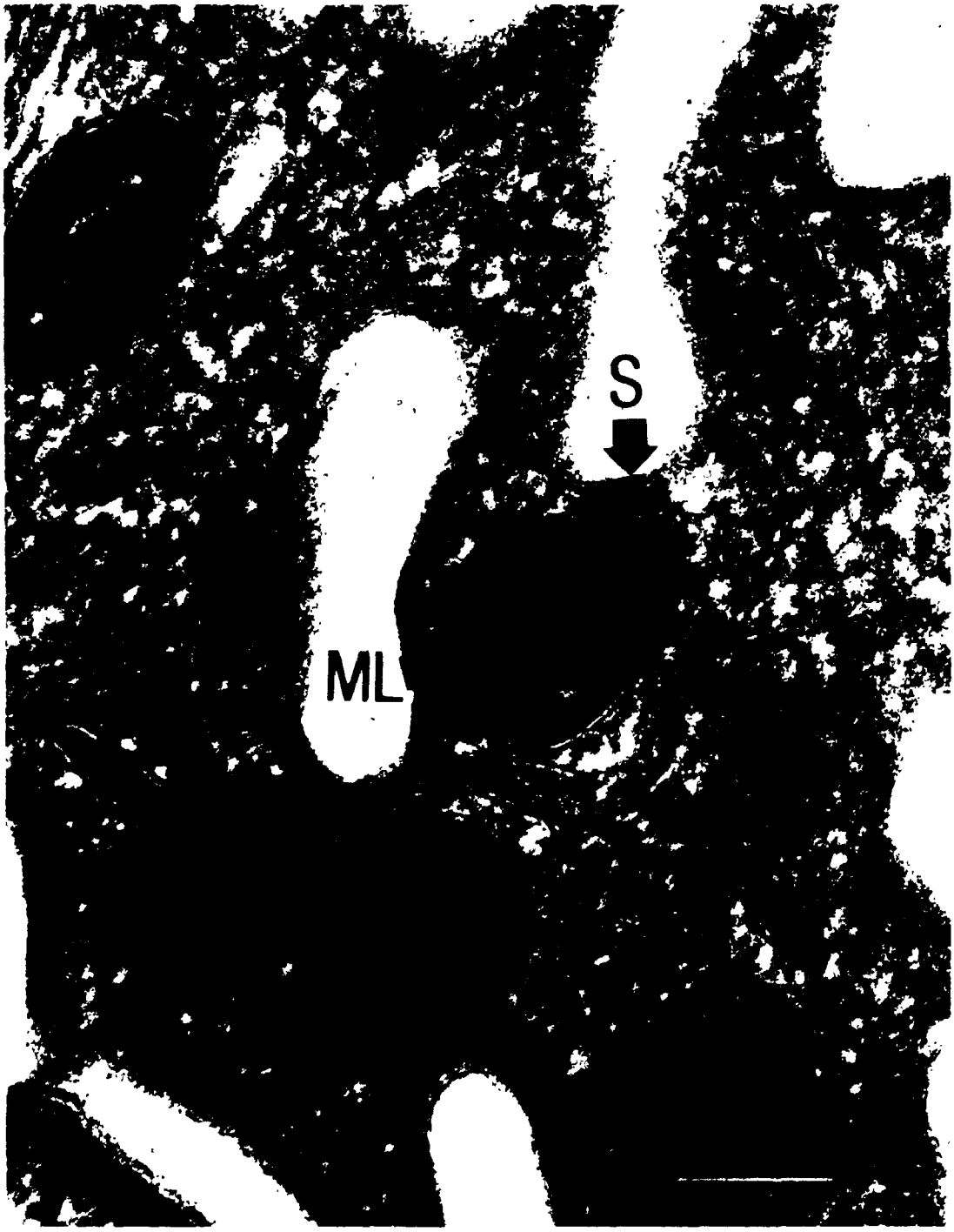
CHAPTER 3

PREPARATION AND PARTIAL CHARACTERISATION OF A MULTILAMELLAR BODY FRACTION FROM SCHISTOSOMA MANSONI

3.1 INTRODUCTION

Schistosoma mansoni is well adapted to its parasitic mode of life and can survive in the host circulatory system for years, despite triggering a specific immune response. Attack by the immune system is directed at the schistosome surface (Rotmans et al., 1981), which has the unusual appearance of a double bilayer structure (Hockley, 1973) and (Figure 3.1). The additional bilayer, external to the plasma membrane, has been referred to as a membranocalyx, as it was thought to be the membranous equivalent of a glycocalyx (Wilson and Barnes, 1974a). However recent evidence suggests that this is not the case and further indicates the presence of a carbohydrate glycocalyx in addition to the outer bilayer (McDiarmid and Podesta, 1983). The term outer bilayer is employed here to refer to the outermost layer. Based on its fixation and staining properties under the electron microscope the outer bilayer and multilamellar bodies (MLB) are considered to be composed primarily of lipid (Wilson and Barnes, 1974a; 1977). The outer bilayer is found only in the blood dwelling flukes and is thought to render them insusceptible to host immune effectors (McLaren and Hockley, 1977). Several mechanisms for this resistance have been proposed, including the binding of disguising host antigens (Smithers and Terry, 1969b), reduced expression of surface antigens, rapid turnover (Wilson and Barnes 1979; Podesta, 1982a), reduced fluidity (Johnson et al., 1982), low adhesive properties (Podesta et al., 1983)

Figure 3.1 Electron micrograph showing multilamellar bodies *in situ* in *S. mansoni*. The multilamellar bodies (ML) are the presumed precursors of the apical surface bilayer complex (S). The function of the other membrane-bound inclusion; the discoid body (D); is not known. Bar represents 0.1 μm.


S
ML

and complement insensitivity (McDiarmid et al., 1983). As the outer bilayer is thought to be of such importance to parasite survival in the immune host, much interest has been centered around its mode of formation and renewal.

Hockley and McLaren (1973) first demonstrated the multilamellar nature of the schistosome surface and suggested that the membranous bodies contributed to the surface, which is continually being broken down and reformed. These membranous bodies have been given several names in the schistosome literature (Morris and Threadgold, 1968; Silk et al., 1969; Smith et al., 1969; Reissig, 1970), but will be referred to here as multilamellar bodies (MLB). We use MLB for the schistosome organelles since this term has been used extensively, for years, to refer to organelles of similar structure and proposed function in type II pneumocytes of mammalian lung. Lamellar structures are common cellular inclusions and may result from one of several different mechanisms (Mason and Williams, 1980). They may arise from autophagy, or the catabolism of intracellular organelles, as is common in cellular injury or nutritional stress. Cells degrade phospholipids slowly and the resulting products may take on a lamellar appearance. Abnormal degradation of specific lipids in cases of lipid storage disease results in lamellar inclusions. Phagocytised phospholipids may also appear lamellar, as in alveolar macrophages. The MLB of type II pneumocytes from mammalian lung represent specific synthesis and storage sites of phospholipid prior to its secretion (Tsao, 1980).

The proposed role of schistosome MLB in secretion of the outer bilayer bears some striking similarities to the mechanism of surfactant production in mammalian lungs. In type II pneumocytes

membrane-bound MLB originate in the golgi complex, fuse with the plasma membrane and release their lamellate contents onto the alveolar surface, where they spread out to form a thin organised layer of lipid surfactant (Massaro and Massaro, 1976). Evidence for a similar role for schistosome MLB comes mainly from transmission and freeze fracture electron microscopy studies of MLB (Torpier et al., 1977; Wilson and Barnes, 1977). Occasionally MLB have appeared to be fusing with the plasma membrane at the base of the surface pits (Bruce et al., 1970), into which they are thought to release their lamellate contents (Torpier et al., 1977). The appearance of MLB in the tegument is concurrent with the formation of the outer bilayer on the surface of developing schistosomula (Hockley, 1973). The staining properties of MLB and the outer bilayer are similar in that both are osmiophilic and stain with uranyl acetate and permanganate, which suggests that both contain phospholipids (Wilson and Barnes, 1974a). Phospholipids are also the major constituent of lung surfactant (Rooney et al., 1975).

There have been two main lines of investigation into the function of schistosome MLB. The path of radiolabelled substrates has been followed and the effects of compounds that inhibit or stimulate secretion have been studied (Wilson and Barnes, 1974b). Results of labelling studies have been inconclusive, partly due to the lack of specific incorporation into MLB or the outer bilayer. Suggestions of a role for MLB in phagocytosis have arisen from experiments where external horseradish peroxidase labelling showed incorporation into MLB (Smith et al., 1969). Incubation of worms with ouabain resulted in a decrease in the number of MLB in the tegument and a flattening of the pitted surface, suggesting a dynamic relationship between MLB and surface membrane pits (Wilson and Barnes, 1974b).

A third approach to the problem, employed with lung MLB (Weibel and Gil, 1977) is the biochemical analysis of MLB and the surface layers that they are thought to produce. This approach has been denied to schistosome workers because of a lack of suitable fractionation procedures. Subcellular fractionation of S. mansoni has not been attempted (Wilson and Barnes, 1979).

The aim of this study was therefore to obtain a relatively pure fraction of MLB, using a technique modified from procedures for isolating lung MLB (Hallman et al., 1976), and to perform initial characterisation of the phospholipids and possible enzyme markers of the fraction.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of multilamellar body fraction

Adult worm pairs were obtained from hamsters 40 - 45 days post infection, as described previously and frozen until sufficient numbers were obtained. Multilamellar bodies were prepared following the method of Hallman et al. (1976), with some modifications. Approximately 6 ml, packed volume, of worms were rinsed in 0.1 M Tris buffer, containing 0.3 M sucrose and 0.1 mM EDTA, pH 7.4. A 40% homogenate (volume/volume) was made with the same buffer. Ten ml of the homogenate was layered over a sucrose density gradient, comprising 10 ml of 0.8 M and 2.5 ml of 0.45 M and centrifuged at 86,000 x g for 40 minutes. The interface (2.5 ml) was collected and adjusted to 0.57 M, by adding 0.58 M Tris-sucrose-EDTA (1:10). A 12 ml aliquot of this was layered onto 10 ml of 0.8 M Tris-sucrose-EDTA and centrifuged at 55,000 x g for 120 minutes. Materials floating at the surface and at

the interface were collected separately, diluted with 0.4 M Tris-sucrose - EDTA and centrifuged at 34,000 x g for 30 minutes. The pellets were resuspended in 0.3 M Tris-sucrose - EDTA and centrifuged at 34,000 x g for 30 minutes. The pellets were then either processed for electron microscopy or resuspended for further analyses. Morphological examination of the pellets allowed the technique to be refined and in later runs the sample was adjusted to 0.55 M Tris-sucrose - EDTA before the second spin and the multilamellar body fraction removed from the interface only.

3.2.2 Electron microscopy

Pelleted material was fixed for electron microscopy, *in situ*, as follows. The pellets were fixed overnight, on ice, in 2.5% glutaraldehyde, 0.1 M Sorensen's phosphate buffer (pH 7.4), containing 3% sucrose. After several rinses in 0.1 M phosphate buffer, with 4% sucrose, the pellets were postfixed for 2 hours in 1% osmium tetroxide in 0.1 M phosphate buffer and then washed repeatedly with distilled water. Following washing, the pellets were incubated at 45°C overnight in 50% saturated aqueous uranyl acetate. Pellets were washed and carefully scraped from the test tubes, before being dehydrated through an acetone series (70%, 80%, 90%, 95%, 2 x 100%) for 10 minutes each, then twice in propylene oxide. The pellets were infiltrated in propylene oxide: resin mixtures, embedded in Araldite 6005, evacuated at 20 lb/inch^2, 45^{o}C overnight and hardened at 60 C. Thin sections (600 - 1,000 Å) were cut, with a diamond knife, on a Philips OMU3 ultramicrotome. Grids were stained with Reynold's lead citrate and examined in a Philips 201 electron microscope.

Freeze thaw fractions of S. mansoni tegument were prepared following the method of Kusel (1972). Approximately 300 worm pairs were frozen in 2 ml KRP, by immersion in liquid nitrogen. Immediately on thawing, the worms were removed by filtration on plastic mesh. The remaining suspension was dropped on to carbon shadowed, formvar coated electron microscopy grids and blotted dry from underneath. The preparations were negatively stained by placing a drop of phosphotungstic acid stain (pH 7.4) on the grids, which were again blotted dry. The grids, with no further staining, were examined as before.

3.2.3 Morphometry

To measure the purity of the MLB in the preparation, point counting methods of stereology were used to determine the volume density (V_v) occupied by MLB, contamination and empty space (Weibel, 1979). Empty space was included as the analysis was conducted on a pelleted fraction rather than a solid tissue.

The optimal print magnification for analysis (M*) was 90,000 x. At magnifications much below this it was difficult to distinguish MLB and multilaminate stacks. Micrographs were taken at the lower left hand corner of randomly selected support grids. A grid of dots, spaced 1.4 cm apart, was drawn on acetate sheets and laid over each 8 by 10 inch micrograph. The distance between the dots (d_1) was chosen such that d_1^2 (is greater than) am, where am is the area in cm^2 of MLB at M*. The number of test points required to be applied to each micrograph (PT_1) was determined from $PT_1 = AT_1/d_1^2$, where AT_1 is the micrograph area available for analysis. A total of 252 test points were applied to each micrograph. The number of

micrographs required to form a representative sample (n) was found to be 10, from the formula $n = P*T/PT_1$. P*T is the number of test points needed to satisfy the precision requirement of 4% expected relative standard error, as a percent of the mean. P*T was determined from a nomogram (Weibel, 1979).

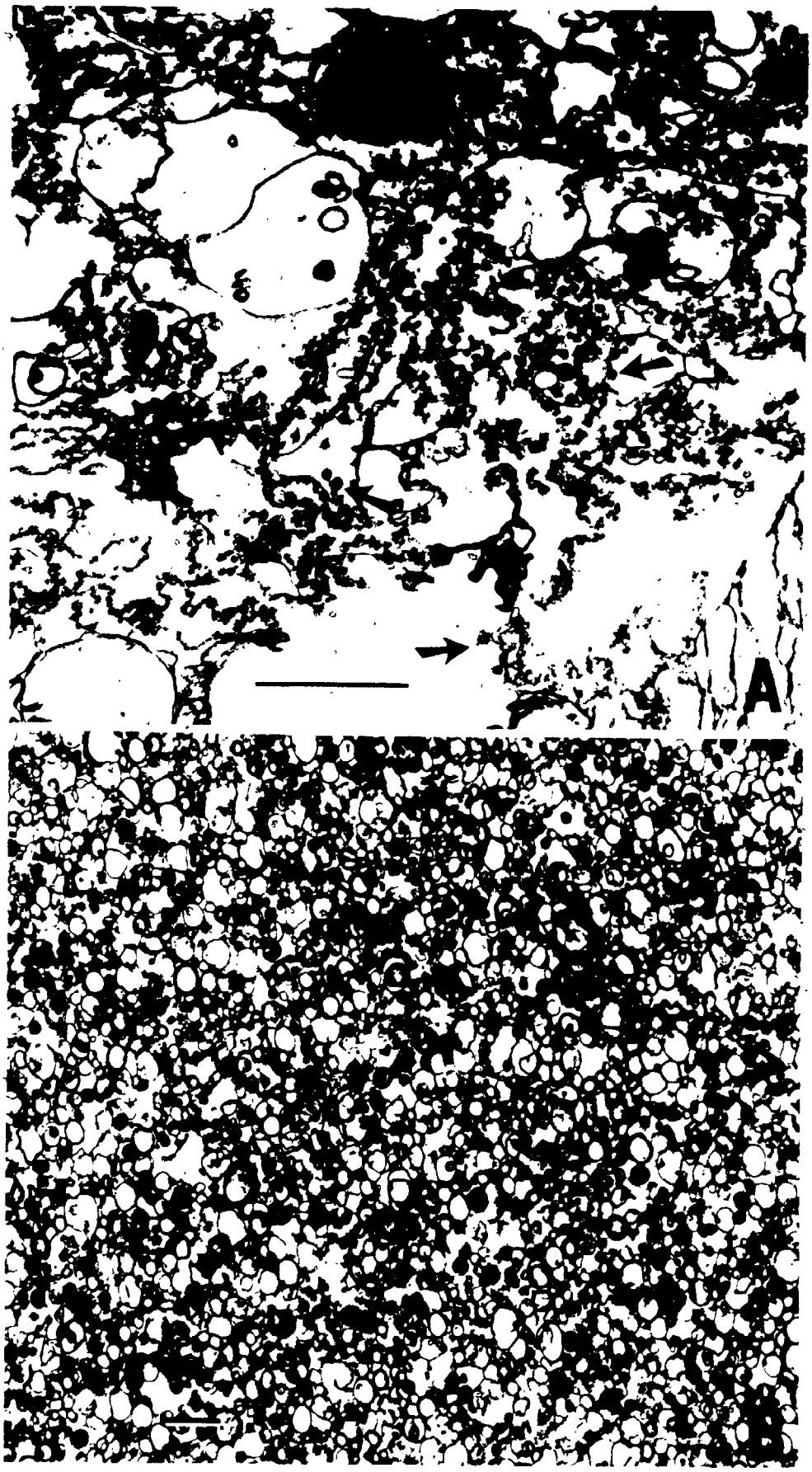
Counts were performed by scoring points overlying a MLB or obviously multilayered stack of bilayers and comparing to scores for contaminating components and empty space. Scores were tallied for each micrograph and the mean of ten micrographs was calculated. Two representative samples were counted, one sectioned from the centre of the pellet and one from the periphery.

3.2.4 Phospholipid analysis

The MLB pellet was resuspended in 0.1 M Tris buffer (pH 7.4) and the lipids extracted by the method of Bligh and Dyer (1959), as described previously. Following extraction the lipids were taken to dryness in a flash evaporator and redissolved in small volumes of 2:1 chloroform-methanol.

Phospholipid classes were separated by means of two-dimensional thin layer chromatography, on silica gel G Rediplates (Fisher Scientific), as described above. Following spraying with H_2SO_4, charred spots were carefully scraped off the plate and the amount of phosphorus in each determined by a modification of the method of Rouser et al., (1970). (See Appendix I). The phosphorus assay was performed in the presence of silica gel, which was removed by centrifugation, before assaying the phosphorus content colorimetrically. Protein was measured by the method of Lowry (1951), using bovine serum albumin as a standard.

Figure 3.2 Electron micrographs showing material pelleted from the sucrose gradient. A, material pelleted from the gradient surface. Arrows indicate neutral lipid droplets and the bar represents 1.0 μm. B, low power view of the multilamellar body pellet, collected from the gradient interface (Bar represents 1.0 μm).


A
B

3.3 RESULTS

The less dense pellet, which floated on top of the sucrose gradient contained almost no MLB, but consisted mainly of small, non-osmiophilic lipid droplets (Figure 3.2a). Loosely organised membrane was also present which, judging by its heptalaminate appearance, probably originated from the worm surface. The denser pellet, from the gradient interface, contained many MLB (Figure 3.2b). Results of the morphometric analysis are shown in Table 3.1. MLB occupied an average of 31% of the pellet volume whilst contamination accounted for 24%, with the remaining volume being empty space. Of the total volume of solid material in the pellet, MLB accounted for 56%, which is a measure of purity of the fraction. Actual purity may be higher than this value as some MLB and stacks of bilayers would not have been recognisable, because of the plane of the section. Large clusters of MLB were membrane-bound (Figure 3.3a), like the aggregations of MLB in the subtegumentary regions of intact cells (Wilson and Barnes, 1974a). Some of the MLB in these vacuoles appeared to be immature since they had homogeneous areas where the lamellae were not fully developed and vesicular inclusions were present.

Many of the osmiophilic membranous vesicles observed at low power were found to consist of lamellate stacks when viewed at higher magnification (Figure 3.3b), suggesting that they were disorganised MLB. The main contamination in the pellet consisted of lipid droplets of various sizes. Unlike the MLB, the lipid droplets did not stain darkly with osmium or uranyl acetate. Small amounts of non-multilaminate membrane might have resulted from surface membrane contamination. No mitochondria were found in the pellet, but the

Figure 3.3 Electron micrographs showing views of the multilamellar body pellet. A, membrane-bound cluster of multilamellar bodies, ranging from the immature homogeneous stage (I) to the more fully formed lamellate type (F). B, multilaminate stacks of bilayers (M). Bars represent 1.0 μm.


A
I
F
B
M

Figure 3.4 Electron micrograph showing the apparent projection core of multilamellar bodies. Arrows indicate the apparent projection core. (Bar represents 0.5 μm).

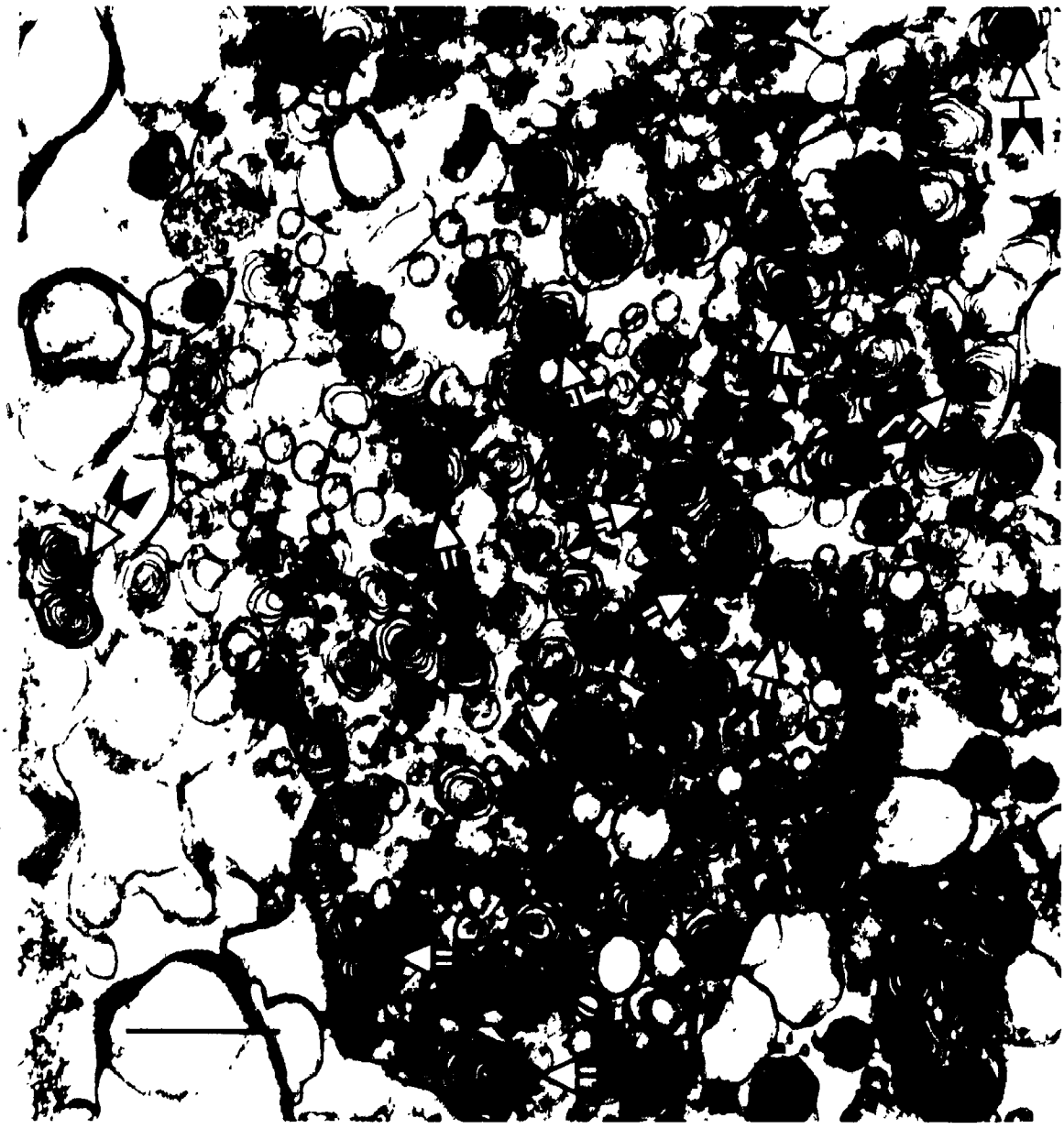


Figure 3.5 Electron micrograph showing a negatively stained preparation of multilamellar bodies. Arrows indicate the apparent projection core of the multilamellar bodies (Bar represents 0.1 μm).

TABLE 3.1

Morphometric analysis of MLB fraction obtained from Schistosoma mansoni.

	% V_v occupied by MLB	% V_v occupied by contamination	% V_v occupied by by empty space
Pellet centre	28	24	48
Pellet periphery	34	25	41
Mean[a]	56	44	44

a Mean values for %V_v occupied by MLB and contamination exclude values for %V_v occupied by empty space.

TABLE 3.2

Protein and phospholipid composition of the multilamellar body fraction.

Substance	Total amount in pellet (µg)[a]
Phosphorus	26 ± 2.39
Phospholipid[b]	772 ± 71.84
Protein	467 ± 19.35
Phospholipid:protein ratio	1.6:1 ± 0.08

a Figures represent mean values ± standard error (n = 3).

b Phospholipid values were derived by multiplying the phosphorus readings by 30. This figure was determined from phosphorus assays on known amounts of standards on thin layer chromatography plates.

TABLE 3.3

Phospholipid classes present in the multimellar body fraction, expressed as a percentage of total phospholipid.

Phospholipid	% of total phospholipid[a]
Phosphatidylcholine	57 ± 2.67
Phosphatidylethanolamine	17 ± 0.66
Sphingomyelin	9 ± 0.58
Lysophosphatidylcholine	9 ± 1.16
Cerebrosides	present

[a] Percentages represent mean value ± standard error (n = 3).

occasional discoid body was observed.

The average size of the MLB was 200 - 300 nm. The width of the inner lamellae was about 7nm, although where they were closely apposed the width appeared less. Spacing between the lamellae varied greatly, from 3nm to 40nm. Where these spaces were narrow, they had a dark fuzzy appearance, rather than that of an empty space between two clearly defined interfaces. The MLB had a trilaminate limiting membrane.

Many of the MLB examined showed a definite protrusion, or area, where the lamellae came together to form a disorganised focus (Figure 3.4). To determine whether this phenomenon was artifactually created during the fractionation and microscopy procedures a freeze-thaw fraction was prepared. This preparation was viewed with negative staining, which omits any fixation or dehydration steps. MLB were located in this preparation (Figure 3.5). Almost all of these MLB displayed a definite core-like area, where the lamellae appeared to converge and exhibited a different, possibly hexagonal, patterning.

The MLB pellet had a phospholipid: protein ratio of 1.6:1 (Table 3.2). The percentage composition of the phospholipid classes can be seen in Table 3.3.

3.4 DISCUSSION

Unlike lung MLB, MLB from S. mansoni did not float on top of the discontinuous sucrose gradient. Collection of MLB at the gradient interface suggests that the MLB of S. mansoni are more dense. These results are consistent with the lower phospholipid-protein ratio of 1.6:1 found for S. mansoni MLB. Phospholipid-protein ratios reported for lung MLB fractions range from 1-12:1 (Page-Roberts, 1972;

Clements and King, 1976). It appears that the lung MLB fractions have a higher phospholipid content and therefore a lower density.

Morphometric analysis revealed that at least 56% (by volume) of the solid material in the pellet was MLB. A major part of the 44% contamination consisted of round droplets, which were not highly osmiophilic or densely stained with uranyl acetate, indicating that they were probably neutral storage lipids (Gil and Weibel, 1970).

Some similar problems have been encountered in the visualisation of lung and schistosome MLB and their surface lining layers. Weibel and Gil first demonstrated the surfactant layer in 1968 by employing lung perfusion and uranyl acetate post fixation. Prior to the use of uranyl acetate as a fixative the MLB and outer bilayer of S. mansoni were poorly visualised with routine glutaraldehyde/osmium fixation (Hockley and McLaren, 1973). This suggests that the schistosome MLB and outer bilayer, like those of lung (Suzuki, 1982) have a high proportion of saturated lipids. Saturated phospholipids are not expected to be well preserved by standard fixation procedures in electron microscopy (Dermer, 1969), since they are not osmiophilic due to the absence of ethylene bonds of cis-unsaturated lipids, which reduce O_sO_4 to black O_sO_2 (Gil and Weibel, 1970). Uranyl acetate is useful for fixing lipid-rich tissues and it helps to avoid loss of phospholipids during extraction (Gil and Weibel, 1970). A protracted uranyl acetate stain (Locke and Huie, 1980) was utilised in this study to obtain maximal staining of phospholipids. Acetone dehydration was employed in processing the pellet for electron microscopy as ethanol tends to extract phospholipids, even after fixation (Hallman et al., 1976). Finley et al. (1968) found ethanol dehydration to decrease the volume of their

lung MLB pellet by 60 - 65%. In addition, ethanol dehydration has been found to drastically alter the appearance of lung MLB and surfactant layers (Douglas et al., 1975; Stratton, 1977). The large empty or homogeneous spaces, previously reported within schistosome MLB (Wilson and Barnes, 1974a), probably represent similar artifacts.

In sections of the pellet many of the MLB exhibited an area showing striking similarities to the projection core of lung MLB (Stratton, 1978). The lamellae, as in the lung, appear to originate from this area. This was not an artifact of preparation as it was clearly visible on MLB from freeze-thaw preparations, which were not fixed prior to viewing (Figure 3.5). The apparent projection core of S. mansoni MLB may represent a growth zone of the lamellae, as in the lung. Alternatively it could be the point of fusion with the plasma membrane, from where the lamellae flow out to form the outer bilayer.

It is of interest at this point to consider the possible developmental sequence giving rise to schistosome multilamellar bodies. The developmental pattern of lung MLB has been traced by autoradiography and has been found to involve a transition from endoplasmic reticulum, to golgi buds, to multivesicular bodies (MVB), to mainly homogeneous MLB with a few lamellae, to MLB packed with lamellae (Massaro and Massaro, 1976). All of the latter stages can be seen in schistosome MLB and the more homogeneous MLB are probably immature, as they are found only in the cell bodies. Stressing the surface of S. mansoni by immune attack results in the appearance of multivesicular-like bodies in the tegument (Simpson and McLaren, 1982). These may represent immature MLB which are released into the tegument as a result of rapid surface membrane turnover to repair the damaged surface.

The analogy between lung and schistosome MLB might be criticized on the grounds that lung surfactant is a very specialised substance with the specific function of reducing surface tension at an air-liquid interface when the surface area is reduced (Hoffman, 1972) - a function hardly applicable to the schistosome surface. However, on examining the phylogeny of lung MLB and surfactant it becomes apparent that surfactant evolved from a covering originally designed to offer protection to exposed cell surfaces (Goniakowska - Witalińska, 1980). The goldfish swim bladder for instance has MLB that produce a protective surface layer, with no surfactant function whatever (Pattle, 1976). The presence of MLB as vehicles for secretion of a protective lipidic covering for cell surfaces at exposed interfaces probably represents a more general phenomenon in animal cell biology.

Since little is known of the biochemistry of S. mansoni organelle fractions (Wilson and Barnes, 1977), it was difficult to verify fraction purity biochemically. However, phosphatidic acid phosphatase (PAPase), utilised as a marker for lung MLB fractions (Meban, 1972; Spitzer et al., 1975), was detected in the pellet (McDiarmid et al., 1982; Rahman and Podesta, 1982). This enzyme had not been described previously from S. mansoni. The high level of activity of this enzyme suggests that MLB are not merely reservoirs of presumptive surface material, but are actively engaged in the synthesis and modulation of this material. PAPase is a specific enzyme with a central and probably regulatory role in phospholipid metabolism (Schultz et al., 1974). It hydrolyses phosphatidic acid to form diacylglycerol - a precursor of phospholipids. As a result of

the extremely large number of worms required to make MLB pellets the pellet could not be screened for other enzymes.

Phosphatidylcholine was found to be the most common phospholipid in schistosome MLB. Phosphatidylcholine is also the major phospholipid in whole worms (Meyer et al., 1970; Young and Podesta, 1982) and in lung MLB (Rooney et al., 1975; King and MacBeth, 1981; Suzuki, 1982). Phosphatidylethanolamine and lysophosphatidylcholine were abundant, at 17% and 9% respectively. Both these phospholipids have been implicated in membrane fusion (Lucy, J.A., 1970; Kolber and Haynes, 1979; Weltzein, 1979) and may facilitate fusion between MLB and the surface plasma membrane.

The probable high saturation of the outer bilayer and MLB phospholipids, discussed earlier, is of interest. Saturation of the fatty acids is a prerequisite for the surface active nature of lung surfactant, as it increases the close packing of the phospholipids within the bilayer (Shinitzky and Barenholz, 1978). If the outer bilayer has a similar high surface tension, then its secretion would be expected to smooth out small irregularities (Gil and Weibel, 1970). Release of the surface active outer bilayer at the base of the membrane pits would result in the flattening out of that pit, thereby presenting fresh material to the worm surface. Schistosomes are known to bind extraneous proteins to their surface (Goldring et al., 1976), which would reduce the surface activity of the outer bilayer and cause pits to form.

The goal in the future studies on schistosome MLB might be to determine the regulatory mechanisms which control the rate of surface turnover. Control could be exerted at three main stages, at the levels of synthesis, transport to the surface and secretion (Podesta,

CHAPTER 4

SEQUENTIAL REMOVAL OF OUTER BILAYER AND APICAL PLASMA MEMBRANE FROM THE SURFACE EPITHELIAL SYNCYTIUM OF SCHISTOSOMA MANSONI

4.1 INTRODUCTION

The human immune response to S. mansoni is directed against surface membrane antigens of the schistosomular and adult stages of the parasite. (Murrell et al., 1977; Rotmans and Mooij, 1982) Future efforts in diagnosis and control of the disease and elucidation of immune evasion mechanisms depend on standardised isolation techniques which will allow the chemical characterisation of the relevant antigens (Simpson and Smithers, 1980; Brink, 1982).

There are three main lines of investigation which have sought to characterise the schistosome surface. First, external labelling procedures, employing lectins or impermeant iodinated markers, have been utilised to characterise surface proteins and carbohydrates. Problems encountered with this technique include low levels of surface labelling (Hayunga et al., 1979), inaccessibility of membrane proteins, dissociation of label, possible penetration of label, labelling of non-specifically adsorbed proteins (Hayunga, 1982; Shah and Ramasamy, 1982) and exposure of parasites and laboratory personnel to gamma radiation.

Second, the prospect of characterising and isolating surface antigens by monoclonal antibodies has arisen (Taylor and Butterworth, 1982; Strand et al., 1982), however at present the antigens involved are ill-defined as the antibodies are raised to heterogeneous preparations. Immunisation with a characterised surface membrane

preparation would presumably allow a much higher yield of monoclonals with specificities restricted to the schistosome surface.

Third, there have been several previous attempts to isolate the surface membranes of S. mansoni, but they have removed varying and often ill-defined amounts of the tegument down to the basal lamina. Kusel (1970; 1972) removed the surfaces of cercariae, schistosomula and adults with 1% Teepol, 1% Tween 80, saturated digitonin, saponin and calcium chloride and freeze thawing. Other workers have also used these procedures (Kusel and MacKenzie, 1975; Cordeiro and Gazzinelli, 1979) which severely disrupt and remove the entire tegument, so that the composition and function of individual membranes within the tegument could not be determined. This is also true for the method of Oaks et al. (1981) employing Triton X-100, where the entire tegument, including parts of the basal membrane were removed. Indeed the levels of Triton X-100 used have been shown to cause muscle damage and inactivation in the subtegumental layers (Depenbusch et al., 1982). Ill-defined surface fractions have also been released by hypertonic potassium chloride (Murrell et al., 1977; Rotmans and Mooij, 1982) and with another detergent Nonidet P40, which releases membrane proteins, (Dissous et al., 1981; Brink, 1982; Hayunga et al., 1982). Simpson et al. (1981) claim to have obtained a preparation highly enriched in the apical membrane complex by incubating the worms in a balanced salt solution at 37°C for five minutes and subsequently pelleting the medium. The purity of this preparation is apparent from its list of contents which include vesicles, red blood cells, MLB, discoid bodies, mitochondria, nuclei and ribosomes.

The studies described above appear to have removed the entire tegument and have not therefore allowed characterisation of the apical membranes. As discussed in Chapter 1, the schistosomes are unusual amongst parasitic flatworms in possessing an additional bilayer on the apical membrane, which has been implicated in protection from immune effectors (Perez and Terry, 1973). The outer bilayer may be involved in various postulated disguise mechanisms (Clegg et al., 1971; Damian et al., 1973; McLaren et al., 1975), which have recently been questioned (Dessein et al., 1981). It may also function as a filtering device, affording a protective covering to the underlying plasma membrane, as in gram negative bacteria (Kanai and Kondo, 1979). Gram negative bacteria also possess an additional bilayer external to the plasma membrane and the hydrophobic - hydrophilic balance of the outer bilayer is frequently responsible for the exclusion of certain molecules, especially those of high molecular weight or hydrophobicity (Mannella and Frank, 1982). The outer bilayer is therefore freely permeable to small solutes and ions, but presents an effective barrier to the larger molecular components of the immune response. Rapid renewal of the surface bilayers has also been suggested as a possible means by which S. mansoni avoids the immune response (Wilson and Barnes, 1977). However, proof of surface membrane function rests ultimately on establishing methods for isolating separately and quantitatively the various membrane components of the syncytium, especially the inner and outer bilayers of the apical membrane.

Other than the present work, there have been two brief reports of attempts to separate the outer and inner bilayers from the apical

surface of S. mansoni. Wilson (1981) compared a freeze-thawed preparation, which was taken as representative of the outer and inner bilayers combined, with an outer bilayer fraction, prepared by binding cationised ferritin to the worm surface and then collecting shed material from the medium. Freeze thawing is known to remove the entire tegument (see above) and no proof for the selective shedding of the outer bilayer was provided. Cesari (1981) removed surface membranes from S. mansoni with the aid of polycationic beads, a technique employed for the isolation of plasma membranes in other systems. While this provides a cleaner membrane fraction it does not address the problem of separating outer and inner bilayers.

Despite the previous lack of success it was felt that the separation of outer and inner bilayers from the surface of S. mansoni was not an unreasonable goal. The reasons for this decision were two fold. The syncytial nature of the surface epithelium and corresponding lack of lateral connecting membranes should, theoretically, make it possible to sequentially strip individual membranes from the tegument, without contamination from intracellular organelles. Furthermore, considerable success in bilayer purification has already been achieved in analogous systems, where an outer bilayer exists external to the plasma membrane. Outer and inner bilayers have been separated from gram negative bacteria (Osborn and Munson, 1974; Mizushima and Yamada, 1975; Gmeiner and Schlecht, 1980; Veslemøy et al., 1980), mitochondria (Schnaitman et al., 1967; Gurtubay et al., 1980) and chloroplasts (Siebertz et al., 1979; Jennings et al., 1980).

This chapter describes experiments that establish criteria for the quantitative separation of inner and outer bilayers from the

apical membrane complex of the surface epithelial syncytium of S. mansoni.

4.2 MATERIALS AND METHODS

4.2.1 Sequential bilayer removal

Adult S. mansoni were obtained from 6-week infected Syrian hamsters, by cardiac perfusion with cold Krebs Ringer Phosphate (KRP, pH 7.4), as described previously. Eluted worms were washed several times in cold 0.1 M Tris buffer, pH 7.4, containing 0.2 M sucrose. The initial exposure to digitonin was performed at 0 °C in a shaking water bath (3 shakes/second), with a hypotonic membrane removal fluid consisting of 0.1% digitonin (Sigma Chemical Company) in 0.1 M Tris buffer (pH 7.4) containing 12 mM sucrose. On completion of the incubation the worms were rinsed repeatedly with 0.1M Tris containing 0.2 M sucrose. The combined membrane removal and wash were filtered through fine stainless steel mesh to remove the worms, or alternatively fluid was decanted with a pasteur pipette and the fluid stored on ice. The worms were returned to a container of fresh membrane removal medium and the incubation and washing procedures repeated. Timing of the digest periods was varied, as detailed below. Products from both digests were spun for 10 minutes at 500 x g to remove debris, and membranes subsequently pelleted at 35,000 x g for 60 minutes at 4 °C.

4.2.2 Electron microscopy

A concanavalin A (Con A)-ferritin conjugate, containing 5 mg/ml ferritin, was prepared according to the methods of Temmink et al.

(1975) and dialysed extensively against phosphate-buffered saline (PBS). Five hundred worm pairs were incubated in 4 ml Con A-ferritin conjugate, at 0°C, with 2 shakes/second for 30 minutes. To remove any unbound conjugate the worms were washed six times in ice-cold KRP, with 3 incubations of 10 minutes each. A small sample of labelled worms was processed for electron microscopy. The remaining worms were subjected to two sequential exposures to digitonin solutions of 20 minutes each, as detailed above. To both membrane suspensions equal volumes of 0.1 M Tris containing 80 mM $CaCl_2$ were added before pelleting. The pellets were washed with 0.1M Tris containing 80 mM $CaCl_2$ and respun.

The following samples were processed for transmission electron microscopy, as detailed previously in Chapter 3: untreated control worms, Con A-ferritin labelled worms, labelled worms after the first and second exposures to the membrane removal fluid and membrane pellets resulting from both exposures.

Negative staining was performed, as described previously in Chapter 3, on outer and inner bilayer preparations, freeze-thaw and saponin surface preparations.

4.2.3 [^{3}H] Con A Labelling

Approximately 500 worm pairs were incubated in a shaking water bath (2 shakes/second) at 0°C for 30 minutes, in 2 ml KRP, containing 2 μ Ci [^{3}H] Con A. To remove unbound label the worms were washed extensively with ice-cold KRP, including 3 incubations for 10 minutes each in a shaking water bath. Sequential incubations in the membrane removal fluid were performed as described above. Pelleted membrane fractions were resuspended in 600 μl water and solubilized overnight

in 1.5 ml NCS (Amersham), at 40°C. Carcasses were divided into several vials and similarly solubilised. All vials received 15 ml Scintiverse (Fisher Scientific) and were counted on a Beckman LS-255 liquid scintillation system. The entire experiment was repeated with male and female worms being processed separately.

4.2.4 [^{125}I] Iodosulfanilic acid labelling.

These experiments were designed to find the optimal duration of exposure to digitonin for the sequential removal of outer and inner bilayers from the surfaces of previously cultured worms. The effects of *in vitro* culture were important as future experiments would necessitate membrane removal from worms previously maintained in culture.

Groups of 100 worm pairs were incubated for 2 hours in medium 199, with 5% heat-inactivated human serum, at 37°C in an atmosphere of 5% CO_2. After several washes in ice-cold KRP the worms were labelled for 15 minutes with 0.1 mCi diazotised [^{125}I] iodosulfanilic acid, an impermeant marker, prepared as per New England Nuclear (iodosulfanilic acid, [^{125}I]-labelling kit). Any unbound label was removed with 6 washes in KRP, containing 1% bovine serum albumin and two washes in KRP alone.

A sample of 5 labelled worm pairs was removed from each lot for total radioactivity determinations. The remaining worms were subjected to the sequential digitonin exposure procedures described previously, to separate the outer and inner bilayers. Lengths of individual exposures were varied from 5 to 25 minutes, whilst the time for completing both exposures was held constant at 30 minutes. Worm carcasses and released products were assayed for gamma emission in a

Beckman Gamma Counter.

4.2.5 Enzyme assays

Samples of inner and outer bilayer preparations (pellets and supernatants) were assayed for the following enzymes. ATPase activity was measured for 15 minutes at 37°C according to the method of Ryre (1975). Phosphate liberated from ATP hydrolysis was determined as per Taussky and Shor (1952). Protein determinations were conducted according to Lowry et al. (1951) and Bradford (1976). Phosphatidate phosphatase was assayed as per Rahman and Podesta (1982). Acid and alkaline phosphatase and 5'-nucleotidase were assayed as per Sigma Chemical Company technical bulletins, numbers 104 and 675 respectively. A series of alkaline phosphatase assays was performed with exposure times varying from 5 to 25 minutes, in conjunction with the [^{125}I]-labelling experiments. Lactate dehydrogenase (LDH) activity was measured in the supernatants of the membrane fractions, spectrophotometrically following the method of Bergmeyer et al. (1965) Supernatants were assayed for hemoglobinase activity, by the method of Timms and Bueding (1959).

Phospholipid separation and quantitation was performed as described previously. Data are presented as means and standard errors of slopes and elevations of linear regression lines. Single classification anova analysis of variance, regression analysis and Student t-tests were used in significance testing (Sokal and Rohlf, 1969).

4.3 RESULTS

4.3.1 Electron microscopy

Initial surveys of the pellets, resulting from two 20 minute exposures of fresh worms to digitonin, revealed both pellets to be composed almost entirely of bilayer material (Figure 4.1). Whilst initial exposure times of 15 to 20 minutes worked well for fresh worms, shorter initial exposure times of 5 to 10 minutes were sufficient for membrane removal from worms previously maintained in culture (see alkaline phosphatase results). Incubation with the Con A-ferritin conjugate did not greatly alter the normal appearance of the worm surface and ferritin was bound to the outer bilayer (Figure 4.2). After the first digitonin exposure there was only a single bilayer remaining on the surface, although occasionally parts of the outer bilayer were observed to be still attached. Sheets and whorls of removed membrane were visible in close proximity to the worm surface. Despite loss of membranous material the integrity and general appearance of the tegument was not altered (Figure 4.2).

After the second exposure to digitonin the tegument was more visibly disrupted, but still intact (Figure 4.2). In most areas the surface was no longer bounded by an apical limiting membrane and, although the ground substance of the tegument had a washed-out appearance, the membrane-bound organelles were still intact. Bilayers with bound ferritin particles were very numerous in the outer bilayer fraction following the first exposure, whereas the inner bilayer fraction contained less ferritin (Figures 4.1; 4.3). The gut epithelium was of normal appearance in control and experimental worms.

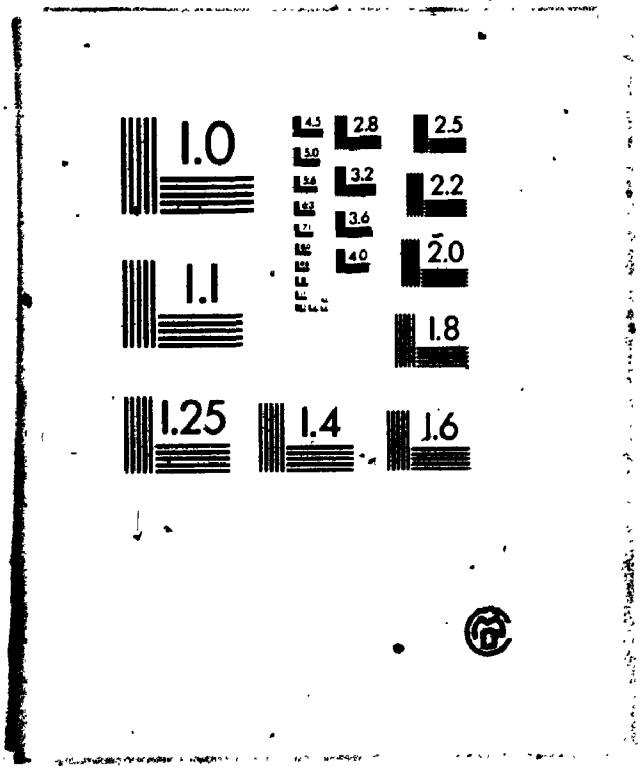
Negatively stained preparations were examined for further characterisation of the fractions. Both outer and inner bilayer fractions showed many membranous vesicles. Those of the outer bilayer

Figure 4.1 Electron micrographs showing outer and inner bilayer pellets. The bilayer fractions were obtained from adult S. mansoni pairs, previously labelled with Con A-ferritin (F) and then subjected to two 20 minute incubations in the digitonin membrane disruption fluid. A, outer bilayer pellet resulting from the first incubation. B, inner bilayer pellet, resulting from the second incubation. Bars represent 0.5 μm.


A
F
B

Figure 4.2 Electron micrographs showing the effects of outer and inner bilayer removal on the surface syncytium of S. mansoni. A, the normal intact surface of freshly perfused worms. (M) indicates the multilamellar bodies in the syncytium (S) and (MF) the underlying muscle fibres. B, the intact parasite surface labelled with Con A-ferritin, (arrows), on the surface of the apical membrane complex. (BB) indicates the apical double bilayer. C, the surface of Con A-ferritin labelled worms after an initial 20 minute incubation in the membrane disruption fluid, to remove the outer bilayer. Note the presence of a single bilayer (SB) on the worm surface and adjacent sheets of removed membrane (R). The ground substance of the syncytium is of normal appearance. D, the surface of Con A-ferritin labelled worms following a second 20 minute incubation in the membrane disruption fluid to remove the inner bilayer. Despite the apparent lack of a surface membrane in many areas the syncytium remains basically intact and in possession of its usual complement of membrane-bound organelles : multilamellar bodies (M) and discoid bodies (D). The underlying muscle fibres (MF) are also intact. Bars represent 1.0 μm in A and D, 0.1 μm in B and C.

2



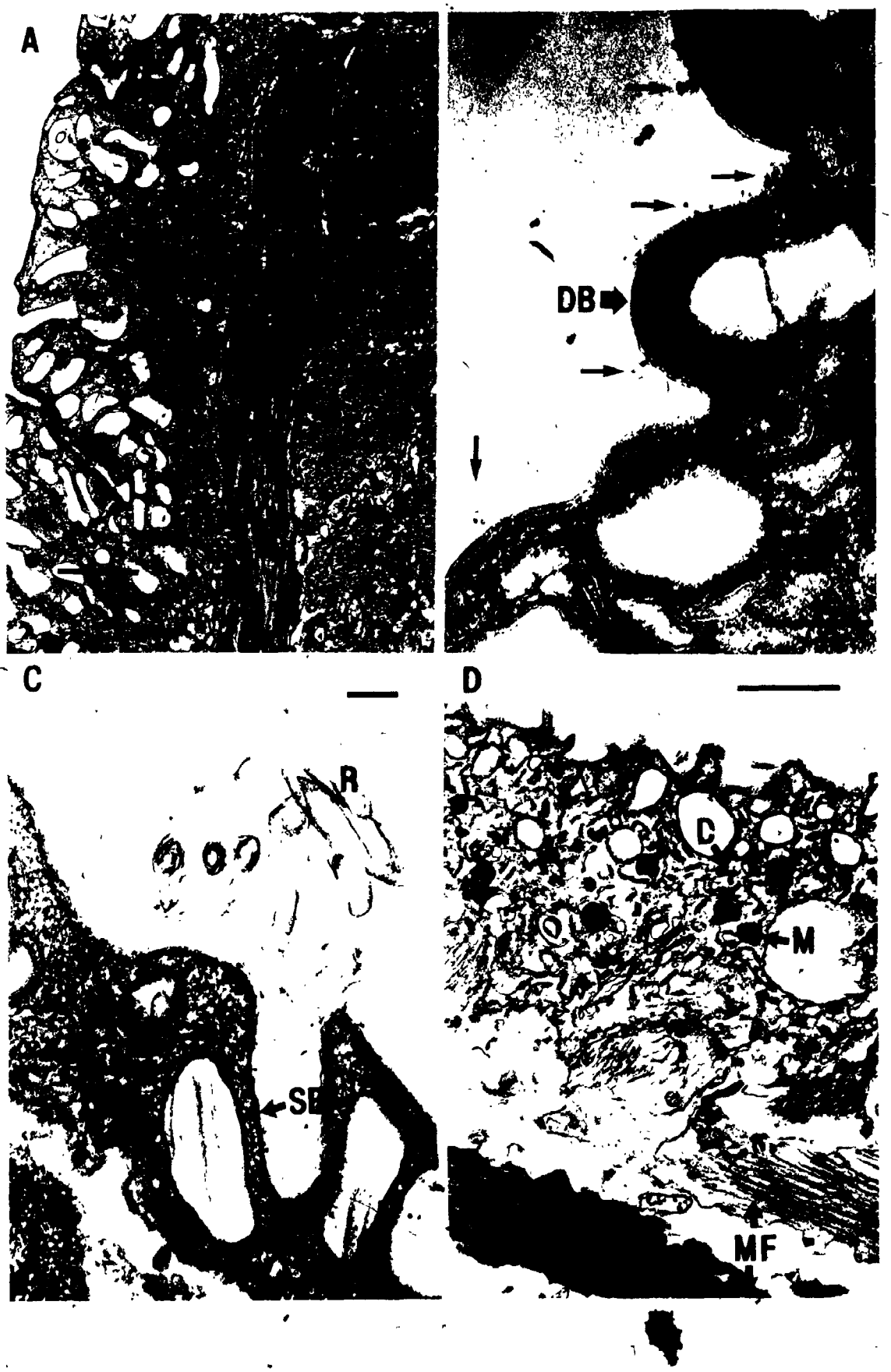

A
DB
C
D
R
SB
C
M
MF

Figure 4.3 Electron micrograph showing Con A-ferritin bound to the outer bilayer. The outer bilayer pellet fraction was removed from intact S. mansoni worm pairs, which had previously been labelled with Con A-ferritin and then subjected to a 20 minute incubation in the digitonin membrane-disruption fluid. Arrows indicate the ferritin molecules. (Bar represents 0.1 μm).

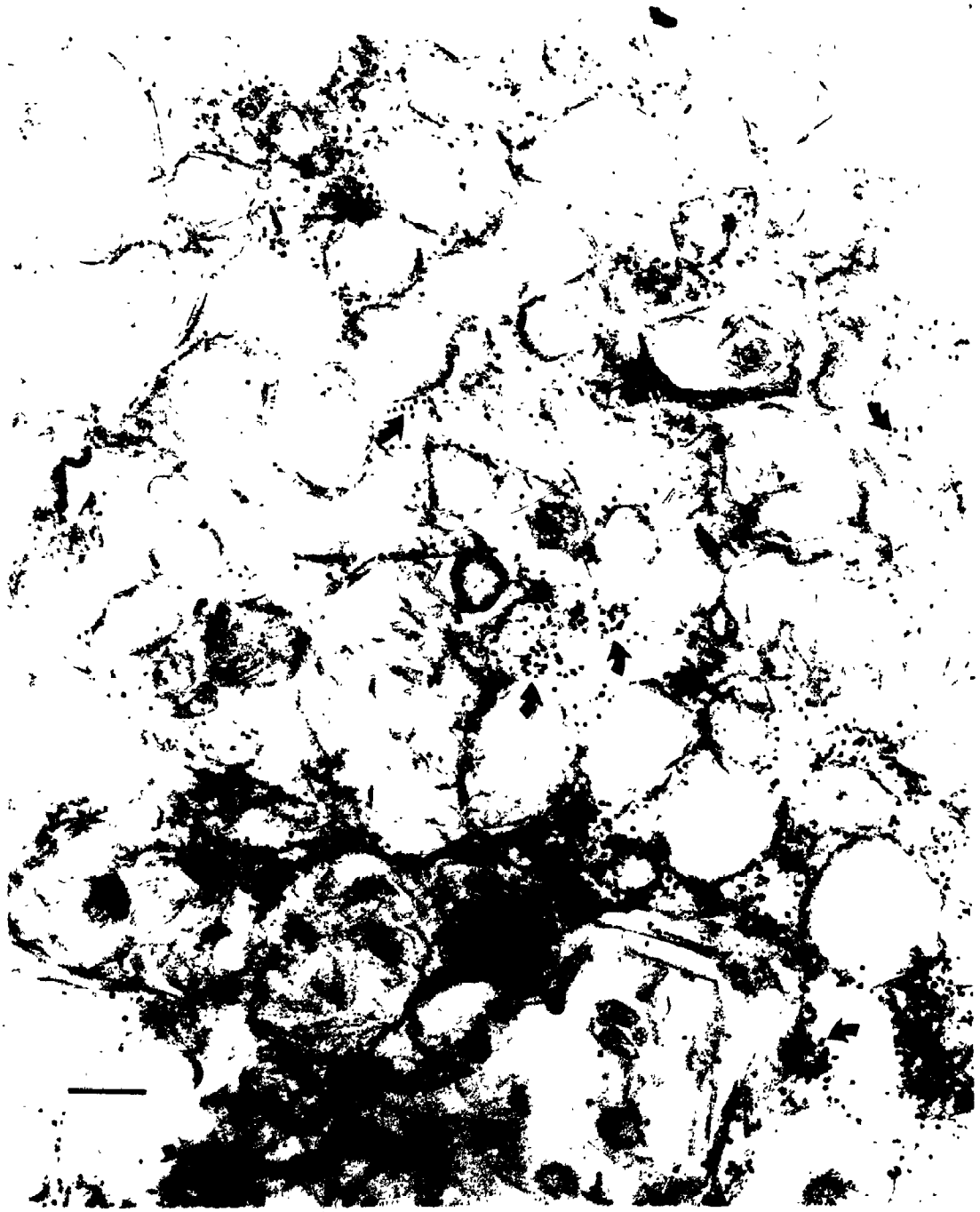


Figure 4.4 Electron micrographs showing negatively stained preparations of outer and inner bilayers. A, outer bilayer preparation resulting from an initial 5 minute exposure to the digitonin membrane disruption fluid. Magnification = 108,750 x . B, inner bilayer preparation resulting from a subsequent 25 minute exposure to the membrane disruption fluid. Magnification = 71,250 x.


A
B

Figure 4.5 Electron micrographs showing negatively stained preparations of the schistosome surface. A and B are preparations of surface bilayer material obtained by a 30 minute incubation in the saponin membrane disruption fluid. Many membranous sheets are apparent, some with evidence of apparent hexagonal patterning (A) and others lack this (B). (P) denotes crystals of the lead stain. C, surface material produced by the freeze-thaw method described in Chapter 2. Note the greater heterogeneity of this preparation and lack of apparent patterning. (Bars represent 0.1 μm).


B
A


B


C

however appeared more disrupted and difficult to visualise (Figure 4.4). Membranous vesicles were also found in the freeze-thaw and saponin-treated preparations (Figure 4.5). The freeze-thaw preparation contained the widest variety of structures. Some of the vesicles resulting from the saponin treatment showed a patterned effect and the possible significance of this is described in the discussion.

4.3.2 [^{3}H] Con A Labelling

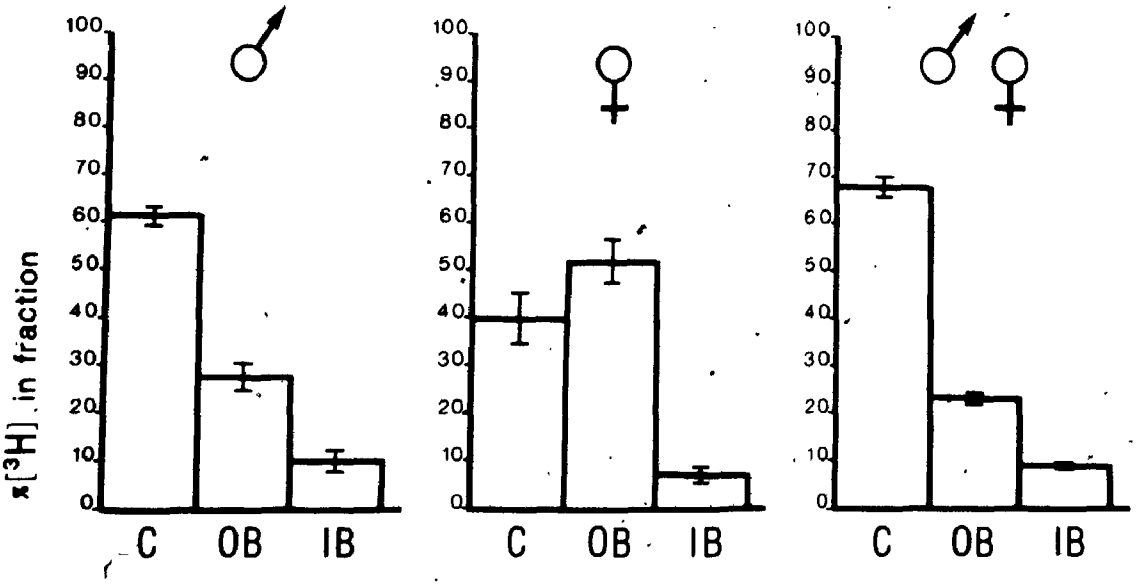
Membrane removal procedures were followed using [^{3}H] Con A labelled worms in the hope that Con A would allow quantitation of the success of outer bilayer removal, in the absence of enzymatic markers for the outer bilayer. In all cases more tritiated label was recovered in the outer bilayer pellet than in the inner bilayer pellet. When the amount of label in these two fractions alone was compared 88, 82 and 72% was found to be in the outer bilayer for females, males and worm pairs respectively. Carcasses remaining after the digest were the most heavily labelled fractions for males or worm pairs, while females yielded the majority of their label in the outer bilayer fraction (Figure 4.6).

4.3.3 [^{125}I] Iodosulfanilic acid Labelling

As [^{3}H] Con A was not ideal as an outer bilayer marker a series of experiments, designed in conjunction with those for alkaline phosphatase, were performed to test an impermeant label, [^{125}I] iodosulfanilic acid, as a marker for the outer bilayer.

The mean value for the total number of counts recovered from lots of 100 worms was $108 \times 10^3 \pm 10 \times 10^3$ (n=10). Of these

Figure 4.6 Histogram showing the distribution of [^{3}H] Con A, expressed as a percentage of total activity in carcasses (C), outer bilayer (OB) and inner bilayer (IB). Males, females or pairs of worms were incubated with [^{3}H] Con A, as a label for the outer bilayer. Groups of labelled worms were then exposed to two sequential incubations in the digitonin membrane disruption fluid to remove outer and then inner bilayers. Activity of [^{3}H] Con A was assayed in bilayer fractions resulting from both incubations and in the remaining carcasses. Values are means $\pm$ standard errors (n = 3).


x[3H] in fraction
100
90
80
70
60
50
40
30
20
10
0
C
OB
IB

counts an average of 4% ± 0.54 remained in the carcasses following membrane removal. Labelling of the inner and outer bilayer preparations varied with the digest times (Figure 4.7). The majority of label was located in the outer bilayer fraction and with increasing length of initial digitonin exposure times increasing amounts of label were recovered in the outer bilayer fraction. Excluding carcass labelling the outer bilayer fraction contained over 90% ± 0.41 (n=4) of the label following initial exposure times of 10 or 15 minutes. At these time points, therefore, the inner bilayer preparation had less than 10% contamination with outer bilayer. An initial digest of 5 minutes resulted in removal of 75% of the label.

4.3.4 Enzyme activity

Initial surveys for enzyme activities of the bilayer pellet fractions suggested that alkaline phosphatase was localised in the inner bilayer. Subsequent experiments were designed to find the specific activity of alkaline phosphatase in outer and inner bilayer pellets. A series of membrane removal experiments, run in conjunction with those for [^{125}I]-labelled worms, were performed using alkaline phosphatase as a quantitative marker for the inner bilayer.

Using p-nitrophenyl phosphate as substrate (pH 9.6), alkaline phosphatase was located predominantly in the inner bilayer fraction (Figures 4.7, 4.8) Specific activity of alkaline phosphatase in the outer bilayer was not significant, having a slope of 0.27 (±0.02) ($P > 0.05$), which was significantly different from that for the inner bilayer at 1.48 (± 0.05) ($P < 0.001$), (Figure 4.8).

With increasing length of the initial exposure to digitonin, increasing amounts of activity were found in the outer bilayer

Figure 4.7 Effect of initial incubation time on the distribution of [^{125}I] iodosulfanilic acid and alkaline phosphatase in outer and inner bilayer fractions. All replicates were performed on pairs of adult worms previously maintained in culture for 2 hours. Following culture, groups of worms were labelled with [^{125}I] iodosulfanilic acid, as a marker for the outer bilayer, and then subjected to two sequential incubations in the digitonin membrane disruption fluid to remove the outer and inner bilayers. Total length of both incubations was held constant at 30 minutes, whilst in individual experiments the length of each digest was varied from 5 to 25 minutes. For both the [^{125}I] and alkaline phosphatase results 100% represents total activity in outer and inner bilayer fractions. In a separate series of experiments on previously cultured worms, run in conjunction with the [^{125}I] labelling experiments, similar membrane removal procedures were followed. Alkaline phosphatase activity was then assayed in both bilayer fractions as a marker for the inner bilayer. Combined results of these two series of experiments therefore allow quantitation of the purity of inner and outer bilayer fractions. The [^{125}I] data are described by the line $Y = 75.35 (\pm 1.09) + 0.87 (\pm 0.08) X$, ($P<0.05$, $n = 10$). The alkaline phosphatase data are described by $Y = 91.05 (\pm 1.92) - 2.02 (\pm 0.28) X$, ($P<0.01$, $n = 41$). Bars indicate standard errors of the mean.

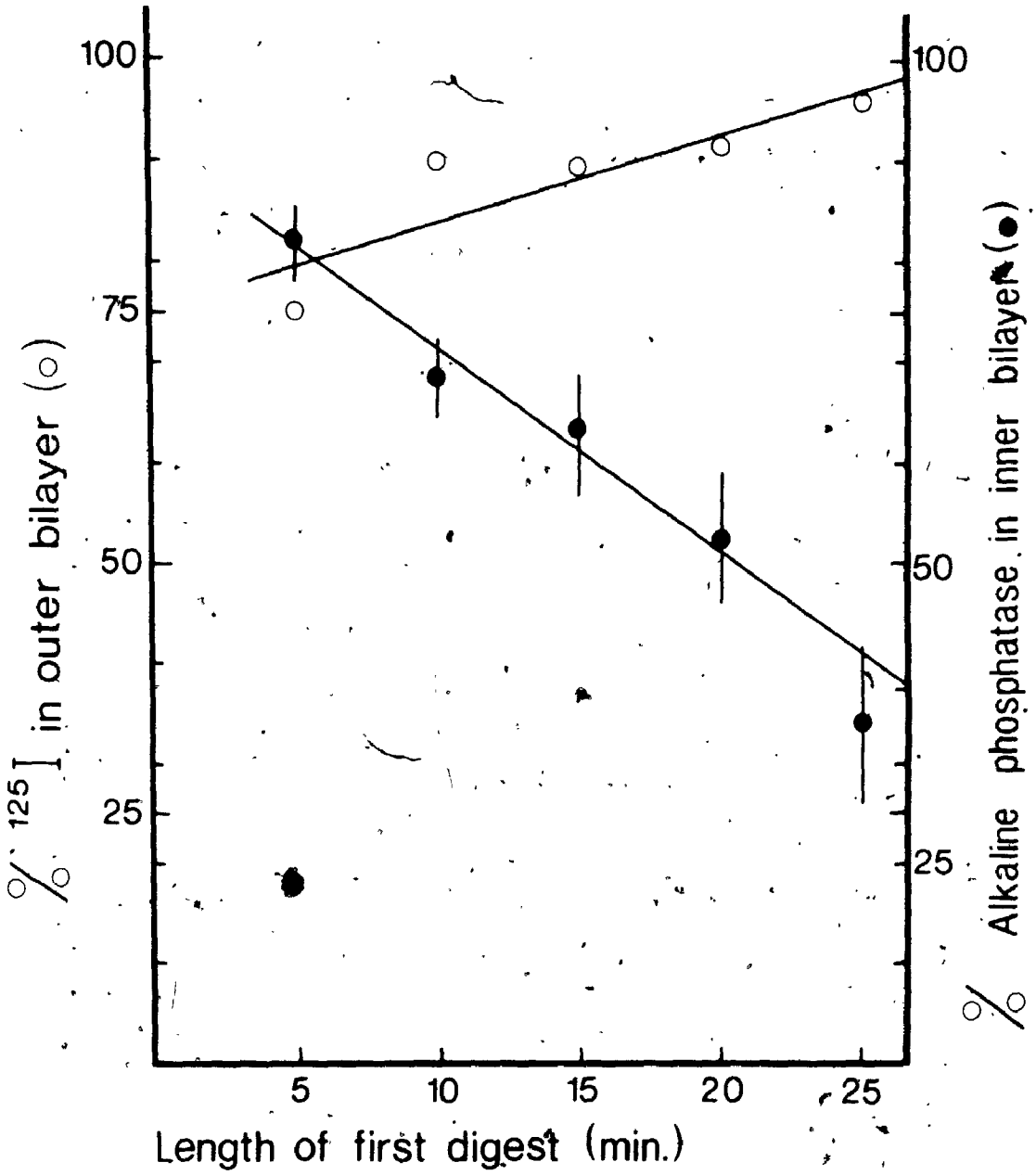
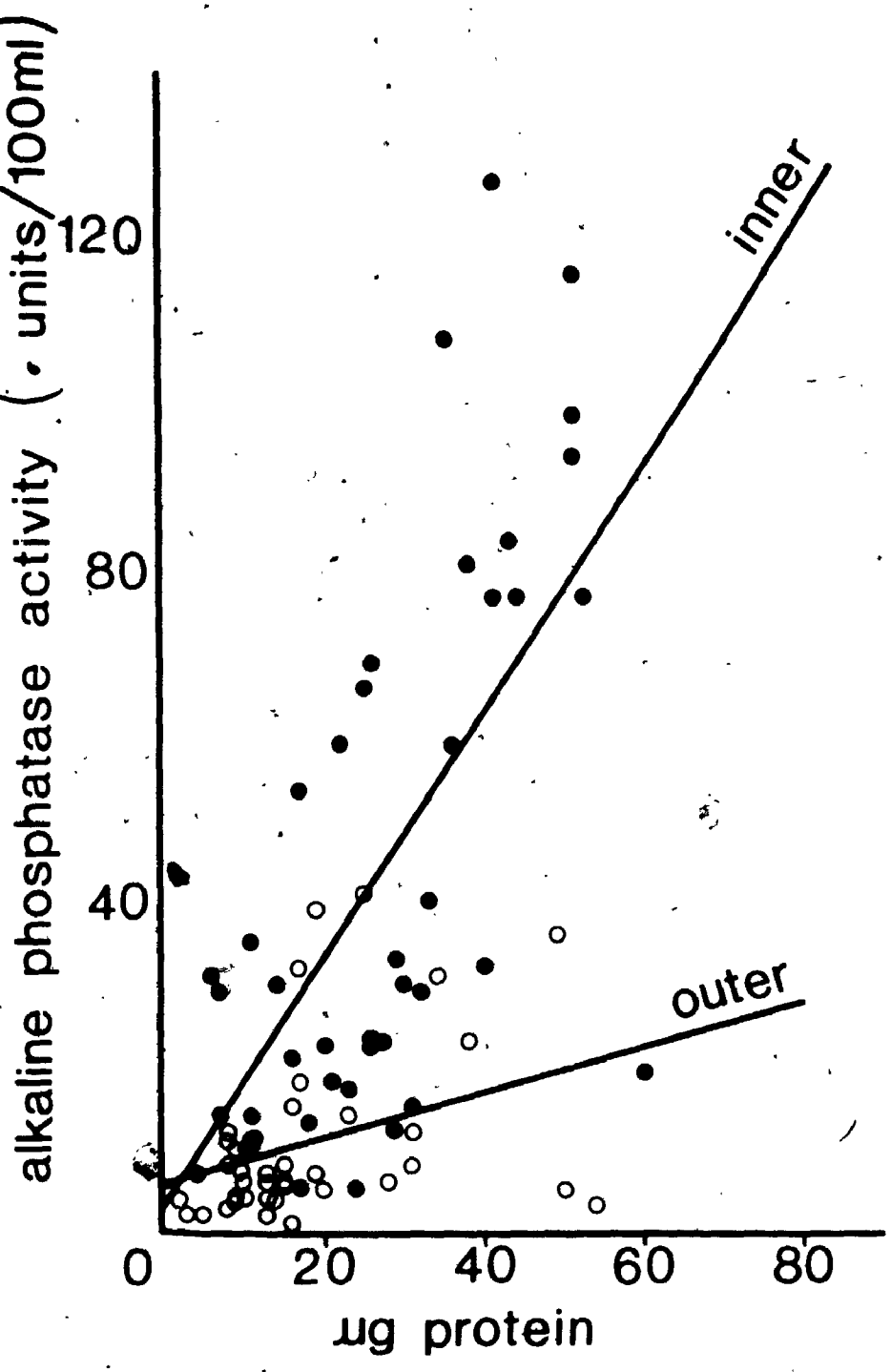

100
75
50
25
100
50
25
% [125I] in outer bilayer (○)
% Alkaline phosphatase in inner bilayer (●)
5
10
15
20
25
Length of first digest (min.)

Figure 4.8 Specific activity of alkaline phosphatase in the outer and inner bilayer fraction pellets. All experiments were performed on pairs of adult worms, previously maintained in culture for at least 2 hours. Outer and inner bilayers were removed by sequential incubations of 5 and 25 minutes in the digitonin membrane disruption fluid. Alkaline phosphatase activity is expressed as Sigma units (σ) per 100 ml. The slopes of the regression lines are equivalent to specific activity of alkaline phosphatase. For the outer bilayer $Y = 6.44 (\pm 0.43) + 0.27 (\pm 0.02) X$, ($P < 0.001$, $n = 39$). The slope for specific activity of alkaline phosphatase in the outer bilayer is not significant at $P = 0.05$. Specific activity of alkaline phosphatase in the inner bilayer is significant ($P < 0.001$) and is 5 times higher than in the outer bilayer. The slopes are also significantly different ($P < 0.001$) indicating that alkaline phosphatase is localised in the inner bilayer.

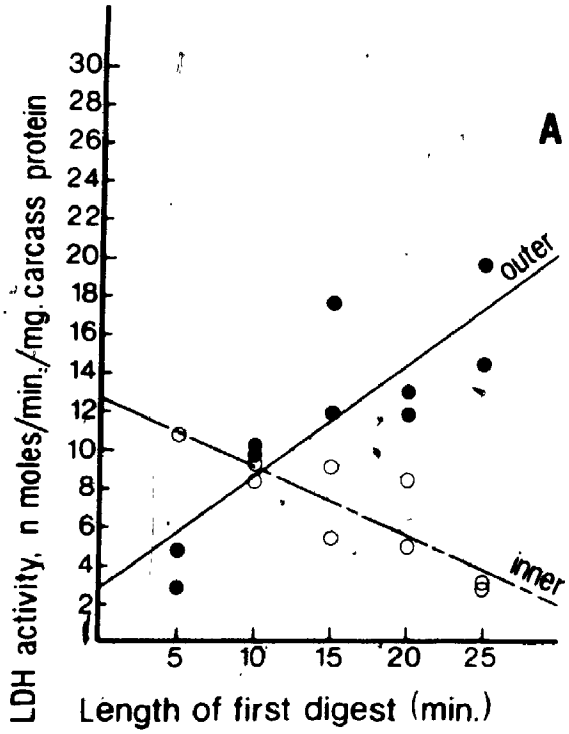

alkaline phosphatase activity (• units/100ml)
120
80
40
0
20
40
60
80
µg protein
inner
outer

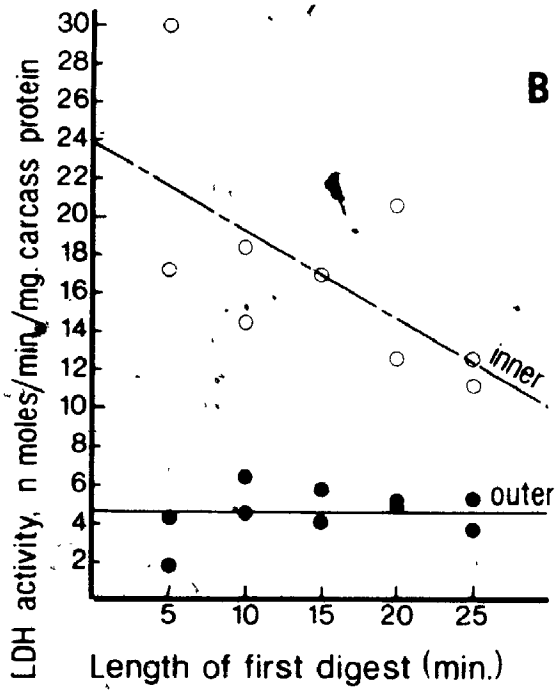
preparations, from previously cultured worms (Figure 4.7). At the initial digitonin exposure times of 5 minutes 81% ± 3% (n=12) of the activity was located in the inner bilayer pellet fraction, suggesting 19% contamination of the outer bilayer fraction with inner bilayer material. For freshly perfused worms, exposure times of 15 minutes followed by 20 minutes yielded 89% ±2% (n=10) of the alkaline phosphatase activity in the inner bilayer pellet. The latter results were significantly different from those obtained for worms cultured for 2 hours prior to membrane removal (Figure 4.7, $P < 0.001$).

The alkaline phosphatase was most likely membrane bound, as material pelleted from the fractions contained 10 to 25 Sigma units/mg protein, whereas the supernatants had less than 1 Sigma unit/mg protein.

LDH, a soluble cytosolic enzyme, was assayed in the supernatants of the bilayer fractions to quantitate protein leakage resulting from damage to the integrity of the worm surface following membrane removal procedures. Increasing levels of LDH activity were found in the outer bilayer supernatant fraction with increasing length of initial exposure to digitonin, whereas LDH activity in the inner bilayer supernatants exhibited the reverse trend (Figure 4.9). However fractions resulting from longer first exposures had a higher degree of cross contamination (Figure 4.7). To correct for the contamination, the decimal fraction (%) of total alkaline phosphatase activity in the outer bilayer was used as a measure of contamination with inner bilayer at each time point. This fraction was subtracted from the LDH results for the outer bilayer and added to those for the inner bilayer (Figure 4.9). This procedure, which assumed that all alkaline phosphatase activity was associated with the inner bilayer

Figure 4.9 Lactate dehydrogenase (LDH) activity in the supernatants of outer and inner bilayer fractions subjected to varying lengths of membrane disruption incubations. All experiments were performed on pairs of adult worms previously maintained in culture for two hours. Lengths of first and second incubations, to remove outer and inner bilayers, were varied in the same manner as in Figure 4.7. LDH, a soluble cytosolic enzyme, was assayed in the supernatants of the bilayer fractions to quantitate protein leakage from the worm surface syncytium. LDH activity was normalised to carcass protein. (A) For the outer bilayer $Y = 2.93 (\pm 0.54) + 0.57 (\pm 0.16) X$, ($P<0.05$, $n = 10$). For the inner bilayer $Y = 12.6 (\pm 0.14) - 0.35 (\pm 0.06) X$, ($P<0.001$, $n = 9$). (B) was derived from A by correcting for bilayer contamination, as quantitated by the alkaline phosphatase results. (Figure 4.7). The contaminating fraction of outer bilayer with inner bilayer was subtracted from the outer bilayer results and added to the inner bilayer results. B therefore illustrates the results expected if the membrane separation technique had worked perfectly. No significant correlation was found between the outer bilayer data and length of the first digest ($P>0.05$, $n = 10$). For the inner bilayer $Y = 24.0 (\pm 0,82) - 0.46 (\pm 0.15) X$, ($P<0.05$, $n = 9$). Protein leakage can therefore be correlated with removal of the inner bilayer.


A
LDH activity, n moles/min./mg. carcass protein
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
outer
inner
5
10
15
20
25
Length of first digest (min.)


B
LDH activity, n moles/min./mg. carcass protein
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
inner
outer
5
10
15
20
25
Length of first digest (min.)

(Figure 4.8), indicated that LDH activity was much lower in the outer bilayer supernatants than in the inner bilayer supernatants. There was no longer a significant correlation between LDH activity in the outer bilayer supernatant and the length of the initial exposure to digitonin. Conversely, for the inner bilayer there occurred a more positive relationship of LDH activity to digest time, as shown by the increased slope of the regression line (Figure 4.9).

Hemoglobinase activity was assayed in the bilayer fraction supernatants as a measure of contamination with gut contents and epithelial membranes. Hemoglobinase activity was not detected in the inner bilayer supernatant and was barely detectable at less than 5 nmol/mg protein in the outer bilayer.

A Na^+, Mg^{2+}-ATPase, described in Chapter 2 and in Podesta and McDiarmid (1982), was detected only in the inner bilayer fraction, with a specific activity of 1.7 ± 1.05 (n=3) µmol phosphorus released per mg protein per minute. Specific activity of the ATPase in the inner bilayer is about five times greater than in the previously described fraction containing both bilayers. Despite its apparent localisation to the inner bilayer the Na^+, Mg^{2+}- ATPase was a less convenient marker enzyme than alkaline phosphatase. The alkaline phosphatase assay is easily performed and the enzyme is present at high specific activity. Tests for acid phosphatase, phosphatidic acid phosphatase and 5'-nucleotidase were negative for both bilayer fractions.

4.3.5 Phospholipids

The phospholipid-protein ratio for the outer bilayer was found to be $\bar{x} = 3.6$ (± 0.71), $n = 8$ and $\bar{x} = 1.5$ (± 0.29, $n = 8$) for the inner

Figure 4.10 Thin layer chromatographs of outer (OB) and inner (IB) bilayer phospholipids. Outer and inner bilayer fractions were obtained by sequential 5 and 25 minute incubations in the digitonin membrane disruption fluid. Lipids were extracted from these fractions, spotted on silica gel G plates and run in 2 dimensions. The solvent system for dimension I consisted of chloroform-methanol - 28% ammonium hydroxide (130:70:10) and dimension II consisted of chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10). Spots were visualised by charring with sulphuric acid. Standards were co-chromatographed in the margins of the plate : a, cerebrosides; b, lysophosphatidyl choline; c, phosphatidylcholine; d, phosphatidylethanolamine; e, sphingomyelin; f, phosphatidylserine. Lipids in the inner bilayer: 1, lysophosphatidylcholine; 2, sphingomyelin; 3, phosphatidylserine; 4, phosphatidyl inositol; 5, phosphatidylcholine; 6, phosphatidylethanolamine; 7, spot co-migrating with cerebrosides. Lipids in the outer bilayer: 1, lysophosphatidylcholine; 2, sphingomyelin; 3, phosphatidylcholine; 4, phosphatidylethanolamine; 5, cerebrosides.

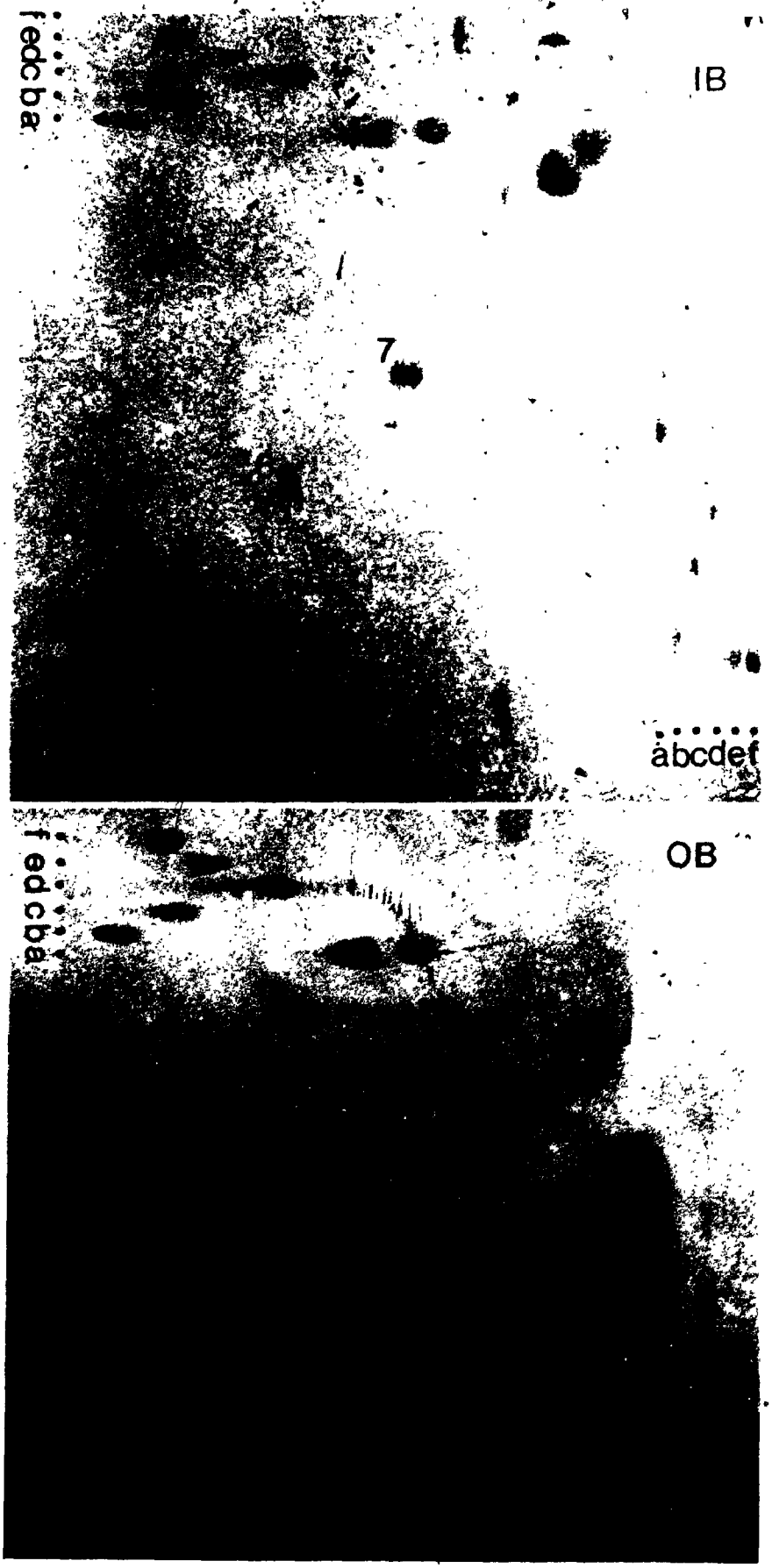
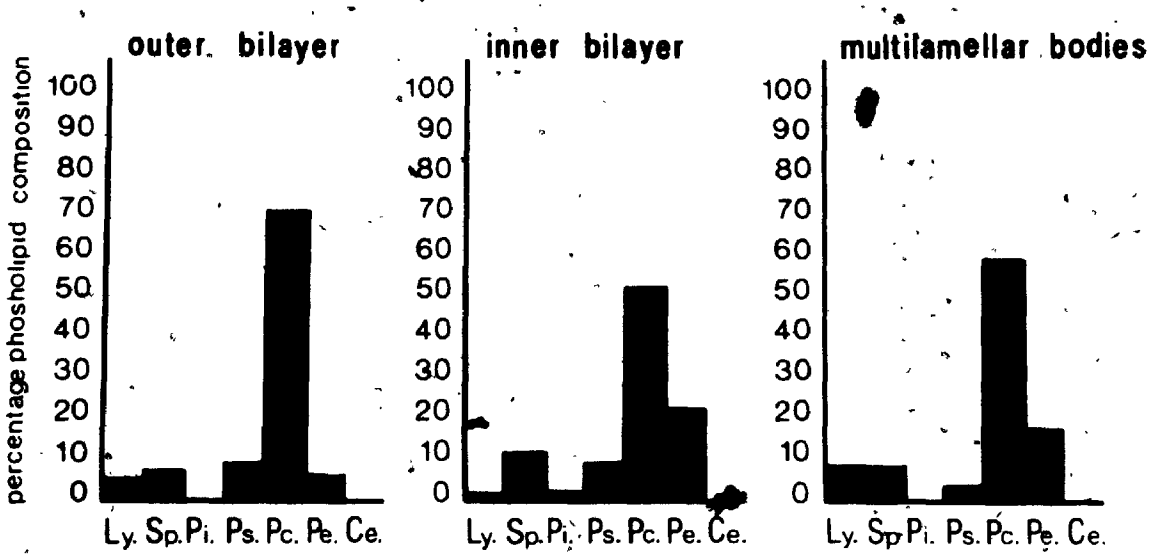

fedcba
IB
7
abcdef
fedcba
OB

Figure.4.11 Phospholipid composition of outer and inner bilayer and multilamellar body fractions from S. mansoni. Data represents the mean values from three experiments. Cerebrosides were detected, but not quantitated.

Ly. - Lysophosphatidylcholine, Sp. - Sphingomyelin, Pi - Phosphatidylinositol, Ps. - Phosphatidylserine, Pc. - Phosphatidylcholine, Pe. - Phosphatidylethanolamine, Ce. - Cerebrosides.


outer bilayer
inner bilayer
multilamellar bodies
percentage phospholipid composition
100
90
80
70
60
50
40
30
20
10
0
Ly. Sp. Pi. Ps. Pc. Pe. Ce.

bilayer. This indicates that the outer bilayer is composed of approximately 78% phospholipid and the inner bilayer of 60% (ignoring neutral lipid). Percentage composition of the phospholipid classes of outer and inner bilayer fractions and, for comparison, the MLB fraction, are shown in Figure 4.11, (See also Figure 4.10).

4.4 DISCUSSION

In the present study an attempt was made to isolate the outer (OB) and inner (IB) bilayers of the apical membrane complex of the epithelial syncytium of S. mansoni, using conventional electron microscopy and marker enzyme quantitation of membrane fractions (Koizumi et al., 1981). Previous efforts to isolate the surface membranes of S. mansoni have resulted in the removal of both inner and outer bilayers together and in most experiments, varying amounts of the tegument and its associated membrane - bound organelles (Kusel, 1972; Brink et al., 1980; Simpson et al., 1980; Oaks et al., 1981). By contrast, following the initial exposure of the parasites to the digitonin solution, used to remove the outer bilayer, the tegument was intact and of normal appearance (Figure 4.2c). Over most of the worm surface only a single bilayer was visible. The remaining bilayer was virtually absent following the second digitonin exposure, used to remove the inner bilayer (Figure 4.2d). Despite the lack of a surface membrane the tegument remained intact and in possession of its usual complement of membrane-bound organelles (discoid and multilamellar bodies). The basal membrane of the epithelial syncytium was intact after both digests.

Persistence of internal structure and membranous inclusions, in

the absence of a limiting membrane, has also been shown in hepatocytes treated with 0.1% digitonin (Fiskum et al., 1980). The integrity of the demembranated cells was attributed to the cell cytoskeleton, while the internal organelle membranes were undamaged as a result of their lower cholesterol content and corresponding reduced susceptibility to digitonin disruption (Bradley et al., 1979; Nakamura et al., 1979 Brocks et al., 1980).

Digitonin is a mild non-ionic detergent, which complexes specifically with cholesterol (Akiyama et al., 1980; Fiskum et al., 1980). It tends not to inactivate membrane-bound proteins (Thang et al., 1980; Rahman et al., 1981) as, at low concentrations, it does not solubilise enzymes out of the membrane, thus separating them from potential obligate phospholipid requirements (Podesta and McDiarmid, 1982). However, the saturated digitonin solutions employed by Kusel (1970) in membrane isolation procedures with S. mansoni probably would solubilise the membrane and extract and denature the proteins (Gurtubay et al., 1980). Digitonin is particularly useful in membrane or metabolite compartmentation studies and has been used to separate the plasma membrane from internal organelle membranes, based on their respective cholesterol contents (Brocks et al., 1980). The outer bilayer of mitochondria and chloroplasts is more susceptible to digitonin than the inner bilayer and again this is correlated with a higher percentage of cholesterol in the outer bilayer (Schnaitman, 1967; Gurtubay et al., 1980; Le Vine et al., 1982). Membrane susceptibility to disruption procedures is also positively correlated with lipid content, with lipid-rich membranes being particularly susceptible (Siebertz et al., 1979). The success of the digitonin membrane removal, described here, implies a difference in the

cholesterol contents of the outer and inner bilayers. As a shorter incubation time is required to remove the outer bilayer than the inner bilayer, the outer bilayer may have a higher cholesterol content (Nakamura et al., 1979). It has been suggested previously that the apical membrane complex of S. mansoni is rich in cholesterol, based on an observed "digitonin shift" in the density of membrane fractions (Simpson et al., 1981b). Cholesterol is the major sterol present in whole worms (Smith and Brooks, 1969). A high cholesterol content of the outer bilayer might help to explain the low fluidity reported for this membrane (Johnson et al., 1982). The presence of high levels of membrane cholesterol is correlated with low fluidity (Hegner, 1976; Schlager and Ohanian, 1979) and also with an increased resistance to the damaging effects of complement (Schlager and Ohanian, 1980a).

Negative staining is routinely employed as a fast and simple method of monitoring membrane fractions (Mizushima and Yamada, 1975; Coulton and Murray, 1979; Kamio and Takahashi, 1980; Veslemøy et al., 1980), although this technique has not been previously applied to schistosomes. Negative staining patterns of the outer and inner bilayer preparations (Figure 4.4) tend to confirm the greater susceptibility of the outer bilayer to digitonin, as these vesicles are more severely disrupted and possibly show some evidence of pit formation resulting from cholesterol: digitonin complexes. This contrasted with the inner bilayer vesicles which were not disrupted and were lacking in pits. The hexagonal patterning of saponin: cholesterol complexes can also be seen in the apical membrane preparations, obtained with saponin, but they are lacking in the detergent-free, freeze-thaw preparations of tegument membranes (Figure 4.5).

Many workers have shown binding of Con A to the surface of S. mansoni (Bennett and Seed, 1977; Murrell et al., 1978; Simpson and Smithers, 1980; Torpier and Capron, 1980). Binding of Con A to adult worms is reported to be specific, restricted to the external surface and stable in non-ionic detergents. Con A has therefore been recommended as a useful marker for the schistosome surface membrane (Oaks et al., 1981). Con A, however, has also been shown to damage the tegument (Simpson and McLaren, 1982). In the present study the use of Con A as a label for the outer bilayer proved to be unsatisfactory. Con A-ferritin did bind to the worm surface, but not in large amounts (Figure 4.2b). The Con A-ferritin complex is larger and less pliable than native Con A and steric hindrance may have prevented it from reaching some membrane sites (Ackerman and Freeman, 1979). In the membrane pellets the conjugate was quite dense, especially in the outer bilayer pellet (Figure 4.3). Although strictly quantitative procedures were not followed, larger amounts of ferritin were present in the inner bilayer than expected and this may indicate dissociation and re-association of label. Re-association, internalisation or trapping of label is also suggested by the results of experiments with [^{3}H] Con A (Figure 4.6). In all cases the outer bilayer was more heavily labelled than the inner bilayer fraction, but for males alone or worm pairs most of the label remained with the carcasses. Label is probably trapped in the gynecophoric canal, which has been shown to be intractable to membrane-removal procedures (Oaks et al., 1981). Even Triton X-100 disruption of the entire tegument failed to remove 20% of the surface bound Con A (Oaks et al., 1981).

Impermeant iodinated markers have provided a useful alternative for the identification of surface membrane components, where the

enzymatic or morphological characteristics of that membrane were unknown (Edwards et al., 1979). Several methods have been used to iodinate the surface components of S. mansoni, both directly (Hayunga et al., 1979; Rumjanek, 1980; Snary et al., 1980; Taylor et al., 1981; for a review see Hayunga and Murrell, 1982) and indirectly by the use of iodinated lectins (Simpson et al., 1980). Bound lectins may alter membrane structure (Torpier and Capron, 1980) or dissociate and re-associate (Simpson et al., 1980). Of the direct methods employed, lactoperoxidase-catalysed iodination of S. mansoni adults has produced varying results (Hayunga et al., 1979; Snary et al., 1980) and may label only a few of the surface components, possibly because of its specificity for tyrosine (Hayunga and Murrell, 1982). Lactoperoxidase will also associate non-specifically with the schistosome surface (Shah and Ramasamy, 1982). The Bolton-Hunter technique has been used to iodinate surface proteins, but it has not been conclusively demonstrated to be impermeant (Hayunga, 1982). However, radioactively labelled diazotised [^{125}I] iodosulfanilic acid has been shown not to penetrate erythrocyte membranes (Edwards et al., 1979). It can be synthesised at high specific activity for use at low concentrations and it binds covalently under mild conditions. The negative charge on the parent molecule retards entry into the cell (Edwards et al., 1979; Hayunga, 1982). Taylor et al. (1981) have successfully labelled S. mansoni surface proteins with this reagent. For these reasons the latter method was used to label the outer bilayer in the present study.

Our results confirm the impermeant nature of [^{125}I] iodosulfanilic acid. Almost all of the bound label was removed during initial exposure to digitonin and only 4% remained in the worm

carcasses after membrane removal. Initial exposure times of 10 minutes or more resulted in over 90% of the [^{125}I] being localised in the outer bilayer fraction (Figure 4.7). This suggests that, at these time points, over 90% of the accessible outer bilayer had been removed. Correspondingly, the inner bilayer pellet would have less than 10% contamination with outer bilayer.

In studies of membrane purification it is necessary to have a marker for identifying the purity of the membrane fractions (Waldman and Chepenik, 1980). This is usually accomplished using enzymes unique to the membranes being isolated. Since the outer bilayer is unusual, we did not assay for unique enzymes but, as discussed above, we used [^{125}I] as a marker for this outer bilayer. If the outer bilayer is a specialised secretion, as suggested by the absence of typical plasma membrane enzyme markers (see below), then the inner bilayer should be analogous to the traditional plasma membrane of other cell types. Alkaline phosphatase and 5'-nucleotidase are two commonly used plasma membrane markers (Waldman and Chepenik, 1980). 5'-Nucleotidase was not detected in either the outer or inner bilayer fraction. The lack of 5'-nucleotidase is not surprising as in other epithelia it has a polarised distribution, being found only in the basal-lateral membranes of the epithelial cells, but not in the apical membrane (Hanna et al., 1979). Alternatively the enzyme may be present, but inhibited by digitonin or enzyme activity may be masked by the high levels of non-specific phosphatase activity. Simpson et al. (1981a) refer to 5'-AMPase activity in the schistosome tegument, but restricted specificity was not demonstrated nor was polarity of the enzyme distribution examined.

Alkaline phosphatase has been located in the tegument of S. mansoni by other workers (Dusanic, 1959; Robinson, 1961; Bogitsch and Krupa; 1971; Cesari, 1974; Cesari et al., 1981), but has not been previously localised to the inner bilayer. Specific activities of alkaline phosphatase in outer and inner bilayers suggests restriction of alkaline phosphatase to the inner bilayer (Figure 4.8) and this may help to explain the inability of Evans and Payares (1981) to iodinate it with the Bolton-Hunter technique. At the initial exposure time of 5 minutes, 83% of the alkaline phosphatase activity was contained in the inner bilayer fraction. Under these conditions, therefore, the outer bilayer fraction contained approximately 17% contamination with inner bilayer material. When these results are combined with those of the iodination experiments (Figure 4.7) the optimal initial exposure time for the sequential removal of both bilayers is 5-6 minutes. Cross-contamination of the fractions at this point is under 20%. Small amounts of the inner bilayer were left on the worm carcasses after both digests, but this was not quantitated as the marker for the inner bilayer, alkaline phosphatase, also occurs at other sites in the worm (Dusanic, 1959; Nimmo-Smith and Standen, 1963). The digitonin incubations did not remove membrane from the schistosome gut epithelium, as evidenced by normal gut morphology and low levels of hemoglobinase activity, detected only following the initial short exposure to remove the outer bilayer.

Freshly perfused worms appear to be more refractory to membrane removal with digitonin, as longer incubation times were required to remove the bilayers. This was evidenced by the higher levels of alkaline phosphatase in the inner bilayer fractions at the longer initial exposure times of 15 or 20 minutes. Maintaining worms in

culture for just two hours would therefore seem to significantly alter their surface membranes, making them more susceptible to separation by exposure to digitonin. Worms transferred from hamsters to culture have been shown to undergo a period of metabolic adaptation (Rotmans et al., 1981) and Hymenolepis diminuta maintained in culture for just 30 minutes has a surface that is more accessible to iodination (Knowles and Oaks, 1979) and whose transport properties change dramatically (Podesta et al., 1977).

Acid phosphatase has been reported from the tegument of S. mansoni, although not in association with membranous inclusions (Watts et al., 1979). No acid phosphatase activity was found in our bilayer pellets. Phosphatidate phosphatase activity was not detected in either fraction, but has been reported from the multilamellar bodies (Rahman and Podesta, 1982). A Na^+, Mg^{2+}-ATPase described from other apical epithelial membranes, was distributed in S. mansoni between the inner and outer bilayers in a pattern similar to that observed for alkaline phosphatase (Podesta and McDiarmid, 1982). Previous descriptions of ATPases from S. mansoni have been from impure preparations, resulting in several ATPases being assayed together (Nechay et al., 1980).

Although the inner bilayer shares several characteristics with apical plasma membranes of other epithelia, the outer bilayer does not appear to be analogous to a traditional plasma membrane. It lacks the ATPase and alkaline phosphatase activities usually associated with apical epithelial membranes. The outer bilayer of gram negative bacteria also has very few known enzymes compared with the inner bilayer (Osborn et al., 1972; Osborn and Munson, 1974). Phospholipases have however been detected in the outer bilayer of

bacteria (Orndorff and Dworkin, 1980). Disruption of the permeability properties of the apical membrane complex and subsequent leakage of soluble cytosolic proteins was quantitated by assaying LDH activity in the supernatants. LDH was used as a high molecular weight marker for cytosolic proteins (Brocks et al., 1980; Fiskum et al., 1980). After the LDH results had been corrected for contamination, by means of the corresponding alkaline phosphatase data, the levels of LDH released following removal of the outer bilayer were minimal. LDH activity released into the supernatant fraction upon removal of the inner bilayer was greater and increased with increasing length of the second digitonin exposure (Figure 4.9). Little LDH was released following removal of the outer bilayer and these results suggest that the outer bilayer is not necessary for the maintenance of a functional barrier to soluble protein leakage. A further indication of this is given by the absence of a "washed-out" appearance of the syncytium after removal of the outer bilayer (Fig 4.2c). Even minor digitonin-induced disruption of the hepatocyte plasma membrane results in protein leakage and a "washed-out" appearance of the cytosol (Fiskum et al., 1980). Pieces of the outer bilayer have been observed to be missing from normal intact worms (Hockley et al., 1975), supporting the idea that the outer bilayer is not essential for surface integrity.

Although further studies are required, it appears that the outer bilayer is not a traditional osmotic barrier characteristic of plasma membranes and, therefore, the usual action of some host immune effectors will be ineffective against the worm. For example, the "hole-punching" mode of action of complement will be ineffective against the outer surface of the adult worm, given that the outer bilayer is not a typical osmotic barrier (Lauf, 1978). The detergent

action of complement on membranes that are not osmotic barriers is much less efficient (Mayer et al., 1981).

It has previously been suggested that the outer bilayer of S. mansoni is enriched in lipid, as it is selectively fixed with uranyl acetate (McLaren and Hockley, 1977), which is known to fix lipids. This is especially noticeable for saturated lipids, which are not osmiophilic (Gil and Weibel, 1970). Up to 90% of externally applied, impermeant, iodinated markers have been recovered in the lipid phase (Wilson, 1981) and Kusel (1972) found it necessary to remove lipids before chloramine-T labelling of the parasites.

The apical membranes of S. mansoni were found to be lipid rich, with an average of 69% phospholipid content (this value does not take the neutral lipids, which were not quantitated, into account). As expected, the outer bilayer contains relatively more phospholipid than the inner bilayer, with a phospholipid-protein ration 3.6:1, compared to 1.5:1 for the inner bilayer. Although bacteria show extreme variation some species have been reported with a higher percentage of phospholipid in the outer bilayer than the inner (Mizushima and Yamada, 1975; Orndorff and Dworkin, 1980). It is also common for the lipids of the outer bilayer to be more saturated (Ishinaga et al., 1979; Gmeiner and Schelcht, 1980), as has been proposed for S. mansoni (McDiarmid et al., 1982). There are some problems inherent in the determination of a phospholipid-protein ratio, especially where detergents are involved. Phospholipid-protein ratios reported from mammalian lung surfactant range from 1-12:1 (Clements and King, 1976). Detergents may selectively extract and solubilise certain proteins and lipids (Thang et al., 1980; Le Vine et al., 1982). The protein content of the outer bilayer may actually be slightly higher

than given here, as sialic acid-containing glycoproteins, which are found in the outer bilayer (Simpson and Smithers, 1980; McDiarmid and Podesta, 1983), are especially susceptible to digitonin solubilisation (Le Vine et al., 1982). Membranes with a high lipid content may also have more proteins solubilised by detergents, such that more protein may be solubilised from the outer bilayer, as it has a higher phospholipid-protein ratio. A similar phenomenon has been found in mitochondria (Gurtubay et al., 1980).

Phosphatidylcholine was the major phospholipid in both outer and inner bilayers and is also the predominant lipid in whole worms (Meyer et al., 1970; Young and Podesta, 1982). Differences between the two bilayers were quantitative, rather than qualitative. The outer bilayer is composed predominantly of phosphatidylcholine, whilst the inner bilayer has a composition almost identical to that reported from other plasma membranes (Koizumi et al., 1981). The high levels of phosphatidylethanolamine in the inner bilayer and MLB (McDiarmid et al., 1982), are of interest, as phosphatidylethanolamine is recognised as a modulator of membrane-membrane contact (Kolber and Haynes, 1979) and may encourage fusion of the MLB with the apical plasma membrane (IB).

Having established at least quantitative dissimilarity between the bilayers it remains a matter of speculation as to how these differences are initiated and maintained. Specific phospholipid transfer proteins may be involved, as in the lung (Tsao, 1980), there may be specific phosopholipases in the bilayers (Osborn, et al., 1972), or the membranes and their individual lipids may have different turnover rates (Ishinaga et al., 1979). Phospholipid turnover in the bilayers is the subject of the next chapter. It is also possible

that, as in bacteria, phospholipids are synthesised or acylated by enzymes of the inner bilayer (White et al., 1971; Osborn and Munson, 1974), allowing intimate control of membrane lipid synthesis. Whatever the mechanism, it is widely recognised that the lipid composition of the surface membrane will greatly influence the outcome of any immune attack (Kanai and Kondo, 1979; Schlager and Ohanian, 1979).

CHAPTER 5

INCORPORATION AND TURNOVER OF PHOSPHOLIPID PRECURSORS IN SCHISTOSOMA MANSONI

5.1 INTRODUCTION

The protective immune response of the mammalian host to Schistosoma mansoni is both stimulated by and directed towards the surface of the parasite (Kusel et al., 1975b; Rotmans and Mooij, 1982; Taylor and Butterworth, 1982). Earlier suggestions that this critical interface, the outer bilayer, might be composed primarily of lipid (McLaren and Hockley, 1977) have recently been confirmed (McDiarmid and Podesta, 1982). The possible significance of the outer bilayer lipids of S. mansoni in immune evasion may be inferred from recent studies of several systems, where the outcome of immune attack is greatly influenced by the lipids of the surface membrane (Kanai and Kondo, 1979; Schlager, 1979; Schlager and Ohanian, 1979). Lipids are no longer viewed as the inert building blocks of membranes, but are widely recognised as direct participants in and modulators of membrane function (Cullis et al., 1980).

Known functions of membrane phospholipids which might facilitate schistosome survival in an immunologically hostile environment include an involvment in calcium transport and membrane fusion (Cullis et al., 1980). The outer bilayer of S. mansoni has the ability to form areas of fusion with the plasma membrane of neutrophils and this has been proposed as a mechanism preventing damage by these cells (Caulfield et al., 1980; Caulfield et al.,

1982). Survival of tumour cells, following attack by antibody and complement, has been correlated with increased lipid synthesis (Schlager, 1979) and with lipid modulation, resulting in a decreased fluidity of the surface membrane (Dahl et al., 1979; Schlager and Ohanian, 1980a). Recent evidence indicates that the outer bilayer of S. mansoni has very low fluidity (Johnson et al., 1982). Resistance to cell-mediated immune attack has also been shown to depend on the lipid composition of the surface membrane (Kanai and Kondo, 1979).

The importance of lipids to the evasion mechanisms of S. mansoni was suggested by the experiments of Rumjanek (1981), where the ability of human serum to confer resistance on young schistosomula was abrogated when the serum was previously de-lipidated. The dynamic role of surface membrane lipids in S. mansoni however, has received little attention. Samuelson and Caulfield (1982) labelled surface glycolipids with periodate oxidation, followed by reduction with Na BH_4 and followed the loss of this label from the surface in vitro. The amount of label was found to decrease with time in culture, with an average half-time of 10-12 hours. This turnover rate was similar to that for glycoproteins, suggesting that the membrane was shed as a unit. [^{125}I] Labelled membrane lipids were also found to be shed into culture media in vitro (Rumjanek and McLaren, 1981; Wilson, 1981), with a half-time of 6 hours, or 3 hours when stimulated (Wilson, 1981). Several workers have proposed that rapid turnover of the surface membranes of S. mansoni might constitute a defense mechanism, by allowing loss of target antigens and the clearance of immunologically compromised areas of membrane (Wilson and Barnes, 1974b; Kusel et al., 1975b; Dessein et al., 1981; Podesta, 1982a). As a general phenomenon rapid membrane turnover is thought to allow

maintenance of membrane in a virgin state, with the surface receptors uncluttered by bound ligands or adsorbed molecules (Doljanski and Kapeller, 1976). Turnover of the surface of S. mansoni is thought to be a rapid process, with most estimates of half-time in the range of 2-10 hours (Wilson and Barnes, 1977; Dean and Podesta, 1982; Samuelson and Caulfield, 1982). Attack by immune cells may accelerate the normal shedding of the surface (Caulfield et al., 1982). Shedding of large amounts of surface membrane antigens may encourage the development of immunological tolerance and circulating immune complexes could block specific receptors on immune effector cells.

Despite the widely recognised importance of the dynamic nature of the schistosome surface, no studies to date have specifically dealt with turnover of the major component of the OB; namely phospholipids (McDiarmid and Podesta, 1982). A controversy exists in cell biology as to whether membrane lipids and proteins turn over at the same rate (Cohen and Phillips, 1980), or with different half-times (Siekevitz, 1972; Doetschman, 1980). In several systems the phospholipids appear to turn over more rapidly than do the proteins (Omura et al., 1967; Pasternak and Bergeron, 1970). The kinetics of phospholipid turnover however, are more complex than for proteins because of transfer proteins (Lumb et al., 1980; Tsao, 1980), exchange reactions and acyl transferase enzymes (Kapeller et al., 1973; Trewhella and Collins, 1973; Rosenthal and Somers, 1979; Khuller et al., 1981). Kinetic analyses are further complicated, as different individual phospholipids and phospholipid moieties can exhibit heterogeneous turnover rates (Pascual de Bazán and Bazán, 1976; Sandra and Ionosescu, 1980).

In general, there have been far fewer studies on lipid turnover

than on protein turnover. Little is known of the origin, synthesis, modulation and turnover of membrane lipids in S. mansoni. The proportions of the major phospholipid classes in the surface bilayers have been determined (McDiarmid and Podesta, 1982), but there is little information available on their kinetics of formation or turnover. Schistosoma mansoni can incorporate lipids and lipid precursors from the external environment in culture (Meyer et al., 1970; Smith et al., 1970; Rumjanek and Simpson, 1980) and presumably from the host in vivo. Platyhelminthes, including S. mansoni, are thought to be incapable of synthesising sterols and long chain fatty acids de novo (Bailey and Fairbairn, 1968; Meyer et al., 1970 and 1979; Smith et al., 1970). [^{14}C] Acetate is incorporated only into the terminal carboxyl group of fatty acids, suggesting a capacity for chain elongation (Jacobsen and Fairbairn, 1967; Meyer et al., 1970). Schistosoma mansoni can synthesise its own complex lipids (Meyer et al., 1970; Young and Podesta, 1982) and must therefore have the ability to determine the lipid composition of its surface membranes. The location of the enzymes involved in surface membrane lipid biosynthesis in S. mansoni is still mainly speculative. Synthesis is presumed to occur in the endoplasmic reticulum and golgi of the subtegumental cells, with subsequent transport, via the MLB, to the surface. However phosphatidic acid phosphatase, a central enzyme of lipid synthesis is now known to be present in the MLB (McDiarmid et al., 1982; Rahman and Podesta, 1982). Rumjanek and Cesari (1981) have suggested that the adult schistosome surface contains high levels of phospholipase activity, which they postulate to be involved in the breakdown and subsequent uptake of external phospholipids. The possibilities of synthesis or acylation at the surface have not been

considered.

Turnover of membrane constituents is most commonly measured with the aid of isotopically labelled metabolic precursors. In S. mansoni such measurements are complicated by the presence of two, closely apposed, surface bilayers, which probably have different turnover rates. Workers, attempting similar measurements of the two suface membranes of mitochondria, realised that kinetic analyses of bilayer components can only be considered valid if cross-contamination is monitored (Bygrave, 1969). Quantitative separation of the outer and inner bilayers of adult S. mansoni is now possible (McDiarmid et al., 1983). For the first time these procedures have allowed the calculation of protein turnover rates for the outer and inner bilayers (Dean and Podesta, 1982). Proteins of the outer bilayer were found to turn over more rapidly, with $t_{1/2}$ = 3 hours, than the inner bilayer, with $t_{1/2}$ = 6 hours (Dean and Podesta, 1982; Dean, 1983). In this chapter the labelling patterns and turnover rates of the surface membrane phospholipids of S. mansoni will be investigated.

5.2 MATERIALS AND METHODS

5.2.1 Thin Layer Chromatography and Autoradiography of Bilayer Phospholipids

Adult worm pairs were metabolically labelled with one of the following isotopes - [^{32}P] 20 uCi/ml (Amersham); [^{3}H] - glycerol (5-10 Ci/mmol, New England Nuclear, NEN) 5 uCi/ml incubation medium; [^{14}C] palmitate (500mCi/mmol, NEN) 1 uCi/ml incubation medium; or [^{14}C] galactose (40-60 mCi/mmol, NEN) 4 uCi/ml incubation medium. Worm pairs were also labelled with [^{125}I] diazotised iodosulfanilic

acid, as described previously. Labelling was accomplished during a 2 hour incubation in medium 199 with 5% heat-inactivated human serum (HIH) at 37°C in a 5% CO_2 atmosphere. Fractions, described in Chapter 4, were prepared from the labelled worms and comprised outer and inner bilayer pellets and supernatants, and homogenates of the remaining carcasses. A sixth fraction was prepared by pelleting the incubation medium at 35,000 x g for 1 hour.

Thin layer chromatography of these fractions was performed, as detailed previously. Spots were visualised in an iodine vapour chamber and a tracing of the spots was made on acetate sheets. The plates were then left until the colouration by iodine had faded. For autoradiography, the x-ray film (X-Omat RP, Kodak) was sandwiched between the TLC plate and a clean glass plate. The two plates were held together firmly with masking tape and stored in the dark to allow selective exposure of the film. Exact positioning of the film, relative to the plate was determined by previously marking small spots on the borders of the TLC plate with radioactive waste material. This allowed subsequent alignment of the TLC plate and the developed autoradiograph. The film was developed automatically in a clinical x-ray film processor.

To determine the radioactivity of individual lipids, radioactive spots were scraped off the plate into scintillation vials. The spots were vortexed vigorously in 1 ml of water, prior to addition of scintillation fluid (Scintiverse, Fisher Chemical Co.). Vials were stored in the dark overnight and counted on a Beckman scintillation counter. All counts were corrected for quenching by automatic external standardisation.

5.2.2 Incorporation of [^{3}H] glycerol into the lipid and water soluble phase of adult worm pairs

Adult worm pairs were incubated in medium 199 with 5% HIH serum, at 37°C, containing 10 µCi/ml [^{3}H] glycerol (5-10 Ci/mmol, NEN). At time intervals ranging from 15 minutes to 6 hours, 20 worm pairs were quickly removed from the medium and immediately homogenised in 0.5 ml ice cold KRP. A 100µl aliquot of the homogenate was stored on ice for subsequent protein determination. The remaining volume was added to 10 ml 2:1 chloroform/methanol and extracted by the method of Bligh and Dyer (1959). The resulting lipid extracts were taken to dryness in scintillation vials and resuspended in Scintiverse for scintillation counting. An aliquot of the aqueous phase was also counted.

5.2.3 Efflux of [^{3}H] Glycerol and [^{14}C] Palmitate from the Lipid and Water Phases of Adult Worm Pairs.

Adult worm pairs were labelled for 2 hours, as above, but with 10 µCi/ml [^{3}H] glycerol and 1 µCi/ml [^{14}C] palmitate in the medium. After labelling the worms were washed extensively in the chase medium (199 + 5% HIH serum, with 10 mM glycerol and 1 mM palmitate). The worms were then incubated in large volumes of the chase medium. At time intervals ranging from 5 minutes to 5 hours, 10 worm pairs were removed and immediately washed in ice-cold KRP, then homogenised in 0.5 ml KRP. As described previously, 100 ml of the homogenate was retained for protein determination and the remainder was used for lipid extraction and scintillation counting. Conventional double isotope counting techniques (Podesta et al., 1977) were used to determine the amount of [^{3}H] and [^{14}C] in the test

samples.

5.2.4 Determination of Turnover Rates of Surface Bilayer Lipids from Adult Worm Pairs Labelled with [^{14}C] Glycerol

Adult worm pairs were labelled during a 2 hour incubation in medium 199 with 5% HIH serum, at 37°C in 5% CO_2, containing 5 μCi/ml [^{14}C] glycerol. The worms were then washed extensively in medium 199, with 10 mM glycerol and put into the chase incubation, consisting of medium 199 with 5% HIH serum and 10 mM glycerol. At time points ranging from 0-4 hours approximately 300 worm pairs were removed to a vial containing 0.2 M sucrose in 0.1 M Tris, (pH 7.4) on ice. The worms were washed in the same medium and surface bilayer fractions obtained, as described previously. Aliquots of the pelleted OB and IB fractions were retained for alkaline phosphatase measurements and the remainder was utilised for lipid extraction and scintillation counting. Carcasses remaining after membrane removal procedures were homogenised and aliquots reserved for protein and alkaline phosphatase determinations and scintillation counting. Materials pelleted from the incubation medium were treated similarly to the bilayer fractions.

Counts in the IB and OB were corrected by means of the alkaline phosphatase data and normalised to carcass protein.

5.2.5 Determination of Turnover Rates of Surface Bilayer Lipids from Adult Worm Pairs Labelled With [^{14}C] Palmitate

Adult worm pairs were labelled as above, in medium containing 1.0 μCi/ml [^{14}C] palmitate. Following labelling the worms were washed and then chased in medium 199 with 5% HIH serum and 1 mM palmitate. At various time intervals during a 4 hour chase incubation

approximately 250 worm pairs were removed, washed and subjected to membrane removal procedures, as detailed previously. The following fractions were obtained: OB and IB pellets, OB and IB supernatants, carcass homogenates and material pelleted from the incubation medium. Aliquots were taken from each of these fractions for protein determinations, alkaline phosphatase measurements and lipid extraction, with subsequent scintillation counting.

The counts obtained for OB and IB pellets were corrected for cross contamination by employing the membrane markers described in Chapter 4. Alkaline phosphatase was utilised as a marker for the IB, and OB contamination was deduced from the results of the [^{125}I] diazotised iodosulfanilic acid labelling experiments. A correction factor (CF) was determined from the formula

$$CF = C_o \left[(a_o/P_i) \,/\, (a_i/P_o)\right]$$

(Dean, 1983) where a_o and a_i represent alkaline phosphastase activity in the OB and IB fractions respectively, C_o represents the uncorrected counts in the OB fraction and P_o and P_i represent protein in the OB and IB fractions. Alkaline phosphatase activity in the OB is therefore used as a measure of contamination of that fraction with IB material. Corrected counts in the OB fraction (cC_o) were derived as follows (Dean, 1983): $cC_o = (C_o - CF) + 0.2\,(C_o - CF)d$

$$= 1.2\,(CO - CF)$$

where 0.2 is a constant derived from the level of 20% contamination of IB with OB at the lengths of digitonin incubation employed (5 and 25 minutes). This 20% level was obtained from the [^{125}I] diazotised iodosulfanilic acid labelling experiments of Chapter 4 and was assumed to be constant throughout. Corrected counts for the IB (cC_i) were determined by $cC_i = C_i + CF - 0.2\,cC_o$. Counts were normalised

to total bilayer protein in both fractions.

5.3 RESULTS

5.3.1 Incorporation of Various Isotopes into the Surface Membrane Phospholipids of Adult Worm Pairs

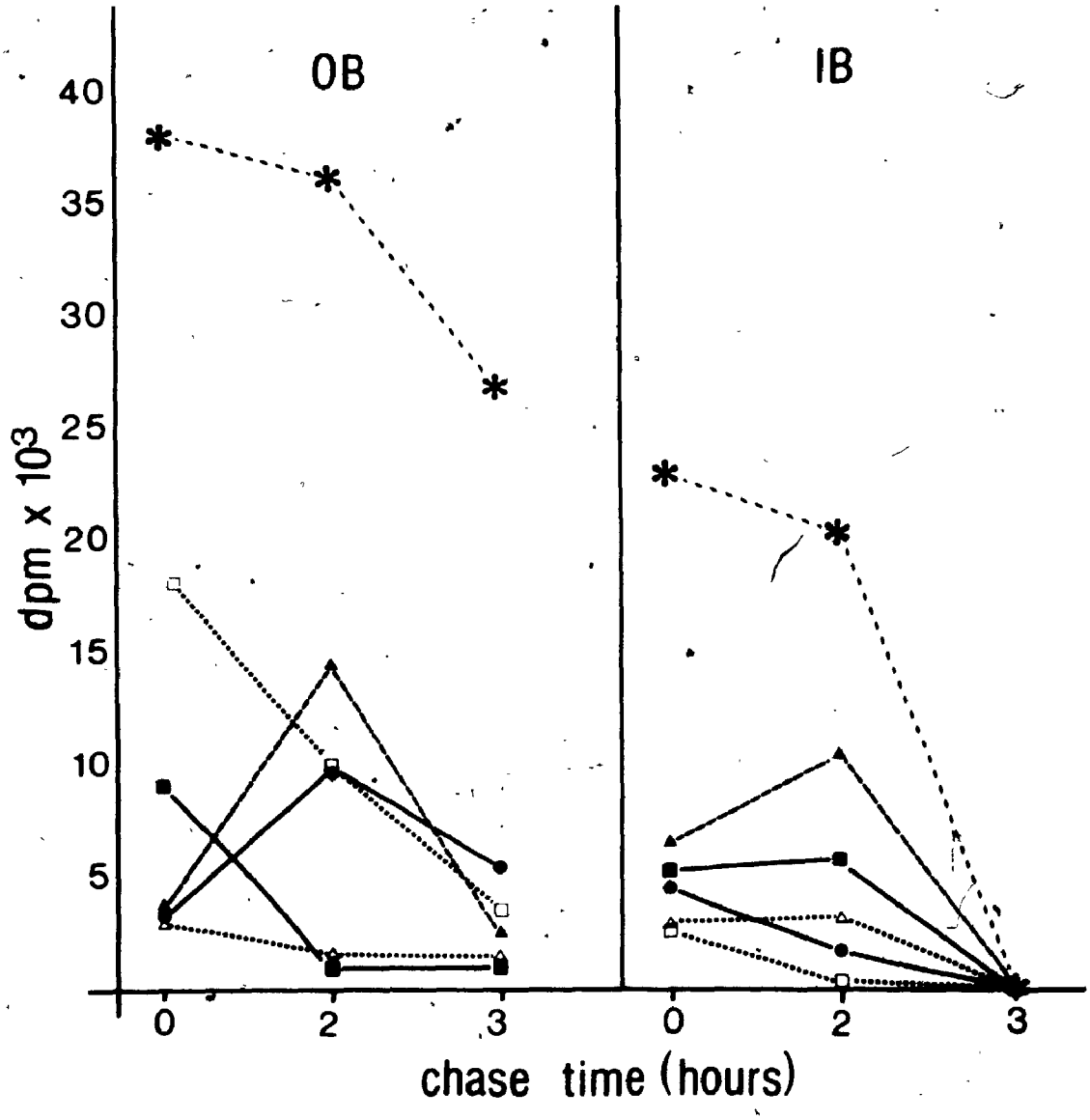
Autoradiographs of OB and IB fraction phospholipids from [^{32}P] labelled adult S. mansoni showed that [^{32}P] was incorporated into the surface membrane phospholipids (Figure 5.1). During a 3 hour chase period the activity of [^{32}P] in both fractions declined (Figure 5.2). Determination of the turnover rates of individual phospholipids proved to be impractical in the present study as insufficient material was available to allow measurement of the specific activity of [^{32}P] in individual phospholipid spots. However the results obtained did indicate some differential turnover rates for the individual phospholipid classes. While activity of most of the phospholipids decreased during the 3 hour chase, the activity of phosphatidylcholine and sphingomyelin increased in the OB during the first 2 hours of chase. Phosphatidylcholine also increased in the IB, but to a lesser extent (Figure 5.2). The label disappeared from the IB at a faster rate than in the OB.

Autoradiographs of OB and IB fraction phospholipids from [^{14}C] glycerol and from [^{14}C] palmitate-labelled adult worms indicated that both of these isotopes were incorporated into the surface bilayer lipids. Autoradiographs of OB and IB supernatant, pelleted material from the incubation medium and homogenised carcass lipid extracts of [^{14}C] palmitate - labelled worms were also examined. Labelled phospholipids were present in each of these

Figure 5.1 Autoradiographs of 2 dimensional thin layer chromatographs of outer bilayer (OB) and inner bilayer (IB) phospholipids. Adult worms were labelled with [^{32}P] 20 μ Ci/ml incubation medium *in vitro* for 2 hours and chased for 2 hours in cold medium. Ly, lysophosphatidylcholine; Pc, Phosphatidylcholine; Sph, Sphingomyelin; Pe, Phosphatidylethanolamine; Ps, phosphatidylserine; X, origin.


OB

Figure 5.2 [^{32}P] Labelling patterns of individual phospholipids in the outer (OB) and inner (IB) bilayers. Adult S. mansoni were labelled during a 2 hour incubation in vitro with 20 μ Ci/ml [^{32}P] and chased in the absence of label for up to 3 hours. Outer and inner bilayers were removed by sequential exposures to the digitonin membrane disruption fluid. Lipid extracts of outer and inner bilayer fractions were run in 2 dimensions on silica gel G thin layer chromatography plates. Labelled lipids were visualised by autoradiography, scraped off the plates and counted in a Beckman liquid scintillation counter. Points represent the means of 3 experiments. Explanation of symbols : ▲ , phosphatidylcholine; ■ , lysophosphatidylcholine; △ , phosphatidylserine; ● , sphingomyelin, □ , phosphatidylethanolamine; ✱, total.


OB
IB
40
35
30
25
20
15
10
5
dpm x 10^3
0
2
3
0
2
3
chase time (hours)

fractions (Figure 5.3). In each case phosphatidylcholine appeared to be the most heavily labelled phospholipid. In both OB and IB supernatants phosphatidylcholine was the only spot visible on the autoradiograph. However, the labelling patterns on these autoradiographs cannot be directly compared since different amounts of label were loaded on each.

[^{14}C] Galactose was incorporated into the lipid phases of OB and IB pellets and supernatants and into the pellet of the incubation medium. The incubation medium pellet had the highest specific activity at 147 dpm/µg protein. The OB supernatant and pellet had 53 and 24 dpm/µg protein respectively, with the IB supernatant and pellet having 15 and 11 dpm/µg protein. However, although over 1,000 worm pairs were used only one spot was visible on most of the autoradiographs. The same spot appeared to be present in all of the fractions except the pelleted incubation medium, where there were 2 species labelled closer to the origin. Owing to the large number of worms required this experiment was only repeated once. The experiment was repeated however with 2.5 µCi/ml [^{14}C] glucose in the labelling medium but the counts in the lipid extracts of OB and IB fractions were not significantly above background.

Autoradiographs of bilayer fractions from worm pairs labelled with [^{125}I] diazotised iodosulfanilic acid indicated the presence of labelled lipids in OB and IB pellet and supernatant fractions. The spot co-migrating with bovine cerebrosides appeared to be particularly densely labelled in the supernatant fractions (Data not shown).

5.3.2 [^{3}H] Glycerol Incorporation into Adult Worm Pairs

[^{3}H] Glycerol incorporation into the water-soluble phase of

Figure 5.3 Autoradiographs of 2 dimensional thin layer chromatographs of outer bilayer (OB), inner bilayer (IB), outer bilayer supernatant (OB SUP), pelleted incubation medium (INC MED) and carcasses (CAR). Adult S. mansoni were labelled with [^{14}C] palmitate 1 μ Ci/ml incubation medium during a 2 hour incubation period in vitro. Pc, phosphatidylcholine; Pe, phosphatidylethanolamine; Sph, sphingomyelin; Ly, lysphosphatidylcholine; Ps, phosphatidylserine; Pi, phosphatidylinositol.

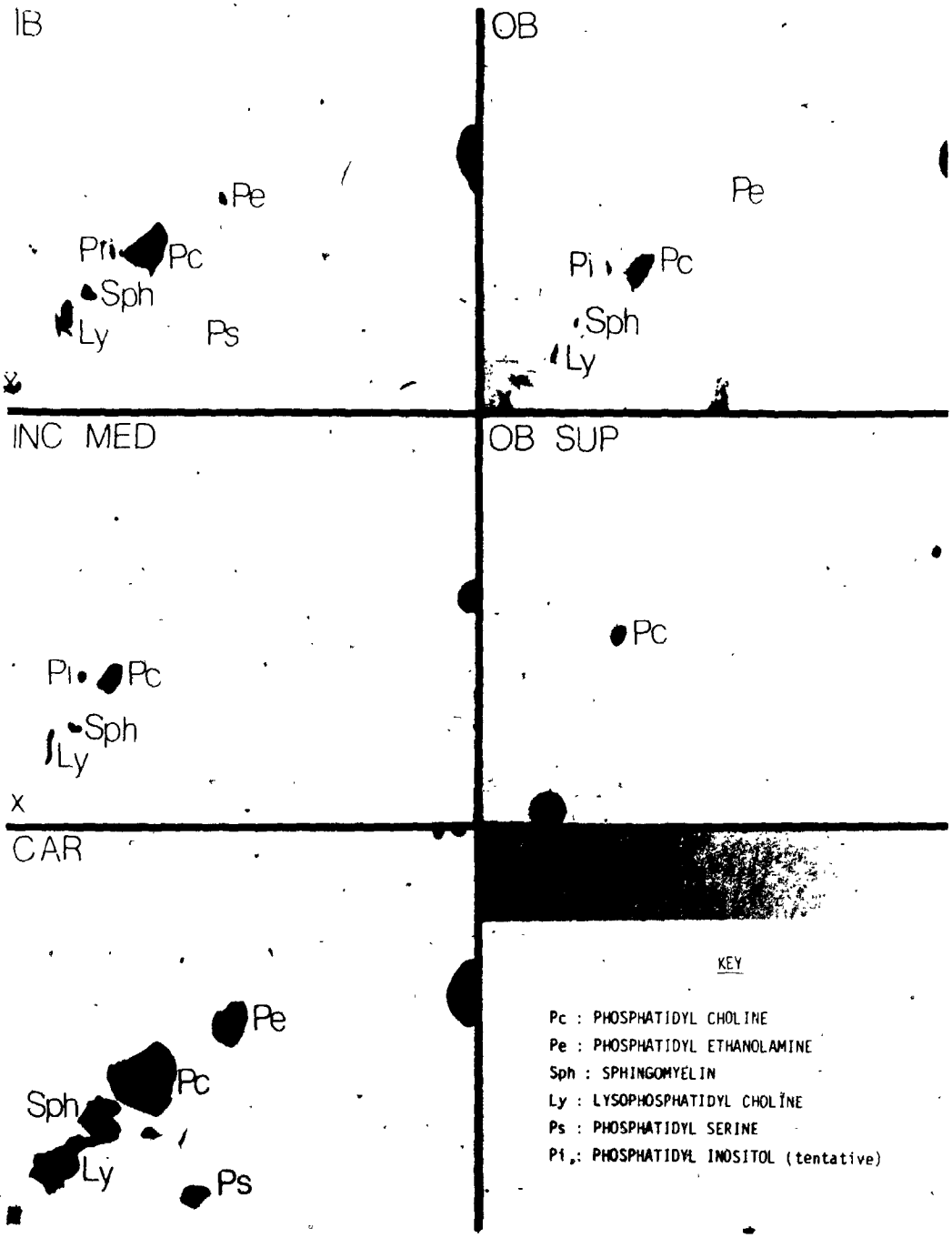

IB
OB
Pe
Pi
Pc
Sph
Ly
Ps
INC MED
OB SUP
Pc
Pi
Pc
Sph
Ly
x
CAR
Pe
Pc
Sph
Ly
Ps
KEY
Pc : PHOSPHATIDYL CHOLINE
Pe : PHOSPHATIDYL ETHANOLAMINE
Sph : SPHINGOMYELIN
Ly : LYSOPHOSPHATIDYL CHOLINE
Ps : PHOSPHATIDYL SERINE
Pi : PHOSPHATIDYL INOSITOL (tentative)

Figure 5.4 The relationship of glycerol incorporation into the water-soluble phase of S. mansoni with time in culture. Adult worm pairs were incubated in medium containing 10 μCi/ml [^{3}H] glycerol. At various time intervals 20 worm pairs were removed and incorporation of label into the water-soluble phase determined. The points shown are mean values from 3 separate experiments and bars indicate standard errors of the mean. The solid line represents the curve fitted to the points by eye. The dotted line represents total incorporation of [^{3}H] glycerol into the lipid and water-soluble phases.

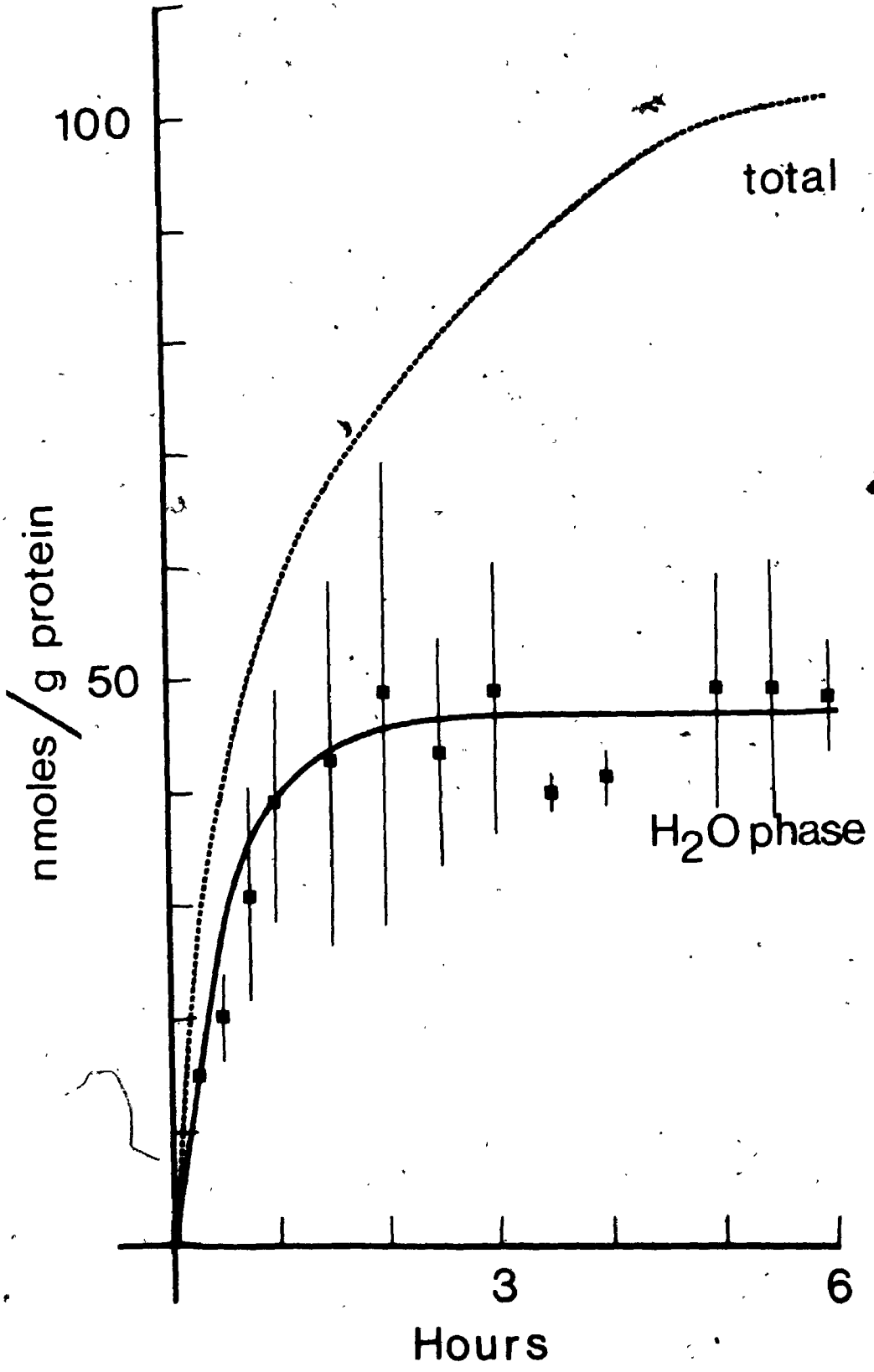
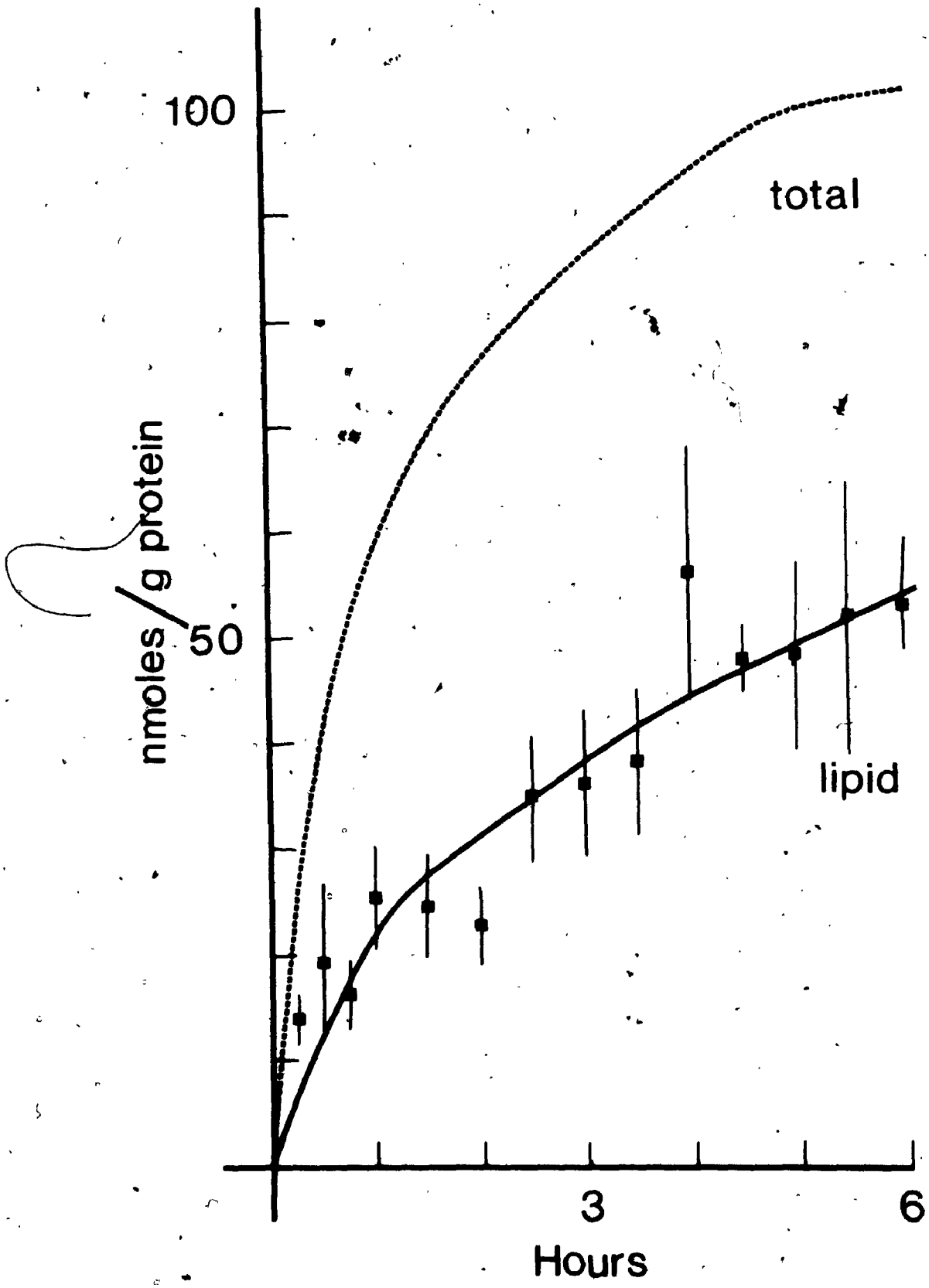

100
50
nmoles / g protein
total
H_2O phase
3
6
Hours

Figure 5.5 The relationship of glycerol incorporation into the lipids of S. mansoni with time in culture. Adult worm pairs were incubated in medium containing 10 μ Ci/ml [^{3}H] glycerol. At the time intervals indicated 20 worm pairs were removed and incorporation of label into the lipid phase determined. The points shown are mean values from 3 separate experiments and bars indicate standard errors of the mean. The solid line is the curve fitted to the points by eye. The dotted line represents total incorporation of [^{3}H] glycerol into the lipid and water-soluble phases.


100
50
nmoles/g protein
total
lipid
3
6
Hours

adult worm pairs increased rapidly with time of incubation up to 1 hour. After 2 hours the incorporation stablised at 46 nmoles/g protein and there was no further increase (Figure 5.4). Although glycerol incorporation into the water-soluble phase of the worms was saturated, total glycerol incorporation continued to increase throughout the labelling period of 6 hours.

The rate of [^{3}H] glycerol incorporation into the lipid phase of adult worm pairs was most rapid during the first hour of incubation (Figure 5). This initial rapid rate of increase however, was much less than that for the water soluble phase. Glycerol incorporation into the lipid phase did not appear to saturate, but continued to increase at a slower rate throughout the incubation period, reaching about 53 nmoles/g protein by 6 hours.

5.3.3 [^{3}H] Glycerol and [^{14}C] Palmitate Efflux from Adult Worm Pairs

Results in this section were plotted as dpm per worm pair or protein versus time of chase incubation, which yielded exponentially decaying curves. Linear functions of this data were derived from log-linear plots, which could be described by the general formula $y = ae^{-bx}$, where a is the intercept, b is the slope and e is the base of the natural logarithm (Sokal and Rohlf, 1973).

A plot of the data for [^{3}H] glycerol efflux from the water soluble phase of adult S. mansoni was non-linear, suggesting the presence of more than one compartment (Figure 5.6). Curve peeling, a simple method of compartmental analysis, was employed to derive the curve for the initial rapid efflux rate (Jacquez, 1972). The procedure involves adding points from right to left, until a

Figure 5.6 Glycerol efflux from the water-soluble phase of *S. mansoni*. Adult worm pairs were labelled during a 2 hour incubation with 10 μ Ci/ml incubation medium [^{3}H] glycerol. Labelled worms were incubated in the chase medium without label for up to 5 hours. At the time intervals indicated 10 worm pairs were removed and [^{3}H] activity in the water-soluble phase determined. Points are the mean values of 3 separate experiments. Line 1 represents a small, slowly exchanging pool ($t_{1/2}$ = 9.8 hours). Line 2 (open symbols) was derived from the original plot (solid symbols) by curve peeling. Line 1 was subtracted from lines 1 + 2 and the difference plotted as line 2, which describes a larger, rapidly exchanging pool ($t_{1/2}$ = 0.15 hours).

Lines are described by an exponential function of the form $y = a_1 \exp b_1 t + a_2 \exp b_2 t$, where a_1 = 47.0 ($\pm$ 0.11); b_1 = -0.071 ($\pm$0.04); a_2 = 142 ($\pm$0.09); b_2 = -4.49 ($\pm$ 0.25). The slope of a_1 is significantly different from zero ($P < 0.001$), and marginally significant ($0.1 > P > 0.05$) for a_2.

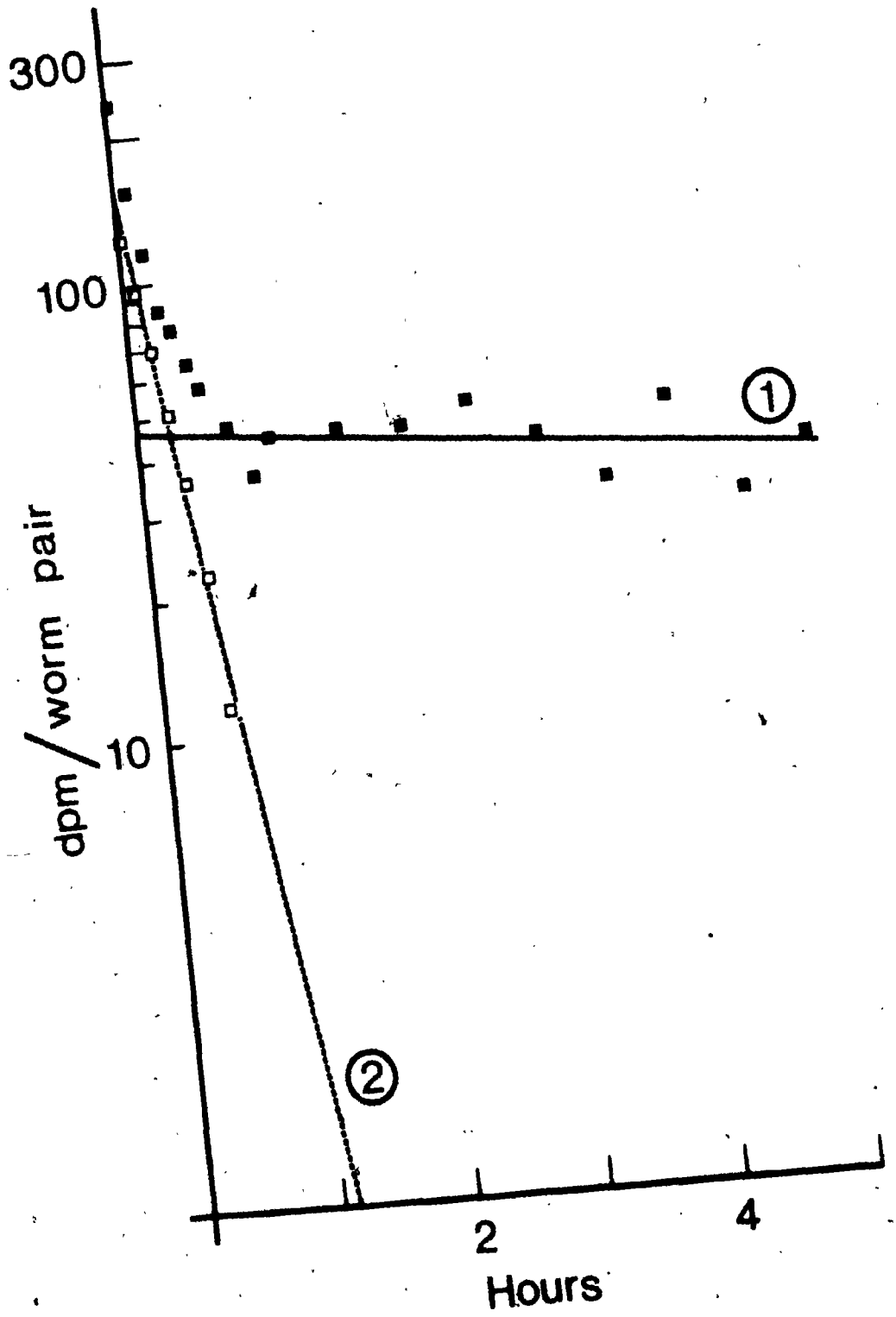
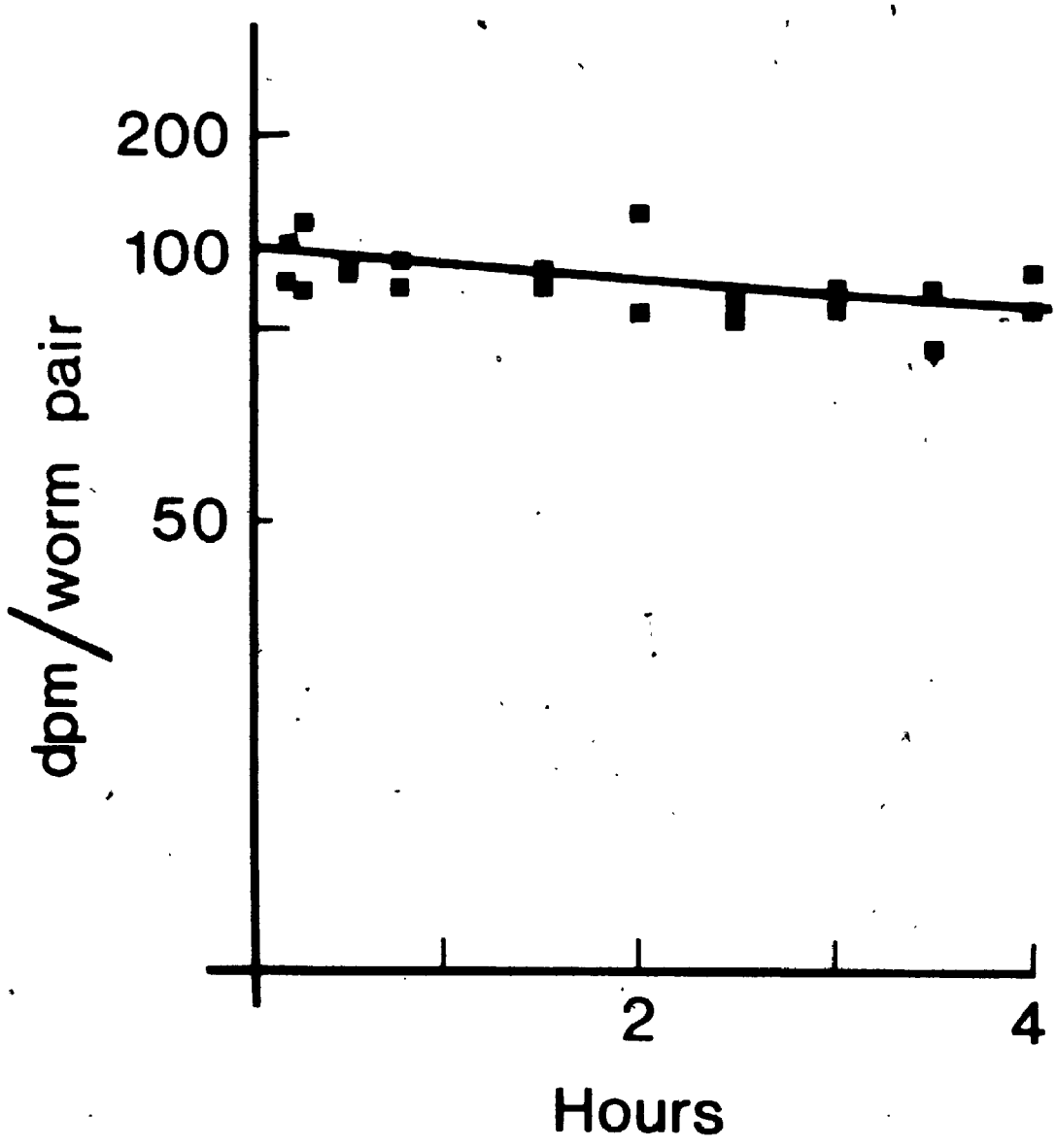

300
100
10
dpm / worm pair
①
②
2
4
Hours

Figure 5.7 Glycerol efflux from the lipid phase of S. mansoni. Adult worm pairs were labelled during a 2 hour incubation with 10 μ Ci/ml incubation medium [^{3}H]- glycerol. Labelled worms were incubated in the chase medium without label for up to 5 hours. At various times 10 worm pairs were removed and [^{3}H] activity in the lipid phase determined. Points show the mean values of 3 separate experiments. The line is described by the exponential function $Y = 127\ (\pm\ 0.04)\ \exp\ -0.05\ (\pm\ 0.02)t$ and represents one compartment with $t_{1/2}$ = 13.9 hours. The slope of the line is significantly different from zero ($0.02>P>0.01$).


200
100
50
dpm/worm pair
2
4
Hours

significant deviation from linearity occurs. If the data points do not deviate significantly from linearity, then one can assume that the label is exchanging with a single compartment (Podesta and Dean, 1982a, 1982b). However, it can be seen from figure 5.6 that there is a significant deviation from linearity and that efflux of [^{3}H] glycerol from the water soluble phase can best be described in terms of 2 compartments - a rapidly exchanging pool, with a half-time of 9 minutes and a more slowly exchanging pool, with a half-time of 9.8 hours. Curve 1 was subtracted from curve 1 + 2 and the difference plotted as curve 2, which describes the rapidly exchanging pool.

A plot of the data for [^{14}C] glycerol efflux from the lipid phase of adult worm pairs yielded a linear relationship, indicating the presence of just one compartment with a half-time of 13.9 hours (Figure 5.7).

[^{14}C] Palmitate, like [^{3}H] glycerol, appeared to efflux from the water-soluble phase of adult worm pairs from 2 compartments (Figure 5.8). For palmitate, the rapidly exchanging pool was small and exchanged with a half-time of 13.8 minutes. The larger and more slowly exchanging pool had a half-time of 11.5 hours. These half-times for the rapid pool were significantly (P 0.001) longer than those obtained with [^{3}H] glycerol.

[^{14}C] Palmitate efflux from the lipid phase of adult worm pairs, like that for glycerol, was linear, suggesting the presence of one compartment. This compartment exchanged with a half-time of 11.5 hours, which was more rapid than that obtained for glycerol, although not significantly so (P 0.5), (Figure 5.9).

5.3.4 Turnover Rates of Surface Bilayer Lipids of Adult Worm Pairs, Labelled with [^{14}C] Glycerol or [^{14}C] Palmitate.

Figure 5.8 Palmitate efflux from the water-soluble phase of S. mansoni. Adult worm pairs were labelled during a 2 hour incubation with 1 u Ci/ml incubation medium [^{14}C] palmitate. Labelled worms were incubated in the chase medium without label for up to 5 hours. At various times 10 worm pairs were removed and [^{14}C] activity in the water-soluble phase determined. Points are the mean values of 3 separate experiments. Line 1 represents a large, slowly exchanging pool ($t_{1/2}$ = 11.5 hours). Line 2 (open symbols) was derived from the original plot (solid symbols) by curve peeling. Line 1 was subtracted from lines 1 + 2 and the difference plotted as line 2, which describes a smaller, rapidly exchanging pool ($t_{1/2}$ = 0.23 hours).

Lines are described by an exponential function of the form $Y = a_1 \exp b_1 t + a_2 \exp b_2 t$, where a_1 = 1.09 ($\pm$ 0.12); b_1 = -0.06 ($\pm$ 0.03); a_2 = 0.56 ($\pm$ 0.07); b_2 = -2.95 ($\pm$ 0.14). The slope of line 1 is not significantly different to zero (0.2 P 0.1), whilst the slope of line 2 is significant (0.01 P 0.001).

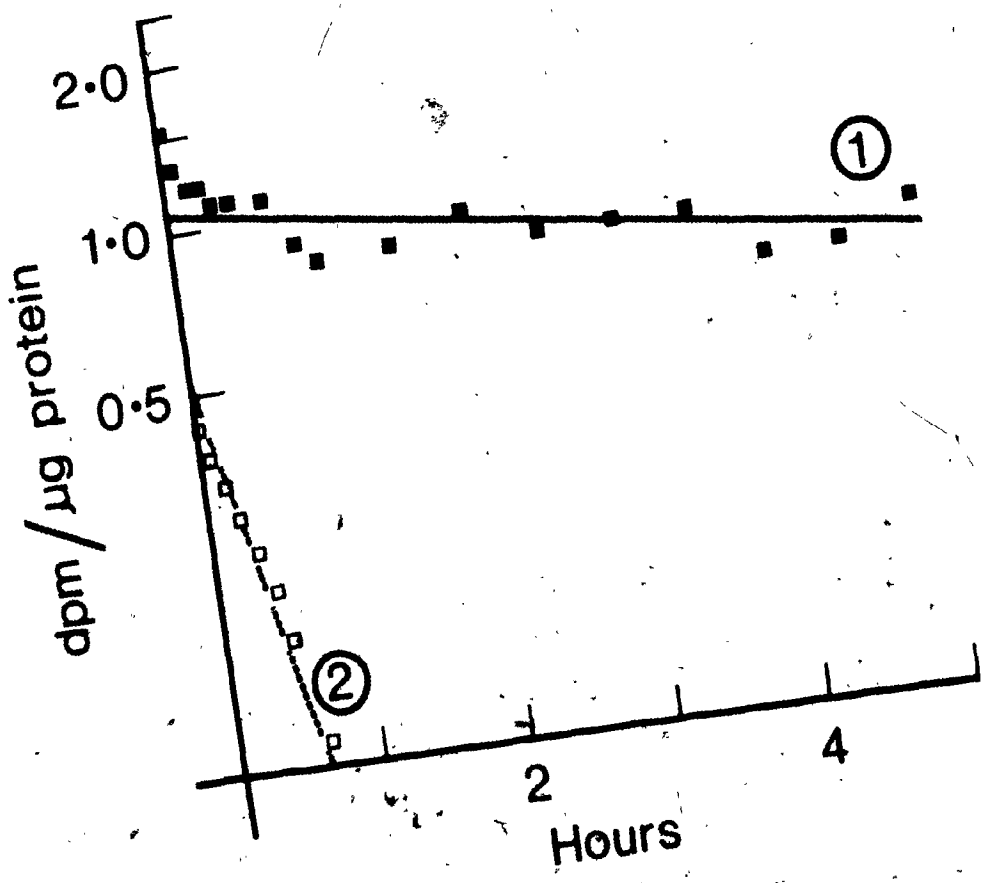

2·0
1·0
0·5
dpm / µg protein
①
②
2
4
Hours

Figure 5.9 Palmitate efflux from the lipid phase of S. mansoni. Adult worm pairs were labelled during a 2 hour incubation with 1 μ Ci/ml incubation medium [^{14}C] palmitate. Labelled worms were incubated in the chase medium without label for up to 5 hours. At the time intervals indicated 10 worm pairs were removed and [^{14}C] activity in the lipid phase determined. Points are the mean values of 3 separate experiments. The line is described by the exponential function $Y = 279\ (\pm 0.06)\ \exp\ -0.06\ (\pm 0.03)\ t$ and represents one compartment with $t_{1/2}$ = 11.5 hours. The slope of the line if significantly different from zero ($0.02>P>0.01$).

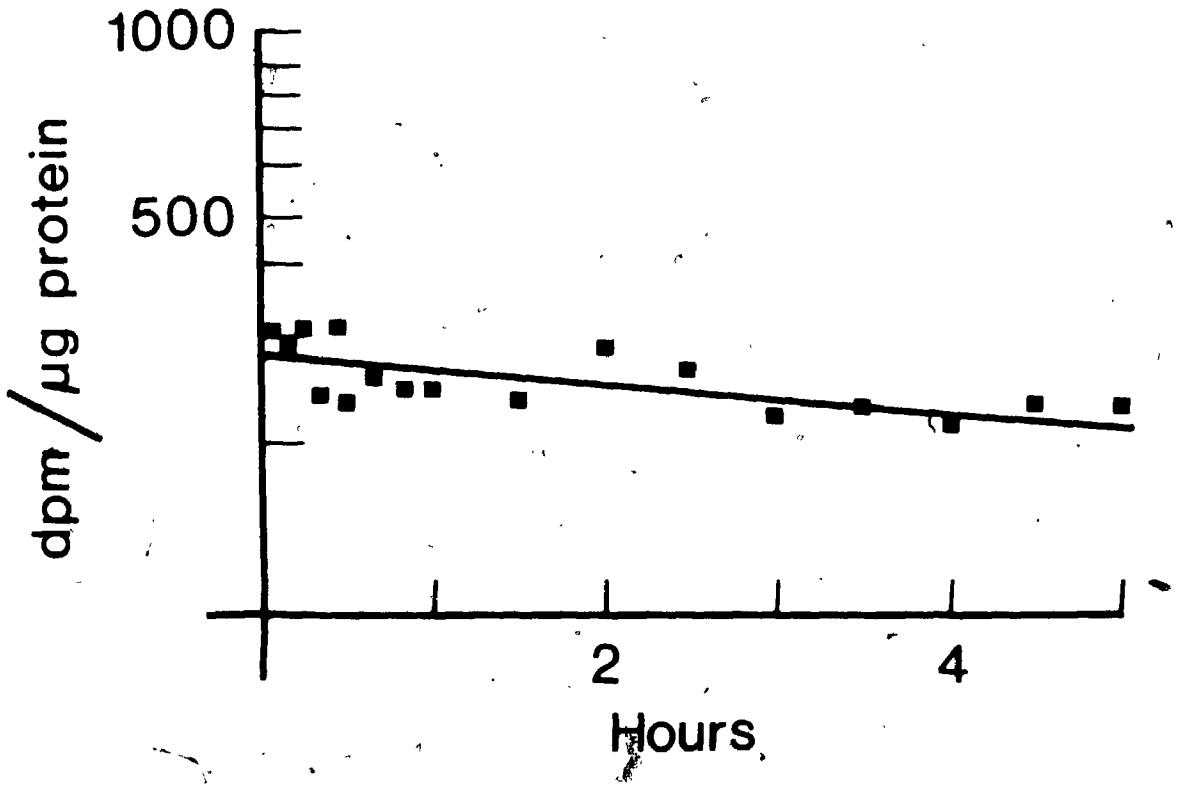

1000
500
dpm / µg protein
2
4
Hours

Figure 5.10 Turnover of glycerol in the outer and inner bilayers. Adult worm pairs were labelled during a 2 hour incubation with 5 μ Ci/ml incubation medium [^{14}C] glycerol. Labelled worms were incubated in the chase medium, in the absence of label, for up to 4 hours. At the time intervals indicated 300 worm pairs were removed and outer and inner bilayer fractions obtained by sequential 5 and 25 minute incubations in the digitonin membrane removal fluid. Activity of [^{14}C] in the outer (OB, clear symbols) and inner (IB, solid symbols) bilayer fractions was determined. Counts in the IB and OB were corrected for cross contamination by means of the alkaline phosphatase data and normalised to carcass protein. Points are mean values. The OB line is described by the function $Y = 23.3\ (\pm 0.27)\ \exp\ -0.06\ (\pm 0.14)\ t$, ($n = 25$) and the IB by $Y = 62.8\ (\pm 0.25)\ \exp\ 0.02\ (\pm 0.13)\ t$, ($n = 25$). The slopes of both lines are not significantly different from zero ($0.9 > P > 0.5$) and neither are they significantly different from each other ($P < 0.5$). Half-time of turnover for the outer bilayer = 11.5 hours, while activity in the IB increases during the 4 hour chase.

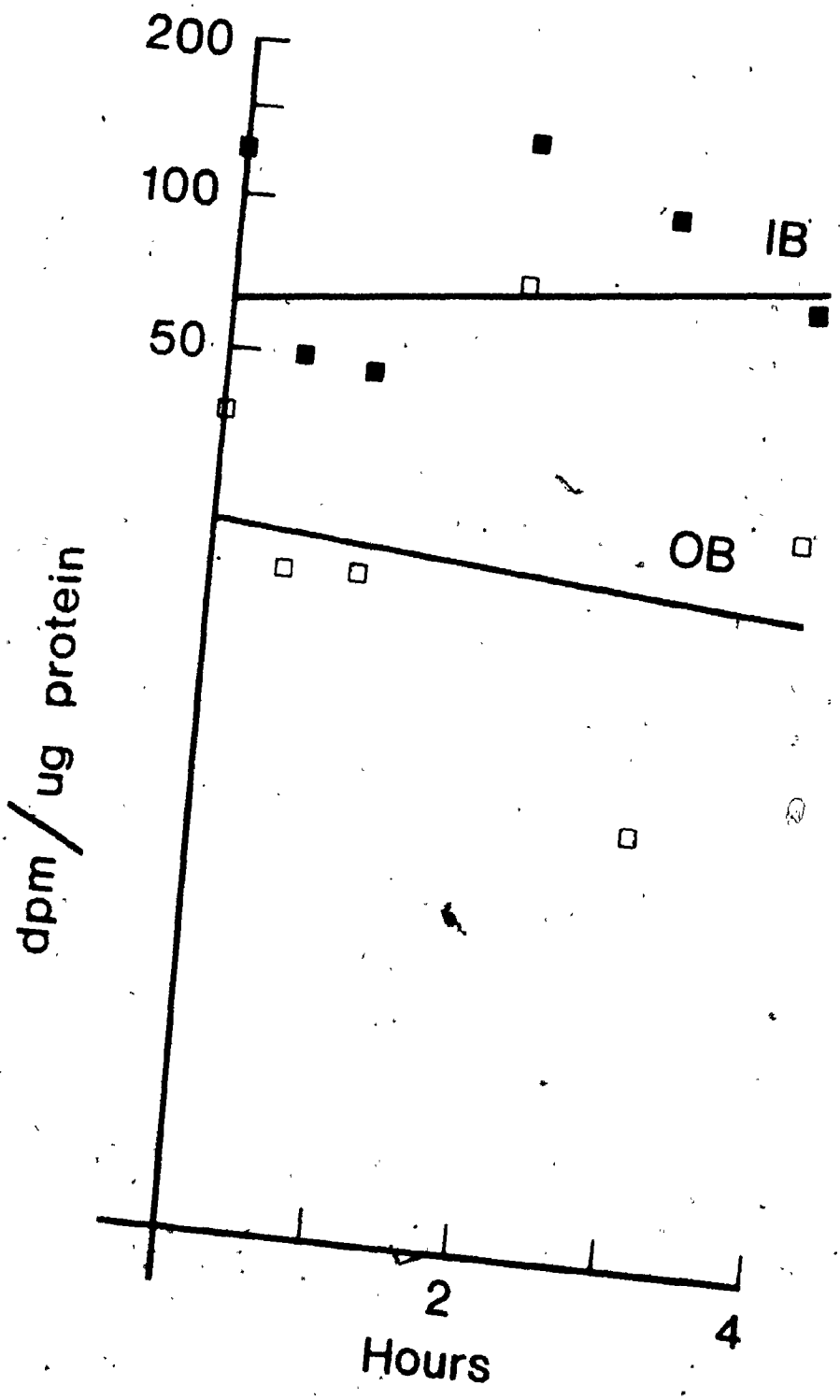

200
100
50
IB
OB
dpm / ug protein
2
4
Hours

Figure 5.11 Turnover of palmitate in the outer bilayer. Adult worm pairs were labelled during a 2 hour incubation with 1.0 μ Ci/ml incubation medium [^{14}C] palmitate. Labelled worms were incubated in the chase medium, in the absence of label, for up to 4 hours. At the time intervals indicated 250 worm pairs were removed and the outer bilayer obtained by a 5 minute incubation in the digitonin membrane removal fluid. Activity of [^{14}C] in the outer bilayer was determined. Counts were corrected for contamination with inner bilayer material by means of the alkaline phosphatase and [^{125}I] data, as described in the text and normalised to total bilayer protein. Points are mean values. The line is described by the function Y = 56.83 (±0.18) exp - 0.47 (± 0.14) t (n = 25). The slope of the line is significantly different from zero ($0.01 > P > 0.001$) and has a half-time of 1.47 hours. Points obtained beyond 3 hours of chase do not fit on this line and were therefore not included in the regression calculation.

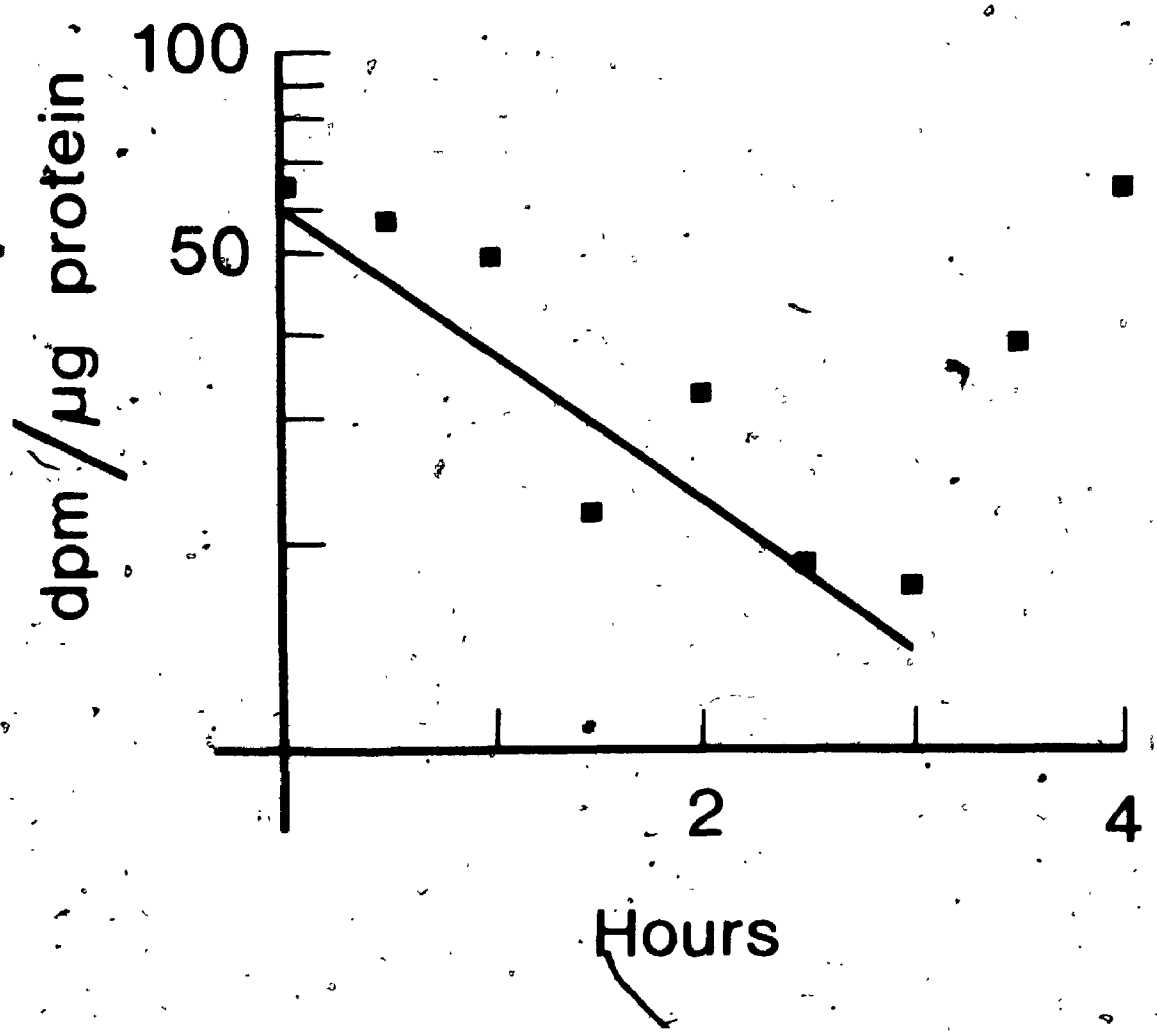

100
50
dpm / µg protein
2
4
Hours

Figure 5.12 Turnover of palmitate in the inner bilayer. Adult worm pairs were labelled during a 2 hour incubation with 1.0 μ Ci/ml incubation medium [^{14}C] palmitate. Labelled worms were incubated in the chase medium, in the absence of label, for up to 4 hours. At the time intervals indicated 250 worm pairs were removed and, after removal of the outer bilayer, the inner bilayer was obtained by a 25 minute incubation in the digitonin membrane removal fluid. Activity of [^{14}C] in the inner bilayer was determined. Counts were corrected for contamination with outer bilayer material by means of the alkaline phosphatase and [^{125}I] data, as described in the text and normalised to total bilayer protein. Points represent mean values. Line 1 represents a small, slowly exchanging pool ($t_{1/2}$ = 17.3 hours). Line 2 (open symbols) was derived from the original plot (solid symbols) by curve peeling. Line 1 was subtracted from lines 1 + 2 and the difference plotted as line 2, which describes a larger, rapidly exchanging pool ($t_{1/2}$ = 0.2 hours).

Lines are described by an exponential function of the form $Y = a_1 \exp b_1 t + a_2 \exp b_2 t$, where a_1 = 137 (± 0.46); b_1 = -0.04 (±0.14); a_2 = 395.4 (±0.14); b_2 = -3.4 (±0.02), (n = 25). The slope of line 1 is not significantly different from zero ($P > 0.5$), whilst the slope of line 2 is significant ($P < 0.001$).

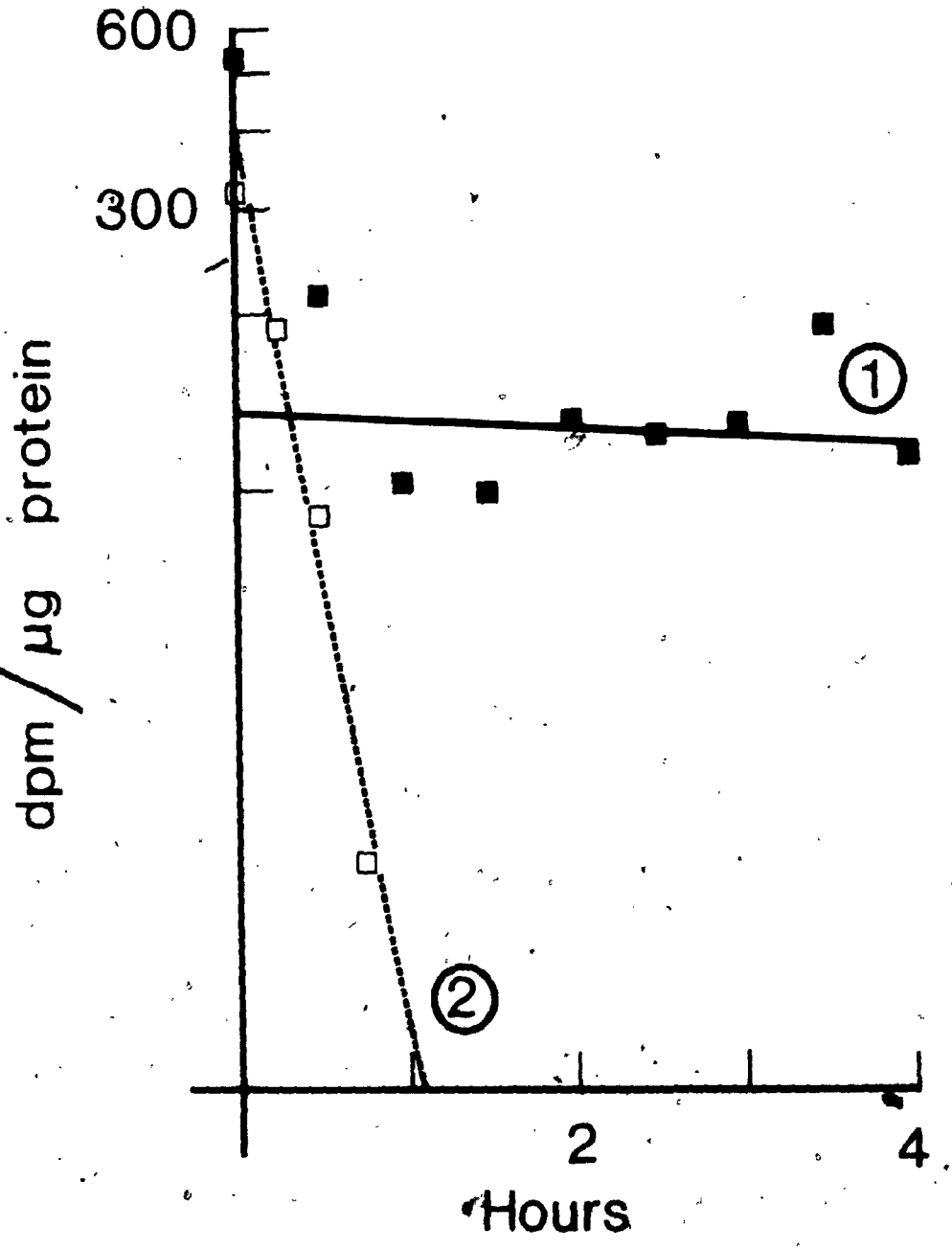
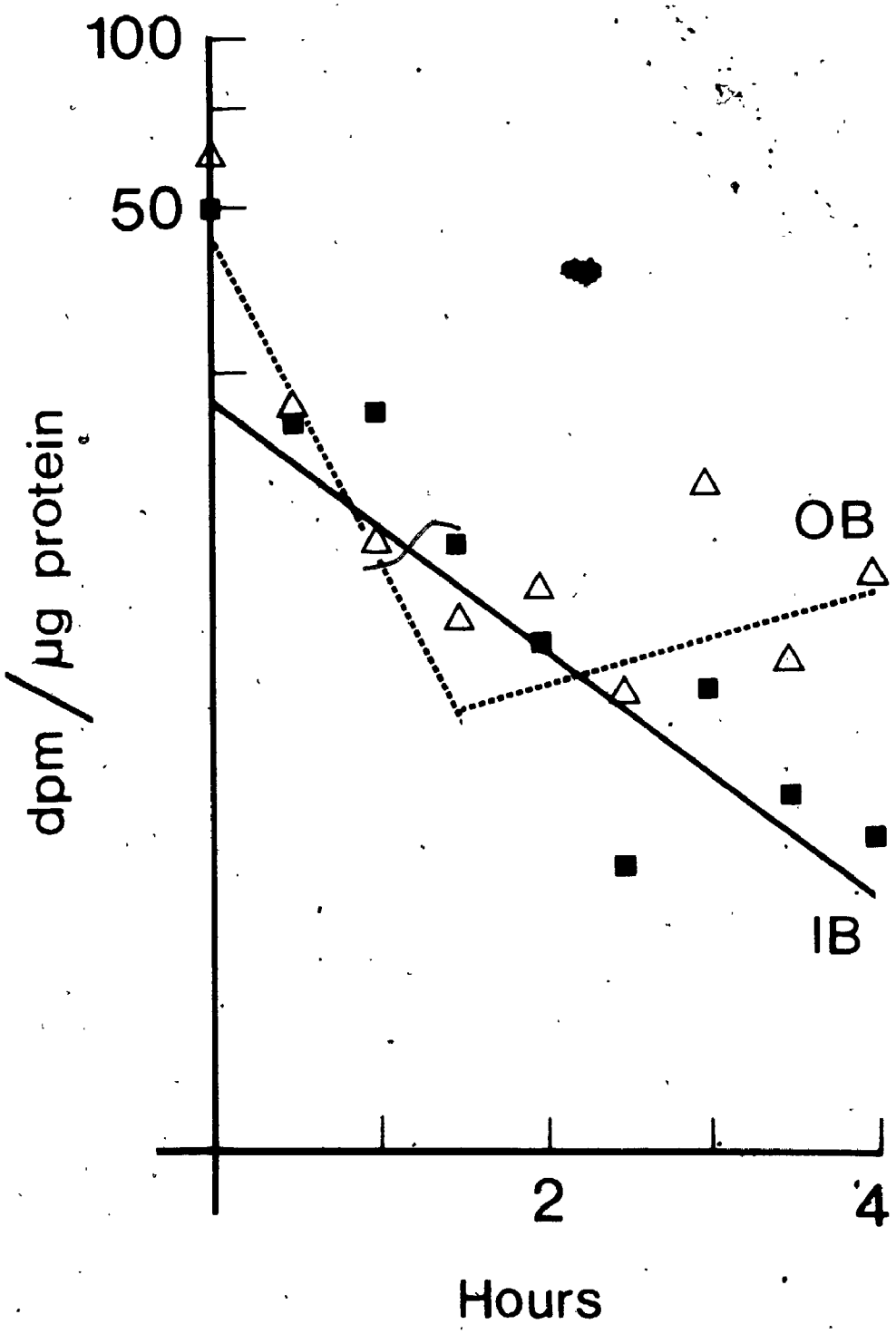

600
300
dpm / µg protein
①
②
2
4
Hours

Figure 5.13 Turnover of palmitate in the supernatants of outer (OB) and inner (IB) bilayer fractions. Adult worm pairs were labelled during a 2 hour incubation with 1.0 μ Ci/ml incubation medium [^{14}C] palmitate. Labelled worms were incubated in the chase medium, in the absence of label, for up to 4 hours. At the time intervals indicated 250 worm pairs were removed and the outer and inner bilayer fractions obtained by sequential incubations of 5 and 25 minutes in the digitonin membrane disruption fluid. Pellets were removed following centrifugation at 35,000 X g for 1 hour. Supernatant lipids were extracted and [^{14}C] activity determined. Counts were normalised to supernatant protein. Points represent mean values. Data for the inner bilayer was described by one line, Y = 21.3 ($\pm$0.23) exp -0.51 ($\pm$0.11)t (n= 43), which had a half-time of 1.36 hours. The outer bilayer supernatant showed a rapid ($t_{1/2}$ = 0.51 hours) decrease until 1.5 hours of chase and then an apparent increase ($t_{1/2}$ = 3.65 hours). Lines are described by an exponential function of the form $Y = a_1, \exp b_1, t + a_2 \exp b_2 t$, where a_1, = 43.4 ($\pm$0.26); b_1, = -1.36 ($\pm$0.32); a_2 = 4.7 ($\pm$0.51); b_2 = 0.19 ($\pm$0.18), (n = 43). The negative slopes of outer and inner bilayer supernatants are significantly different from zero ($P<0.001$), whilst the apparent increase in the outer bilayer is not significant ($0.4>P>0.2$).


100
50
dpm / µg protein
OB
IB
2
4
Hours

The regression lines obtained for the turnover of [^{14}C] glycerol in the lipid phase of OB and IB pellets proved not to be significantly different from zero (0.9 P 0.5) (Figure 5.10), owing to the large amount of scatter in the data, suggesting possible inadequacy of correction or normalisation procedures. The results also suggested that [^{14}C] glycerol might not be suitable as a label for determining surface bilayer lipid turnover.

Results for the turnover of [^{14}C] palmitate in the OB and IB pellet fractions were corrected for cross-contamination, by means of the formula described in 5.2.5 and were normalised to total protein in OB and IB pellet fractions. Data points for the OB pellet lipids were linear up to 3 hours of chase incubation and had a half-time of 1.5 hours (Figure 5.11). However, two points obtained at 3.5 and 4.0 hours showed increased activity. Turnover data for [^{14}C] palmitate in the IB pellet was alinear and suggested the presence of two compartments (Figure 5.12). One compartment was large consisting of 74% of total and had a rapid turnover rate of 12 minutes, whilst the other was much smaller (36%) and exchanged more slowly at $t_{1/2}$ = 17.3 hours. [^{14}C] Palmitate turnover in the IB supernatant fraction appeared to consist of one compartment, with $t_{1/2}$ = 1.4 hours (Figure 5.13). The turnover rate of [^{14}C] palmitate in the OB supernatant had a half-time of 30 minutes during the first hour and a half of chase; after that point the scattered data is inconclusive and the slope not significantly different from zero.

5.4 DISCUSSION

The concept of metabolic turnover, whereby components of the cell are replenished, has gained widespread acceptance (Thompson,

1973). Turnover refers to the overall processes of synthesis and degradation, which are equivalent in the steady state (Doyle and Tweto, 1975). Experiments in this chapter were designed to provide information on the rates of turnover of the outer and inner bilayer phospholipid components. As has been discussed previously, general measurements of surface turnover in S. mansoni have been performed with two types of label. Problems involved in the use of external, non-metabolised markers may include alteration of turnover rates by the marker, selective sampling of membrane constituents, association and disassociation of label and an inability to distinguish turnover rates of OB and IB. The method of choice for determining the turnover of membrane constituents is the measurements of the loss in activity of a metabolically-incorporated, isotopically-labelled precursor administered as a discrete pulse (Schimke, 1975; Zak et al., 1979). Some of the assumptions and problems in this method will be outlined later in this discussion.

A related parasite, Spirometra mansonoides is known to be able to incorporate [^{32}P] into it's phospholipids (Meyer et al., 1966). Labelling with in the present study indicated that phosphatidylcholine (Pc) and phosphatidylethanolamine (Pe) were the most heavily labelled phospholipids, 65% of the activity being present in Pc and 25% in Pe. The synthesis of membrane phospholipids is shown by results of the present experiments, which show the incorporation of [^{32}P] into the phospholipids of the outer and inner bilayers (Figure 5.1). Synthesis of surface glycolipids is suggested by the incorporation of galactose into bilayer lipids.

There is currently some disagreement in the literature as to whether membrane phospholipids show homogenous turnover (Cohen and

Phillips, 1980; Hallman et al., 1981) or heterogeneous turnover, where individual phospholipids exhibit unique turnover times (Thompson, 1973; Freysz and Mandel, 1980; Sandra and Ionosescu, 1980). A definitive answer to this question was unobtainable as insufficient material was available to allow the calculation of specific activity of individual phospholipids. This problem is not uncommon, where supplies of material are limited, and results in an inability to distinguish between metabolic activity and pool size (Smith et al., 1980). However, as the percentage contribution of the different phospholipid classes to the total phospholipid content of OB and IB is known (Figure 4.1), the relative synthetic activities can be deduced. Figure 5.2 shows the labelling patterns of the major surface membrane phospholipids following a 2 hour incubation with [^{32}P] and subsequent 3 hour chase period. The rate of decay of total label appears to be faster in the IB than in the OB, raising the possibility that IB phospholipids turn over more rapidly than those in the OB. Phosphatidylcholine, although the major phospholipid of whole worms (Young and Podesta, 1982) and of the OB (McDiarmid and Podesta, 1982; Figure 4.1), is not the most heavily labelled in the OB at the start of the chase. Phosphatidylcholine in the IB however is the most heavily labelled phospholipid throughout the chase time. It has frequently been noted that Pc and Pe are the most heavily labelled lipids, following in vitro or in vivo administration of [^{32}P] (Marinetti et al., 1957). In the OB both lysophosphatidylcholine (LPc) and Pe have higher levels of activity at 0 hours of chase than would be expected from their relative contributions to the OB (Figure 5.2). These two phospholipids also exhibit the most rapid decay with time of chase. Phosphatidylethanolamine has been found to incorporate

relatively more label than expected in hepatocytes (Dallner et al., 1966) and shows very rapid decay in liver (Cohen and Phillips, 1980). The more extensive decay of Pe in myeloma cells was considered not to be an artifact of [^{32}P] labelling, as it was found to occur regardless of the precursor species employed. The rapid decrease in activity of Pe and concomitant increase in Pc during the first 2 hours of chase suggests the possible existence of a methylation pathway. Production of Pc by the methylation of Pe is not usually a major synthetic pathway in eukaryotic cells, with the exception of hepatocytes (Trewhella and Collins, 1973; Kawamoto et al., 1980) and no evidence for this pathway has been found in whole S. mansoni (Young and Podesta, 1982). The methylation pathway, although not important for the bulk synthesis of Pc, is important in calcium transport, fusion and secretion, lipid translocation and signal transduction at surface membranes (Hirata and Axelrod, 1980). The methylation pathway in S. mansoni may have remained undetected as phospholipid formed by transmethylation generally represents a very small, but metabolically active pool (Hirata and Axelrod, 1980).

Phosphatidylcholine may be synthesised de novo, by methylation of Pe and also via the acylation of LPc (Kawamota et al., 1980). Involvment of LPc acylation is suggested by the high initial activity in the OB, followed by a rapid decrease (Figure 5.2), similar to that for Pe and by the relatively high activity of LPc in the IB. Lysolipids play an important modulatory role in membranes (Weltzein, 1979) and the synthesis of Pc from LPc, rather than de novo, represents a more energy efficient pathway (Mansbach and Parthasarathy, 1979).

Although the experiments with [^{32}P] have raised some

interesting possibilities, the further use of this label for the determination of bilayer turnover times was not considered prudent. The reasons for this decision stemmed from the assumptions inherent in the method used for the determination of turnover rates. In the steady state rates of synthesis and degradation are equal, so that measurements of degradation allow calculation of turnover time (Doyle and Tweto, 1975). The rate constant of degradation may be measured by following the loss of label from a fraction as a function of time after the single administration of a radioactively labelled precursor. The major problem with this, apparently simple, technique is that it assumes that the radioactive precursor is administered as a short pulse, which enters and leaves the precursor pool almost instantaneously (Doyle and Tweto, 1975; Schimke, 1975). This in turn requires that the isotope should be rapidly incorporated and should then not leave the molecule or be reutilised (Poole, 1971; Doljanski and Kapeller, 1976). Large membrane precursor pools are a problem and permit extensive reutilisation (Scanlin and Glick, 1977), which masks heterogeneity of turnover (Koch, 1962) and increases the apparent half-times (Poole, 1971). [^{32}P], owing to its ubiquitous distribution in the biosynthetic pathways of the cell, is not an ideal marker for the quantitation of turnover rates. [^{32}P] recycles from slowly turning over precursor pools (Benjamins and McKhan, 1973; Cohen and Phillips, 1980) and its prolonged incorporation yields longer turn over times for phospholipids than do other labelled precursors (Hallman et al., 1981).

Radiolabelled glycerol was chosen as one precursor of phospholipids, which might allow calculation of OB and IB phospholipid turnover times. Glycerol is a common precursor for membrane

phospholipids and is incorporated rapidly, at high specific activity, into the structural backbone (Pascual de Bazán and Bazán, 1976; Scanlin and Glick, 1977; Cohen and Phillips, 1980). The use of glycerol as a precursor is therefore thought to provide an accurate measurement of de novo synthesis and degradation of membrane phospholipids (Omura et al., 1967; Baker and Thompson, 1972).

Before measuring turnover of bilayer phospholipids, the ability of S. mansoni in culture to incorporate [^{3}H] glycerol was determined. Incorporation into the aqueous phase was initially rapid, but stabilised by 2 hours (Figure 5.4). Incorporation into the lipid phase increased throughout the 6 hours of incubation, although at a somewhat slower rate after the first hour (Figure 5.5). A very similar pattern of glycerol incorporation into hepatocytes was found by Omura et al. (1967) and they suggested that the hydrophilic portion of the lipids had a shorter half-life. However they seem to have ignored the fact that free glycerol is soluble in water (Bailey and Fairbairn, 1968). A more likely explanation is that the rapid initial rise in the water soluble phase represents free glycerol present in internal pools (Bailey and Fairbairn, 1968; Podesta et al., 1977). Label from [^{3}H] glycerol is not usually incorporated into proteins within the time span of the present experiments (Benjamins and McKhan, 1973; Cohen and Phillips, 1980). In Hymenolepis diminuta, a related parasite, glycerol uptake is rapid and accomplished by two mechanisms, one of which is mediated and the other is non-facilitated diffusion (Bailey and Fairbairn, 1968; Pittman and Fisher, 1972; Uglem et al., 1974; Pappas and Read 1975). Radiolabelled glycerol is incorporated into the phospholipids of H. diminuta and yields a pattern of labelling on thin layer chromatography similar to that for [^{32}P]

(Oldenborg et al., 1975). Incorporation of [^{14}C] glycerol into the phospholipids of S. mansoni (including OB and IB), following a 2 hour labelling period, was likewise confirmed in the present study by TLC and autoradiography. From these studies a 2 hour labelling period was deduced to be sufficient to allow saturation of any internal glycerol pools and for label to be incorporated into the OB and IB phospholipids.

The turnover rate of the total lipid fraction of S. mansoni, as measured with [^{14}C] glycerol, was found to have a $t_{1/2}$ of 13.9 hours (Figure 5.7). The slope of the line through the data points is equal to the rate constant of degradation (Kd), which is customarily expressed in terms of half-time, where $t_{1/2} = 0.693/Kd$ (Koch, 1962; Thomas and Rhodes 1970; Doyle and Tweto, 1975; Schimke, 1975). The straight line fit through these points indicates exponential decay with first order kinetics and suggests that the decay process is random (Doyle and Tweto, 1975). However in other systems several metabolic pools of phospholipids have been detected (Doetschman, 1980; Freysz and Mandel, 1980; Kannan et al., 1980; Kawamoto et al., 1980). The apparent simplicity of lipid turnover in intact S. mansoni may result from the heterogeneity of cell types present or reutilisation of label (Koch, 1962; Doyle and Tweto, 1975). Whether or not figure 5.7 represents a simplification of lipid turnover in S. mansoni, it is apparent that these parasites are capable of very rapid lipid metabolism, of a similar time scale to rapidly dividing pre-fusion muscle cells in vitro (Sandra and Ionosescu, 1980).

The loss of [^{3}H] glycerol label in the water soluble phase of entire S. mansoni was found not to follow first order kinetics (Figure

5.6), suggesting heterogeneous or non random turnover (Schimke, 1975; Cancedo et al., 1982). The data indicates the presence of two metabolically distinct pools, one with a very rapid turnover of $t_{1/2}$ = 0.15 hours and a slower one, with $t_{1/2}$ = 9.8 hours. The rapid initial pool probably represents the free glycerol pool and the initial water-soluble products of glycerol metabolism, such as glycerol-3 - phosphate (Bailey and Fairbairn, 1968; Sestoft and Fleron, 1975).

Lipid metabolic turnover is unlike the more intensively studied process of protein turnover, in that it is not an all or none event. There may be enzymatic replacement of only a certain molecular component, whilst the greatest part of the complex lipid is reutilised (Thompson, 1973). The loss of radioactivity from labelled phospholipid pools therefore is not a simple function of the intracellular rates of lipid synthesis, but depends also on the sizes of intracellular precursor pools (Smith and Kikkawa, 1979) and a variety of exchange reactions (Jobe, 1979; Vance and Choy, 1979; Rock and Jackowski, 1982). Considering the multitude of exchange reactions detected in phospholipid metabolism it is not surprising to find that different moieties of phospholipids may exhibit different turnover rates (Pascual de Bazán and Bazán, 1976; Khuller et al., 1981). As a corollary to this, the apparent turnover rate of cell phospholipids is often dependent upon the labelled precursor employed (Siekevitz, 1972; Scanlin and Glick, 1977).

Following from the above arguments it was important to determine the rates of lipid degradation in S. mansoni with another precursor, in addition to glycerol. [^{14}C] Palmitate was chosen for this study as palmitic acid has been widely utilised in studies of

phospholipid turnover (Sun and Horrocks, 1969; Nardone and Andrews, 1979; Rosenthal and Somers, 1979; Smith et al., 1980). Palmitate is generally the major saturated fatty acid of platyhelminths, including S. mansoni (Meyer et al., 1966; Smith et al., 1966). It is transported in a similar manner to glycerol (Pittman and Fisher, 1972) and is rapidly incorporated into the lipid fraction (Ginger and Fairbairn, 1966).

During a 2 hour incubation [^{14}C] palmitate was incorporated into the bilayer phospholipids (Figure 5.3). Labelled Pc was detected in the bilayer supernatant fractions, indicating dissociation of Pc from the membrane possibly by the formation of micelles (Samuelson and Caulfield, 1982), or by its specific interaction with extracted membrane proteins. Labelled phospholipids were also detected in the pelleted incubation medium, providing further support for the proposed shedding of the surface bilayers (Samuelson et al., 1982). Caution should be exercised here however as the presence of labelled compounds in the medium might also be indicative of damage or disruption (Doljanski and Kapeller, 1976).

Lipid turnover in S. mansoni, measured with [^{14}C] palmitate, exhibited first order kinetics and had a $t_{1/2}$ of 11.5 hours (Figure 5.9). The use of [^{14}C] palmitate as a precursor therefore yielded a slightly more rapid turnover time than with glycerol although not significantly so ($P > 0.5$). This finding was somewhat unexpected as palmitate is usually subject to more extensive reutilisation than glycerol (Scanlin and Glick, 1977), which would tend to prolong turnover times (Poole, 1971; Doyle and Tweto, 1975). However free palmitate, unlike glycerol, is soluble in the organic phase and initially much of the label disappearing from the lipid phase may be

due to free palmitate. Palmitate is readily adsorbed to the cell surface and this external compartment is in rapid equilibrium with the intracellular free fatty acid pool (Kannan et al., 1980). The difference in turnover rates obtained with the two precursors was not significant ($P > 0.5$) and the accelerated apparent turnover with palmitate may be an artifact of the free fatty acid pool and its solubility in the organic phase.

Activity of [^{14}C] palmitate in the aqueous phase of S. mansoni during the chase incubation was very low and exhibited biphasic kinetics, with a small rapidly turning over pool ($t_{1/2}$ = 0.23 hours) and a larger pool with $t_{1/2}$ = 11.5 hours (Figure 5.8). As the major pool has the same turnover time as the lipid phase it suggests that they constitute the same compartment. Appearance of low levels of label in the aqueous phase indicates some metabolism of phospholipids, probably by oxidation to acetate, which can then enter various metabolic pools (Rosenthal and Somers, 1979).

Turnover rates of the OB and IB, as measured with [^{14}C] glycerol are shown in figure 5.10. Despite correction for cross contamination the data were subject to considerable variation, but distinct trends were apparent for the two bilayers. The OB exhibited a turnover time of 11.5 hours, while during the 4 hour chase period the IB showed no evidence of decay and indeed gave some indication of continued incorporation. These results verify the suggestions that the OB and IB exhibit individual turnover rates, with the OB having the more rapid rate (Wilson and Barnes, 1974a; Podesta 1982b). Although the OB has a higher percentage lipid composition, the IB was more heavily labelled. This pattern has also been found in bacterial and mitochondrial membranes (Kamio and Takahashi, 1980) and suggests

synthesis or modulation of phospholipids at the IB. The lack of decay in the IB may be explained by a very slow turnover rate, the presence of extensive membrane precursor pools (Scanlin and Glick, 1977), exchange or recycling between the IB and internal labelled pools (Doyle and Baumann, 1979; Thilo and Vogel, 1980; Meldolesi and Ceccarelli, 1981) and synthetic activity at the inner bilayer (Ishinaga et al., 1979). Surface membranes may contain their own phospholipid synthesising enzymes and in gram negative bacteria these are usually located in the IB and not the OB (White et al., 1971; Osborn and Munson, 1974). Turnover of the IB may reflect exchange and recycling with internal membrane pools rather than true turnover and loss of label. Wilson and Barnes (1974b) suggested that the surface membrane recycled to the basal invaginations, but it is difficult to reconcile this idea with the asymmetry of the epithelium (Podesta and McDiarmid, 1982). In mammalian lung there is a bidirectional flux of phospholipids between the MLB and surfactant (Hallman et al., 1981), mediated in part by specific phospholipid transfer proteins (Lumb et al., 1980; Tsao, 1980) and a similar mechanism may be present in schistosomes.

The OB has a more rapid apparent turnover rate than the IB although not significant at $P = 0.5$ and the half-time of 11.5 hours is in general agreement with estimates of surface turnover, which range from 2-10 hours for S. mansoni (Wilson and Barnes, 1977; Dean and Podesta, 1982; Samuelson and Caulfield, 1982) and 1 to 24 hours for cells in general (Kapeller et al., 1973). However it is less rapid than the half time reported for protein turnover in the OB, ($t_{1/2}$ = 3 hours), (Dean and Podesta, 1982). Surface membrane phospholipids usually exhibit more rapid turnover rates than the protein component

(Omura et al., 1967; Thompson, 1973). Glycerol therefore appears to have greater metabolic stability or to be subject more to reutilisation in the OB than leucine. Glycerol may not be a good precursor for measuring the dynamic state of the bilayer phospholipids because of its incorporation into the diglyceride and triglyceride lipid-precursor pools (Pascual de Bazán and Bazán, 1976).

The turnover rates obtained for OB and IB phospholipids when [^{14}C] palmitate was utilised as the precursor are very different from those found with glycerol (Figures 5.11 and 5.12). The IB phospholipids exhibited biphasic turnover, having a large rapidly turning over pool ($t_{1/2}$ = 12 minutes) and a slower pool with a half-time of 17.3 hours. The turnover rates of membrane phospholipids are frequently reported to be biphasic (Omura et al., 1967; Doetschman, 1980; Freysz and Mandel, 1980), however in some cases the data has not been log transformed and so assumptions of biphasic turnover are not substantiated (Pasternak and Bergeron, 1970; Benjamins and McKhan 1973; Cohen and Phillips, 1980). Explanations of biphasic turnover include the effects of cell handling, the presence of different cell types, membrane domains, phospholipids with unique turnover rates (Doyle and Baumann, 1979; Freysz and Mandel, 1980) and reserve precursor pools of membrane (Scanlin and Glick, 1977). Schistosoma mansoni has extensive precursor pools, in the form of MLB (McLaren, 1980; McDiarmid et al., 1983), the bilayer phospholipids do appear to have individual turnover rates (McDiarmid and Podesta, 1982) and there is considerable heterogeneity of the surface in adult worm pairs (McDiarmid and Podesta, 1983).

The very rapidly decaying compartment of the IB was not detected with the glycerol precursor and therefore probably does not

represent de novo lipid synthesis (Giusto and Bazan, 1979). This implicates the IB as a site of post-synthetic phospholipid modulation. It is not uncommon for the structural specificity of phospholipids to be determined, after de novo synthesis, by exchange reactions (Trewhella and Collins, 1973; Rooney, 1979). Many plasma membranes, including the IB of gram negative bacteria, have transacylating enzymes (White et al., 1971; Rock and Jackowski, 1982). Acyltransferases can rapidly modify membrane phospholipids, changing the physiological properties of the surface membrane (Thompson, 1973). The ability to rapidly modify the fatty acyl content of surface membranes is very important in modulating enzyme activity (Hegner, 1976; Merisko et al., 1981), membrane fluidity (Wieslander et al., 1980) and subsequent resistance to immune attack (Dahl et al., 1979; Schlager and Ohanian, 1980a). Rapid turnover of plasma membrane palmitic acid has been associated with protection from complement (Schlager and Ohanian, 1980b). Even small changes in the fatty acid composition of lipid pools may exert important regulating effects (Smith and Kikkawa, 1979; Dise et al., 1980). Stimulated lipid turnover may not involve glycerol moieties, but turnover of phosphate and acyl groups may be important signals in stimulus-secretion coupling (Shearer and Richards, 1981).

Another possible explanation for the initial rapid rate of turnover in the IB and subsequent slow rate is that they represent artifactual turnover rates resulting from the in vitro manipulations (Smith et al., 1980; Rotmans et al., 1981). Phospholipid synthesis has been noted to decline with length of time in culture (Gerard et al., 1982). However the de novo synthesis of phospholipids in the OB and IB did not exhibit biphasic turnover (Figure 5.10). Whatever the

explanation, the question remains as to the fate of the components in these rapidly metabolising compartments. The extremely rapid rate of turnover of the fast compartment in the IB suggests that there is little recycling or reutilisation of this pool. These results, combined with the rapid turnover of the OB ($t_{1/2}$ = 1.5 hours) raise the interesting possibility that phospholipids or acyl groups may be translocated from the IB to the OB, as in gram negative bacteria (Ishinaga et al., 1979). The different turnover rates of the OB and IB might help to maintain the individual phospholipid content of each.

Phospholipids of the OB turn over very rapidly, at least up to 3 hours of chase. Beyond this time the effects of in vitro manipulation may be changing. The rapid turnover of the OB supports the concept that the surface is shed in culture (Murrell et al., 1974; Kusel et al., 1975a; Tarleton and Kemp, 1981; Samuelson and Caulfield, 1982) and would explain the appearance of labelled phospholipids in the incubation medium (Figure 5.3).

Turnover of [^{14}C] palmitate in the supernatants of OB and IB fractions was rapid (Figure 5.13). The initial rapid decline in the OB supernatant may be the result of adsorbed free palmitate being released. The slope of the subsequent increase in activity was not significant, at P = 0.2. Rapid turnover of the IB supernatant phospholipids may reflect the selective turnover of those components of the bilayer which were extracted during digitonin treatment.

The results of this chapter, when viewed as a whole, provide support for the hypothesis that survival of S. mansoni adults depends on their ability to regulate and rapidly renew the surface, with the least expenditure of energy (Podesta, 1982a). It is apparent that the phospholipids of the surface bilayers are capable of very rapid

turnover. The suggested involvement of phospholipid modulation, with the reutilisation of certain molecular components, would allow this rapid turnover to be as economical as possible.

CHAPTER 6

IDENTIFICATION OF A SIALIC ACID CONTAINING GLYCOCALYX ON THE SURFACE OF SCHISTOSOMA MANSONI

6.1 INTRODUCTION

The resistance to host immune effectors that is gradually acquired as schistosomula mature from 0-5 days, is thought to be related to alterations in the composition of the outer bilayer (McLaren, 1980). The biochemical basis of this protection, however, is not known. Seeking an explanation for this phenomenon, the general literature was examined for an analogous situation. Although the presence of an additional bilayer beyond the plasma membrane has been considered unique to the blood flukes (McLaren, 1980), a similar structure is present in a variety of other organisms, particularly gram negative bacteria (Costerton et al., 1981).

With respect to the composition of the outer bilayer it is now widely recognised that most gram negative bacteria have a glycocalyx (Costerton et al., 1981). In bacteria, the glycocalyces exert a profound effect on the interactions of the invading organism with the host immune system (Joiner et al., 1982; Markham et al., 1982) and immune effector cell adhesion (Henricks et al., 1982). As immune killing of schistosomes is mediated by similar systems it is also desirable to know whether or not schistosomula and adult S. mansoni possess a glycocalyx. Despite considerable evidence to the contrary, they are considered not to have a glycocalyx (McLaren, 1980). Indeed, the outermost bilayer, which is considered to play a crucial role in the avoidance of host immune effector mechanisms (McLaren and Hockley,

1977), is postulated to have replaced the glycocalyx of other trematodes (Wilson and Barnes, 1977). Data supporting the presence of a glycocalyx on adult S. mansoni include histochemical studies showing the binding of Periodic Acid-Schiff (PAS), colloidal iron, cationised ferritin and possibly alcian blue (Morris and Threadgold, 1968; Stein and Lumsden, 1973; Wilson and Barnes, 1974a; Wheater and Wilson, 1976) and numerous studies showing lectin binding to the surface (Simpson and Smithers, 1980; Torpier and Capron, 1980; Linder and Huldt, 1982). Biochemical studies have also revealed surface glycoproteins (Hayunga et al., 1983) and glycosyl transferases (Rumjanek and Smithers, 1978) and results of the previous chapter indicate galactose incorporation into the surface bilayers. The present study was therefore prompted by inconsistencies between the unusual absence of a glycocalyx and the ample evidence for the presence of surface carbohydrates.

Previously the glycocalyces of many bacterial species remained undetected due to the dependence of the delicate, hydrated polysaccharide matrices on hydrogen bonding (Costerton et al., 1981), causing the coat to collapse against the surface membrane during dehydration for electron microscopy. Ruthenium red, which binds anionic moieties of polysaccharide matrices in the external coat, may be used to prevent such collapse during dehydration (Sideri et al., 1982). In the present chapter the use of this method is reported to produce similar observations for the effect of ruthenium red on the surface of S. mansoni males, females and schistosomula.

6.2 MATERIALS AND METHODS

Adult S. mansoni were gently expressed from the cut ends of the

hepatic portal vein of Syrian hamsters, infected 40-45 days previously. The worms were processed for electron microscopy either immediately or after extensive washing of the parasites in Kreb's Ringer Phosphate (KRP), pH 7.4 at 4°C. Mechanically obtained schistosomula (MS) were obtained from cercariae following the method of (Brink et al., 1977). Briefly, cercariae were vortexed at high speed for 2 minutes, incubated in minimal volumes of medium 199 for 30 minutes and vortexed as before. These schistosomula were incubated in medium 199, without serum, at 37°C in 5% CO_2 for 24 hours before use. Serum was omitted from this preparation to allow comparison of schistosomula which had not been exposed to host macromolecules with those which had, the skin penetrated schistosomula. Skin schistosomula (SS) were produced by allowing cercariae, in aged water, to penetrate through isolated and shaved hamster skin, which was tightly clamped over a tube of medium 199 with 5% heat inactivated human serum (HIH), at 37°C (Clegg and Smithers, 1972). The preparation was incubated for 3 hours and schistosomula were subsequently concentrated by centrifugation of the medium at 120 x g for 2 minutes. Before use SS were incubated for 24 hours as above, with the addition of 5% HIH serum. Lung stage schistosomula were obtained from the lungs of hamsters, infected 6 days previously by subcutaneous injection of 2,000 cercariae, by the method of Brink (1982). After perfusion via the right ventricle, to remove excess blood cells, the lungs from 8 hamsters were finely minced and incubated for 6 hours in Eagles Minimal Essential Medium with 5% HIH serum, containing 0.1 mg/ml gentamycin sulphate, at 37°C in 5% CO_2. Lung debris was removed by filtration through stainless steel mesh (20 mesh/cm) and the schistosomula spun down at 80 x g for 30 minutes.

For neuraminidase treatment, adult S. mansoni were incubated at 37°C in KRP, pH 6.4 with 3.3 units/ml neuraminidase (Clostridium perfringens type V, Sigma Chemical Co.). Control incubations were similarly performed in the absence of neuraminidase.

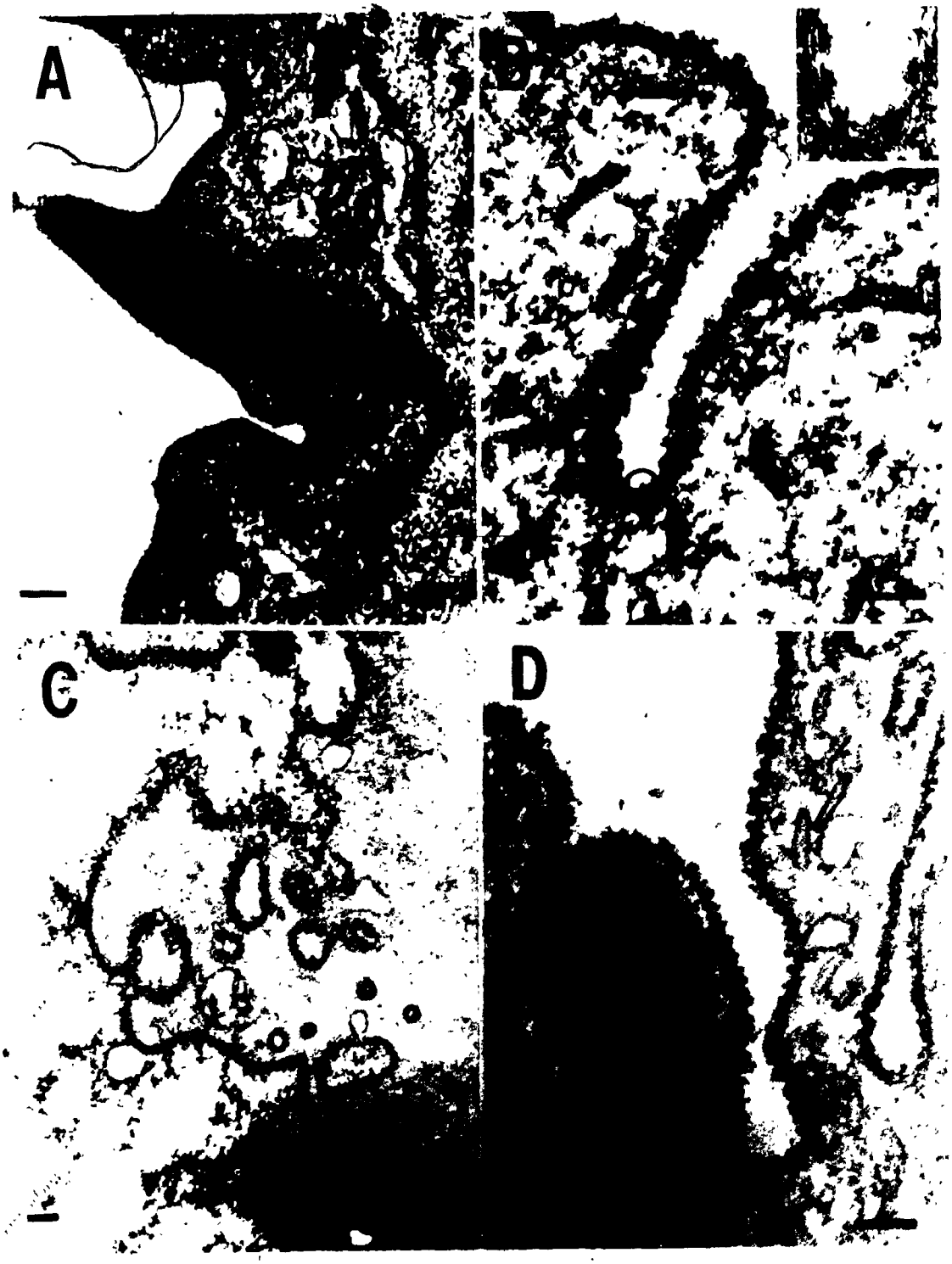
Schistosomula and adults were prepared for electron microscopy following the method of Luft (1971). Fixatives contained equal proportions of 3.6% glutaraldehyde, 0.2 M cacodylate buffer and 1,500 ppm ruthenium red (Polysciences Inc.). Specimens were post-fixed in 1.7% osmium tetroxide and 500 ppm ruthenium red buffered in 0.07 M sodium cacodylate, dehydrated in acetone, embedded in Araldite, sectioned with a diamond knife. Grids were examined in a Philips 201 electron microscope unstained or stained with lead citrate and uranyl acetate.

6.3 RESULTS

Ruthenium red fixation of adult flukes revealed the existence of a negatively charged layer external to the outer bilayer, indicating, for the first time, the presence of a glycocalyx on the schistosome surface which was similar to the surface coat of other cell types (Figure 6.1). Ruthenium red has also preserved the integrity of the outer bilayer of S. mansoni in the absence of uranyl acetate post-fixation.

Much of the adult surface exhibited a regularly spaced array of dense aggregated material (Figure 6.1). The aggregates were approximately 21 nm wide and 11.5 nm in height and appeared to correspond with clumps of electron dense material in the interbilayer space. Some further indication of possible communication between the two bilayers was found in areas where thin dense lines appeared to

Figure 6.1 Electron micrographs showing visualisation of the glycocalyx of S. mansoni with ruthenium red. a, 24 hour skin penetrated schistosomulum, with only a single bilayer visible on the surface. b, dense aggregates on the adult female surface. Encircled area is enlarged to show patterning of the bilayers. c, male dorsal surface, showing extensive surface glycocalyx. d, male ventral surface with aggregated surface coat material. Grids a and b were stained with lead citrate and uranyl acetate, while c and d were not stained. Bars represent 0.1 μm.


A
B
C
D

traverse each bilayer in close alignment (Figure 6.1b).

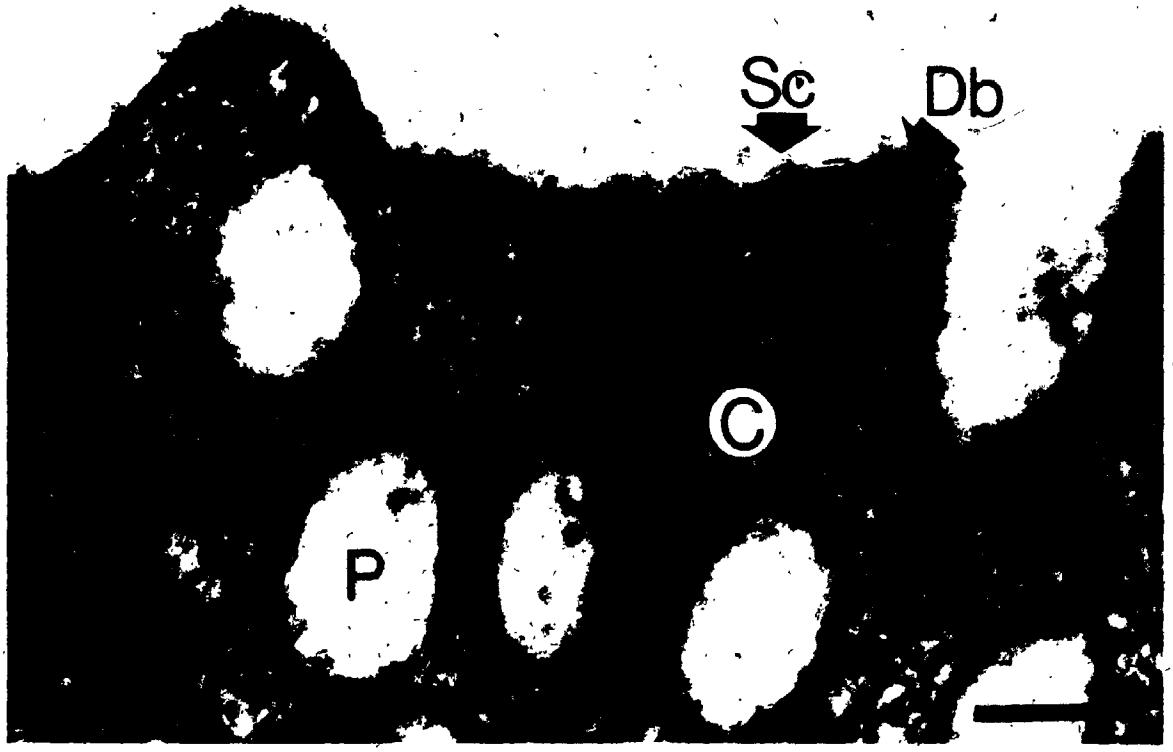
Regional and sexual differences were found in the patterning of the cell surface coat (Figure 6.1). The aggregated distribution, discussed above, was characteristic of the male ventral and female surfaces. Although the male dorsal surface exhibited some patterning, the coat was usually denser and thicker (up to 150 nm), forming a more continuous covering. With ruthenium red fixation none of the 24 hour schistosomula showed development of the outer bilayer and surface coat comparable to the adults. Only occasionally were areas of double bilayer evident, some exhibiting small clumps of surface coat. Ruthenium red did bind to the surface of lung stage schistosomula (Figure 6.3). The surface coat again had a clumped distribution, exhibiting a circular appearance in places, depending upon the angle of sectioning.

Control worms in the neuraminidase experiments were of normal appearance, except for some larger aggregations of surface coat material (Figure 6.2a). Neuraminidase treatment however, removed most of the negatively charged surface coat material and caused an apparent increase in the prevalence of multiple stacking of bilayers (Figure 6.2b). The glycocalyx was no longer identifiable over most of the surface, regardless of the number of bilayers present. General tegument morphology was also somewhat altered, although the integrity of the cytoplasm and outer membrane was preserved. Basal vacuolation was observed in addition to increased amounts of bilayer material and variant discoid bodies, the latter appearing to be involved in a previously undescribed secretory process, possibly related to the surface coat (Figure 6.2c).

Figure 6.2 Electron micrographs of the surface of S. mansoni adults, showing the effects of neuraminidase treatment on ruthenium red staining patterns of the glycocalyx. In addition to ruthenium red fixation, these specimens were post-fixed overnight in half-saturated aqueous uranyl acetate. None of the sections were stained. In a and b the scale bars represent 0.1 μm and 1.0 μm in c (10nm in enlargement). a, control S. mansoni, following incubation at pH 6.4 for 30 minutes in the absence of neuramindiase - note the presence of surface coat material. b, the effects of neuraminidase treatment. Adults were incubated at pH 6.4 for 30 minutes with 3.3 units/ml neuraminidase. Multiple bilayers are evident and the surface coat is lacking. c, the effects of neuraminidase treatment. Adults were treated as in (b). Large numbers of discoid bodies, amorphous vesicles and basal vacuolations are evident in the tegument. Enlargement shows a discoid body apparently involved in a secretory process.


A

Figure 6.3 - Electron micrograph showing ruthenium red staining of surface coat material (Sc) on 6 day old schistosomulum. Note surface coat in pits (P) and also the dense staining of the tegument cytoplasm (c). (Db) indicates the double bilayer of the apical membrane. Bar represents 0.1 µm.


Sc
Db
C
P

6.4 DISCUSSION

Although the surface of S. mansoni is known to have exposed glycoproteins and glycolipids and to bind colloidal iron and a variety of lectins, there has been no prior morphological identification of a glycocalyx (McLaren, 1980). Indeed the complete bilayer external to the plasma membrane has previously been visualised only with uranyl acetate post-fixation and has been postulated to replace the glycocalyx of other trematodes (Wilson and Barnes, 1977). Surprisingly the ruthenium red used in the present study, in addition to stabilising the glycocalyx, has preserved the integrity of the entire outer bilayer in the absence of uranyl acetate. In some of the sections (Figures 6.1b and 6.3), especially those of schistosomula, the internal structures appeared to have been stained by ruthenium red, indicating some penetration of the stain.

A clumped distribution was evident over large areas of the adults. A similar patterning of the cell coat has been reported from bacteria (Costerton et al., 1981) and another syncytial membrane - that of the mammalian placenta (Sideri et al., 1982). Although clumping could represent a fixation artifact, the clumped distribution on bacterial surfaces has been explained by the presence of domains in the outer bilayer. These zones of adhesion are thought to communicate with the inner bilayer, to be involved in signal transduction across the bilayers and to involve the export of protons, ions, lipopolysaccharides, phospholipids and outer bilayer proteins (Magnusson and Bayer, 1982). The corresponding aggregation of surface and interbilayer material in S. mansoni may result from a permanent association between the two, or both may have clumped simultaneously during fixation. Although we can not presently distinguish between

these possibilities the results do point to possible mechanisms for material and information transfer between and across the two bilayers, as was proposed for bilayer phospholipids in Chapter 5.

The observed regional and sexual differences in surface coat morphology may represent structural heterogeneity, or the viscous nature of the male dorsal surface (Johnson et al., 1982) may have prevented the lateral aggregation of surface material during fixation. The functional heterogeneity of the schistosome surfaces is evident from the results of other studies showing preferential binding to the male dorsal surface of lectins (Torpier and Capron, 1980; Linder and Huldt, 1982), complement (McGuinness and Kemp, 1981), dialysed iron (Morris and Threadgold, 1968), and PAS (Wheater and Wilson, 1976). In addition, the differential adhesiveness of the surfaces of S. mansoni (Podesta et al., 1983), as estimated from interfacial free energy determinations (Boyce et al., 1983), can be explained on the basis of glycocalyx morphology discussed in this study. The male dorsal surface is exposed to immune effector cells and presents a thick hydration layer of low interfacial free energy and hence adhesiveness (Podesta, 1982a). In contrast the sequestered male ventral and female surfaces (Phillips and Colley, 1978) are more adhesive, as indicated by the thin discontinuous surface coat (Figure 6.1), higher interfacial free energies (Podesta et al., 1983) and the non-specific adherance or trapping of bacteria and red blood cells to the male ventral surface (Kemp et al., 1977; 1980). Again a similar correlation is present in bacterial systems between interfacial free energy, adhesiveness and the extent and aggregation of the surface glycocalyx (Edebo et al., 1980).

Although the outer bilayer of developing schistosomula was

described as being almost complete by 3 hours (McLaren, 1980), other workers have found that in vitro this process may take from 6-48 hours (Murrell et al., 1978; Taylor et al., 1981; Butterworth et al., 1982). The lack of an outer bilayer in skin penetrated and mechanically transformed schistosomula from this study may reflect slow development of this layer or, in the absence of a glycocalyx, the OB may not be stabilised by ruthenium red. Further studies may differentiate between these two phenomena, but it is obvious that the young schistosomula in this study did not possess a glycocalyx comparable to the adult flukes.

The poorly developed surface coat may help to explain why schistosomula under 48 hours post-transformation bind more antibody and immune effector cells (Dessein et al., 1981) and express surface antigens to a greater degree than older parasites (Butterworth et al., 1982). They also activate complement by the alternate pathway (Dessein et al., 1981) and are more susceptible to immune effectors (Dean, 1977).

Lung stage schistosomula however were found to possess a surface coat. During maturation the schistosomula undergo a decline in antigenicity and susceptibility (Bickle and Ford, 1982), which is independent of any host antigen disguise (Sher and Moser, 1981). Gram negative bacteria with reduced glycocalyces are also known to be more vulnerable to immune effectors, especially to cell-mediated damage (Edebo et al., 1980; Okamura and Spitznagel, 1982). The lack of a complete glycocalyx on young schistosomula and its subsequent development by 6 days may be a key to our understanding of concomitant immunity, where the resident adult population survives in the face of an immune response which kills invading schistosomula (Smithers and

Terry, 1969a).

Results of the experiments utilising parasites exposed to neuraminidase suggest the presence of surface sialic acid, this conclusion being consistent with previous studies (Simpson and Smithers, 1980; Samuelson and Caulfield, 1982). The lack of staining, following neuraminidase treatment, argues for the contribution of sialic acid to surface charge, as has been found in other systems (Sherbert, 1978). Simpson and Smithers (1980), however, using indirect lectin-binding techniques on fixed worms concluded that sialic acid, although present, did not contribute significantly to the negative surface charge. In bacterial and other systems surface carbohydrates, especially sialic acids, afford protection from the effects of complement and prevent its activation by the alternative pathway (Liang-Takasaki et al., 1982; Markham et al., 1982). Recent evidence however, suggests that adult S. mansoni will bind C_3 via the alternative pathway of complement activation (Tarleton and Kemp, 1981; Linder and Hoildt, 1983). The protective nature of the schistosome glycocalyx may, therefore, be in prevention of the insertion of the terminal hydrophobic and lytic components of the complement system, as in some bacterial systems (Joiner et al., 1982). Many areas of multiple bilayers were evident following neuraminidase treatment, suggesting either an increased rate of secretion of the outer bilayer or inhibition of surface shedding.

A very recently published paper provides support for the results presented in this chapter. Simpson et al. (1983) concluded, from increased lectin binding following neuraminidase treatment, that lung stage and adult worms have surface sialic acid, whilst young schistosomula do not. Coincidentally the lung and adult stages had a

negative surface charge implicating sialic acid as a contributer to surface charge. In conclusion it is apparent from the present study that the host-parasite interface consists of a complex, hydrated, charged carbohydrate coat and that the function of the outer bilayer can not be to replace the glycocalyx of other cells. Similar to microbial systems the glycocalyx of schistosomes probably affords protection from the immune response by a variety of mechanisms, including decreased cellular adhesion and protection from the effects of complement. Although the outer bilayer of S. mansoni is thought to develop within 3 hours (McLaren, 1980) the glycocalyx is not complete at 24 hours and this may account for the previously unexplained susceptibility of young schistosomula to immune clearance.

CHAPTER 7

CONCLUSIONS

The initial aims of the thesis, to develop techniques for separating the surface bilayers and to investigate turnover of the surface bilayer phospholipids, have been accomplished and the following conclusions may be made.

: The proposed asymmetry of the apical and basal membranes of the surface syncytium was confirmed following the separation of these layers.

: Multilamellar bodies (MLB), the presumed precursors of the apical surface bilayers, may be quantitatively prepared from S. mansoni employing techniques similar to those for isolating multilamellar bodies from mammalian lung.

: Schistosome MLB possess an apparent projection core, possibly analogous to those of mammalian lung multilamellar bodies.

: Schistosome MLB contain the same major phospholipids as the apical surface bilayers, providing further support for their presumed function.

: The phospholipid to protein ratio of schistosome MLB is lower than that of lung multilamellar bodies.

: [^{125}I] diazotised iodosulfanilic acid can be employed as a marker for the outer bilayer (OB) of the apical membrane complex and alkaline phosphatase is a useful marker enzyme for the inner bilayer (IB).

: Lactate dehydrogenase release may be monitored as a measure of cytosolic leakage during membrane removal procedures.

: Markers characterised for the outer and inner bilayers allow the quantitative separation of the two bilayers by sequential exposures to a digitonin membrane disruption fluid.

: The outer bilayer has a higher percentage composition of phospholipid than the inner bilayer.

: Differences in phospholipid composition of outer and inner bilayers are quantitative rather than qualitative.

: The inner bilayer is analogous to the traditional plasma membrane of other cell types.

: An intact outer bilayer does not appear to be necessary for the maintenance of cytoplasmic integrity.

: [^{32}P] is incorporated in vitro into the phospholipids of the outer and inner bilayers.

: [^{32}P] labelling patterns of outer and inner bilayer phospholipids during an in vitro chase period give preliminary indications of heterogeneous turnover of phospholipids.

: Adult S. mansoni can incorporate glycerol into lipid and water-soluble phases in vitro.

: Glycerol effluxes from two compartments in the water-soluble phase, from a large pool and from a smaller less rapidly exchanging pool.

: Glycerol effluxes from one compartment in the lipid phase.

: Palmitate effluxed from two compartments in the water-soluble phase, from a small rapid pool and from a larger less rapidly exchanging pool.

: Palmitate effluxed from one compartment in the lipid phase.

: Metabolic precursor labelling of lipids, when combined with outer and inner bilayer separation procedures may be employed to determine turnover rates of outer and inner bilayer lipids.

: Utilising [^{14}C] palmitate as a precursor, lipids of the outer bilayer turnover with an apparent half time of 1.5 hours.

: Lipids of the inner bilayer turned over from a rapidly exchanging pool ($t_{1/2}$ = 0.2 hours) and from a smaller slower pool ($t_{1/2}$ = 17.3 hours)

: Lipids of the outer and inner bilayers turned over at significantly different rates.

: Supernatant bilayer lipids exhibited different kinetics to those that remained membrane-bound.

: S. mansoni is capable of rapid turnover and modulation of surface bilayer phospholipids and this may be correlated with protection from immune effector mechanisms.

: Ruthenium red can be used to visualise a negatively charged glycocalyx on the surface of S. mansoni.

: The glycocalyx is most extensive on the male dorsal surface and has an aggregated appearance on the male ventral and female surfaces.

: Young schistosomula lack a glycocalyx, whilst lung stage and adult parasites possess a glycocalyx and this may help to explain the phenomenon of concomitant immunity.

: Sialic acid may be responsible for the negative charge of the schistosome surface.

Appendix I. Phosphorus assay, based on the method of Rouser et al. (1970), for the quantitation of phospholipids.

All glassware used in this assay was rendered phosphate-free by soaking in acid and washing in phosphate-free detergent.

Protocol: - Evaporate sample

- add 0.1 ml concentrated H_2SO_4 and char over an open flame
- add 0.05 ml perchloric acid and heat over flame until clear (completely oxidised).
- add 2 ml H_2O
- add 1 ml 1% ammonium molybdate
- add 1 ml 4% ascorbic acid
- incubate for 10 minutes at 70° C.
- read optical density at 820 nm.

The standard curve was prepared from a solution of 0.1 mg/ml potassium dihydrogen phosphate and ranged from 0.2 - 14.4 µg phosphorus.

APPENDIX II

Molecular and Biochemical Parasitology, 6 (1982) 225–235
Elsevier Biomedical Press

ENRICHMENT AND PARTIAL ENZYME CHARACTERIZATION OF ATPase ACTIVITY ASSOCIATED WITH THE OUTWARD-FACING MEMBRANE COMPLEX AND INWARD-FACING MEMBRANE OF THE SURFACE EPITHELIAL SYNCYTIUM OF *SCHISTOSOMA MANSONI*

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(Received 2 February 1982; accepted 2 July 1982)

The outward-facing (OFM) and inward-facing (IFM) membranes of the surface epithelial syncytium of *Schistosoma mansoni* were separated by sequential exposure to saponin solutions. The OFM, containing both inner and outer bilayers, contained ATPase activity that was stimulated by Mg^{2+} and Na^+, but not K^+ or HCO_3^-, and was inhibited by Ca^{2+} and ethacrynic acid. The OFM enzyme was unaffected by ouabain, oligomycin, SCN^- and azide and had a pH optimum of 7.5. The OFM ATPase therefore has properties similar to ATPases characterized from the apical membrane of a variety of epithelial cells where it is thought to augment the regulatory cell volume decreasing function of $(Na^+ + K^+)Mg^{2+}$-ATPase. The IFM contained ATPase activity that was stimulated by Mg^{2+}, Na^+ and K^+, and was inhibited by ouabain indicating that the IFM enzyme was the Na^+-pump ATPase. The results are discussed in terms of the transepithelial transport function of the surface epithelial syncytium and a Ca^{2+}-ATPase reported previously from the OFM of *S. mansoni*.

Key words: *Schistosoma mansoni*, Epithelial membranes, ATPase, Membrane enzymes, Membrane isolation.

INTRODUCTION

A number of attempts have been made to isolate the surface membranes of parasitic platyhelminths, particularly the surface membranes of *Hymenolepis diminuta* [1] and *Schistosoma mansoni* [2]. However, the purity of the membrane fractions obtained in these studies has not been tested with the use of enzyme markers for contaminating membranes. Isolation of the surface membranes must exclude contamination by membranes of mitochondria and other cell organelles and, since the surface of endoparasitic platyhelminths is a polarized epithelial layer [3, 4], cross-contamination between surface

Abbreviations: IFM, inward-facing membrane; OFM, outward-facing membrane; MLB, multilamellar bodies.

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(outward-facing, OFM) and basal (inward-facing, IFM) membranes of the epithelial syncytium requires caution. However, due to the syncytial nature of the surface epithelial layer, it should be possible to sequentially remove the surface membranes without the necessity of further purification routines which would be required using homogenized cells.

The most commonly used marker enzymes in epithelial cell membranes are the adenosine triphosphatases (ATPases). The basal membranes of epithelial cells contain a Mg^{2+}-ATPase which can be distinguished from mitochondrial ATPase by its sensitivity to ouabain [5]. The ATPase found in the apical membrane (OFM) of epithelial cells is also insensitive to ouabain and has recently been distinguished from mitochondrial ATPase by the sensitivity of the latter to azide [6].

In the present study we obtained membrane fractions from the surface epithelial syncytium of *S. mansoni* that were enriched with either the OFM (plasma membrane and outer membranous surface coat) or the IFM. We report on the partial characterization of ATPase activities in these membrane preparations. In separate reports we describe experiments in which we isolated the multilamellar bodies [7] and associated phosphatidic acid phosphatase activity [8], and separated the surface plasma membrane and associated membranous surface coat [9].

MATERIALS AND METHODS

Membrane removal: Adults of both sexes of *Schistosoma mansoni* (Puerto Rican Strain) were obtained from infected Syrian hamsters 40–45 days post-infection by cardiac perfusion with cold Krebs-Ringer phosphate (pH 7.4) using conventional techniques. After washing in Krebs-Ringer phosphate, the worms were incubated in the membrane disruption fluid at 4°C for 5 min. The membrane disruption fluid contained 0.5% saponin (Sigma Chemical Co.) and 3.0% $CaCl_2$ in 0.1 M Tris/sucrose buffer, at pH 7.4. This solution had an osmolarity of approximately 350 mosM (Instrumentation Laboratories osmometer). Following incubation the worms were vortexed for 30 s at high speed and then filtered over a plastic mesh. The fluid resulting from the first incubation was stored on ice and the worms placed in fresh membrane disruption fluid for a second incubation of 7 min. Worms from the second incubation were vortexed and filtered as before. After each digest several worms were removed and stored on ice for electron microscopy.

The suspensions from both incubations were spun at 500 × *g* for 10 min to remove any larger particles. Membranes from both fractions were pelleted at 20 000 × *g* for 1 h at 4°C. For enzyme analysis, the membrane pellets were resuspended in Tris buffer.

Electron microscopy: Untreated *S. mansoni* adults and sample carcasses from the first and second incubations were processed for electron microscopy. Pellets or worms were fixed for 3 h, at 4°C, in 2.5% glutaraldehyde/0.05 M cacodylate buffer with 3% sucrose and 2.0 μM calcium acetate. After several rinses in 0.05 M cacodylate, with 12% sucrose, the fractions were postfixed in 1.0% osmium tetroxide for 1.5 h, washed in distilled

water and left in 50% saturated aqueous uranyl acetate overnight. Fractions were washed and dehydrated through an ethanol series to propylene oxide. They were then infiltrated in propylene oxide: resin mixtures and embedded in Araldite 6005.

Thin sections (600–1000 Å) were cut, with glass knives, on a Philips OMU 3 ultramicrotome. Grids were stained with uranyl acetate and Reynold's lead citrate and examined in a Philips 201 electron microscope.

Enzyme assays: ATPase activity was measured for up to 20 min at 37°C according to the method of Ryre [10]. Phosphate liberated from hydrolysis of ATP was determined by the method of Taussky and Shorr [11] in a final volume of 4 ml. Protein determinations were conducted according to Lowry et al. [12]. When Na^+-free reaction mixtures were required, 60 mM disodium ATP (Boehringer) solutions were rendered Na^+-free according to Rahman et al. [13, 14].

RESULTS

Electron microscopy: Electron micrographs of the surface epithelial syncytium of *S. mansoni* prior to membrane removal and after removing the OFM and IFM, with associated pelleted material, are shown in Fig. 1. The syncytium is bounded by an OFM and IFM and contains membrane bound organelles, including the multilamellar bodies (MLB) and discoid bodies (Fig. 1A). Following the first exposure to the saponin suspension, the OFM was removed, leaving intact most of the syncytial material including the spines and IFM (Fig. 1B). The pellet derived from the first saponin exposure contained mostly membrane material (Fig. 1D), which was characterized by having two bilayers (Fig. 1F), a feature consistent with the OFM being a heptalaminate barrier. Following the second saponin incubation, the surface of *S. mansoni* was devoid of syncytial material (Fig. 1C) and the pellet was characterized by the presence of spines and membrane material (Fig. 1E). Comparisons of Fig. 1D and E indicated that the pellet of the first saponin treatment contained membranes with far less contaminating material than that obtained from the second exposure to saponin. However, since the enzyme data derived from these pellets were consistent with the epithelial nature of the epithelial syncytium (see below), the primary objective of this study was satisfied and no further purification of the membrane material was attempted.

ATPase activity: The ATPase activities of both the OFM and IFM membrane fractions were linear with respect to reaction time up to 20 min and protein in the reaction mixture up to 70–80 μg per 0.1 ml of membrane suspension (Fig. 2). The activity in both fractions was dependent upon Mg^{2+} concentration up to 3.5 mM Mg^{2+}, beyond which no further stimulation occurred (Fig. 3). Dependence of the ATPase activity on the pH of the reaction mixture is demonstrated in Fig. 4. The optimum pH in both the IFM and OFM fractions occurred in the pH range of 7.0–7.5, although the curve for the IFM indicated a broad range of pH over which the ATPase activity was at or very near optimal levels (pH 6.0–7.5).

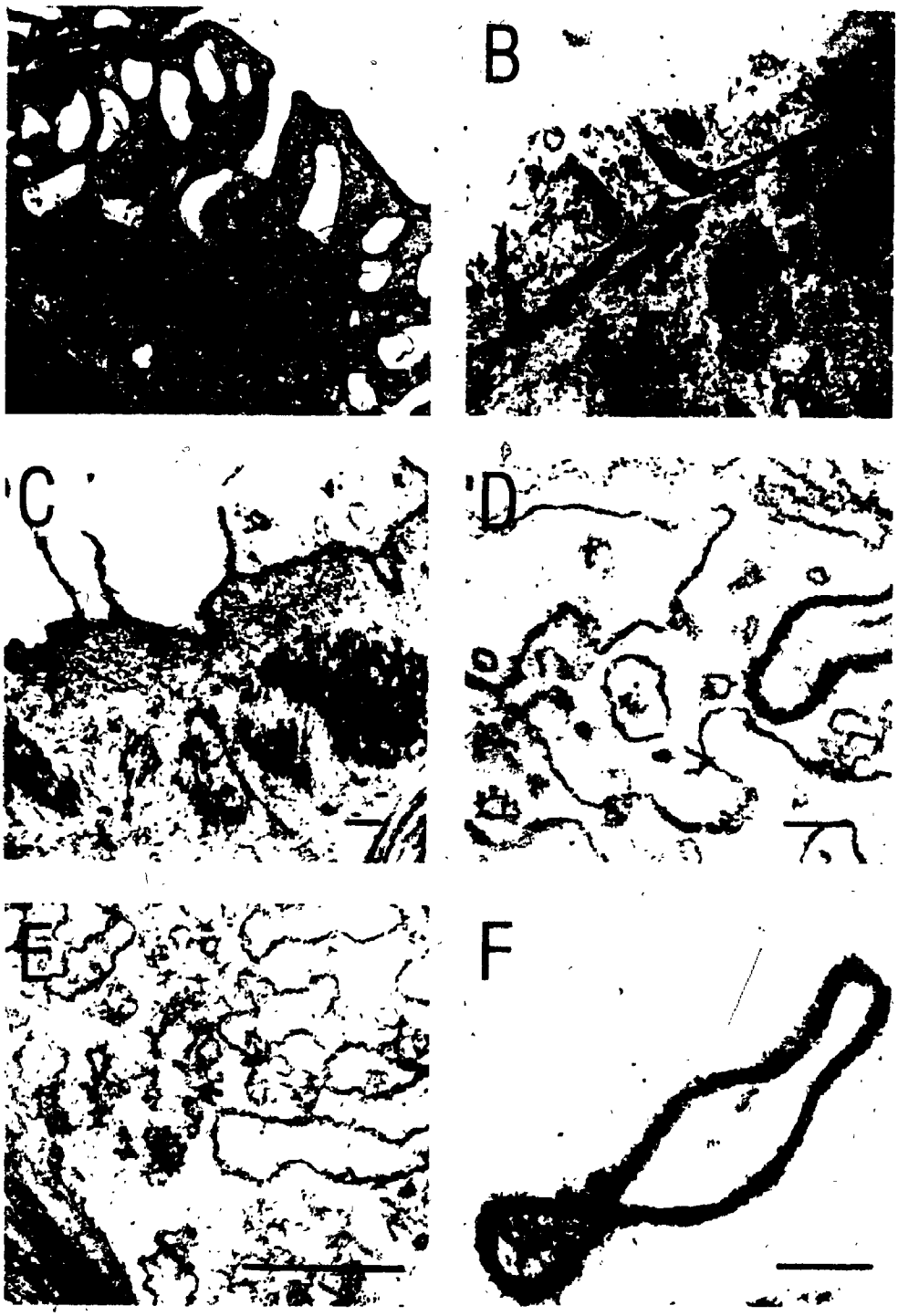

Fig. 1. Electron micrographs of the surface epithelial syncytium of *Schistosoma mansoni* prior to and after sequential removal of the outward-facing (OFM) and inward-facing (IFM) membranes. A, prior to incubation in the membrane disruption fluid; B, surface after the first incubation in the membrane disruption fluid; C, surface after the second incubation in the membrane disruption fluid; D and F, membrane pellet after first incubation; E, membrane pellet after second incubation. A, B, C, E, bar represents 1.0 μm; D and F, bar represents 0.1 μm.

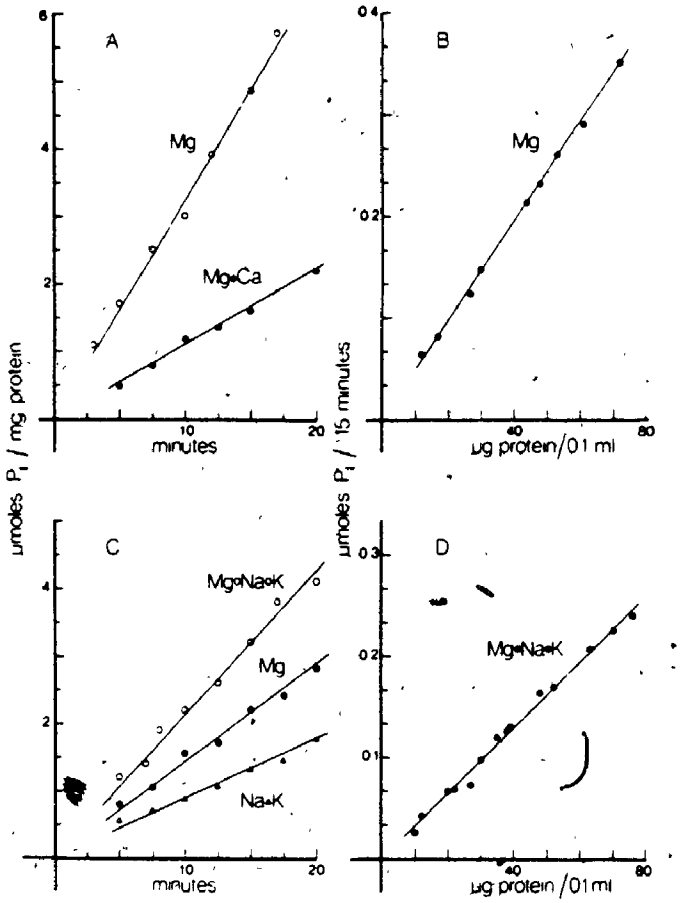

Fig. 2. Dependence of ATPase activity on time of reaction and protein in reaction mixture obtained from outward-facing (OFM, A and B) and inward-facing (IFM, C and D) membranes of the surface epithelial syncytium of *Schistosoma mansoni*. The reaction mixture contained 48 mM Tris-HCl (pH 7.5), sucrose to 300 mosM, and when added, 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl and 5 mM $CaCl_2$, each added at the expense of sucrose.

The ion-dependence of the ATPase activities from the IFM and OFM preparations are indicated in Table I and Fig. 2. The OFM enzyme was stimulated by all ion combinations except when K^+ or HCO_3^- was the co-ion with Mg^{2+} and when Ca^{2+} was included in the reaction mixture producing an inhibition of the enzyme activity. Maximum activity occurred in the presence of both Na^+ and Mg^{2+}. Similar results were obtained for the IFM enzyme except that K^+ also stimulated the activity when in combination with Mg^{2+}. Maximum activity occurred in the presence of Mg^{2+}, K^+ and Na^+ (Table I and Fig. 2).

Distinguishing between the enzymes from the two membrane preparations was made easier using inhibitors (Table II). Ouabain inhibited only the enzyme in the IFM, ethacrynic acid inhibited only the OFM enzyme and, as an indicator of contamination by mitochondrial ATPase, azide inhibited only the ATPase activity of the IFM prepara-

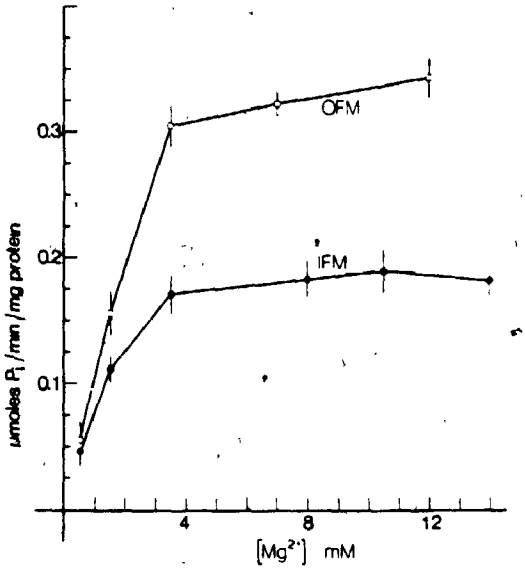

Fig. 3. Mg^{2+}-dependence of ATPase activity obtained from membrane fractions of outward (OFM)- and inward (IFM)-facing membranes of the surface epithelial syncytium of *Schistosoma mansoni*. The reaction mixture contained 48 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20 mM KCl, 4 mM ATP, 100 μl of membrane suspension and 35 mM sucrose. Sucrose was replaced with $MgCl_2$ to desired Mg^{2+} concentration.

tion. Oligomycin and SCN^- had little or no effect on the enzymes in either membrane preparation.

DISCUSSION

It has been shown using the results from membrane transport studies that the surface of endoparasitic platyhelminths, in particular *Hymenolepis diminuta* and *S. mansoni*, is a polarized epithelial syncytium capable of vectorial transepithelial transport of solutes and water [3, 4, 15–19]. Transepithelial transport is possible only if the limiting membranes of the epithelial layer are asymmetrical with respect to their permeability properties and the enzyme activities associated with the differential permeabilities [3,4]. Although asymmetry has been demonstrated with respect to ion, water and nonelectrolyte transport, this is the first study showing asymmetry of enzyme activities associated with ion transport in *S. mansoni*. As supported below, the OFM of *S. mansoni* contains ethacrynic acid inhibited ATPase activity similar to that observed in the OFM of several other epithelia [6] while, as in all transporting epithelia so far examined, the IFM contains ATPase activity that has properties similar to the classical Na^+-pump, or (Na^++K^+)-Mg^{2+}-ATPase.

Our first problem was to obtain fractions enriched with OFM and IFM. Using the saponin method of Kusel [2], under rigorously controlled incubation times, we were able to sequentially remove the syncytium in two time-dependent fractions, one con-

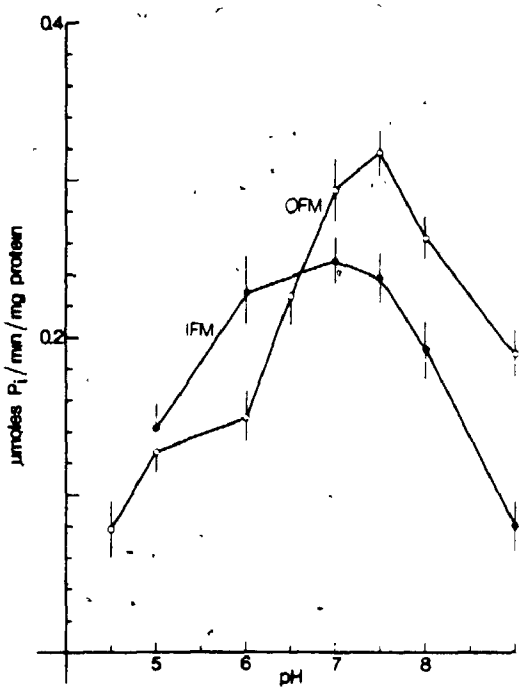

Fig. 4. Dependence of ATPase activity on pH of reaction mixture using enzyme from membrane fractions obtained from the outward-facing (OFM) and inward-facing (IFM) membranes of the surface epithelial syncytium of *Schistosoma mansoni*. The reaction mixture contained 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 100 μl of membrane suspension, 4 mM ATP, 48 mM Tris-HCl buffer in range of pH 6.0–9.0 or 0.1 M 4-morpholinoethanesulfonic acid buffer below pH 6.0.

taining IFM and the other containing OFM membranes. The first exposure to saponin removed approximately the apical half of the syncytium containing the OFM but leaving the IFM and spines intact (Fig. 1). The absence of ouabain-sensitive and azide-sensitive ATPase activity in this fraction rules out contamination by IFM and mitochondrial membranes (Table II). Although we did not observe intact multilamellar bodies (MLB) in this fraction, contamination by these organelles is likely if the MLB are disrupted. We have recently isolated a MLB fraction from *S. mansoni* in which it was shown that membrane material from disrupted MLB have a double bilayer appearance characteristic of the OFM [7]. However, if the MLB are the precursors of the OFM [7, 8], then contamination of our OFM fraction by MLB will not alter our interpretation of the ATPase activities associated with the epithelial syncytium of *S. mansoni*. Double bilayer structures were not observed in the IFM fraction.

The second exposure to saponin removed the remainder of the surface epithelial syncytium, including the spines and the IFM. The latter is supported by the presence of ouabain-sensitive ATPase activity associated with this fraction, although contamination by mitochondrial ATPase was evident from the partial inhibition (18%) by azide (Table II). However, this fraction was not as clean a preparation as that obtained from the first saponin exposure and the lower ATPase activity (Fig. 2) per mg protein from the IFM is probably a reflection of contaminating protein.

TABLE I

Ion dependence of ATPase activity associated with membrane fractions isolated from the surface epithelial syncytium of *Schistosoma mansoni*

Ion composition[a]	OFM[b]	IFM[b]
Mg^{2+}	0.281 ± 0.014	0.141 ± 0.011
Ca^{2+}	0.033 ± 0.009[c]	0.023 ± 0.007[c]
Mg^{2+}:Ca^{2+}	0.058 ± 0.010[c]	0.051 ± 0.009[c]
Na^{+}	0.096 ± 0.012[c]	0.076 ± 0.009[c]
Mg^{2+}:Na^{+}	0.326 ± 0.017[c]	0.176 ± 0.010[c]
K^{+}	0.062 ± 0.011[c]	0.071 ± 0.008[c]
Mg^{2+}:K^{+}	0.269 ± 0.015	0.188 ± 0.016[c]
Na^{+}:K^{+}	0.114 ± 0.013[c]	0.089 ± 0.011[c]
Mg^{2+}:Na^{+}:K^{+}	0.318 ± 0.019[c]	0.205 ± 0.012[c]
HCO_3^-	0.088 ± 0.009[c]	0.059 ± 0.009[c]
Mg^{2+}:HCO_3^-	0.294 ± 0.014	0.136 ± 0.009
Mg^{2+}:Na^{+}:K^{+}:HCO_3^-	0.324 ± 0.013[c]	0.217 ± 0.010[c]

[a] The reaction mixture contained 48 mM Tris-HCl (pH 7.5), 100 µl of membrane suspension made up to 300 mosM with sucrose. When added, the reaction mixture contained (at the expense of sucrose) 5 mM $MgCl_2$, 100 mM NaCl, 5 mM $CaCl_2$, 20 mM KCl and 25 mM K_2HCO_3 (equilibrated with 5% CO_2).

[b] Units: µmol P_i · min^{-1} · mg^{-1} protein.

[c] Significantly different from results with Mg^{2+} alone at least at 5% level of significance with at least 5 degrees of freedom.

TABLE II

Effects of inhibitors on ATPase activity associated with epithelial membranes of *S. mansoni*

	OFM[b]	IFM[b]
Inhibitor		
Control[a]	0.331 ± 0.011	0.229 ± 0.009
Ouabain (1.0 mM)	0.320 ± 0.011	0.076 ± 0.015[c]
Oligomycin (25 µg in ethanol)	0.318 ± 0.013	0.206 ± 0.012
SCN^- (1.0 mM)	0.304 ± 0.010	0.215 ± 0.011
Ethacrynate (2.0 mM)	0.115 ± 0.016[c]	0.211 ± 0.014
Azide (5.0 mM)	0.321 ± 0.012	0.188 ± 0.010[c]

[a] Reaction mixture contained 48 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20 mM KCl and 5 mM $MgCl_2$.

[b] Units: µmol P_i · min^{-1} · mg^{-2} protein.

[c] Significantly different ($P > 0.005$) from control with 6 degrees of freedom using Student's *t*-test.

The effects of detergent on membrane ATPases have been well characterized. Generally, below the critical micellar concentration, detergents have little effect on ATPase activity and may even stimulate enzyme and transport activity [5, 20–22]. Above the critical micellar concentration detergents often have deleterious effects on the activity of ATPases [20–22]. Saponin, at concentrations below the critical micellar concentration has been shown to enhance ATPase activity, has less inhibitory effects at higher concentrations and has been used to protect enzyme activity from more severe effects of other ionic or non-ionic detergents [20]. Membrane enzyme activity is enhanced by saponin at low concentrations due to the detergent making membrane vesicles more permeable thereby increasing the accessibility of ions and substrates to the catalytic site of the enzyme [5, 21, 22]. For these reasons we used saponin to prepare the membrane fractions enriched in IFM and OFM.

More recently, a number of phosphohydrolyzing enzymes has been detected in double apical membranes prepared from material shed by *S. mansoni* in vitro [23]. All the enzyme activity releasing phosphate in these shed membranes had pH optima in excess of pH 9–10 [23], which is unusual for transport ATPase activities [21,22]. Unlike the Mg^{2+}-ATPase obtained from OFM prepared in the present study, the Mg^{2+}-ATPase of the shed membranes was activated by Ca^{2+} and was inhibited by 11% using ouabain in the absence of Mg^{2+} and Ca^{2+}. The latter observation suggests that the entire syncytium may have been shed since the presence of ouabain-sensitive ATPase activity is indicative of contamination of OFM by IFM membrane (Table II). Unlike Ca^{2+}-Mg^{2+}-ATPases from other membrane systems [21, 22], the ATPase from shed membrane material was inhibited by phosphate and delipidation by gel filtration using Ultrogel AcA 22 columns did not alter the specific activity of the enzyme [23]. Plasma membrane Ca^{2+}-Mg^{2+}-ATPases generally have a requirement for phospholipids, particularly phosphatidylserine and particularly when solubilized in Triton-X100 [21, 22], the detergent used to solubilize the membrane shed by *S. mansoni* in vitro [23].

The results of the present study are in support of the epithelial transport function of the surface epithelial syncytium of *S. mansoni* and other endoparasitic flatworms [3, 4, 15]. The OFM contains ethacrynate-sensitive, Na^+-Mg^{2+}-ATPase activity which is thought to augment the regulatory cell volume decreasing function of the Na^+-pump ATPase [6, 14]. The IFM contained ATPase activity similar to that associated with plasma membrane (Na^++K^+)-Mg^{2+}-ATPase observed in many other cell systems [5, 22, 23]. The polarized distribution of this enzyme in epithelial cells is a prerequisite for transepithelial transport of Na^+, water and Na^+-coupled sugar and amino acid transport observed in this and other parasitic flatworms [3, 4].

In a separate study we show that the ATPase and alkaline phosphatase activities associated with the OFM of *S. mansoni* are confined to the inner bilayer and not the outer bilayer of this surface membrane complex [9].

ACKNOWLEDGEMENTS

This study was supported by grants to R.B.P. from the Natural Sciences and Engineering Research Council of Canada and J.P. Bickell Foundation.

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