ISOLATION OF SURFACE MEMBRANES AND TEGUMENTAL ORGANELLES FROM *SCHISTOSOMA MANSONI*

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To Elaine and Lump

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ABSTRACT

Isolation of surface membranes and tegumental organelles from *Schistosoma mansoni* by A N MacGregor.

Methods were developed for isolation of surface membranes, tegumental spines and discoid granules from *Schistosoma mansoni*. The surface membrane of the parasites was disrupted by freezing worms in liquid nitrogen and allowing them to thaw. As a consequence of morphological and biochemical analyses of released material and parasite carcasses, the surface membrane isolation method was gradually improved. Tris(hydroxymethyl)methylamine buffers were shown to cause alterations in tegument morphology; the parasites were therfore suspended in balanced salt solution immediately prior to freezing. Upon thawing the parasites were agitated to release surface membranes. Vortexing was shown to be a more efficient and less damaging method of releasing surface membranes than sonication. The basal membrane of the tegument appeared to remain intact after freeze-thaw and vortexing. Surface membranes were released as large sheets which were readily separated from other released material by differential centrifugation and could be further purified by density gradient centrifugation.

Enzyme assays suggested some contamination of surface membranes with internal membranes may have occurred as a result of excessive damage caused by freeze-thaw. Rates of freezing and thawing were varied and cryoprotective compounds were used in order to reduce freeze-thaw damage. This damage, which was

assessed using assays for internally located enzymes, was greatly reduced by addition of ethan-1,2-diol to worms before freezing. The released surface membranes were however, less easily sedimentable.

Tegumental spines were separated from the surface membrane by density gradient centrifugation. Discoid granules were isolated by differential centrifugation from the material released by freeze-thaw and vortexing. Their constituent proteins and glycoproteins were compared with those of the surface membrane, spines and tegumental cytoplasm. This showed some correlation between surface membrane and discoid granule glycoproteins. The possible function of discoid granules in aiding surface membrane turnover is discussed.

ABBREVIATIONS

CCR	cytochrome c reductase
ConA	concanavalin A
DWH	denuded worm homogenate
FITC	fluorescein isothiocyanate
FTS	freeze-thaw supernatant
GFH	glucose-free Hanks' balanced salt solution
HBSS	Hanks' balanced salt solution
MHC	major histocompatability antigens
NGS	normal goat serum
PBS	phosphate-buffered saline
PVP	polyvinylpyrrolidone
RCF	relative centrifugal force
SDH	succinate dehydrogenase
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEM	scanning electron microscope
SS	sonication supernatant
TBS	tris-buffered saline
TCA	trichloroacetic acid
TEM	transmission electron microscope
tris	tris(hydroxymethyl)methylamine
VS	vortex supernatant

GENERAL INTRODUCTION

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I.1 Schistosomiasis

It is generally accepted that 200 million people are infected with the disease schistosomiasis. Three times that number are at risk in the seventy countries where the disease is found.

There are three major species of human schistosomes. *Schistosoma mansoni* is found throughout Central and South America and Africa. *S haematobium* also occurs in Africa and, in addition, the Middle East, while *S japonicum* is found in South-East Asia.

The life-cycle of *S mansoni* is as follows. Adult females in the hepatic portal system, lay eggs. These have a sharp spine which, in conjunction with the peristaltic motion of the intestine, and probably enzymic secretions (Asch and Dresden, 1979), aids penetration of the mesenteric vein and intestinal walls. Eggs in the lumen of the gut are then voided in the faeces. If they reach fresh water they will hatch. The resulting free-swimming larva is a miracidium infective for certain species of planorbid snails. Miracidia infiltrate the snail tissue and each forms a mother sporocyst. This multiplies asexually, producing many daughter sporocysts which in turn give rise to cercariae.

In response to light, cercariae leave the intermediate host and swim in the water until they encounter a human. On burrowing into the skin the cercaria loses it's tail and is known as a schistosomulum. This migrates through the skin until it enters

a blood vessel. It then passes to the lungs. The worms elongate to allow them to migrate along the lung capillaries. They enter the systemic circulation and are probably then carried in the bloodstream until they reach another capillary bed (Miller and Wilson, 1980). The schistosomula slowly move through the capillaries and eventually are returned to the lungs. A number of circuits of the systemic-pulmonary blood system may be required until, by chance, the schistosomula enter the hepatic portal vein. They probably remain in this location because their size prevents passage through the liver capillaries.

Schistosomula commence blood feeding in the hepatic portal system and mature into adults. Schistosomes are unusual among trematodes in that they are dioecious. Females are cylindrical while males conform to the normal flatworm pattern. The lateral edges of the male body curl in a ventral direction to form the gynaecophoric canal. The female lies in the canal (Figure 1).

The most serious effects of the disease are caused by the eggs. These become embedded in capillaries and tissues of the liver and intestine, where they induce inflammatory reactions. Fibrous granulomata result. Capillaries become blocked and necrosis and ulceration occur. This leads to periportal cirrhosis and portal hypertension. Splenomegaly occurs due to proliferation of lymphocytes, the large number of eggs lodged in the spleen and the congestion of the liver. Fluid accumulates in the abdominal cavity. Symptoms of the disease include abdominal pain and diarrhoea with blood and mucus.

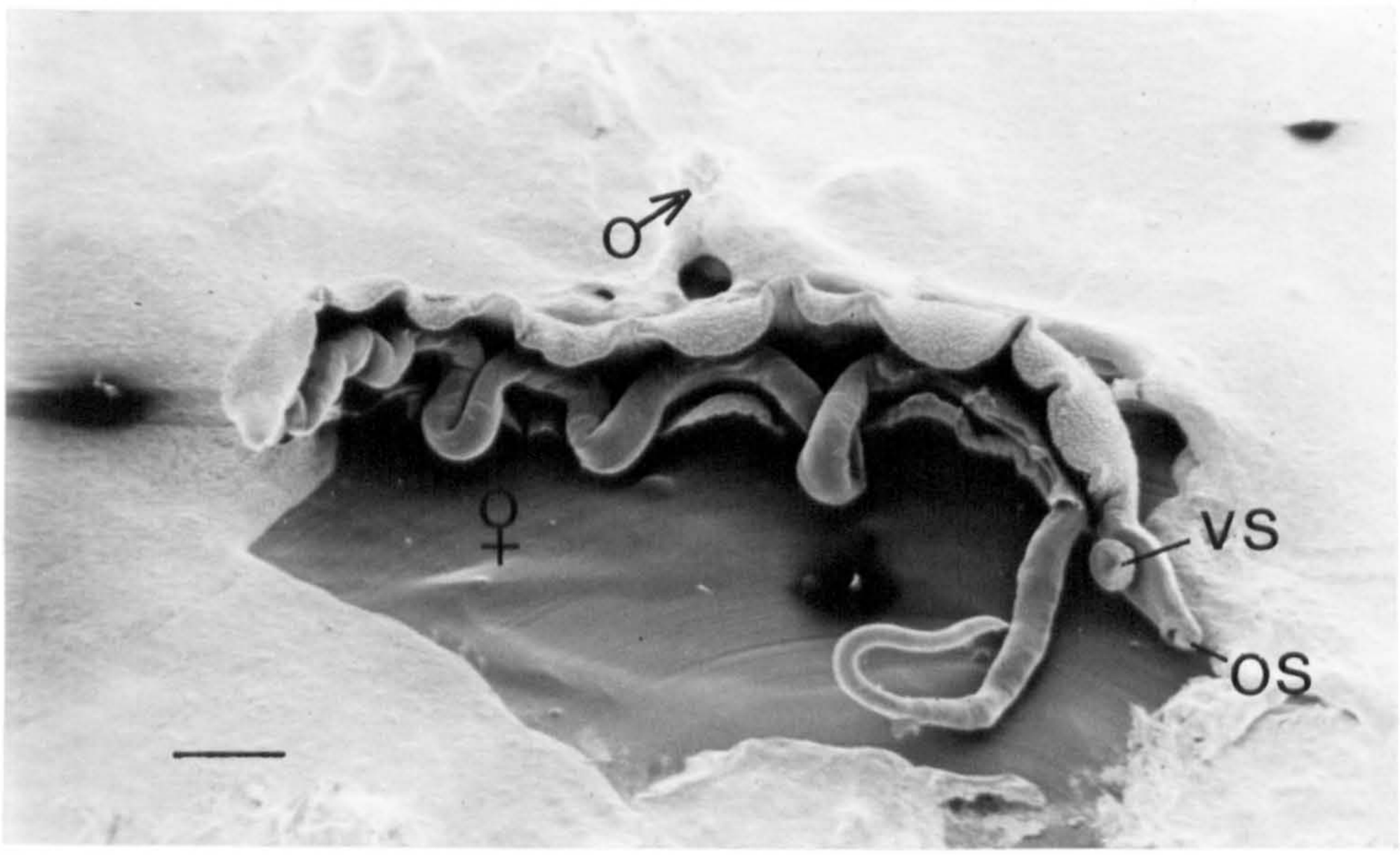


Figure 1. Male and female *S mansoni*.

vs, ventral sucker; os, oral sucker.

Bar = 1 mm.

I.2 Schistosome morphology

Adult schistosomes are about 1 cm long. They have a bifurcating gut and feed on erythrocytes. This was suggested by Rogers (1940) who noted that the gut is filled with the black pigment haematin and argued that this was derived from the proteolytic degradation of haemoglobin. The main cell type in the body is parenchyma. Glycogen is stored in these cells (Reissig, 1970). Reproductive organs lie within the parenchyma. The parenchyma is invested with circular and longitudinal muscles which in turn are covered by the tegument.

I.3 Ultrastructure of the tegument

A number of ultrastructural studies have been performed on the tegument of adult worms (Morris and Threadgold, 1968; Silk, Spence and Gear, 1969; Smith, Reynolds and von Lichtenberg, 1969; Hockley and McLaren, 1973; Wilson and Barnes, 1974a). It was soon established that the tegument is a living syncitium and not an inert layer. This syncitium (Figure 2) covers the entire body and extends down through the muscle layers in cytoplasmic connections which link the tegument with cell bodies lying within the parenchyma. The tegument above the muscle layers is about one to three microns thick. There are pits in the tegument and the cytoplasm is electron dense. The cell bodies contain the nuclei of the tegument. They are also sites of synthetic activity (Wheater and Wilson, 1976) containing Golgi apparatus, endoplasmic reticulum and most of the tegumental mitochondria. It is probable that two tegumental organelles are synthesised in the cell bodies (Wilson and Barnes, 1974a) and transported in the

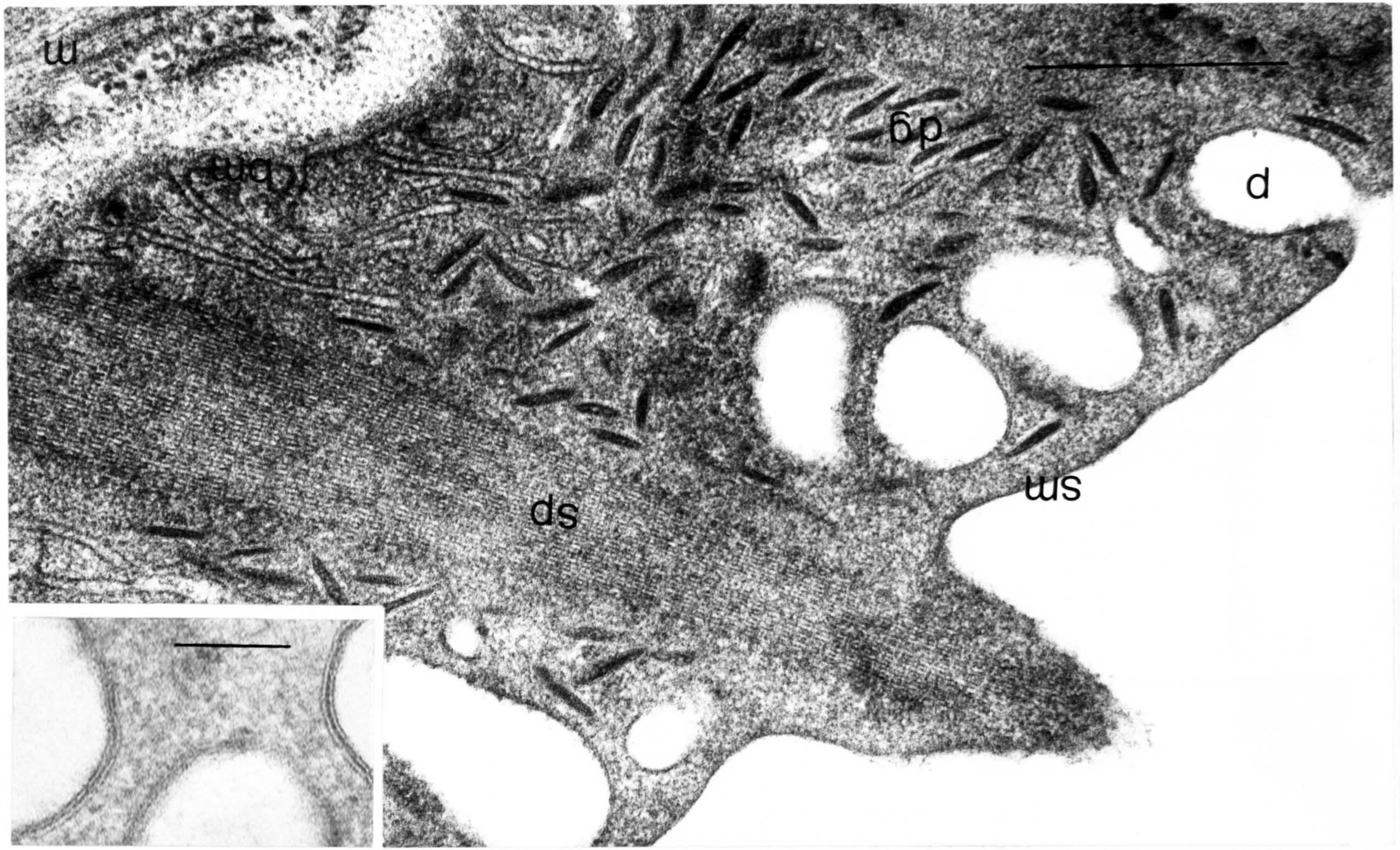
Figure 2. The schistosome tegument.

sm, surface membrane; bm, basal membrane; p, pit; dg, discoid granule; sp, spine; m, muscle.

Bar = 0.5 μm.

Inset - the double outer membrane fixed by uranyl acetate.

Bar = 0.1 μm.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cytoplasmic connections to the tegument. These organelles are discoid granules and multilaminate vesicles. Discoid granules are membrane-bound, have electron-dense contents and are known to contain carbohydrate (Wilson and Barnes, 1974a). Their function has not yet been determined. It seems they may break down and contribute their contents to the cytoplasm (Wilson and Barnes, 1974b) but other suggested functions are that they contribute to the spines (Smith *et al*, 1969) or to the surface membranes (McLaren, 1980). Multilaminate vesicles are membrane bound. They appear to contain whorled membrane. These vesicles are thought to fuse with the surface membrane of the tegument and contribute their membranous contents on to the surface (Hockley and McLaren, 1973; Wilson and Barnes, 1974a,b). Ultrastructural observations on the surface membrane showed it to have regions of pentalaminate appearance (Smith *et al*, 1969; Spence and Silk, 1970). The use of uranyl acetate as a tertiary fixative after conventional glutaraldehyde and osmium fixation revealed that the outer surface of the parasite has a heptalaminate appearance in the electron microscope, showing it to be two closely apposed lipid bilayers (Hockley and McLaren, 1973). These will be referred to as the outer and inner bilayers of the surface membrane. Schistosomula and adults have a double outer membrane (Figure 2). The outer bilayer is probably in a state of constant sloughing and is replenished by multilaminate vesicles (Hockley and McLaren, 1973; Wilson and Barnes, 1974a,b). The significance of a double membrane at the schistosome surface and its turnover will be discussed later. Large spines are found in the tegument. These are believed to be composed of paracrystalline protein (Smith *et al*, 1969; Morris and Threadgold, 1968) and probably help

to anchor worms in blood vessels (Crabtree and Wilson, 1981).

The basal membrane of the tegument has invaginations into the cytoplasm. These invaginations and the pits found in the tegument suggest that nutrient absorption may be a function of the tegument, although the main digestive organ is the gut. There is some evidence that the tegument serves such a role (Senft, 1968; Chappell, 1974; Rogers and Bueding, 1975; Ernst, 1976; Carlisle and Weisberg, 1978).

The tegument is separated from the muscle layers by a basal lamella composed of elastin-like microfibrils (Kohn, Cotta-Pereira, Lopez-Alvarez and Kattenbach, 1979).

I.4 The tegument and evasion of the immune response.

Schistosomes survive in an environment which is potentially extremely hostile. The reasons for their survival have yet to be fully elucidated. Several possible explanations have been advanced, all of which depend on the properties of the tegument and, in particular, of the surface membrane.

I.4.1. The host antigen disguise. Smithers, Terry and Hockley (1969) showed that adult worms transferred from mice to monkeys previously sensitised against mouse antigens were killed by immunological attack directed against the tegument. Since this killing did not occur in non-immunised monkeys (Smithers and Terry, 1967; Smithers *et al*, 1969) it must have been due to attack by the immune system of the monkey against mouse antigens on the

surface of the parasites. If host antigens are present on the schistosome surface, then they might provide an antigenic mask which protects the worms. The host would recognise the worm as self, rather than foreign and the parasites would survive. Clegg, Smithers and Terry (1970) and Perez and Terry (1973) provided evidence that host antigens on the parasite surface were of similar nature to antigens on the surface of erythrocytes. The mechanism of acquisition of these antigens is not known, but they firmly adhere to the worm surface (Clegg *et al*, 1970).

Schistosomula cultured in medium containing human blood were shown to acquire glycolipids or glycoproteins associated with blood group antigens (Clegg *et al*, 1971; Goldring, Clegg, Smithers and Terry, 1976). They can also acquire Forsmann antigens (Dean and Sell, 1972) which are glycolipids. Using erythrocytes previously labelled in their carbohydrate moities, Goldring, Kusel and Smithers (1977) demonstrated that only glycolipids were transferred to schistosomula. They also provided evidence that schistosomula were unlikely to synthesise host-like molecules. Further apparent evidence for the glycolipid nature of acquired host antigens comes from the use of lactoperoxidase catalysed iodination to label externally exposed proteins on schistosomula and adult worms (Snary, Smith and Clegg, 1980). All of the proteins labelled on adult worms were found on artificially transferred, *in vitro* maintained schistosomula. No new proteins were found in adults suggesting that host antigens are unlikely to be proteins.

Schistosomula can acquire intercellular substance in

penetrating mouse skin (Smith and Kusel, 1979). This host antigen which is a glycoprotein (Miyagawa, Hojo, Ishii, Yoshioka and Samamoto, 1977) is soon lost from the surface however, since it is absent from worms recovered from mouse lungs.

Among the host molecules discovered on the surface of *S mansoni* are products of the K, D and I regions of the murine major histocompatibility (MHC) gene complex (Sher, Hall and Vadas, 1978; Gitter and Damian, 1982; Gitter, McCormick and Damian, 1982). These antigens were found on schistosomula from mouse lungs. They could be acquired *in vitro* (Sher *et al*, 1978) and were not synthesised by the parasites (Simpson, Singer, McCutchan, Sacks and Sher, 1983)..

The discovery of MHC antigens on the parasite surface exposes two anomalies. Firstly, these antigens are glycoproteins (Schwartz, Kato, Cullen and Nathenson, 1973; Cullen, Freed and Nathenson, 1976) and secondly, K and D antigens are involved in recognition of foreign material by the host (Zinkernagel and Doherty, 1974; Doherty and Zinkernagel, 1975). Their presence on schistosomes should presumably encourage rejection of the parasites by the host.

The importance of host antigens on the parasite surface has been questioned by the work of Dean (1977) and Dessein, Samuelson, Butterworth, Hogan, Sherry, Vadas and David (1981). They found that incubation of freshly transformed schistosomula in defined culture medium for a number of days resulted in the acquisition of resistance to antiserum, antiserum in the presence of eosino-

phils and eosinophils in the presence of complement. This resistance to susceptibility was independant of uptake of host antigens.

There are therefore, some inconsistencies in the available data. Although there can be no doubt that host antigens are present on the schistosome surface, there is as yet no reason to believe they are protective.

The theory of the host antigenic disguise has been used to explain the apparent death of later schistosome infections without any obvious effect on an existing infection (Smither and Terry, 1965). It is proposed that the newly invading parasites are unable to acquire host antigens before being killed. The immune response directed against them is triggered by schistosome antigens in the existing infection, but that infection is safe because of its host antigenic disguise. The destruction of a challenge infection by an immune response provoked, but not affecting the primary infection, has been termed concomitant immunity (Smithers and Terry, 1969). Recent work suggests that concomitant immunity may have to be reappraised. It seems that death of challenge parasites could be due to the pathological effects of the primary infection which prevent sequestration of the invading parasites in the liver (Wilson, Coulson and McHugh, 1983).

I.4.2. The double bilayered surface membrane. A double surface membrane is now known to be a common feature of blood-dwelling flukes while gut flukes have a single membrane (McLaren and Hockley, 1977). Schistosome cercariae have a single membrane.

Formation of the second membrane begins immediately after penetration of host skin and by three hours after penetration most of the schistosomula surface membrane is heptalaminate (Hockley and McLaren, 1973). Although this is apparently an adaptation to existence in blood, it is not yet known precisely what function it has in evasion of the immune response. It has been suggested that the outer bilayer may be essentially free of worm antigens and that this would confer some protection to the worm by rendering it immunologically inert (Wilson and Barnes, 1977). Since uranyl acetate stabilises lipids (Silva, Guerra and Magalhães, 1968) is seems likely that the outer bilayer is lipid rich and may therefore contain few schistosome antigens. This is supported by the observation of Snary *et al* (1980) that the adult surfaces have only very low amounts of externally exposed protein.

Schistosomula in culture medium can lose lipids to and acquire others from, serum in the medium (Rumjanek and McLaren, 1981). The nature of the modulation depends on the serum used. The subsequent response to immune attack also varies with the serum used, human serum apparently providing greater protection than foetal calf serum. The implication of this work is that the lipid composition of the surface membranes may be altered during migration of the schistosomula in such a way as to provide protection to the parasites from the host immune system. A lipoprotein receptor has been discovered on the surface of schistosomula and it is thought that lipids may be transferred from host to parasites by the binding of low density lipoproteins and the reutilization of theirlipids.(Rumjanek, McLaren and Smithers, 1983).

Ultrastructural observations suggested that the outer bilayer might be in a state of constant turnover (Hockley and McLaren, 1973; Wilson and Barnes, 1974a). It is obviously a less stable membrane than the inner bilayer as judged by the difficulty in fixing it. Perez and Terry (1973) presented electron microscope evidence that appeared to show fragments of membrane being cast off and renewed in adult worms taken from mice and cultured *in vitro* in the presence of monkey anti-mouse serum. Additional evidence for turnover of the outer bilayer comes from an ultrastructural study of dynamic processes in the tegument using compounds which disrupt secretory processes (Wilson and Barnes, 1974b). Incubation of adult worms in medium containing ouabain led to flattening of the parasite surface and a virtual absence of multilaminate vesicles. Some multilaminate vesicles were detected in surface pits. This appeared to confirm that these vesicles contribute their contents to the worm surface. Cyclic AMP seemed to speed up tegument function in the same way as immune serum and the supply of multilaminate vesicles and discoid granules was rapidly exhausted.

It seems then, that the outer bilayer of the schistosome tegument is in a state of constant turnover and that rate of turnover can be altered in response to environmental conditions. Some attempts have been made to quantify turnover rate. Wilson and Barnes (1977) incubated adult worms in the presence of cationised ferritin. This protein will bind to negative charges on membrane surface. After removal from that medium worms were examined ultrastructurally at various times. This indicated that the ferritin, which is electron dense, was lost first from the

tegumental pits, then the apical surface of the tegument and finally from the spines. This appears to be the route of movement of the outer bilayer. It probably receives new material from multilaminate vesicles which fuse with the pits. This material moves up the pits into the surface, up the spines and finally off into the medium. The experiments suggested a half-life of two to three hours for the outer bilayer under these conditions. Kemp, Brown, Merritt and Miller (1980) also concluded that surface membrane turnover was rapid. They noted the loss of host immunoglobulins on the surface of adult worms after six hours in culture.

Kusel, Mackenzie and McLaren (1975) and Kusel, Sher, Perez, Clegg and Smithers (1975) demonstrated the appearance of radio-labelled proteins from the surface of adult schistosomes in culture medium. Using a double isotope labelling technique, Kusel and Mackenzie (1975) showed that membrane proteins may be released as a result of a slow membrane turnover and a rapid secretory process. The half-life of schistosome surface membranes was estimated at 2 days (Kusel, Sher *et al*, 1975). This compares favourably with the turnover of mammalian cell membranes (Arias, Doyle and Schimke, 1969). This has led to speculation that the rapid secretory process might be due to turnover of the outer bilayer at the worm surface while the slower turnover may be due to the inner bilayer (McLaren, 1980) which, in this respect, acts more like a normal plasma membrane. Turnover of the outer bilayer may prevent binding of antibody or immune effector cells.

I.4.3. Lung-stage schistosomula. Lung-stage schistosomula may

possess another survival mechanism. The surface of these worms appears to have some unique properties. For instance, although drugs may increase the susceptibility of skin-stage schistosomula to anti-serum, lung-stage worms are not so affected (Dean, 1977). In addition, radiation-attenuated lung-stage worms are substantially less immunogenic than cercariae or skin-stage worms (Sher and Benno, 1982) and lung schistosomula are not killed by eosinophils in the presence of anti-host antigen antibody although older worms are (McLaren and Terry, 1982). The survival mechanism has not yet been identified and indeed, there may be more than one. The findings of Dean (1977) and Sher and Benno (1982) might be explained by the possession of an inert surface layer at this stage. However, since anti-host antigen antibody does bind to worms (McLaren and Terry, 1982) another mechanism must exist to explain the failure of eosinophils to damage the schistosomula.

I.4.4. Summary. In summary, the schistosome surface is the target of the hosts immune system. That the parasites withstand attack is evidenced by the survival of a single infection for many years in human hosts. Evasion of the immune response is probably due to a number of factors, including :

a) the acquisition of host molecules which may or may not act as an antigenic disguise;

b) turnover of the outer bilayer of the surface membrane which may prevent the binding of antibody and immune effector cells;

c) the existance of few parasite antigens in the outer bilayer, making this layer immunologically inert or at least only mildly antigenic;

d) modulation of the lipid content of the outer bilayer which

may also confer some protection.

I.4.5. Conclusions. In order to make a complete study of the properties of this unusual membrane it is essential that it be isolated. The proteins and lipids which serve to make up the membrane could then be separated by conventional techniques. These molecules are responsible for the membrane's properties and so the relative importance of the factors listed above may be elucidated from knowledge of the properties of the molecules and unknown properties may be discovered. Isolated membranes may provide evidence on the identity of host molecules attached to the membrane and on the nature of their attachment.

The molecules of parasite origin on the surface may be identified and studies can be undertaken to determine which of these is antigenic. Identification of parasite antigens exposed on the surface has important implications for the development of a vaccine against schistosomiasis.

Since schistosomula of different ages appear to have varying susceptibility to immune attack, comparison of the molecules present in the surface membrane at different stages may yield useful information on the reasons for this variation.

I.5 General principles of plasma membrane isolation.

Plasma membrane isolation has been the subject of a number of reviews (Steck and Wallach, 1970; Hinton, 1972; Wallach and Lin, 1973; De Pierre and Karnovsky, 1973; Neville, 1975, 1976; Glick, 1976). An examination of these permits one to define a number of basic principles. Although most of the information gathered concerns isolation of cell membranes from mammalian tissues or from cultured cells, the principles are no less valid when applied to schistosomes.

In order to isolate plasma membranes, cells must first be disrupted in order to extrude their contents. This is difficult to control. After disruption the plasma membranes may exist in many shapes and forms from large sheets to small vesicles. This can lead to isolation of membrane parts which are not representative of the whole. Often methods give one extreme or the other. Most reviewers prefer the membrane to be broken into large sheets as these are more easily separated from the rest of the cell contents. It is possible, however, to remove plasma membranes from a heterogeneous mixture of vesicles derived from many sources (Steck and Wallach, 1970).

Isolation of plasma membranes follows disruption. This usually involves a crude separation of organelles by sedimentation rate followed by separation according to density. Some methods use only differential centrifugation but sedimentation coefficients of cell organelles are not sufficiently dissimilar to allow their purification without also using density gradient

centrifugation (De Pierre and Karnovsky, 1973).

Criteria must be used to assess purity of fractions. Morphological examination can show the main components of fractions. It is often used to demonstrate purity but this may not be valid. All smooth membranes look alike in the electron microscope so it is impossible to distinquish between the vesicles formed by lysosomes, peroxisomes, Golgi apparatus, smooth endoplasmic reticulum and those formed by plasma membranes. Electron microscopic examination is useful, however, if the plasma membranes to be isolated have distinctive features making their unequivocal identification possible. Such identification must be used with care since it can lead to selective isolation of particular membrane fragments. Quantitation in microscopy is difficult and significant results cannot be obtained without the use of laborious techniques. Morphology is only useful therefore, in certain situations and even then, its uses are limited. Some workers have used morphological criteria alone in assessing the purity of preparations but this is condemned by the reviewers.

The various membranes of cells possess different enzymes and the enzyme profile of a particular membrane is probably unique to that membrane. If an enzyme is specific, or nearly so, to one membrane type then its presence in a subcellular fraction identifies the presence of that membrane. Thus enzymes may be used as membrane markers. Enzyme markers are easily quantifiable and rapidly assayed. Plasma membranes appear to have a great variation in enzymes and those which are characteristic of one plasma membrane need not be characteristic of

another. There is no ubiquitous marker enzyme for plasma membranes. Possible marker enzymes can sometimes be detected using cytochemistry or by assaying ectoenzymes (enzymes exposed on the surface of cells). Although enzymes are the most commonly used markers there are problems associated with them. For example, activity of 5'nucleotidase can often be confused with other phosphatase activities (Glick, 1976). This was the case in one study of isolated schistosome surface membranes (Simpson, Schryer, Cesari, Evans and Smithers, 1981). Enzymes may be extracted, activated or inactivated depending on isolation conditions. Rupture or purification may cause small fragments of certain activities to be lost or soluble enzymes to be added. It is possible, even likely, that the marker enzymes will also be present to some extent in subcellular organelles. These difficulties make it preferable that multiple markers be used. This may also help prevent isolation of uncharacteristic membrane fragments.

Other markers have been suggested. Chemical composition of fractions was once thought to be useful. The molar ratio of cholesterol to phospholipid may be an important characteristic of plasma membranes as other membranes contain little cholesterol. The distinction however, is not all that clear and cholesterol may not be a good marker. Similarly, it was thought that sialic acid was almost exclusive to plasma membranes. This was believed because pre-treatment of cells with neuraminidase (this enzyme liberates terminal sialic acid molecules from oligosaccharides) removed nearly all of the sialic acid. More recently it has been shown that neuraminidase penetrates cell membranes and the

previous results are therefore in doubt (Wallach and Lin, 1973; De Pierre and Karnovsky, 1973).

It is possible to bind markers to membranes before rupturing cells. These may be lectins, antibodies or covalent labels. These have the supposed advantage that they are only present on the plasma membrane and remain bound to it during fractionation. Some reviewers doubt whether ^{125}I binds only to the plasma membrane, however (Neville, 1976; Glick, 1976) and it may label proteins selectively (Glick, 1976). Although in general, these extrinsic labels are well received, Neville (1976) points out that if a plasma membrane represents 1 % of the cell protein, and if there is just 1 % cell breakage during labelling, then this could lead to errors as high as 50 % in purity of membranes.

Hormone and viral receptors can be used as markers where the relevant data is at hand.

It seems that although criteria of purity are vital when preparing plasma membrane fractions the only ones generally available are morphology, enzymes and artificially applied labels. None of these is perfect and results must be interpreted with caution. Whatever markers are used it is most important that specific activities of markers in membrane fractions are related to specific activity in homogenates. If this is not done it is impossible to gauge purity of fractions. The reviewers stress the importance of providing a complete balance sheet showing the fate of markers in all fractions. Many authors report only specific activity of markers in the purified membrane

preparation and it is impossible to judge purity with this alone.

Contamination of fractions by organelles must be assessed. If nuclei, lysosomes or mitochondria are disrupted, their contents may absorb to, co-sediment with or degrade the plasma membrane. They must therefore be tested for biochemically. If disruption does not take place nuclei can be effectively removed using low centrifugal force or their high density. Mitochondria, lysosomes and peroxisomes are generally more dense than plasma membranes and therefore separating these organelles is not difficult. Endoplasmic reticulum causes more problems as its density may overlap that of plasma membrane. If large membrane sheets are formed on cell rupture these can generally be separated from endoplasmic reticulum by rate sedimentation as the latter vesiculates on cell rupture. Markers for organelle contamination are usually enzymes but, where appropriate, other molecules can be assayed. For example, nucleic acids can be assayed to assess contamination by nuclei and ribosomes. Most organelles have characteristic enzymes which can serve as markers in many systems. Again, the complete distribution of markers within the various fractions must be known in order to judge the extent of contamination in the purified membrane fractions.

I.6 Methods used in isolation of schistosome surface membranes.

A study of the methods used for isolation of surfaces from helminth parasites shows that in many cases there are significant omissions from the general principles outlined above. In the case of helminths, the objective is not normally to isolate

all plasma membranes. Only those on the external surface are of interest. This necessitates a departure from the standard procedures of tissue disruption followed by purification of plasma membranes from the homogenate. At least one attempt to fractionate schistosome homogenates has been made, however. Smithers, Roodyn and Wilson (1965) separated schistosome homogenates into five fractions on the basis of rate sedimentation. Morphological characterisation showed that one of the fractions was rich in tegument but the surface membrane enzyme marker was not concentrated in this fraction. More recently refined approaches to the isolation of helminth surface membranes have been made. The main problem is to separate the outer membrane from the rest of the worm. It is axiomatic that to isolate any membrane the membrane must first be damaged in order to allow its separation from the material it encloses. In the case of the erythrocyte, cells can be lysed and their contents washed away. The membrane reseals and probably is little affected by the procedure. With helminths however, it is essential that plasma membranes within the body of the worm (ie, all plasma membranes other than those at the surface) be retained in the worm body. If released they are likely to co-purify with the surface membrane. Therefore, the procedure used to damage the surface should be sufficiently gentle to prevent excessive damage to underlying components which would result in release of much unwanted material. This initial step in isolation of surface membranes may be the most crucial. Subsequent purity of fractions probably depends on this more than anything else.

Six basic methods have been used to remove surface membranes

from helminths for subsequent analysis :

a) the use of detergents. Originated by Kusel (1970, 1972) this is the most commonly used method of isolating surfaces of schistosomes and cestodes. After a few minutes incubation in medium containing detergent, worms are washed in a sieve and released surface is removed from the medium by centrifugation. Kusel (1970) isolated surfaces from cercariae and schistosomula of *S mansoni* with digitonin. He used saponin in 1972 to release membranes from these stages as well as adult schistosomes. Extending Kusel's technique to the cestode *Hymenolepis diminuta*, Oaks, Knowles and Cain (1977) released brush border fragments with saponin. More recently the saponin technique as applied to *H diminuta* was apparently improved. Ultrastructural studies (Rahman, Mettrick and Podesta, 1981) indicated that much less contaminating cytoplasm was released when saponin was used in hypotonic medium. The surface brush border was apparently released without tegumental cytoplasm but no attempt was made to quantify the increase in purity of the final preparation. More recently, Podesta and McDiarmid (1982) used saponin and McDiarmid, Dean and Podesta (1983) used digitonin to remove surface membranes from schistosomes.

Interference with protein and carbohydrate assays by impurities in saponin and difficulties with characterisation of purified membranes led Knowles and Oaks (1979) to use the non-ionic detergent Triton X-100 to release surface membranes from *H diminuta* and this detergent was later used on schistosomes (Oaks, Cain, Mower and Raj, 1981; 1983). Both saponin and Triton X-100 have recently been applied to the cestode *Echinococcus*

granulosus (McManus and Barrett, 1985);

b) freezing and thawing. Kusel (1972) observed that the surface could be removed from adult schistosomes by freezing in liquid nitrogen and then thawing. Surface fragments were separated from the denuded bodies by sieving and then sedimented by centrifugation. This disruption method has also been used in schistosomes by Oaks *et al* (1983) and in cestodes by McManus and Barrett (1985). Repeated freezing and thawing in hypotonic medium released surface membranes from schistosomula of *S mansoni* (Taylor and Wells, 1984);

c) disruption by phosphate buffered saline. Simpson, Cesari, Evans and Śmithers (1980) and Simpson, Schryer, Cesari, Evans and Smithers (1981) incubated adult *S mansoni* in a phosphate buffered saline. This induced "shedding" of surface membranes into the medium. The method utilises the fact that tegumental damage occurs in certain media (Smith *et al*, 1969; Wilson and Barnes, 1974b; Simpson and McLaren, 1982; Carlisle, Weisberg and Bentley, 1983). This membrane shedding is not simply turnover of outer bilayer. The damage occuring with this method is clearly considerable as 40 % of the surface membranes are released by five minutes incubation (Simpson *et al*, 1981);

d) polycationic beads. These have been used to isolate plasma membranes from cells (Jacobson, 1977; Jacobson and Branton, 1977). The beads bind to anions on the cell surface and the plasma membrane can then be sheared, leaving the membrane bound to the beads. Cesari, Torpier and Capron (1983) recently applied this technique to adult schistosomes and were able to isolate the surface membrane by vortexing the bead-coated worms to shear the

membrane and then sedimenting the beads;

e) calcium chloride disruption. This method was used by Bennett and Seed (1977) to remove surface membranes from adult *S mansoni*. The technique is based on the observation by Kusel (1970) that the surface of schistosomula separated from underlying tissues when worms were incubated in calcium chloride solutions. The method has recently been characterised by means of enzyme assay and electron microscopy (Oaks *et al*, 1983);

f) osmotic shock. It has been found that the surface of schistosomula of *S mansoni* also separates from the rest of the body when the parasites are subjected to hypertonic conditions. This forms the basis of surface membrane isolation methods reported by Kusel, Gazzinelli, Colley, De Souza and Cordeiro (1984) and Levi-Schaffer, Tarrab-Hazdai, Schryer, Arnon and Smolarsky (1984).

Of the methods of surface membrane isolation published prior to the commencement of the present study (Kusel, 1972; Bennett and Seed, 1977), none used markers to assess fraction purity. Thus little was known of the efficacy of these techniques. There was clearly room for improvement in the methods since density gradient centrifugation had not been employed in the purification of released material.

I.7 Isolation of tegumental organelles.

Principles of isolating organelles are similar to those of plasma membranes. The method of cell rupture should keep organelles intact if possible. Separation is by differential and density gradient centrifugation. Markers are, of course, essential.

There is one published method for the isolation of organelles from the schistosome tegument. McDiarmid, Podesta and Rahman (1982) described the isolation of multilaminate vesicles from worm homogenates. They used a method previously developed for isolation of multilamellar bodies (which are morphologically similar to multilaminate vesicles) from lung tissue (Hallman, Miyai and Wagner, 1976). Multilaminate vesicles were separated from other components solely on the basis of density. No enzyme markers for multilaminate vesicles are known so fraction purity was assessed by quantitative morphometric analysis. This showed that just over half the solid material in the multilaminate vesicle fraction was multilaminate vesicles. It is probable that fraction purity might have been increased by isolating the vesicles from released tegument following either freeze-thaw or detergent treatment. Similarly, some improvement in purity might have been achieved if some differential centrifugation was employed although the amount of material required probably precluded this. All purification steps tend to reduce yield and multilaminate vesicles are not numerous in schistosomes.

The multilaminate vesicle pellet contained the enzyme phosphatidic acid phosphatase. No details are given as to the activity of this enzyme or its enrichment in the fraction. The authors suggest that phosphatidic acid phophatase in multilaminate vesicles may serve to modulate the lipids on the surface of the parasite or to synthesise new ones.

Discoid granules are estimated to be fifteen times more numerous than multilaminate vesicles (Wilson and Barnes, 1974a).

This may reflect their importance to the parasite, although their function has yet to be elucidated. It may be possible to discover their function by comparing their composition with that of surface membranes and tegumental cytoplasm. If they contribute to one of these this may be discernible by a similar composition. It therefore seems worthwhile to attempt to isolate them.

I.8 Thesis contents.

The first chapter of the thesis describes the detailed analysis of a surface membrane isolation procedure based on freezing and thawing worms. The principles outlined earlier were applied. Analysis was by electron microscopy of worm bodies and "surface membrane" pellets at various stages in the isolation procedure, and by assaying marker enzymes for surface membranes as well as internal components. As a result of these analyses, improvements were made in the method. The technique finally arrived at will be compared to those of other workers with particular emphasis on the effects of the disruption method on the purity of the final preparation.

It became clear during the course of the above work that great damage was caused to worms by the freeze-thaw process. This could have led to contamination of surface membrane preparations with internal components. The second chapter of this thesis describes attempts to reduce freeze-thaw damage by alteration of freezing rate and thawing rate and by the use of compounds known to reduce freeze-thaw damage.

The third chapter presents details of methods for isolation of spines and discoid granules from the tegument of *S mansoni*. These organelles were extracted from the material released following freeze-thaw. Proteins and glycoproteins from the discoid granules were compared with those from the surface membrane and the cytoplasm of the tegument.

A morphological analysis of the material released from adult worms after binding of cationised ferritin to the surface forms an Appendix to the thesis.

CHAPTER ONE

ISOLATION OF SURFACE MEMBRANES

1.1 INTRODUCTION

The work described here takes as its starting point a method of isolating surface membranes from schistosomes, developed by E Wells (unpublished) and based on the freeze-thaw technique of Kusel (1972). Following freezing and thawing the worms were vibrated and the released material purified by differential and density gradient centrifugation. Little was known about the effects of the disruption method on the worms or the extent of contamination of the various fractions. It was thus necessary to fully characterise the steps in the procedure and to that end morphological analysis was conducted on worms and released material at all stages and results were compared with biochemical assays.

The surface membrane marker used was alkaline phosphatase. This enzyme has been known for some time to be present in the tegument of *S mansoni* (Nimmo-Smith and Standen, 1963; Wheater and Wilson, 1976). Its localisation in the surface membrane was confirmed cytochemically (Morris and Threadgold, 1968; Bogitsh and Krupa, 1971; Ernst, 1976). It is not unique to this membrane however, being found in the plasma membrane of tegumental cells, parenchyma and suboesophageal cell bodies (Ernst, 1976). By assaying activity of whole, unbroken worms and comparing to homogenised worms, it was estimated that one third of the total activity was located in the surface membrane (Roberts, MacGregor, Vojvodic, Wells, Crabtree and Wilson, 1983). This figure was therefore adopted as the target in experiments involving surface membrane liberation. If one third of the total activity could be removed then this would probably represent all of the surface

membrane alkaline phosphatase. If more than one third was released it would suggest that internal membranes had been liberated.

In some experiments markers for internal components were employed. Succinate dehydrogenase is a marker enzyme for mitochondrial inner membrane and this was assayed. NADH cytochrome c reductase is an electron transport enzyme found in mitochondria and endoplasmic reticulum (Sottocasa, 1976) and this was assayed. The protease found in the schistosome gut (Timms and Bueding, 1959) was also assayed. As well as quantifying organelle contamination of fractions, markers for internal components may give an indication of the possible extent of contamination by other plasma membranes within worms for which specific markers were not available.

As a result of morphological analysis and biochemical assays it was possible to make improvements to the original method and to assess the purity of the final preparation.

1.2 MATERIALS AND METHODS

Individual experiments are described in 1.3 Experiments and Results. The present section contains technical details of procedures frequently used in the experiments.

1.2.1 Reagents

Chemicals and biochemicals were normally obtained from Fisons and Sigma respectively unless otherwise indicated and were generally of the highest available quality.

1.2.2 Parasites

Parasites were recovered from infected Lac A mice, following administration of anaesthetic doses of Sagatal (May and Baker), by perfusion of the venous system with Eagle's medium (MEM Wellcome) containing heparin (5 units/ml). Blood was removed from the medium by rinsing at least five times with Eagle's medium alone. Worms were carefully examined under a dissecting microscope and mouse hairs or tissue and any damaged worms removed. Treatment of worms thereafter is described in the results section. The parasites were transferred to various buffers of pH 7.4 by rinsing five times in that buffer and aspirating. All steps after transfer from Eagle's medium to buffer were at $4^{0}C$.

1.2.3 Sonication

In some experiments worms were sonicated following freeze-thaw. Sonication was carried out in a MSE 100W Ultrasonic Disintegrator using a 1/8 inch diameter microprobe at an amplitude of 4 μm.

1.2.4 Centrifugation

Centrifuges and rotors used for each sedimentation rate are shown below. Average and maximum relative centrifugal force (RCF) is quoted for each centrifugation step used. In the remaining sections only maximum RCF values will be given.

RCF		Centrifuge	Rotor	RPM
xg_{av}	xg_{max}			
75	100	MSE Mistral 4L	62303	635
5 000	7 200	MSE HS 25	16 x 15 ml inner ring	8 600
54 000	73 500	MSE HS 25	16 x 15 ml outer ring	25 000
58 000	82 000	MSE HS 25	4 x 20 ml swing out	24 000
80 000	115 000	Beckman L2-65B	SW27.1	25 000

1.2.5 Transmission Electron Microscopy

This was used to examine worms and released material. Initially the following fixation technique, based on the methods used by Hockley and McLaren (1973) and Wilson and Barnes (1974a), was employed. The primary fixative used for worms and pellets of released material was 4 % (w/v) glutaraldehyde (Taab) in 0.1 M

sodium phosphate, pH 7.4 at 4^0C. Fixation was for 4 hours and material was broken into small pieces after about 45 minutes in fixative. Compact pellets were fixed by addition of primary fixative to the centrifuge tube. Where pellets were not compact, eg where 100 x *g* sedimentation was used, they were resuspended in buffer then centrifuged for 15 minutes in an Eppendorf 5414 bench centrifuge and fixed. Following primary fixation the material was rinsed three times in phosphate buffer, stored in this buffer overnight, then fixed in 1% (w/v) osmium tetroxide (EMscope) in 0.1 M phosphate buffer for 2 hours at 4^0C. Worms and pellets were then rinsed three times in twice-distilled water. Uranyl acetate was used as a tertiary fixative as it stabilised multilaminate vesicle and surface membrane structure, (Hockley and McLaren, 1973). Material remained in 0.5 % (w/v) uranyl acetate containing 45 mg/ml sucrose for 1.5 hours at 4^0C. It was then rinsed in distilled water and dehydrated in a series of ethanols. One per cent (w/v) uranyl acetate was added to ethanols from 70 % to 100 %. The material was then cleared in propylene oxide and embedded in Epon-Araldite (Taab).

Results indicated that multilaminate vesicle and surface membrane structure was not stabilised by this fixation technique. The failure was attributed to the use of phosphate buffer, in the early steps, which causes uranyl acetate to precipitate (McLaren, personal communication). A second fixation method was therefore used. Cacodylate buffer (0.1 M) was substituted for phosphate buffer in the above schedule and uranyl acetate fixation was carried out in darkness.

Thin sections were cut using a Reichert Om U3 ultramicrotome, stained in uranyl acetate and lead citrate and viewed in a Kratos Corinth 500 electron microscope.

1.2.6 Scanning Electron Microscopy

Worms were fixed for 4 hours in 4 % gluteraldehyde in 0.1 M phosphate buffer, pH 7.4 at 4^0C. They were then rinsed three times in twice-distilled water, dehydrated in an acetone series, impregnated with liquid carbon dioxide, critical point dried (Polaron E3000), sputter coated with gold (using a Polaron E5000 SEM coating unit) and viewed in a Cambridge Stereoscan 600 electron microscope (Crabtree and Wilson, 1980).

1.2.7 Biochemical Assays

Protein was assayed either by the ninhydrin method of Kunkel and Ward (1950) or the Folin-phenol method of Lowry, Rosebrough, Farr and Randall (1951)

Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate as substrate. Final concentrations during the reaction were 10 mM *p*-nitrophenyl phosphate, 50 mM glycine, 1 mM $MgCl_2$, 0.5 % (w/v) Triton X-100 (Koch-Light), pH 10.5. The reaction was carried out at 37^0C for not more than 15 minutes and stopped by addition of 0.8 ml 0.5 M NaOH. Absorbance of each solution was determined at 420 nm and compared to *p*-nitrophenol standards. Figures for no-enzyme blanks were subtracted from the results. One unit of enzyme liberated one µmole of *p*-nitrophenol from

p-nitrophenyl phosphate per minute.

Succinate dehydrogenase was assayed by the method of King (1967) except that Triton X-100 was added to the reaction mixture and the total volume was reduced to 1 ml. This colorimetric method uses dichlorophenolindophenol (final concentration 50 µM) and phenazine methosulphate (final concentration 3 mM) as electron acceptors. One unit of enzyme activity caused a decrease of 1.0 absorbance units per minute at 25°C.

NADH cytochrome c reductase was assayed by the method of Beaufay, Amar-Costesec, Feytmans, Thinès-Sempoux, Wibo, Robbi and Berthet (1974) in a final volume of 1 ml. The reduction of cytochrome c was monitored spectrophotometrically at 550 nm for 2 to 3 minutes. One unit of enzyme activity caused an increase of 1.0 absorbance units per minute at 37°C.

Protease was assayed by the method of Dresden and Deelder (1979), at pH 3.9 using a final concentration of 10 mg/ml Azocoll (Calbiochem) as substrate, in the presence of 10 mM dithioerythritol. A sample of 0.2 ml was incubated with an equal volume of substrate at 37°C in sealed tubes. The reaction was stopped by placing the tubes on ice, adding 0.6 ml Hanks' balanced salt solution and sedimenting the substrate by centrifugation. One unit of activity caused an increase of 1.0 absorbance units per hour at 37°C.

1.3 EXPERIMENTS AND RESULTS

1.3.1 Freezing, thawing and vortexing worms in hypotonic tris buffer.

The method of surface membrane isolation developed by Wells (unpublished) and based on that of Kusel (1972) involved freezing worms in liquid nitrogen, then allowing them to thaw. The worms were vibrated to release the surface membranes and the supernatant removed from the worm bodies and centrifuged. The resulting pellet was then resuspended and centrifuged on a sucrose density gradient. The first experiment examined, by transmission electron microscopy, the effects of this procedure on the worm bodies and the nature of the pellet derived by sedimenting the released material.

Fifty worm pairs, recovered and cleaned as described in Materials and Methods, were suspended in 0.84 % (w/v) NaCl, buffered with 10 mM tris (hydroxymethyl) methylamine/HCl (TBS), in a plastic test tube. Eagle's medium was removed by rinsing worms in this buffer. TBS was then replaced by 10 mM Tris/HCl and the volume reduced to 3 ml. Hypotonic buffer was used as it was believed it would weaken the tegument and thereby render it more readily disruptable. The worms were frozen by plunging the tube into liquid nitrogen and holding it there until the buffer solidified. The worms were then allowed to thaw by maintaining the test tube at room temperature but care was taken to ensure the temperature of the buffer did not exceed 4^0C.

The parasites were sedimented by low speed centrifugation for 30 seconds, then rinsed with 10 mM tris (hydroxymethyl) methylamine (tris)/HCl and vibrated in 3 ml of this buffer. Vibration is required to remove the tegument, freeze-thaw simply loosens it. This step was carried out using a bench vortex mixer. The tube was firmly touched against the side of the rotating cup until a vortex was created and was then immediately withdrawn. This was done 10 times, with care being taken to keep the buffer cold. Worm bodies were sedimented and part of the supernatant was removed for morphological examination of released material. This aliquot was centrifuged at 73 500 x *g* for one hour and the pellet processed for electron microscopy. The remaining worm bodies were rinsed with 10 mM tris-HCl and homogenised in 6 ml of this buffer. The experimental scheme is summarised in Figure 3. Aliquots of the various supernatants were removed and assayed for alkaline phosphatase activity.

Table 1 shows the assay results. Release of alkaline phosphatase was lower than expected. Only 10 % was released although some 33 % is in the surface membranes. This was probably due to inadequate vibration of the worms.

Ultrastructural inspection of worms removed for fixation before the tube was frozen, revealed gross damage. The normally electron-dense cytoplasm of the tegument was bleached and large whorls of lamellate material were present (Figure 4). Multi-laminate vesicles were present in low numbers. Elongate electron-dense structures, assumed to represent discoid granules whose appearance had been altered, were found. Internal structure

Worms recovered from mice by perfusion of the hepatic portal system with Eagle's medium. Hairs and mouse tissue were removed from the medium and 50 worm pairs transferred to tris-buffered saline by rinsing five times.

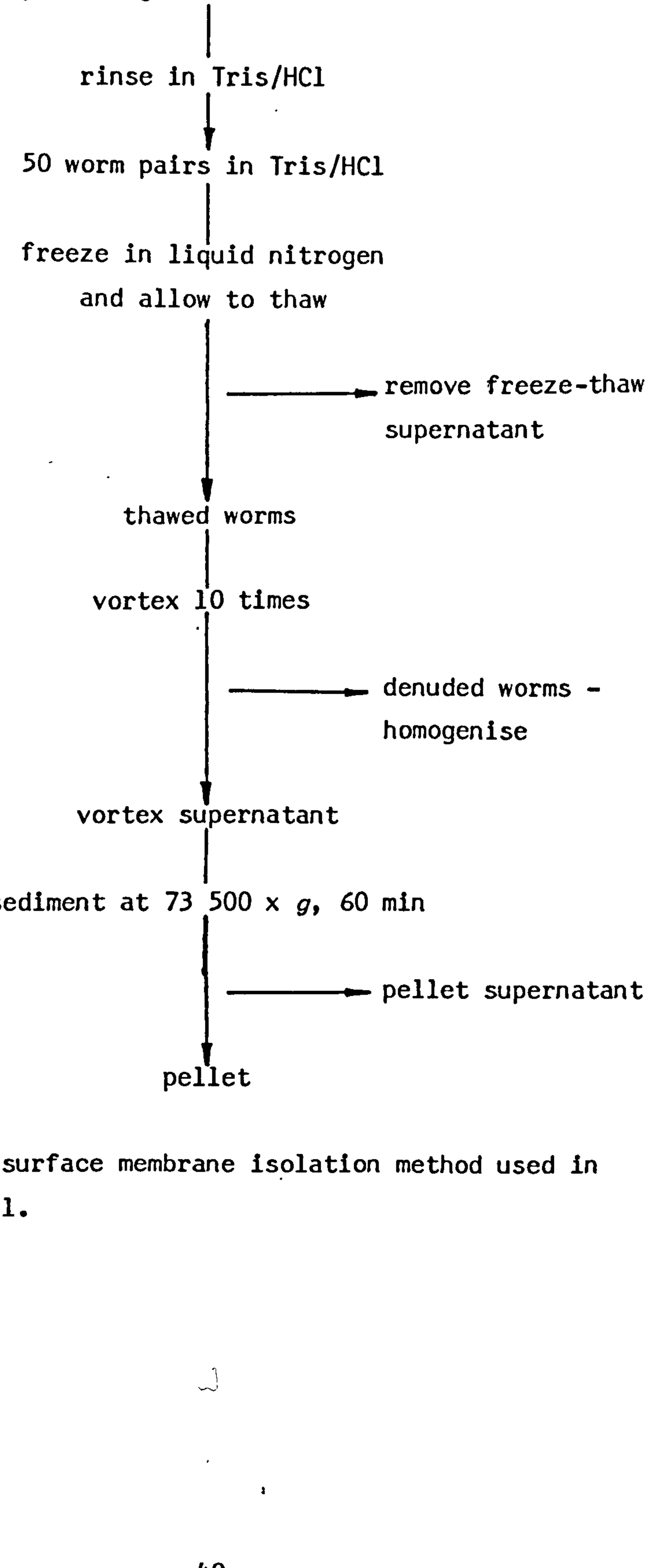


Figure 3. The surface membrane isolation method used in 1.3.1.

Table 1 Distribution of alkaline phosphatase in fractions produced by freezing, thawing and vortexing worms in hypotonic tris buffer.

	units*	%
freeze-thaw supernatants	0.002	0.4
vortex supernatant	0.050	10.2
denuded worm homogenate	0.439	89.4
total	0.491	
vortex supernatant pellet (73 500 x *g*)	0.0	

* *p*-nitrophenol liberated from *p*-nitrophenyl phosphate at 37°C (μmole/minute).

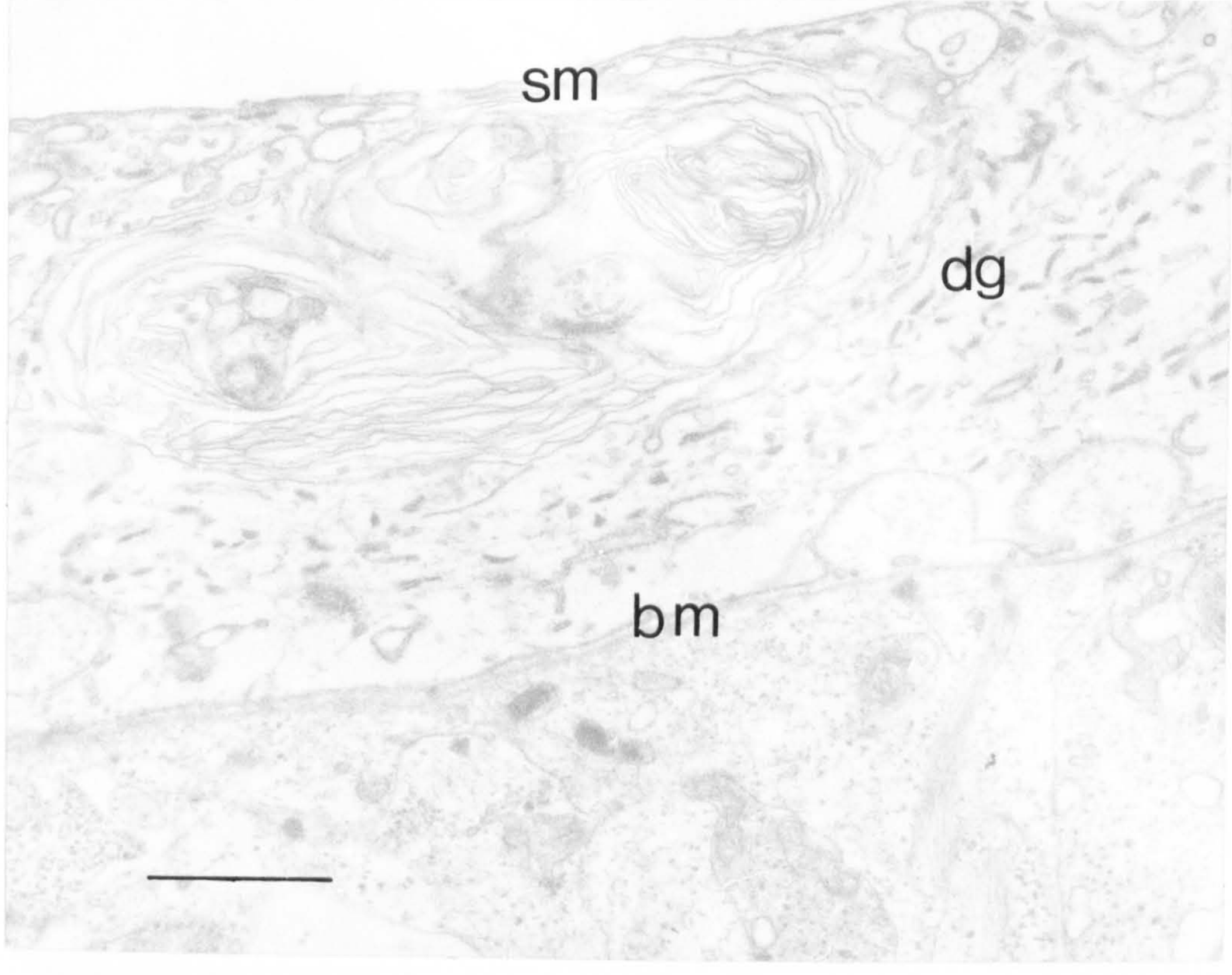


Figure 4. Effect of tris buffer on the schistosome tegument. sm, surface membrane; bm, basal membrane; dg, discoid granule? Bar = 1 μm.

was greatly disrupted. Plasma membranes were broken and organelles intermixed. The basal membrane of the tegument appeared intact, however.

The condition of worms after freeze-thaw and after vortexing was similar to that of the worms just described. The tegument was missing in some places (Figure 5) but was clearly not wholly released. This low liberation of surface membrane was consistent with the results of the alkaline phosphatase assay. It was impossible to quantify release of surface membrane by TEM without laborious serial sectioning of large numbers of worms.

Ultrastructural examination of the pellet indicated that the surface membrane was released in large sheets (Figure 6). Much of the material present was clearly recognisable as sheets of surface membrane, but multilaminate vesicles, free spines and mitochondria were also found. Some myelin-like membrane was seen.

1.3.2 Freezing, thawing and sonicating worms in isotonic tris buffered saline.

The previous experiment demonstrated that the amount of tegument released could not be adequately assessed by examination of worms by transmission electron microscopy. In order to assess surface membrane release more accurately it was decided to employ scanning electron microscopy. Since vortexing gave inadequate agitation to worms for complete release of surface membranes, sonication was used in the next experiment. This was thought

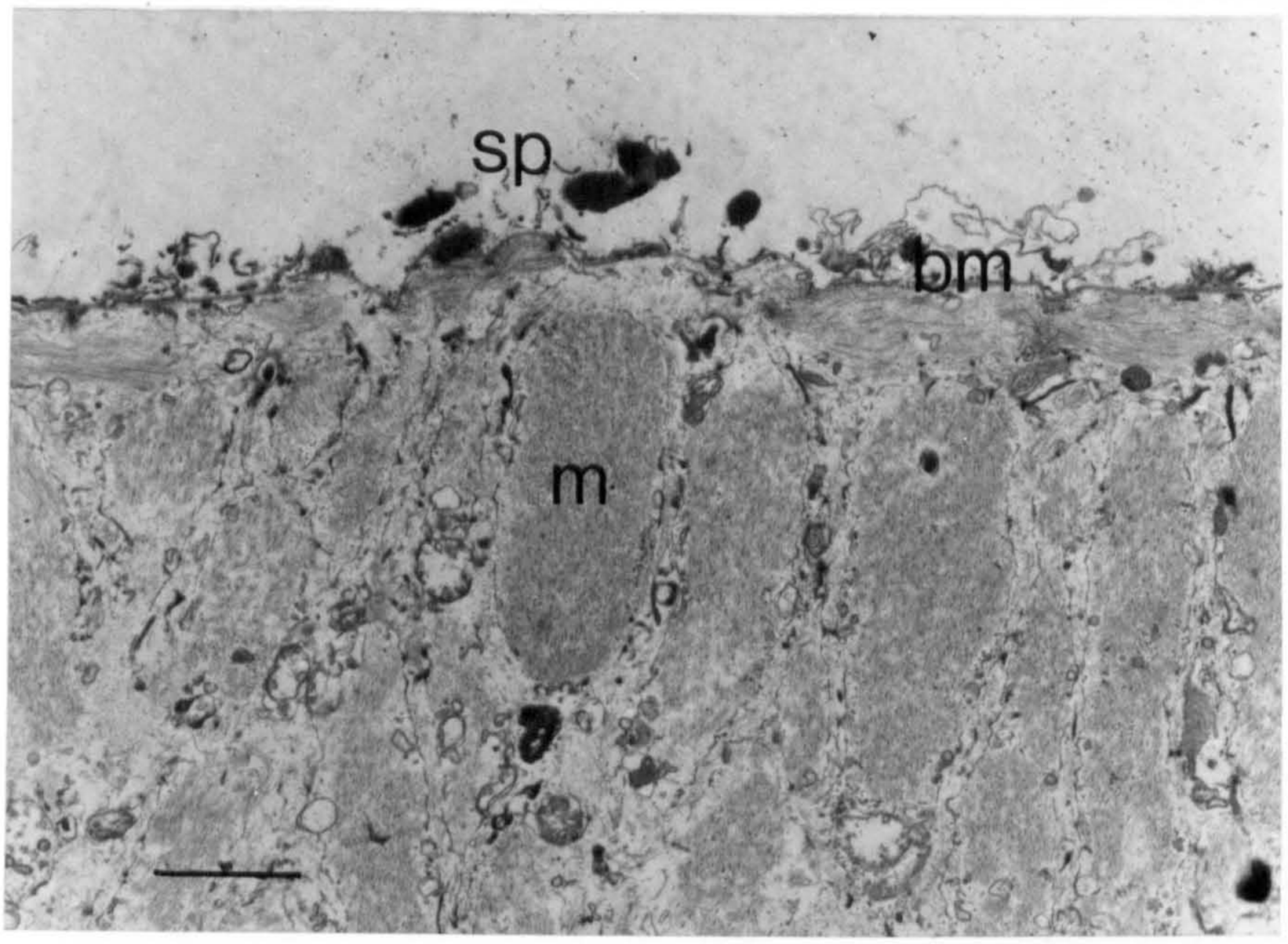


Figure 5. Denuded worm after freeze-thaw and vortexing.

bm, basal membrane; m, muscle; sp, spine.

Bar = 2 μm.

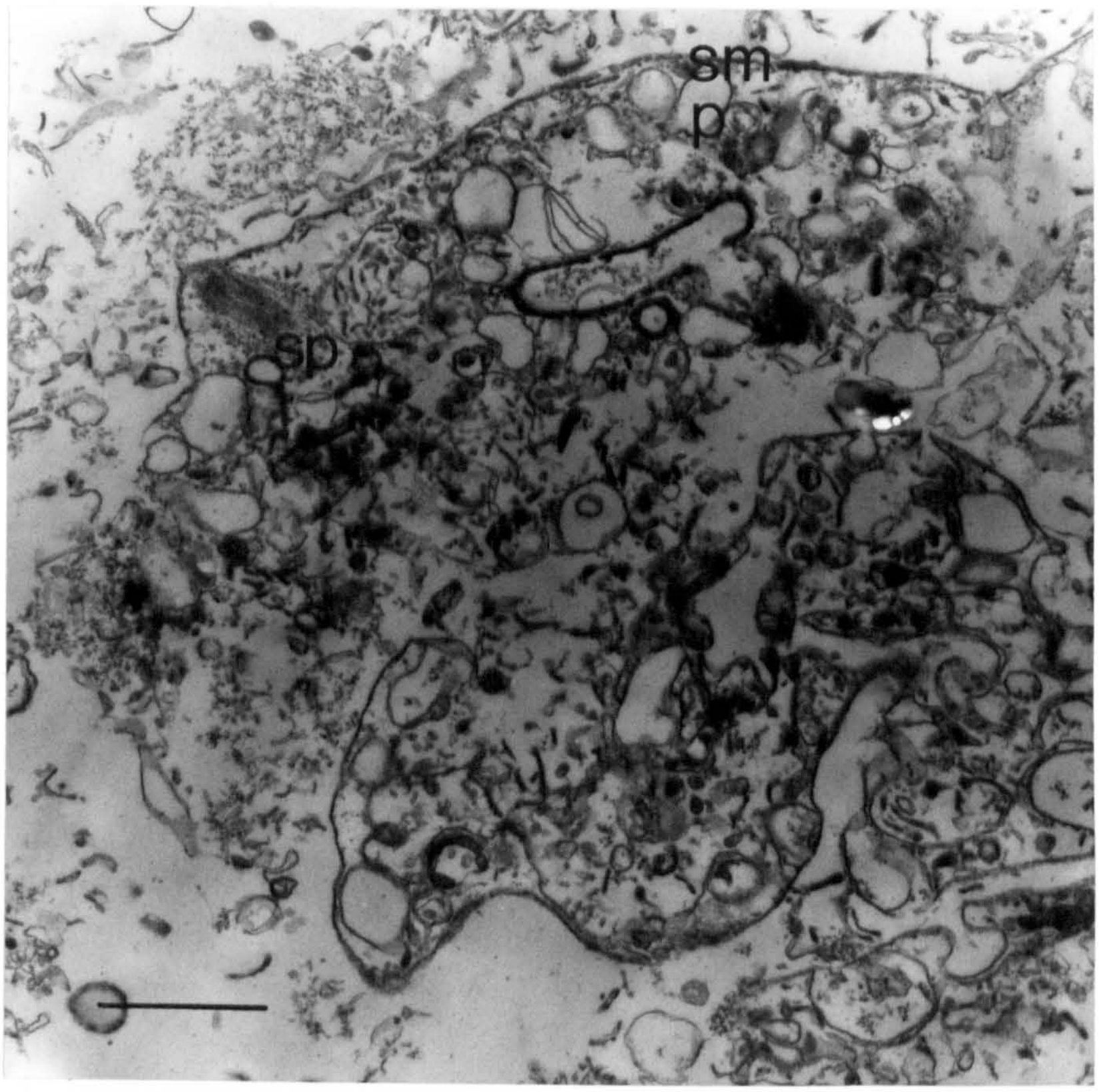


Figure 6. Pellet derived by centrifugation of material released by vortexing after freeze-thaw in hypotonic tris buffer.

sm, surface membrane; p, pit; sp, spine.

Bar = 1 μm.

to be more easily controlled and therefore, more reproducible. The use of hypotonic tris buffer was discontinued. Isotonic tris buffered saline (TBS) was used throughout the experiment as it was thought that hypotonic buffer may have caused the extensive disruption of worms in the previous experiment.

One hundred pairs were frozen in 4 ml of TBS. After thawing and sedimenting, the worms were rinsed in TBS and sonicated for 20 seconds in 1.5 ml buffer, as described in Material and Methods. The vortex supernatant was removed and centrifuged at 73 500 x *g* for one hour and the bodies homogenised.

Table 2 shows results of alkaline phosphatase and protein assays. Some 20 % of alkaline phosphatase was released as was 27 % of protein. Most of the protein in the sonication supernatant appeared soluble.

The pellet was dark brown in colour, which suggested the presence of haematin from the gut. It contained many membrane-bound vesicles, apparently filled with cytoplasm (Figure 7). Some muscle and mitochondria were found as were bodies similar in appearance to the laminate whorls previously seen in the tegument (Figure 8). Worms examined by scanning electron microscopy showed considerable tegumental denudation (Figure 9 *cf* normal male dorsal surface, Figure 10). Denudation appeared most severe in females with the male gynaecophoric canal being fairly well protected.

Table 2. Distribution of alkaline phosphatase and protein in fractions produced by freezing, thawing and sonicating worms in isotonic tris-buffered saline.

	protein		alkaline phosphatase			
	mg	%	units*	%	specific† activity	relative§ enrichment
freeze-thaw supernatant	1.15	15.5	0.077	6.7	0.067	0.44
sonication supernatant	0.89	12.0	0.158	13.9	0.177	1.15
denuded worm homogenate	5.40	72.5	0.909	79.4	0.100	0.65
total	7.44		1.144		0.154	
sonication supernatant pellet (73 5000 x *g*)	0.6		0.0			

* *p*-nitrophenol liberated from *p*-nitrophenyl phosphate at 37°C (µmole/minute)

† enzyme units/mg protein

§ specific activity of fraction/total specific activity

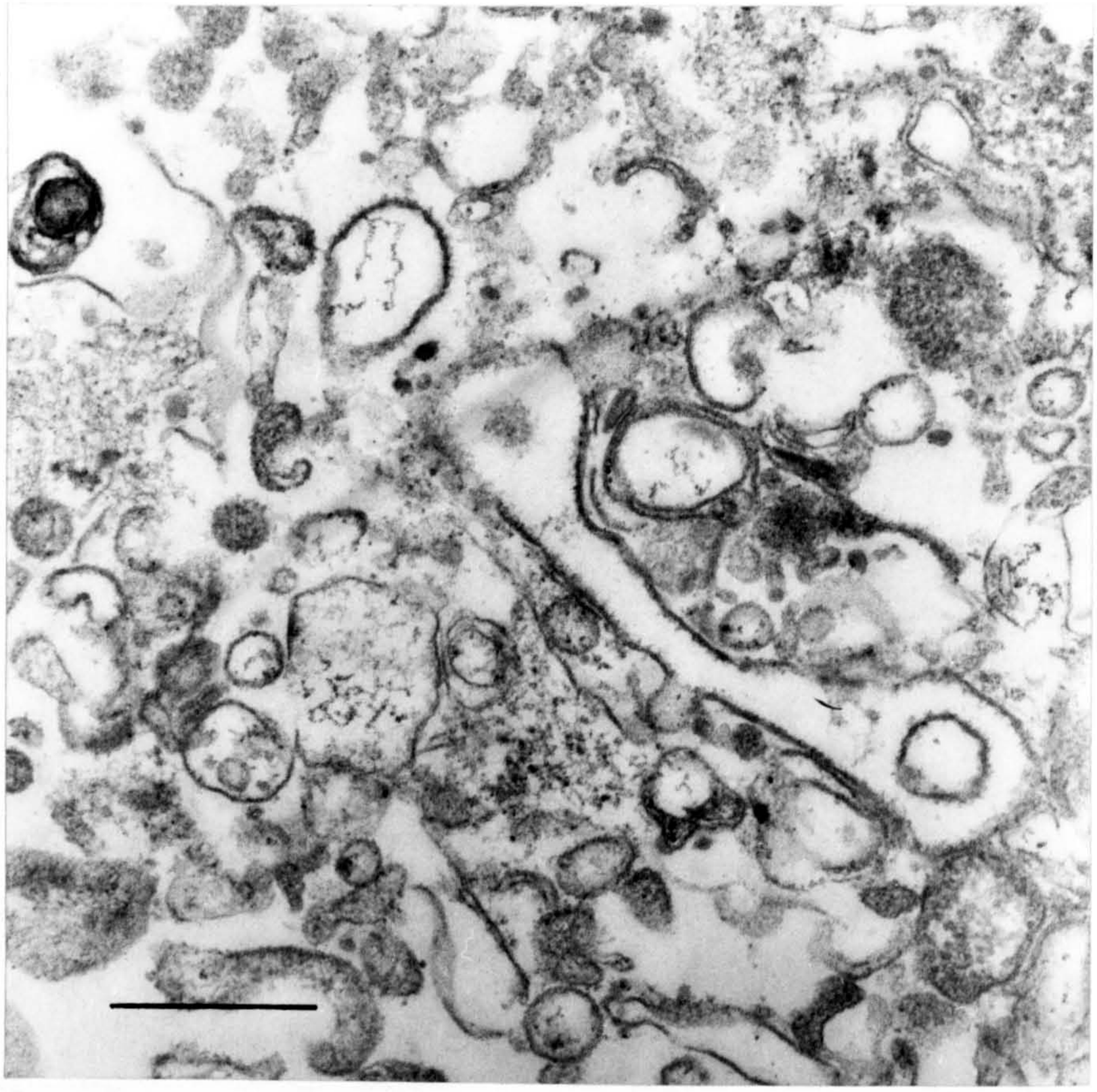


Figure 7. Membrane pellet produced by freezing, thawing and sonicating worms in isotonic tris-buffered saline. Bar = 0.5 μm.

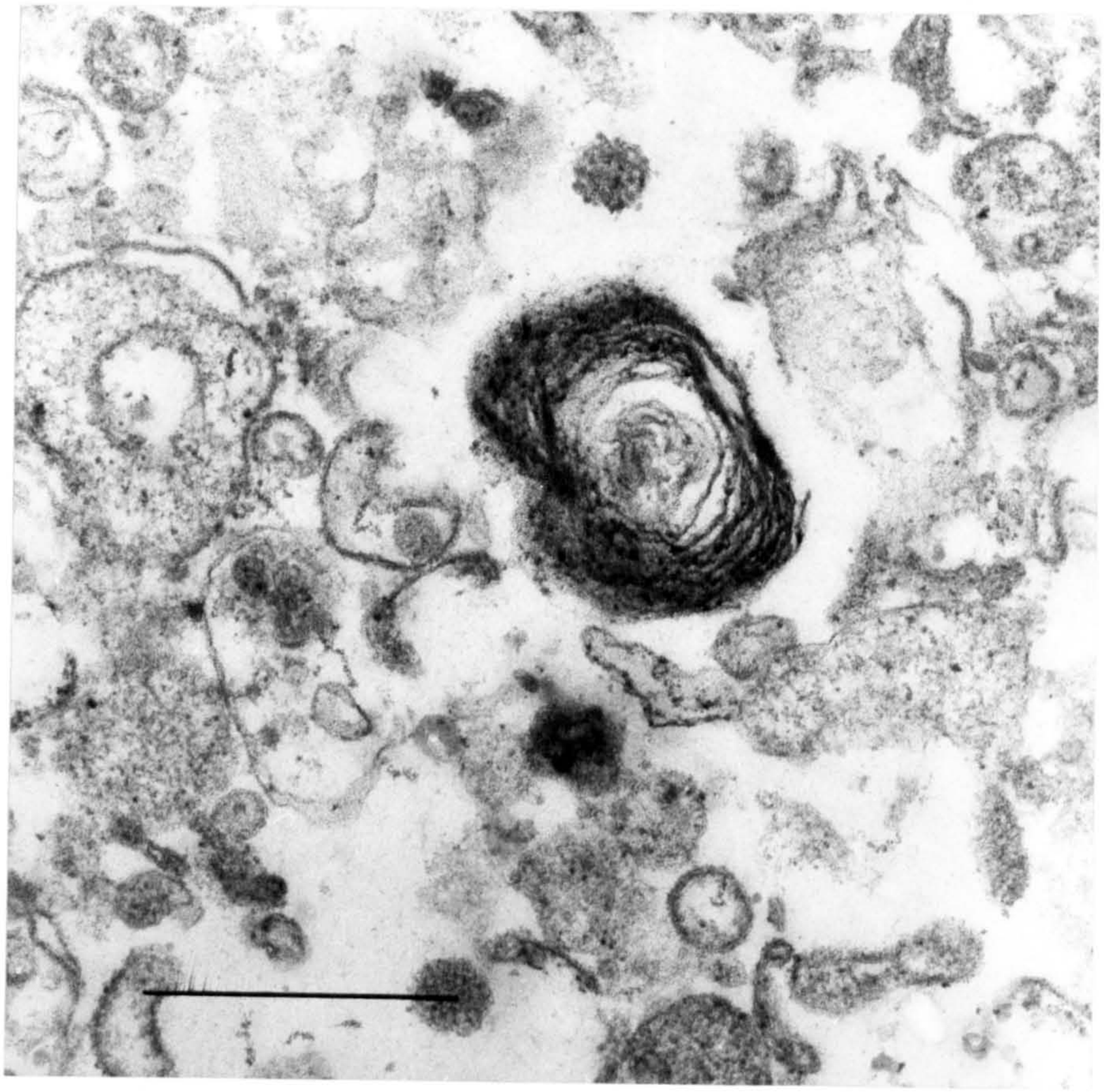


Figure 8. Lamellate material in the pellet derived by centrifugation of the sonication supernatant.

Bar = 0.5 μm.

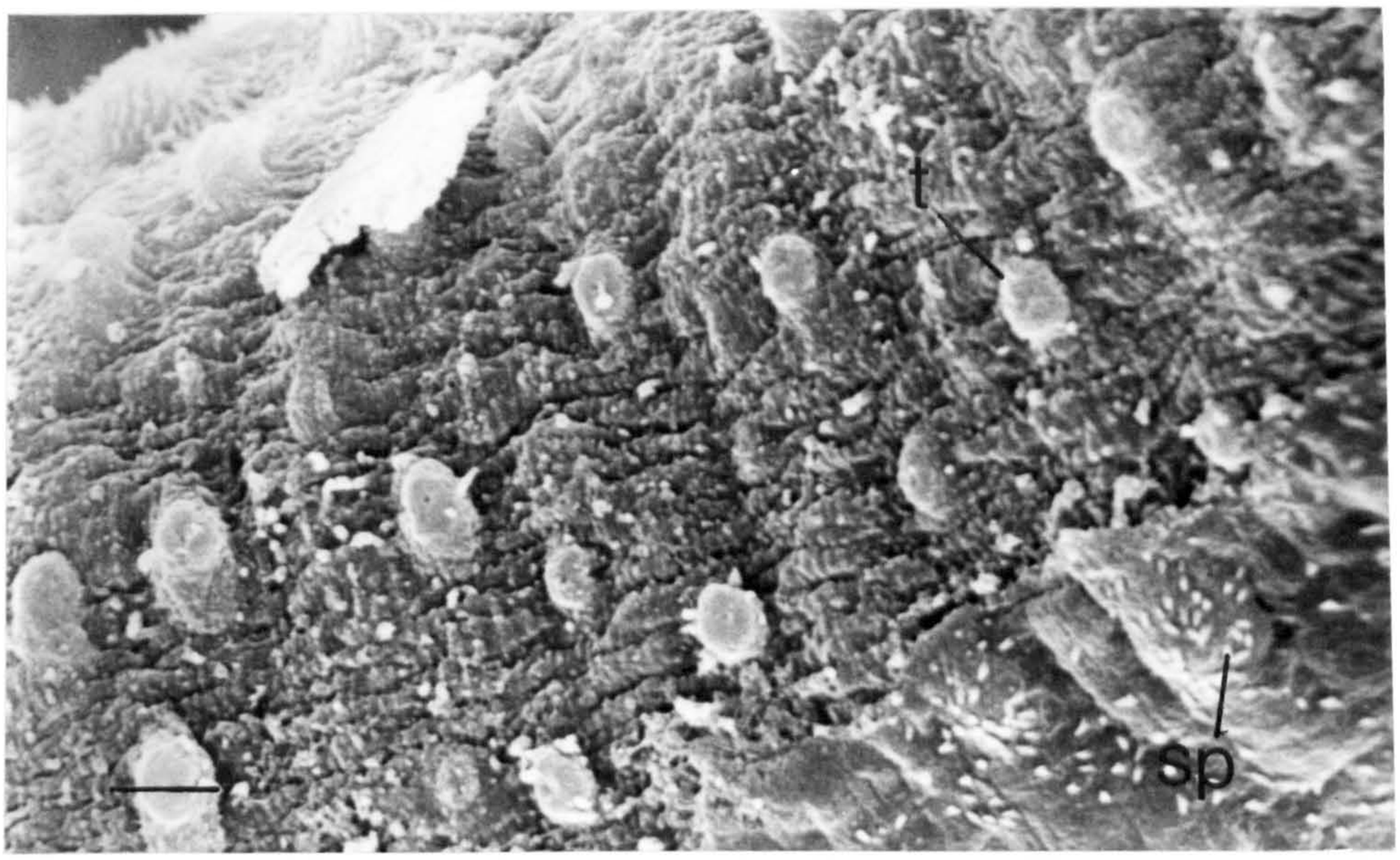


Figure 9. Dorsal surface of adult male *S mansoni* showing partial removal of the tegument.

t, tubercle; sp, spine.

Bar = 10 μm

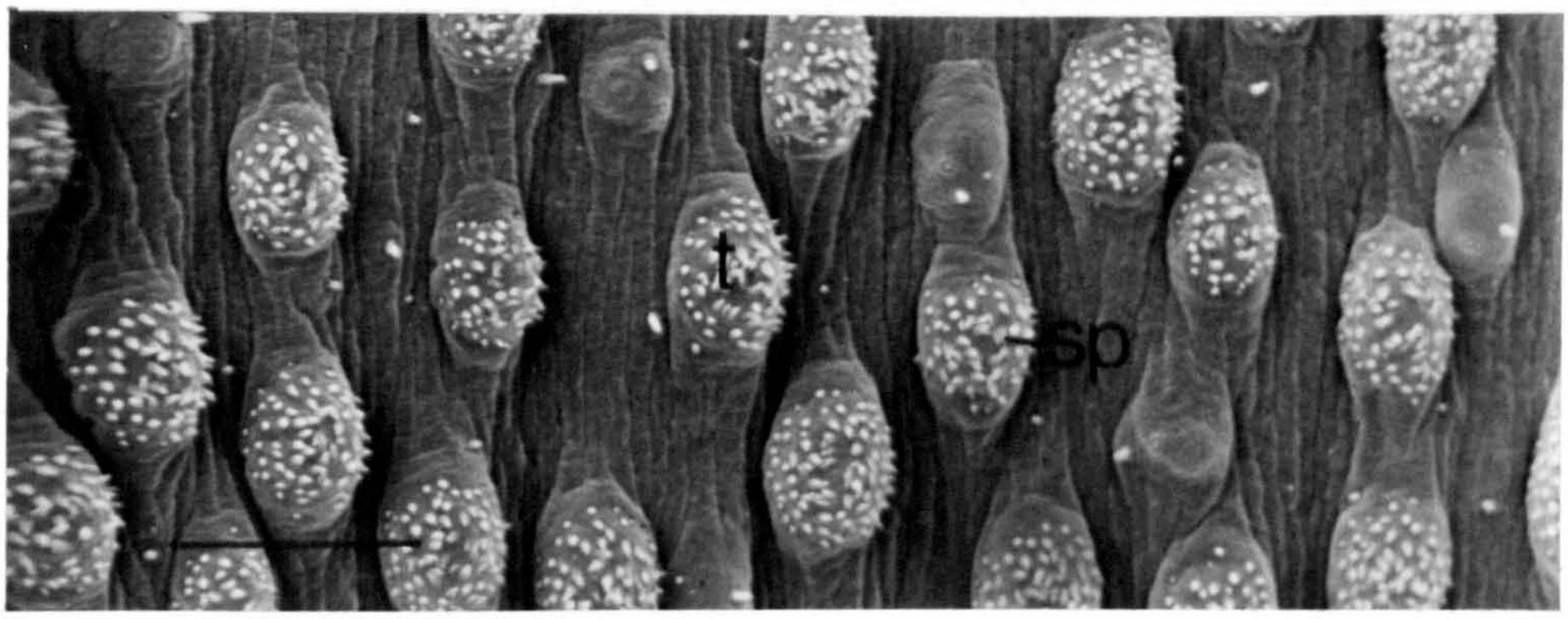


Figure 10. Dorsal surface of untreated male *S mansoni*.

t, tubercle; sp, spine.

Bar = 100 μm.

1.3.3 Freezing, thawing and sonicating worms in Hanks' balanced salt solution.

The presence in the surface membrane pellet of bodies resembling the whorled lamellae earlier seen in the tegument (Figure 4), suggested that isotonic TBS might be exerting a similar effect to hypotonic tris buffer and damaging the worms. It was therefore decided to freeze worms in a medium which was known to have little effect on their ultrastructure. Wilson and Barnes (1974b) showed that Hanks' balanced salt solution (HBSS) caused only some vacuolation of the tegument after 30 minutes incubation at 37°C. Worms held in this medium for 2 minutes at 4°C (following perfusion of mice with Eagle's medium and collection in Eagle's medium for up to 45 minutes), were examined. They showed some tegumental vacuolation (Figure 11), but there was little effect on the rest of worm structure. Incubation of worms in HBSS had no effect on topography. HBSS was used in all succeeding experiments. Mitochondria had been observed in the material released in the experiment described in 1.3.1 so it was decided to assess mitochondrial contamination by assaying succinate dehydrogenase.

In order to examine the released material after it was purified on a sucrose density gradient, a large number of worms were used.

Five batches of about 250 worm pairs were each frozen in 2 ml HBSS and stored at -20°C until required. Upon thawing the buffer was removed from each tube and pooled. The total freeze-

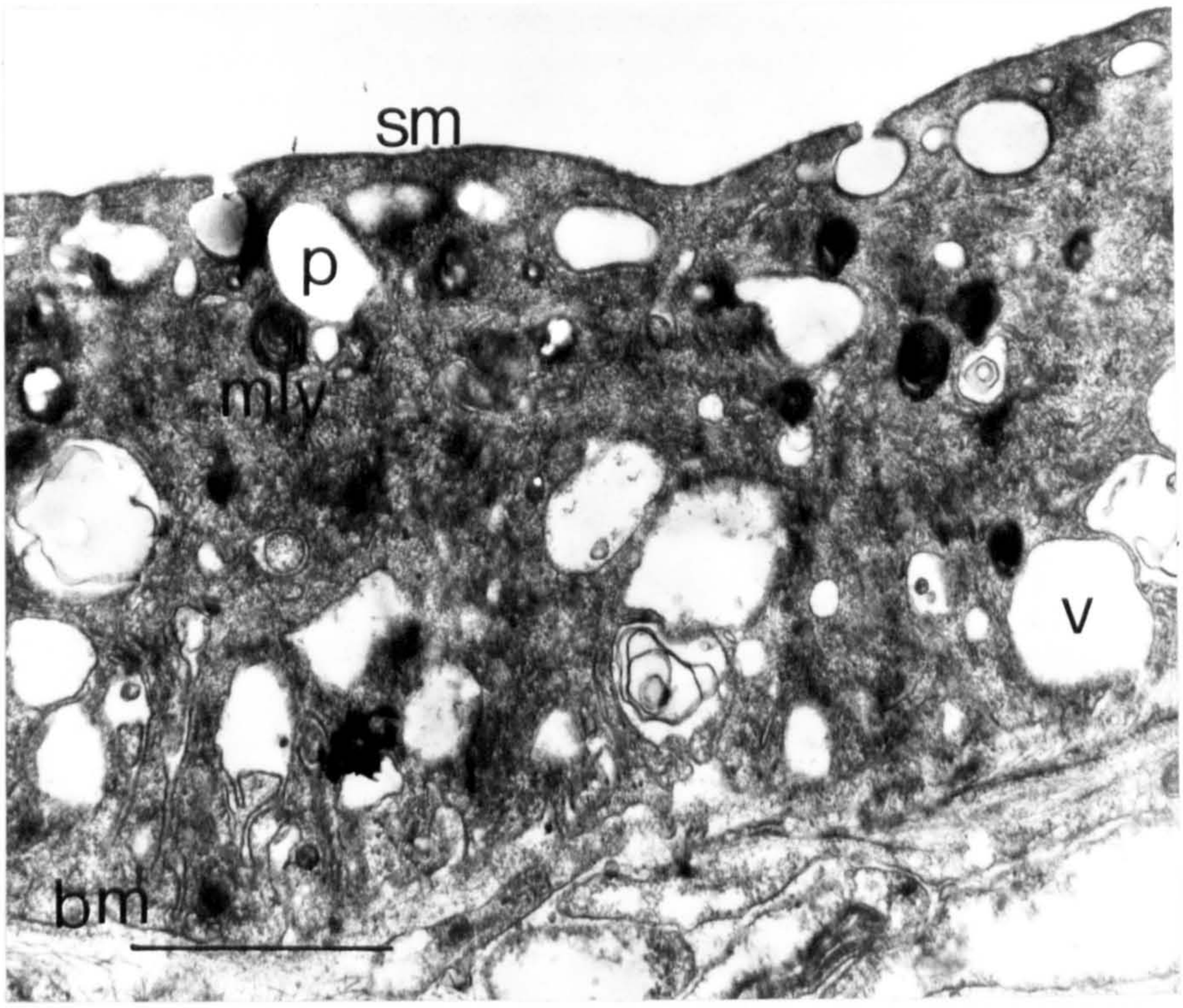


Figure 11. The tegument of worm kept in Eagle's medium for 45 minutes and Hanks' balanced salt solution for 2 minutes.

sm, surface membrane; bm, basal membrane; p, pit; mlv, multi-laminate vesicle; v, vacuole.

Bar = 1 µm.

thaw supernatant was made to 25 ml by repeated careful rinsing of worms and addition of the rinsing buffer to that previously removed. In this way, soluble material could be removed from the tube. The volume of HBSS in each tube was taken to 2 ml and each was sonicated for 15 seconds. The sonication supernatants were again pooled and made to 25 ml. The sonication supernatant was centrifuged at 7 200 x *g* for 25 minutes and the pellet resuspended in 3 ml HBSS. A 0.3 ml sample of the suspension was centrifuged at 73 500 x *g* for 30 minutes and fixed for electron microscopy. A small aliquot was removed for assays and the rest of the suspension was layered on a freshly prepared 15 ml 20 - 60 % continuous sucrose gradient, made up in 10 mM tris/HCl. The gradient was centrifuged at 115 000 x *g* for 90 minutes and the centrifuge was allowed to decelerate without braking. Fractions of about 1 ml were removed by piercing the base of the centrifuge tube with a hypodermic needle connected to a peristaltic pump and a fraction collector. A small aliquot from each 1 ml fraction was assayed for alkaline phosphatase activity. Figure 12 shows the activity profile of the gradient. The two fractions with greatest activity, which contained 33 to 38.5 % (w/v) sucrose, were pooled and the material pelleted by centrifugation at 73 500 x *g* for 30 minutes. The pellet was fixed for transmission electron microscopy.

Table 3 shows the results of assays. Some 20 % of total alkaline phosphatase was released in this experiment. The enzyme was enriched 4.5 times in the pellet derived from the sonication supernatant. Just under one third of the total protein was released from worms by freeze-thaw and sonication

Figure 12. Alkaline phosphatase activity in 1 ml fractions from a sucrose density gradient after centrifugation of material released by freeze-thaw and sonication in Hanks' balanced salt solution.

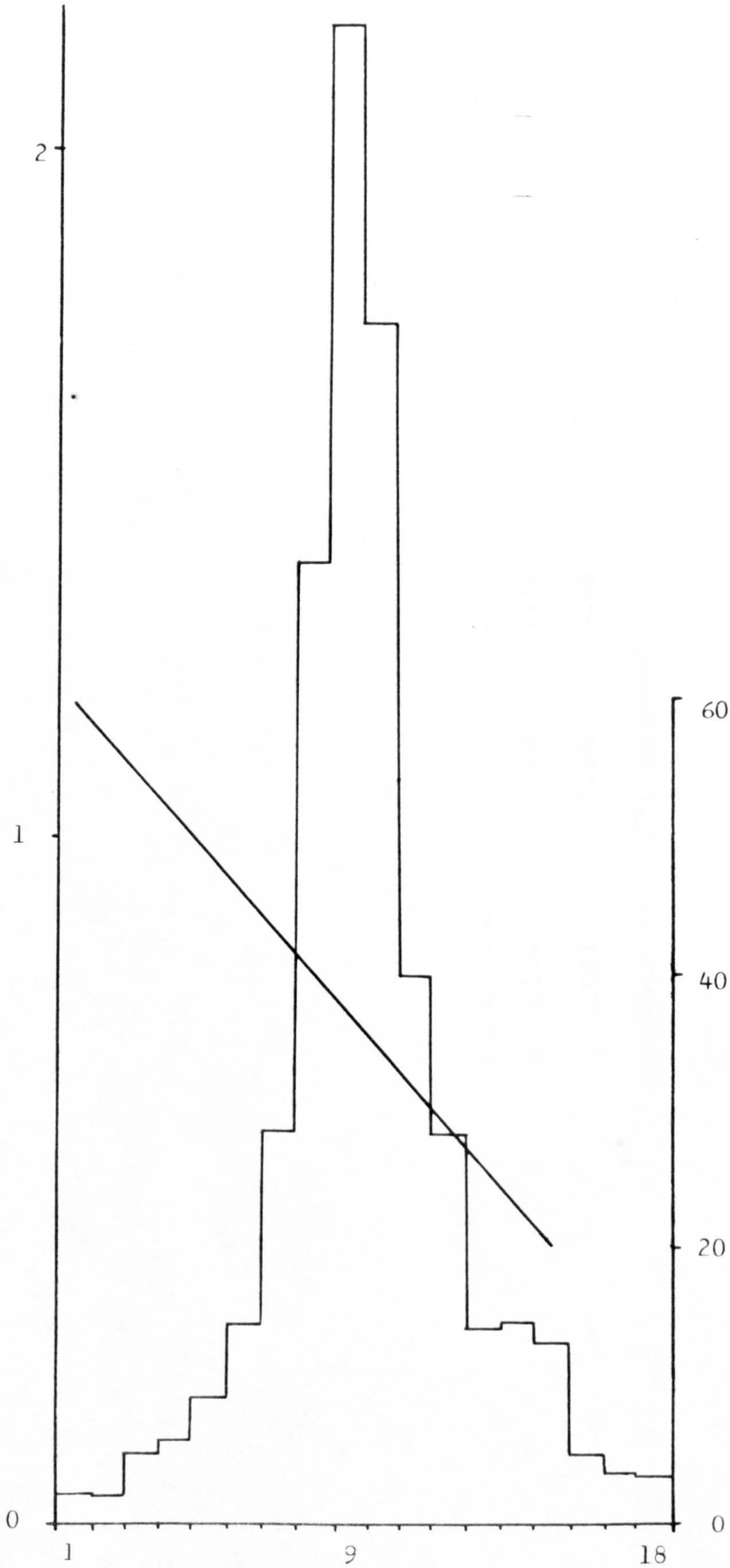

2
1
0
Absorbance at 420nm
60
40
20
0
Sucrose Concentration (% w/v)
1
9
18
Fraction Number

Table 3. Distribution of protein, alkaline phosphatase and succinate dehydrogenase in fractions produced by freezing, thawing and sonicating worms in Hanks' balanced salt solution.

	protein		alkaline phosphatase				succinate dehydrogenase		
	mg	%	units*	%	specific† activity	relative§ enrichment	units∞	%	specific† activity
freeze thaw-supernatant	22.60	21.5	1.024	6.1	0.045	0.23	2.25	9.4	0.010
sonication supernatant (SS)	11.89	11.3	2.373	14.2	0.200	1.04	0.0	0.0	0.0
denuded worm homogenate	70.57	67.2	16.725	79.7	0.237	1.23	21.60	90.6	0.306
total	105.06		20.122		0.192		23.85		0.227
SS 7 200 x *g* supernatant	11.214		0.134		0.030	0.16	0.0		
SS 7 200 x *g* pellet	1.19		1.023		0.860	4.48	0.0		

* *p*-nitrophenol liberated from *p*-nitrophenylphosphate at 37°C (µmole/minute)

† enzyme units/mg protein

§ specific activity of fraction/total specific activity

∞ absorbance decrease per minute of dichlorophenolindophenol and phenazine methosulphate at 600 nm, 25°C

and, of the protein in the sonication supernatant, little sedimented. Nine per cent of the succinate dehydrogenase activity was released by freezing and thawing. There was no further release during sonication and thus there was no detectable activity in the pellet derived from the sonication supernatant. Samples of the final preparation were not assayed as the entire pellet was processed for electron microscopy.

Worms examined by scanning electron microscopy after freeze-thaw and sonication were even more extensively denuded of their tegument than in the previous experiment even though alkaline phosphatase release was similar. It was clear, however that only about two thirds of the visible male tegument was missing and this correlated well with the alkaline phosphatase assay. In males the dorsal surface appeared most affected (Figure 13). Tubercles were usually uncovered and appeared to have collapsed slightly. The dorso-lateral margins were mostly free of tegument (Figure 14) although in places the tegument was present butmuch disrupted, with spines lying on the surface (Figure 15). It was difficult to examine the ventral surface as the gynaecophoric canal was often closed. Where the ventral surface was visible, stripping appeared to have occured in patches (Figure 16). Females were extensively denuded (Figure 17 *cf* Figure 18); about 90 % of the tegument was missing.

Inspection by transmission electron microscopy of worms subjected to freeze-thaw and sonication showed there was extensive disruption of cells (Figure 19). Muscle blocks were displaced. The supernatant after sonication was brown in colour

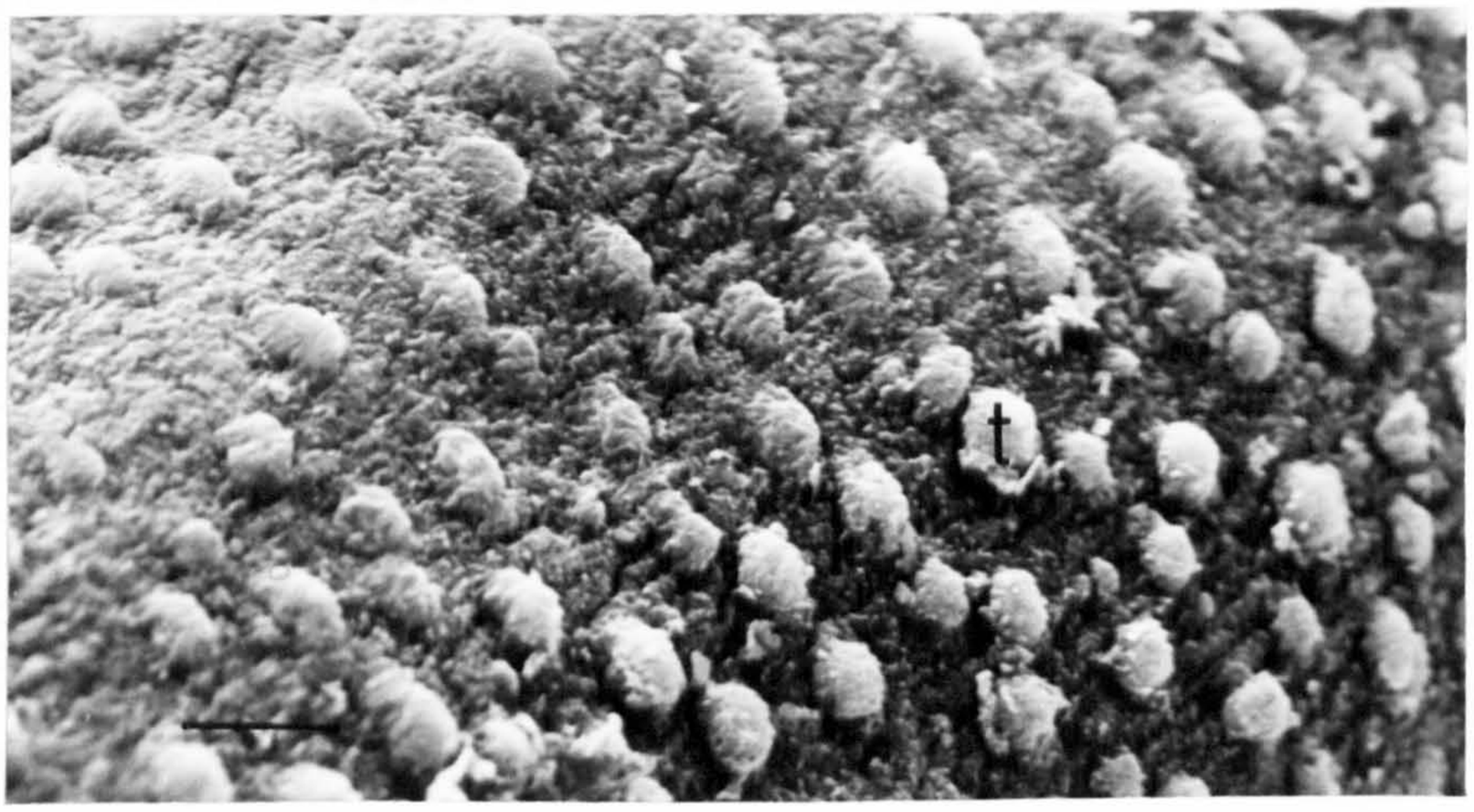


Figure 13. Dorsal surface of male *S mansoni* after freeze-thaw and sonication in Hanks' balanced salt solution.

t, tubercle.

Bar = 20 µm.

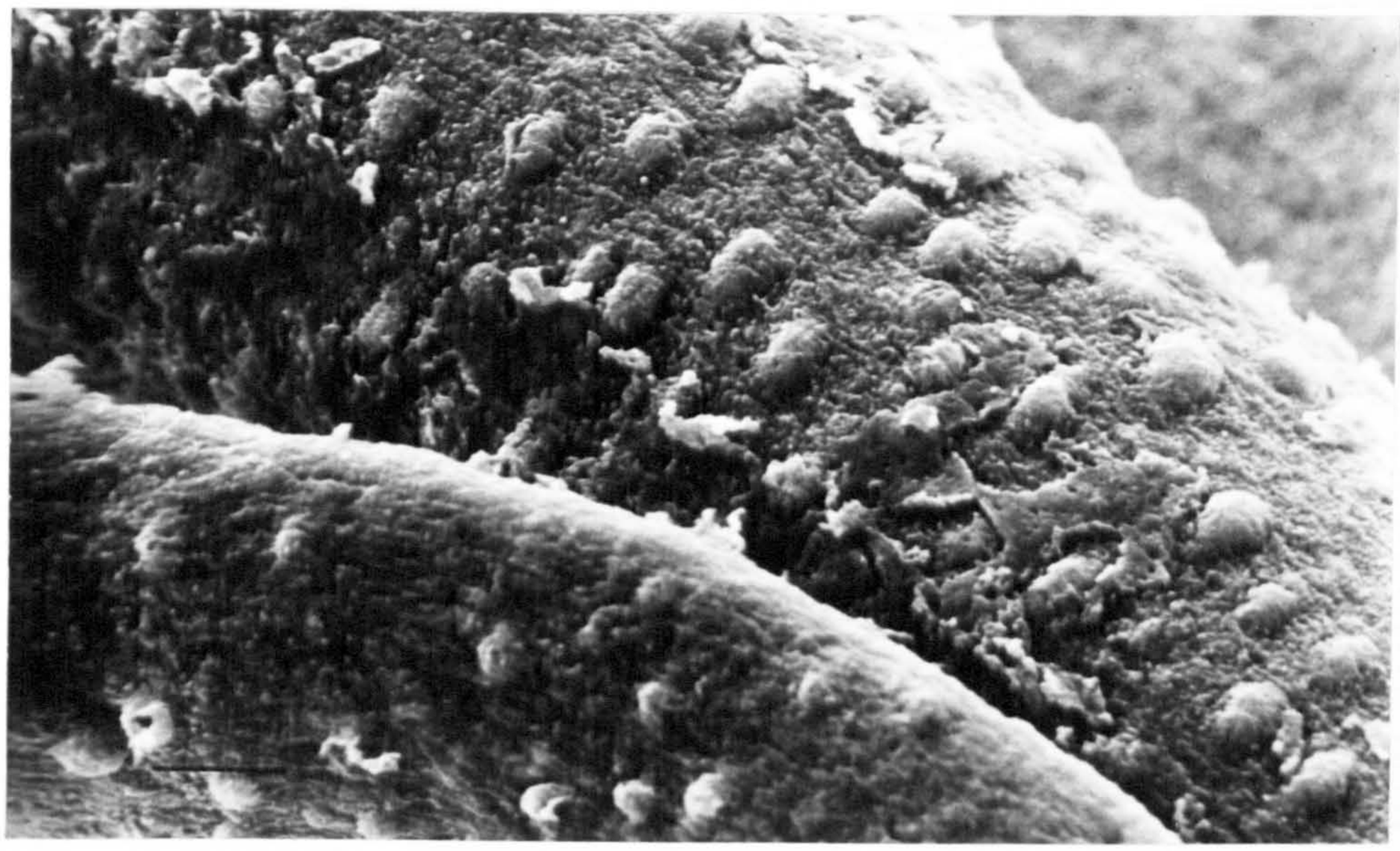


Figure 14. Dorso-lateral margin of male *S mansoni* after freeze-thaw and sonication in HBSS.

Bar = 20 µm.

Figure 15. Spines on the surface at the lateral margin of a male *S mansoni* after freeze-thaw and sonication in HBSS.

sp, spine.

Bar = 100 µm.

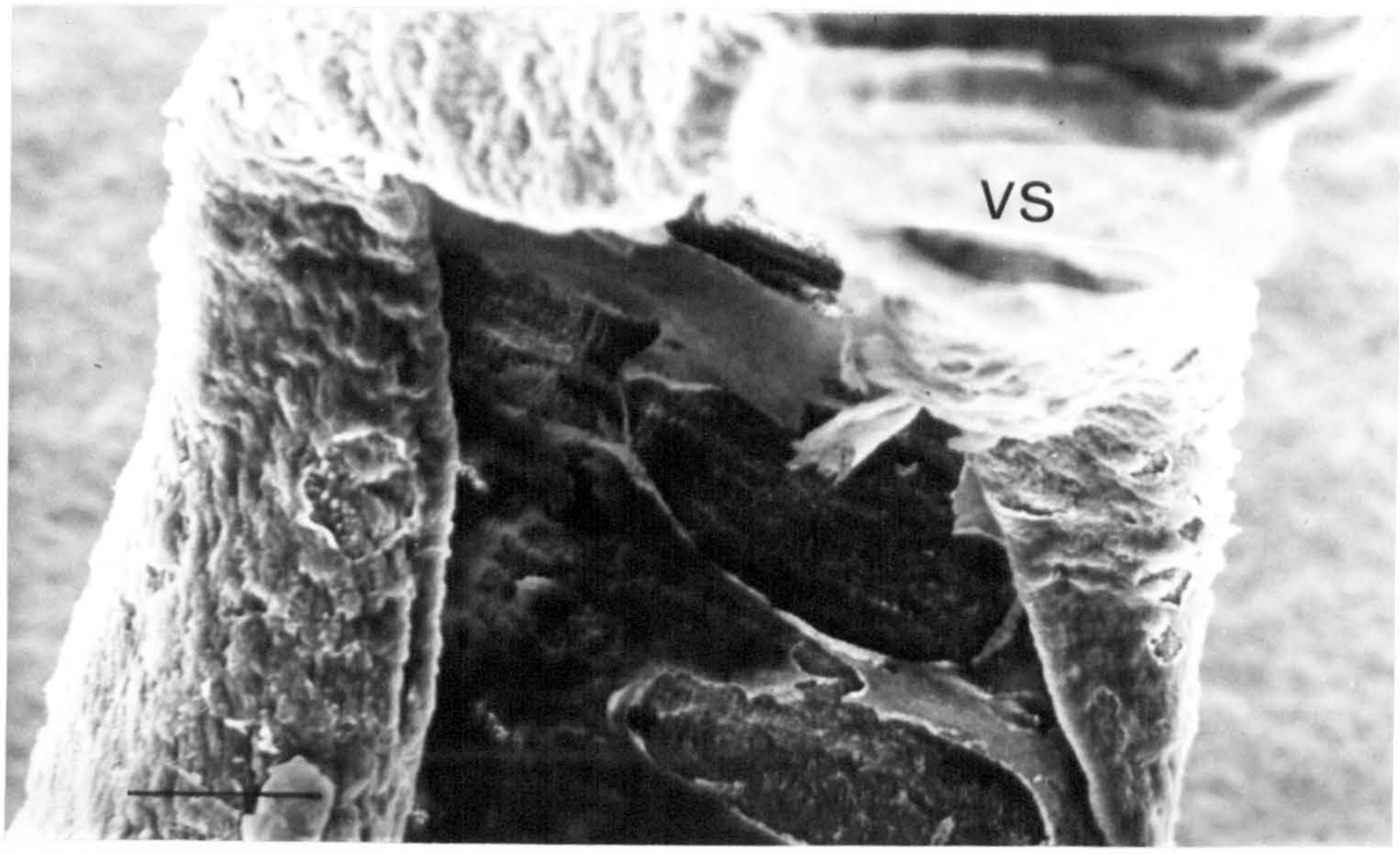


Figure 16. Ventral surface of a male *S mansoni* after freeze-thaw and sonication in HBSS. Patches of tegument have been removed.

vs, ventral sucker.

Bar = 50 µm.

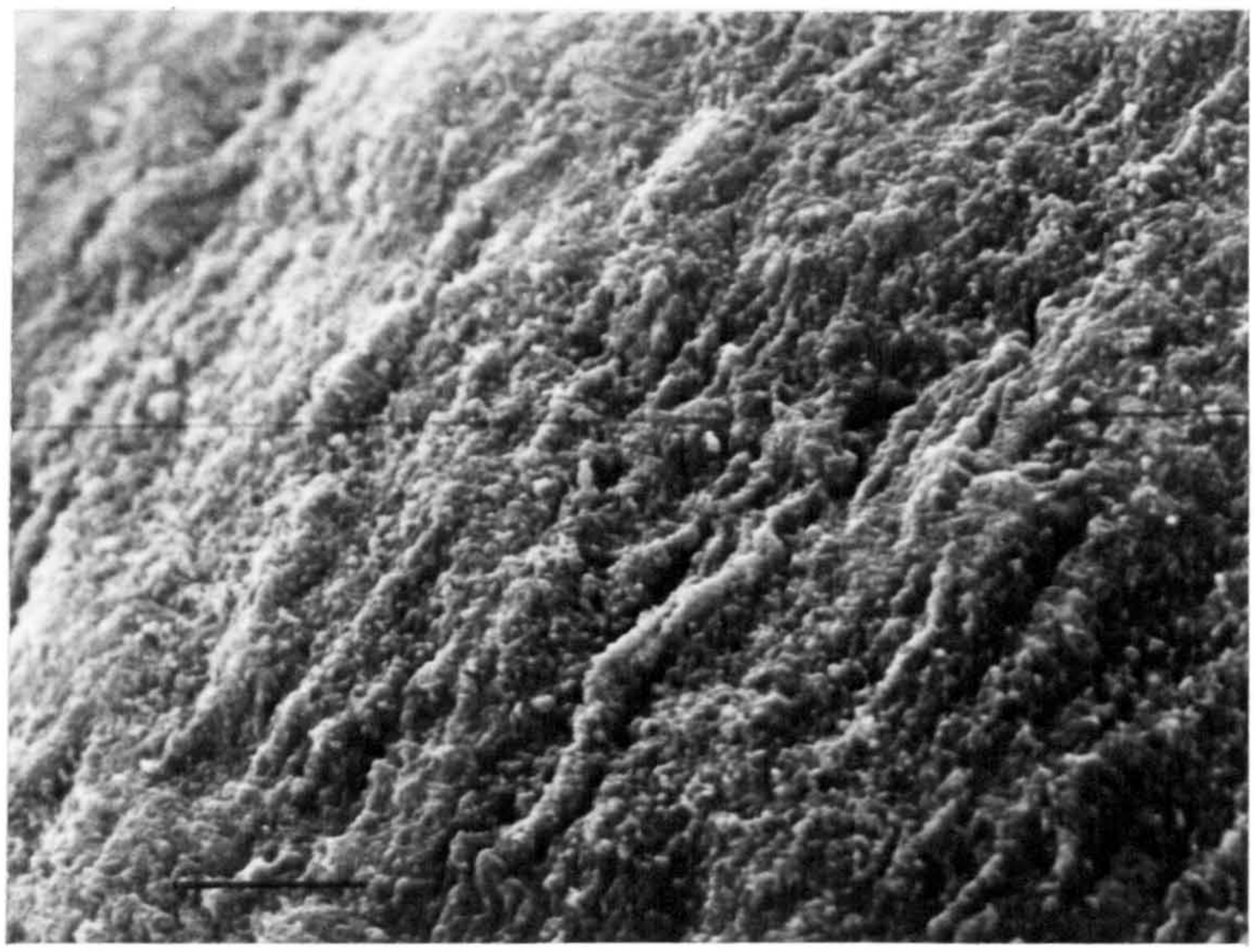


Figure 17. Female *S mansoni* denuded of tegument by freeze-thaw and sonication.

Bar = 5 µm.

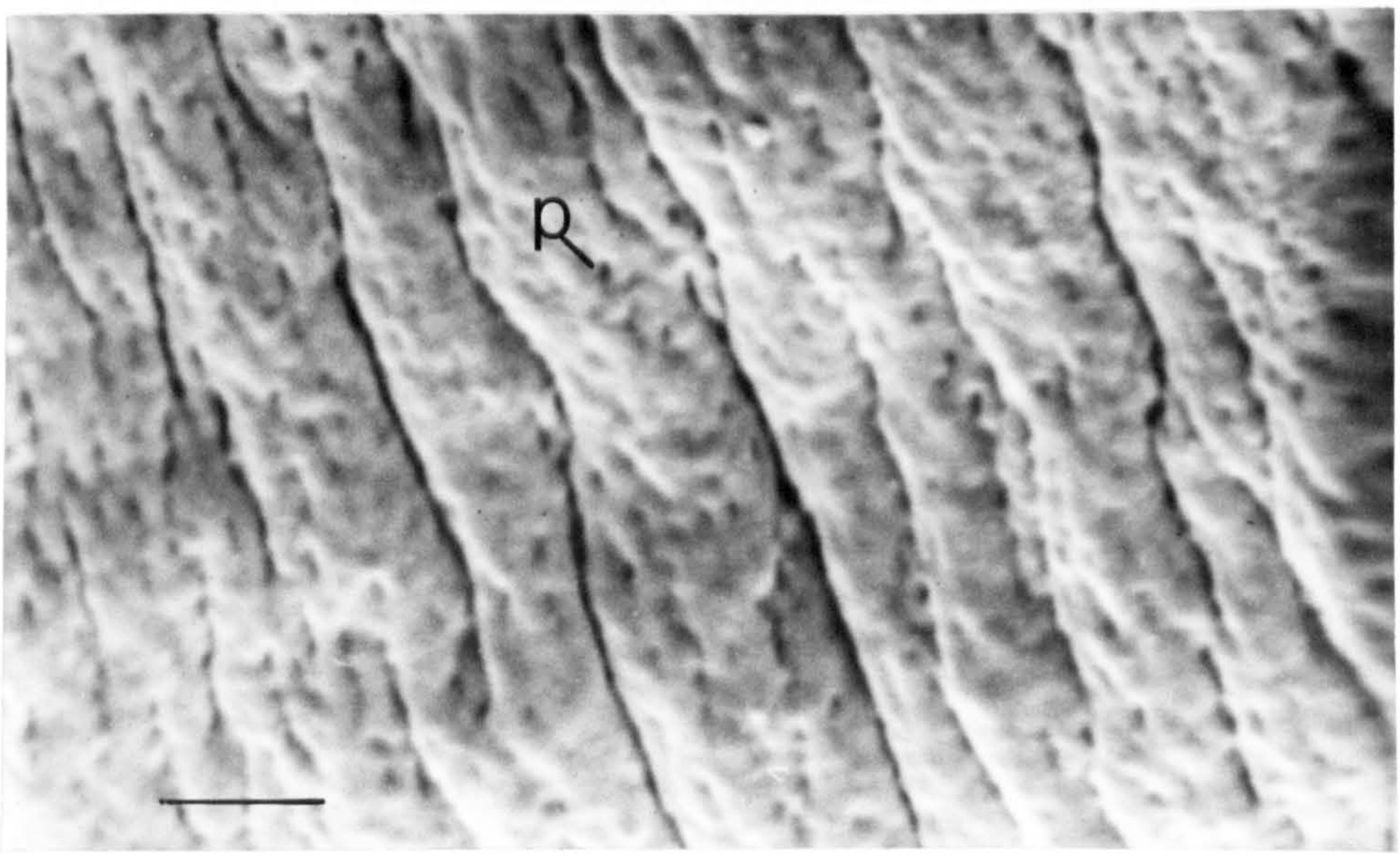


Figure 18. Dorsal surface of untreated female *S mansoni*.

p, pit.

Bar = 2 µm.

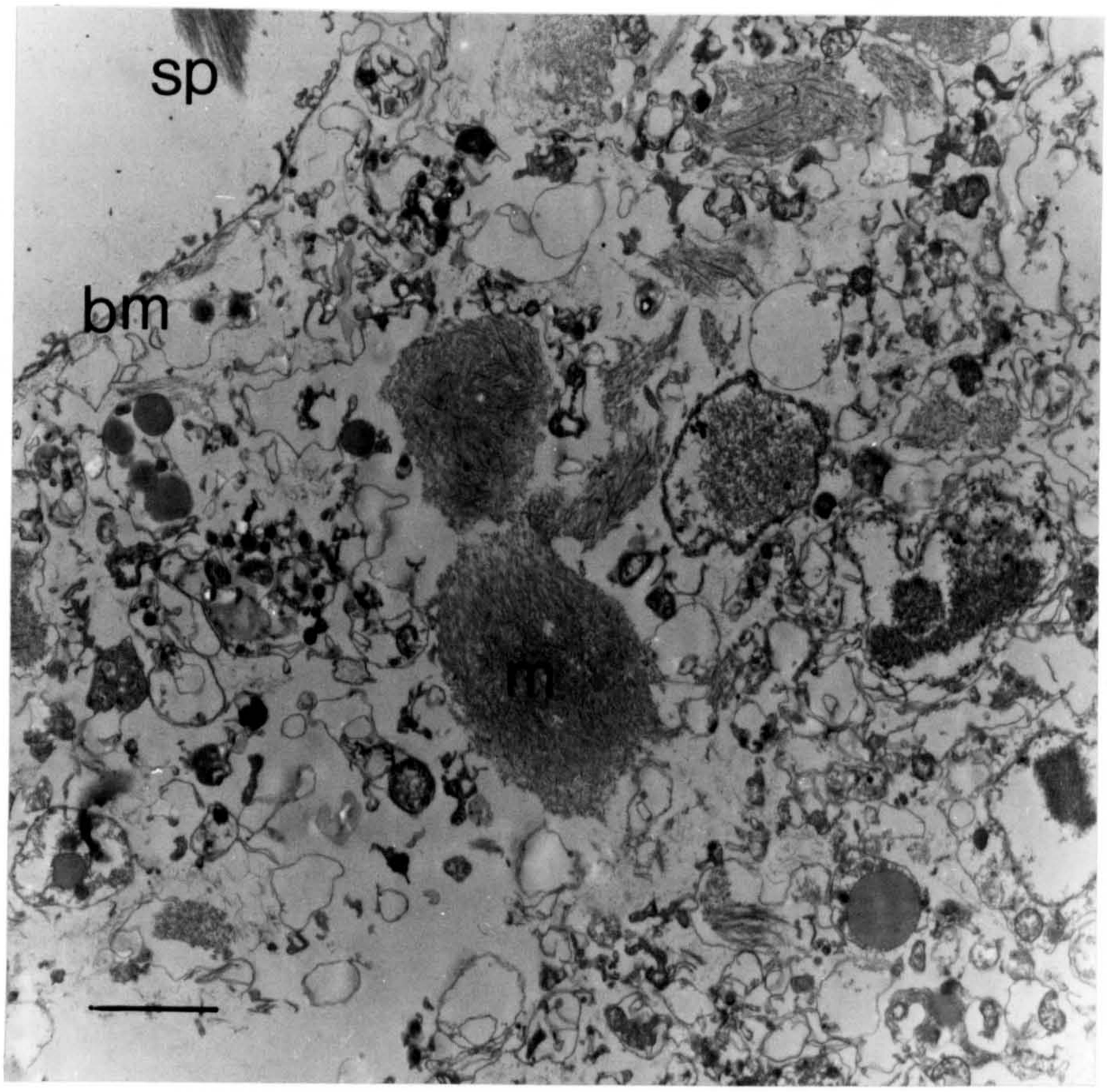


Figure 19. Transmission electron micrograph showing the surface of a worm subjected to freeze-thaw and sonication in HBSS.

bm, basal membrane; sp, spine; m, muscle.

Bar = 2 µm.

as was the resultant pellet. This again suggested the release of haematin from the gut of the parasites so, clearly, sonication caused considerable damage. Figure 20 shows the male dorsal surface denuded of tegument but with the basal membrane apparently still intact. In some places membrane whorls were seen (Figure 21 and 22). The pellet derived by centrifugation of the sonication supernatant was found to contain much membrane in the form of vesicles (Figures 23 & 24), spines, muscle, rough endoplasmic reticulum and myelin-like membranes. The sheets of surface membrane described in section 1.3.1 and illustrated in Figure 6 were not found in this pellet. After density gradient centrifugation spines were not found in the final membrane pellet and neither was muscle. Most of the material present was membrane bound vesicles (Figure 25). There was great heterogeneity of vesicle size and multilaminate vesicles were present. Some of the membrane was in the form of myelin-like vesicles. Sometimes small vesicles were found inside larger ones. Rough endoplasmic reticulum contaminated this pellet.

1.3.4 A reappraisal of the vortexing method

The presence of muscle and rough endoplasmic reticulum in pellets suggested that worms had been severely damaged by sonication. Damage to worms was not nearly so severe when vortexing was used as the agitation method (1.3.1). Vortexing released membranes in large sheets whereas sonication yielded an heterogeneous array of vesicles, which were not readily identifiable and not easily separated from other released material. It seemed probable that the sheets of tegument yielded by vortexing

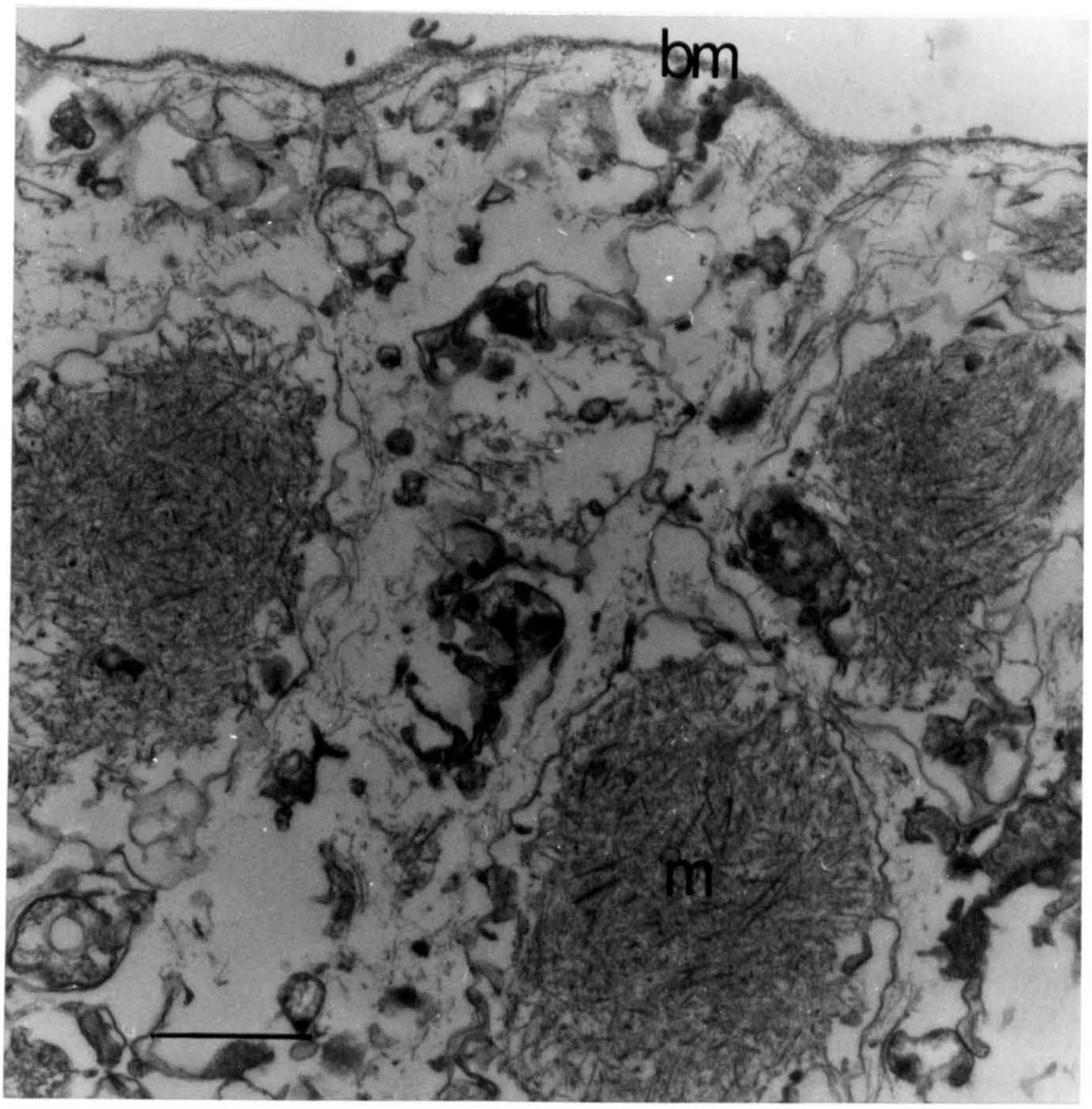


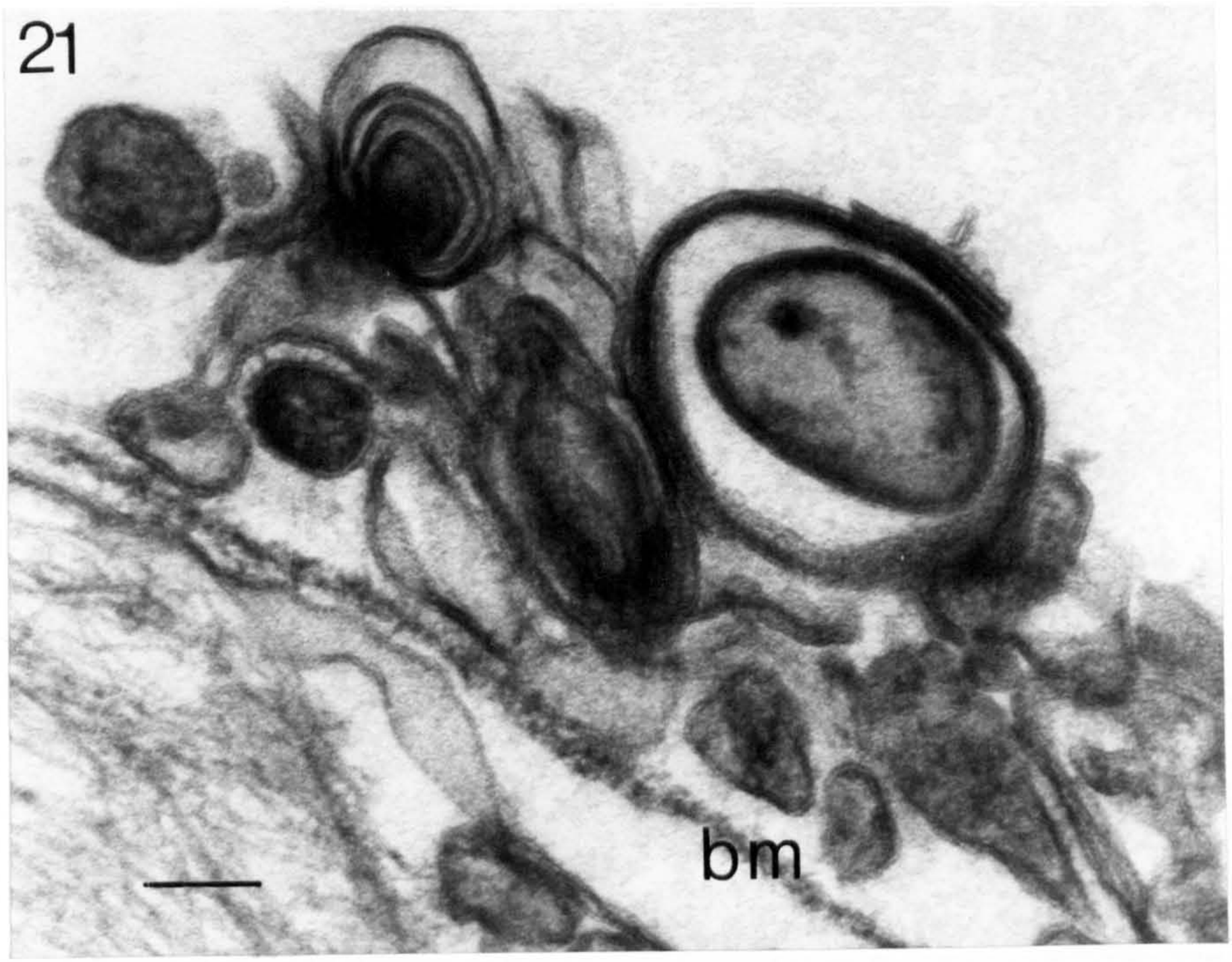
Figure 20. Intact basal membrane after freeze-thaw and sonication in HBSS.

bm, basal membrane; m, muscle.

Bar = 1 μm.

Figure 21
and Figure 22. Whorls of membrane seen at the surface of worms subjected to freeze-thaw and sonication in HBSS. bm, basal membrane.
Bar = 0.1 μm.

21



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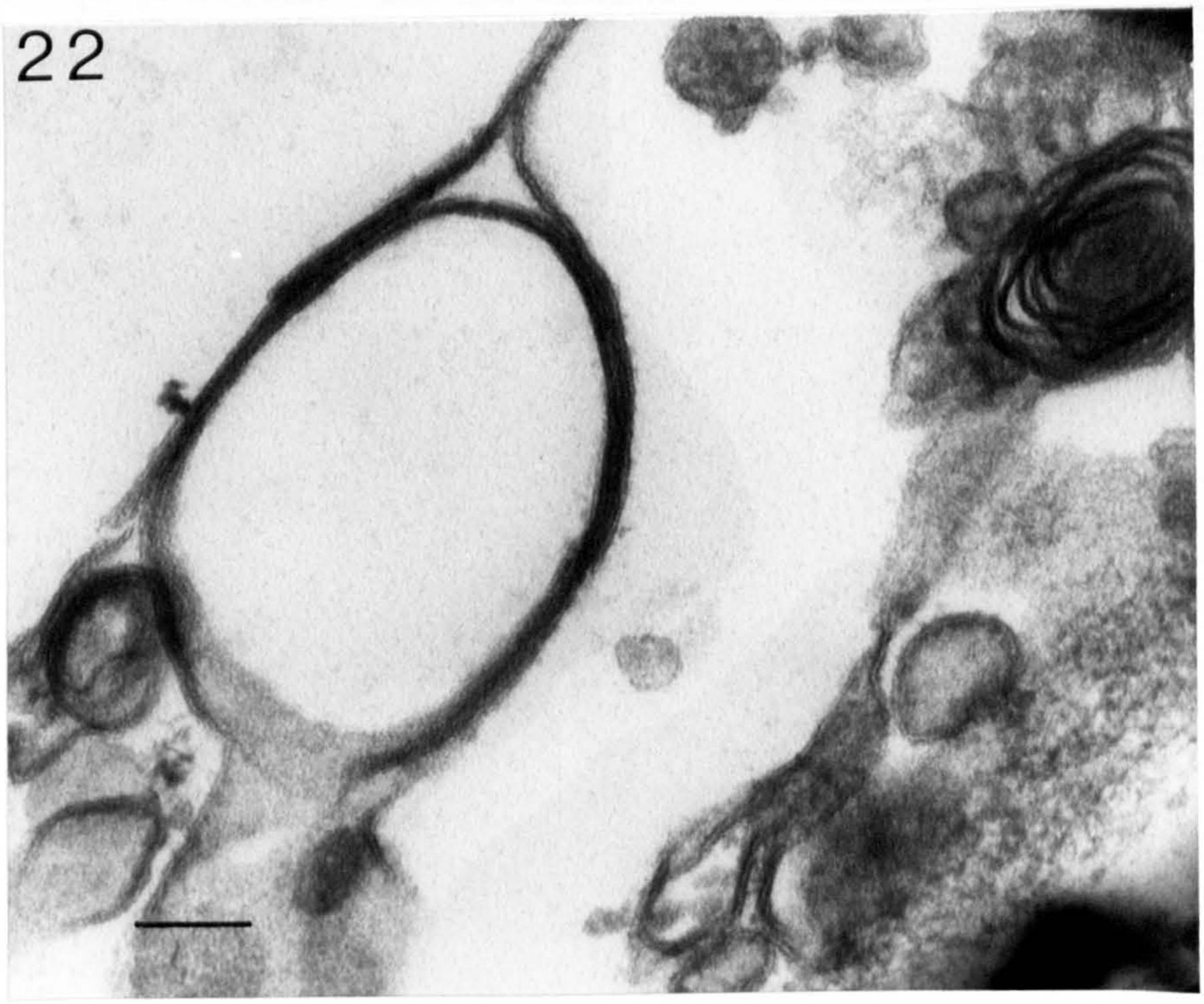


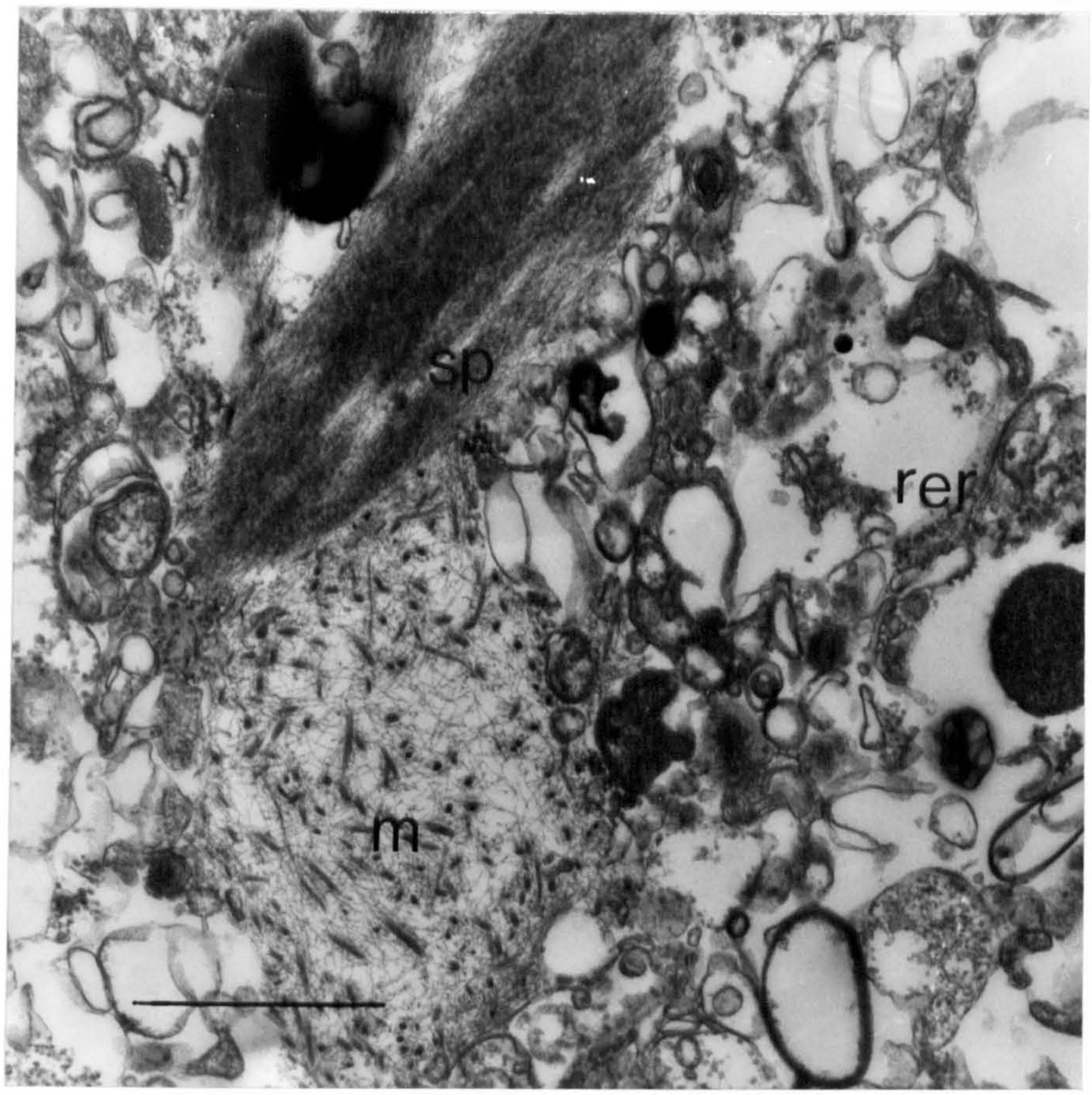


Figure 23. Pellet derived by centrifugation of the sonication supernatant after freeze-thaw in HBSS. sp, spine; m, muscle; rer, rough endoplasmic reticulum.

Bar = 1 μm.

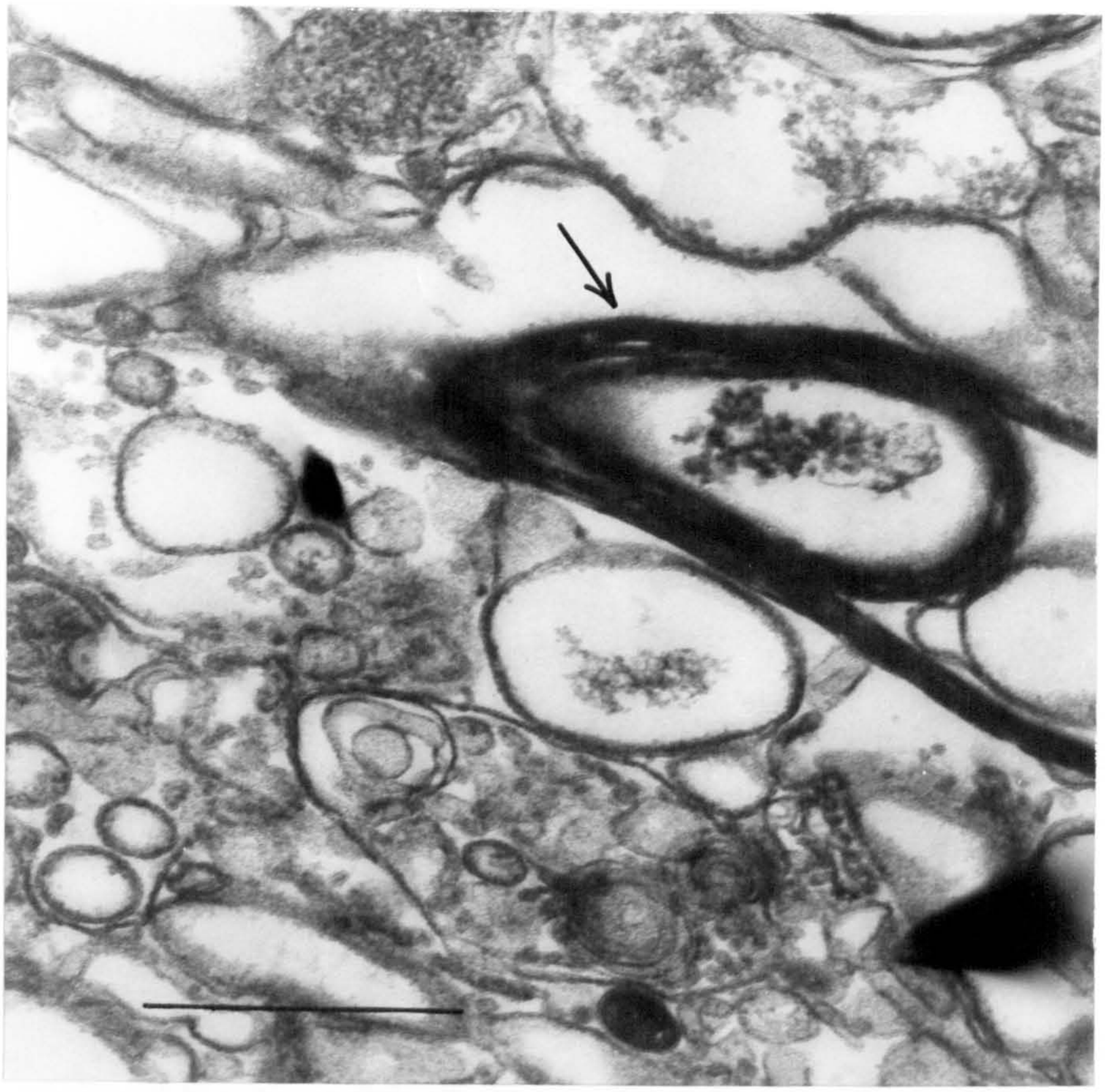


Figure 24. Myelin-like vesicle (arrow) in the pellet derived by centrifugation of the sonication supernatant after freeze-thaw in HBSS.
Bar = 0.5 µm.

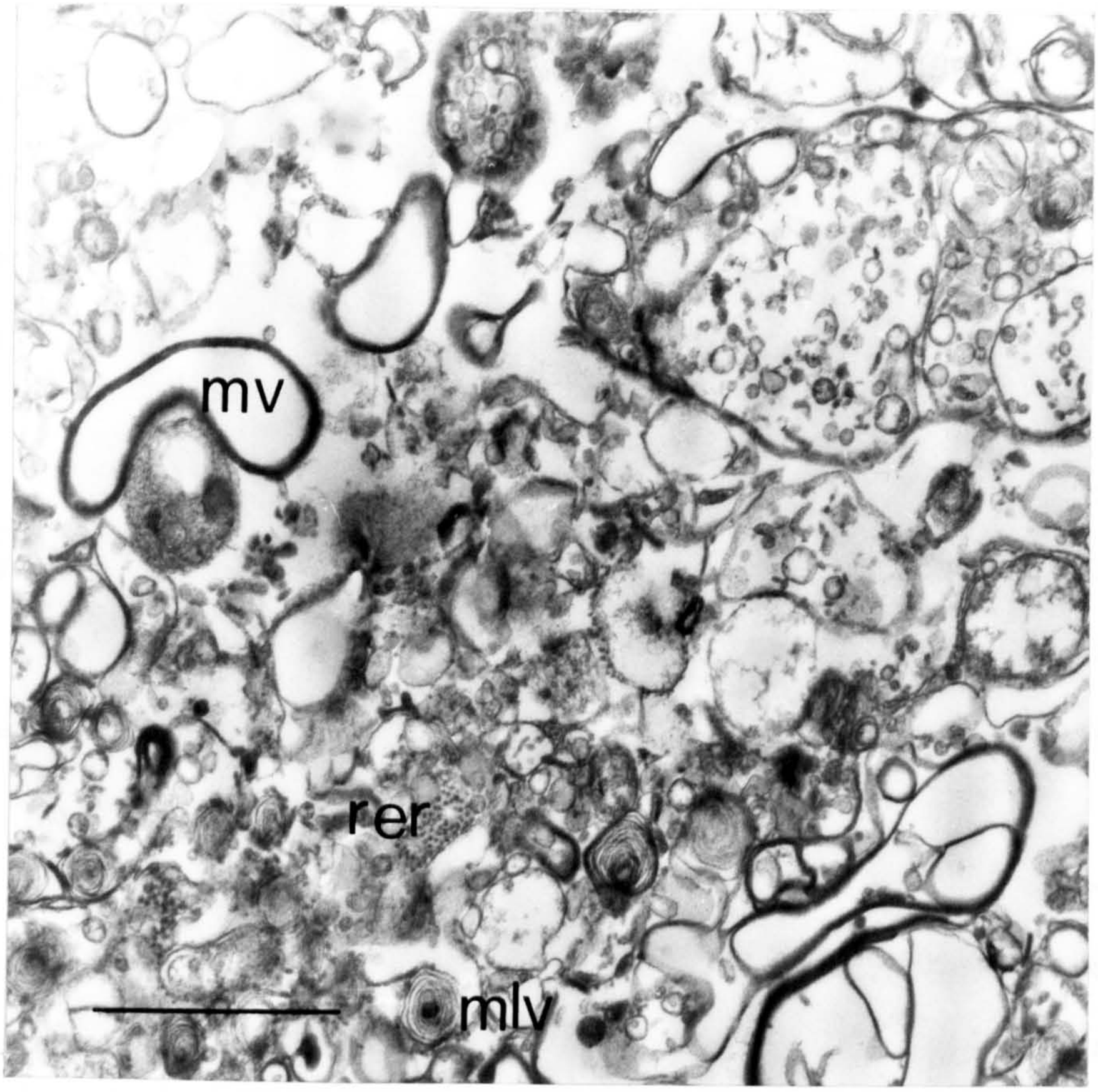


Figure 25. Resultant material after density gradient centrifugation of the material in Figure 23. mv, myelin-like membrane; rer, rough endoplasmic reticulum; mlv, multilaminate vesicle. Bar = 1 μm.

might be separated from other material by low speed centrifugation. However, previous results with vortexing showed that little surface membrane was liberated. It therefore seemed logical to reappraise the vortexing method. To that end an experiment was conducted to show, firstly, the minimum number of vortices required for total release of tegument and, secondly, whether it might be possible to maximise release of alkaline phosphatase while minimising damage to worms by using a particular degree of vibration.

A tube of about 300 worm pairs was thawed and the freeze-thaw supernatant removed. The tube was then vortexed 32 times and a sample of HBSS removed after 1, 2, 4, 8, 16 and 32 vortices. An equal volume of fresh HBSS was added to replace the sample.

Figure 26 shows results of assays for alkaline phosphatase, protein and succinate dehydrogenase. These results were corrected to allow for the removed samples. Release of alkaline phosphatase and protein reached a maximum after four vortices and SDH after only two. One third of the total alkaline phosphatase was released by four vortices. Clearly the vigour with which worms were vortexed was much greater than in 1.3.1 since only a fraction of the tegument was released after 10 vortices in that experiment. No optimum number of vortices was demonstrated to cause maximum alkaline phosphatase release with minimum liberation of protein and SDH. However, it was shown that the use of a greater number of vortices than required probably does not lead to further damage to worms.

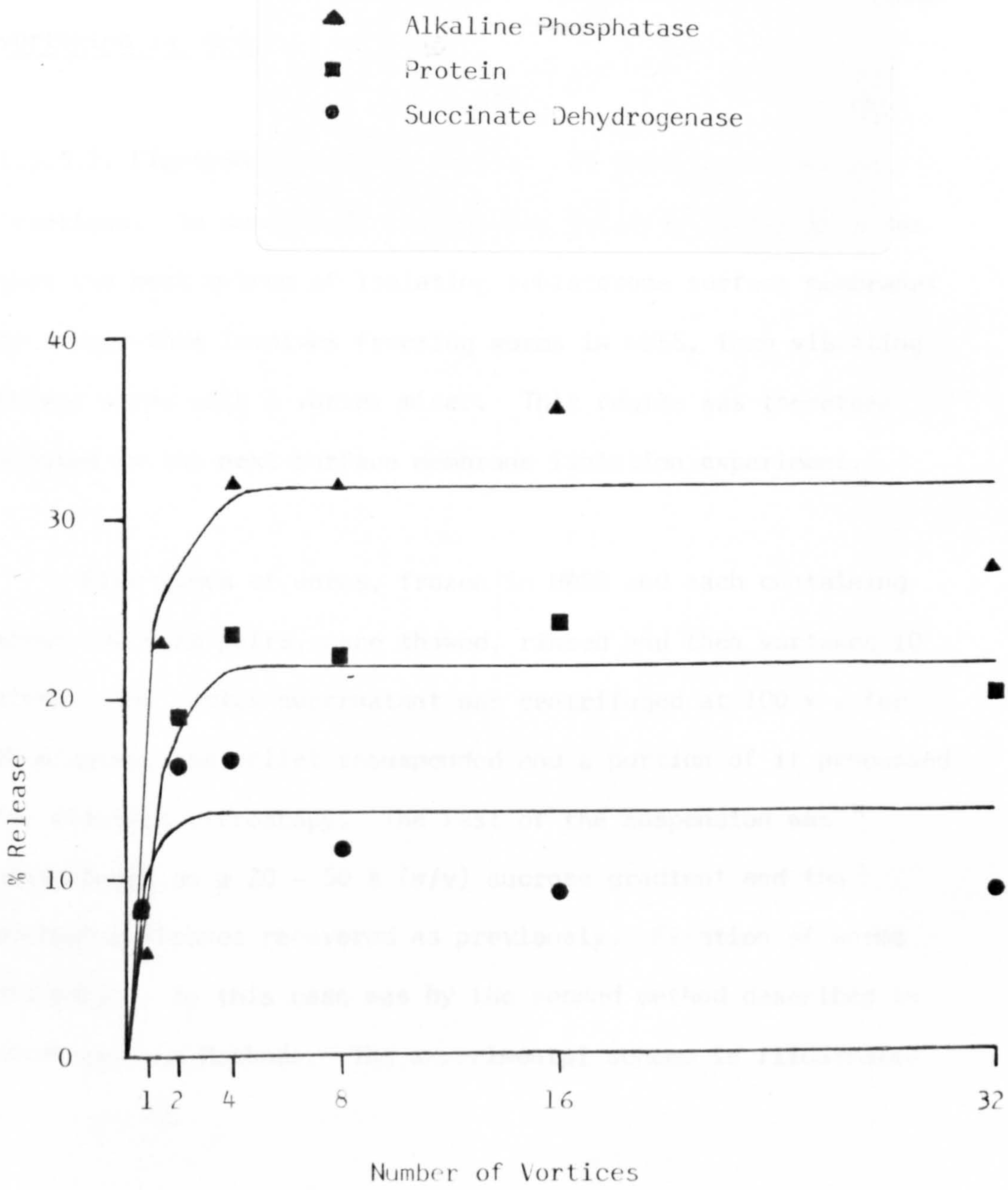


Figure 26. Release of alkaline phosphatase, succinic dehydrogenase and protein with increasing number of vortices. Total release of markers was :-

	% Alkaline Phosphatase	% Protein	% SDH
FTS	0.8	13.8	25.0
VS	31.8	22.6	13.3
DWH	67.5	63.7	61.7

1.3.5 Isolation of surface membranes by differential and density gradient centrifugation of material released by freeze-thaw and vortexing in HBSS.

1.3.5.1. Electron Microscopic analysis of worms and membrane fractions. To summarise the results detailed so far it seems that the best method of isolating schistosome surface membranes by freeze-thaw involves freezing worms in HBSS, then vibrating thawed worms with a vortex mixer. This regime was therefore adopted in the next surface membrane isolation experiment.

Five tubes of worms, frozen in HBSS and each containing about 250 worm pairs, were thawed, rinsed and then vortexed 10 times. The vortex supernatant was centrifuged at 100 x *g* for 30 minutes, the pellet resuspended and a portion of it processed for electron microscopy. The rest of the suspension was centrifuged on a 20 - 50 % (w/v) sucrose gradient and the surface membranes recovered as previously. Fixation of worms and pellets in this case was by the second method described in Materials and Methods. The experimental scheme is illustrated in Figure 27.

Worms were examined by TEM after vortexing and found to be denuded of tegument. The basal membrane appeared to be intact (Figure 28). However, some empty spaces were seen in tubercles and this may mean that some material was lost from these structures. Internal cells were much disrupted.

The pellet derived from the vortex supernatant contained

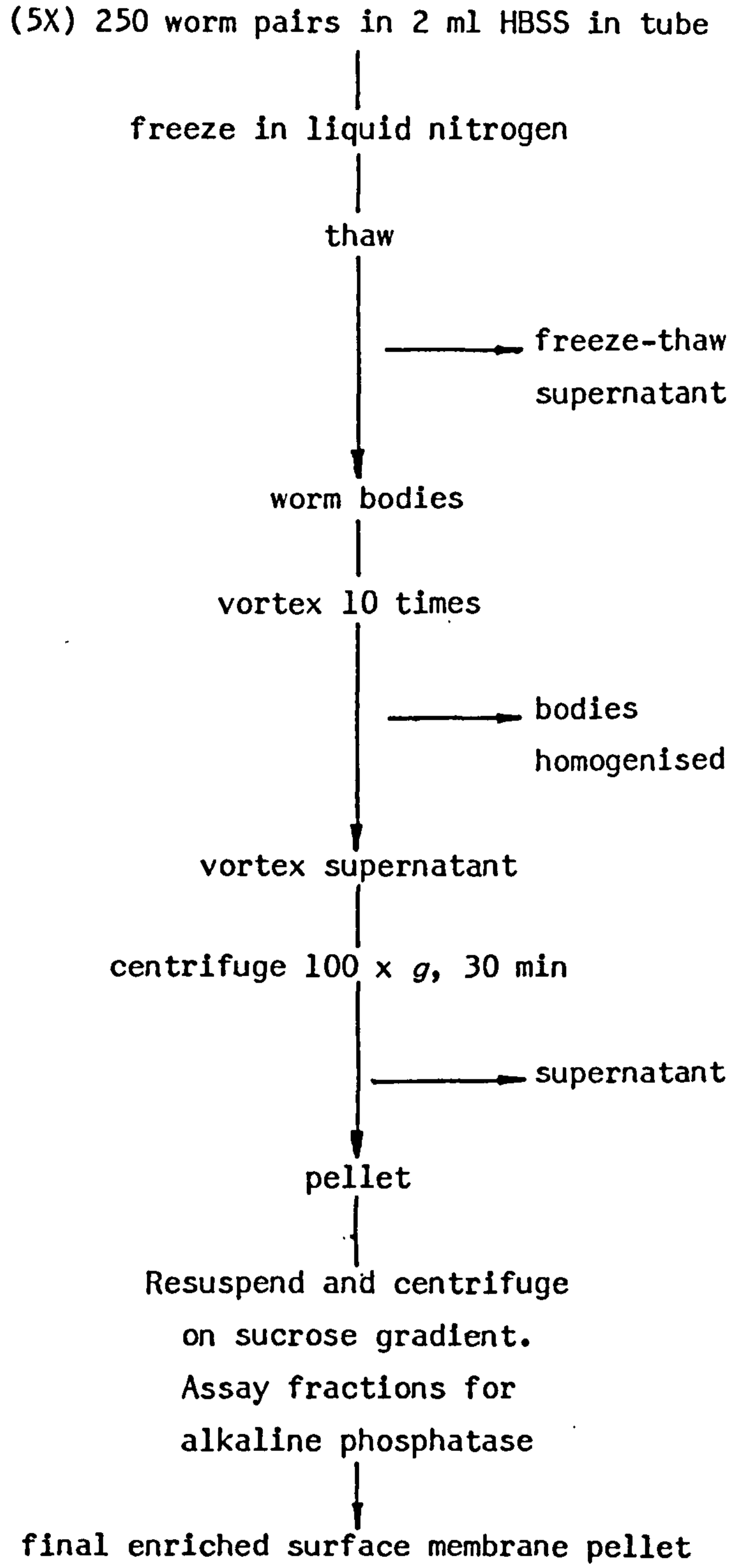


Figure 27. Experimental scheme used in 1.3.5.

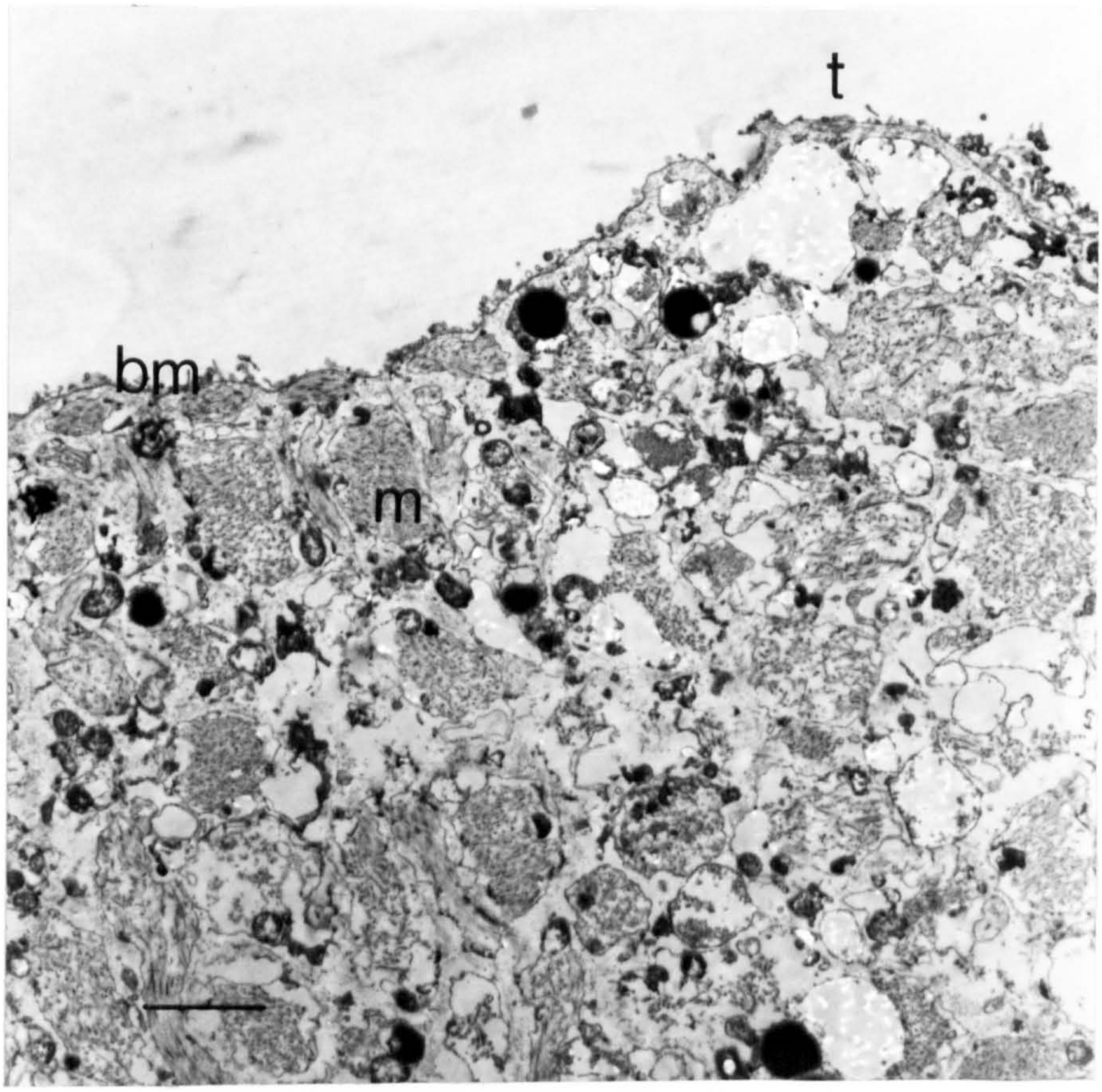


Figure 28. The dorsal surface of a male *S mansoni* after removal of the tegument by freeze-thaw and vortexing in HBSS.

t, tubercle; bm, basal membrane; m, muscle.

Bar = 2 µm.

sheets of surface membrane (Figure 29) many of which had a multilaminate appearance (Figures 30 and 31). Multilaminate vesicles were present but few discoid granules were detected. Some spines were present, but these were not apparently attached to the surface membrane. Myelin figures were again found.

The material in the final pellet is shown in Figures 32 and 33 . Sheets of surface membrane were clearly visible. Much membrane was present in the form of small vesicles whose origin is not known. There appeared to be little other contamination. Spines were absent from this pellet.

1.3.5.2. Succinate dehydrogenaseassay. In order to assess the purity of fractions in 1.3.5.1. a number of enzyme assays were performed. This is reported in the next section. First, problems associated with one assay will be detailed.

All of the SDH released by freeze-thaw and sonication in 1.3.3 was found in the freeze-thaw supernatant (Table 3) and most of the released SDH in 1.3.4 was in this supernatant (Figure 26). More SDH activity was consistently found in the freeze-thaw supernatant than in the sonication or vortex supernatants (Wells, personal communication) but only low quantities of surface membrane were released by freeze-thaw alone, as judged by assay of alkaline phosphatase activity. These results did not conform to the logical assumption that surface membranes must be liberated first to allow underlying structures to be released. Soluble components might be extracted before surface membranes were liberated but particulate components ought not to be.

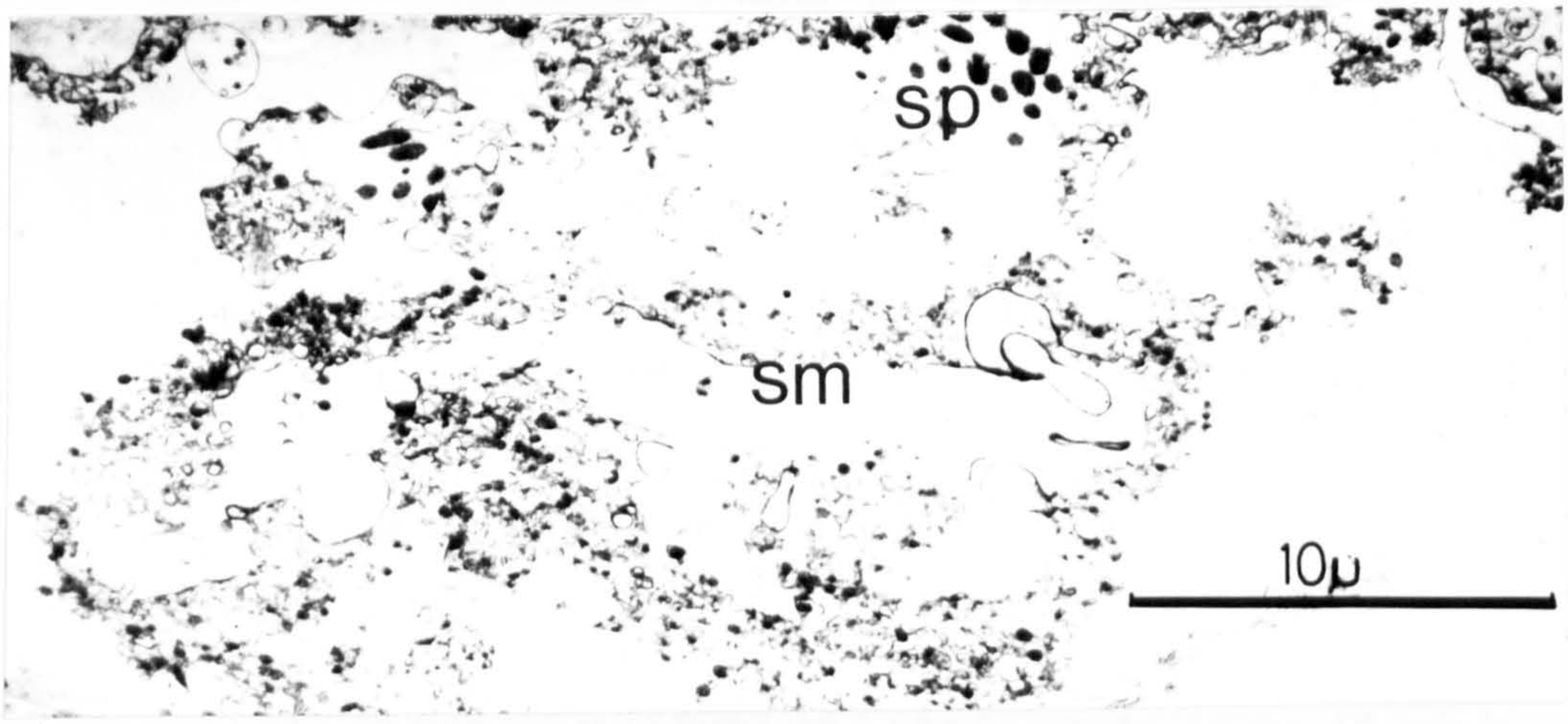


Figure 29. The pellet derived from the vortex supernatant after freezing and thawing worms in HBSS.

sp, spine; sm, surface membrane.

Bar = 10 μm.

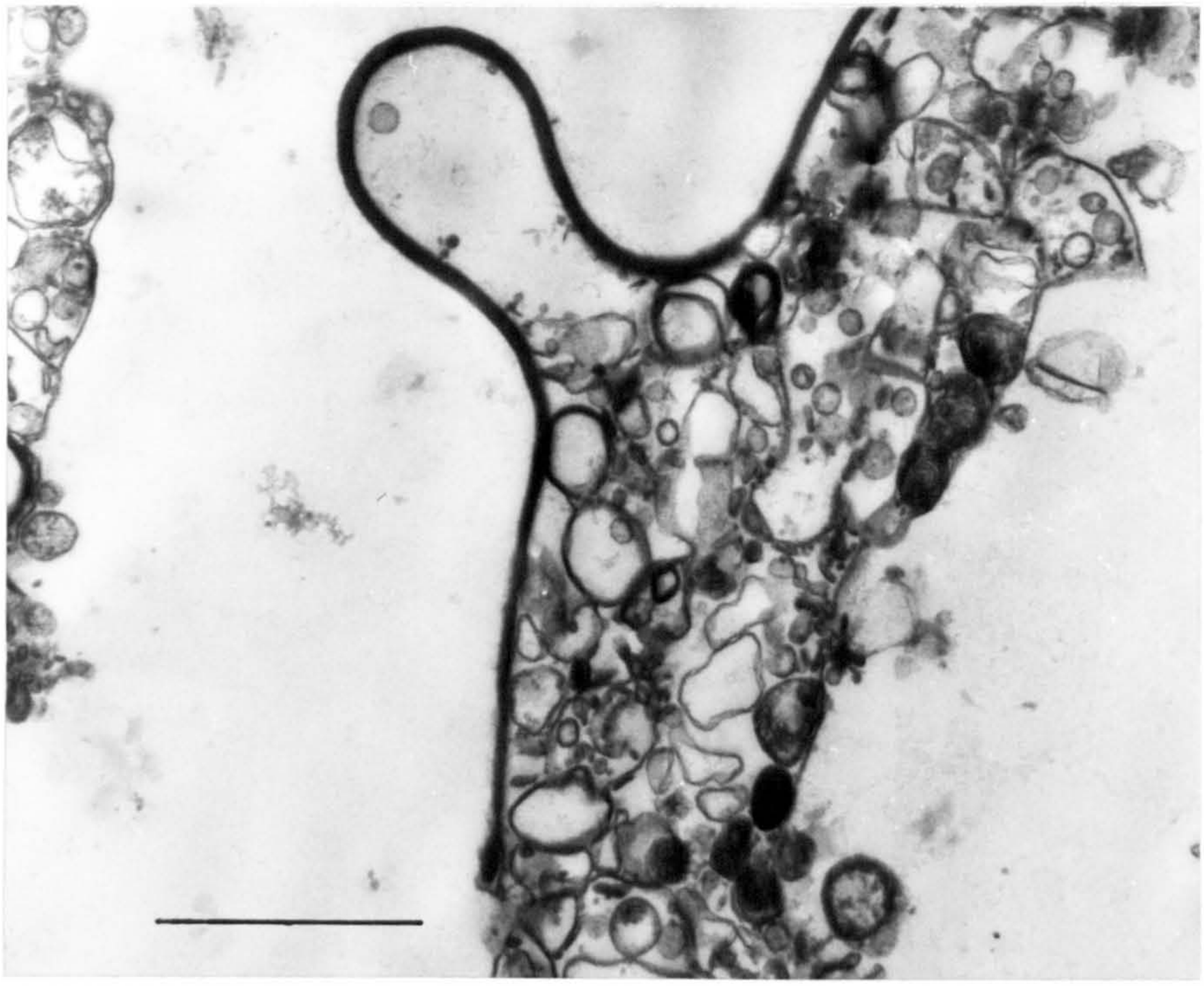


Figure 30. Multilaminate surface seen in isolated membrane.

Bar = 1 μm.

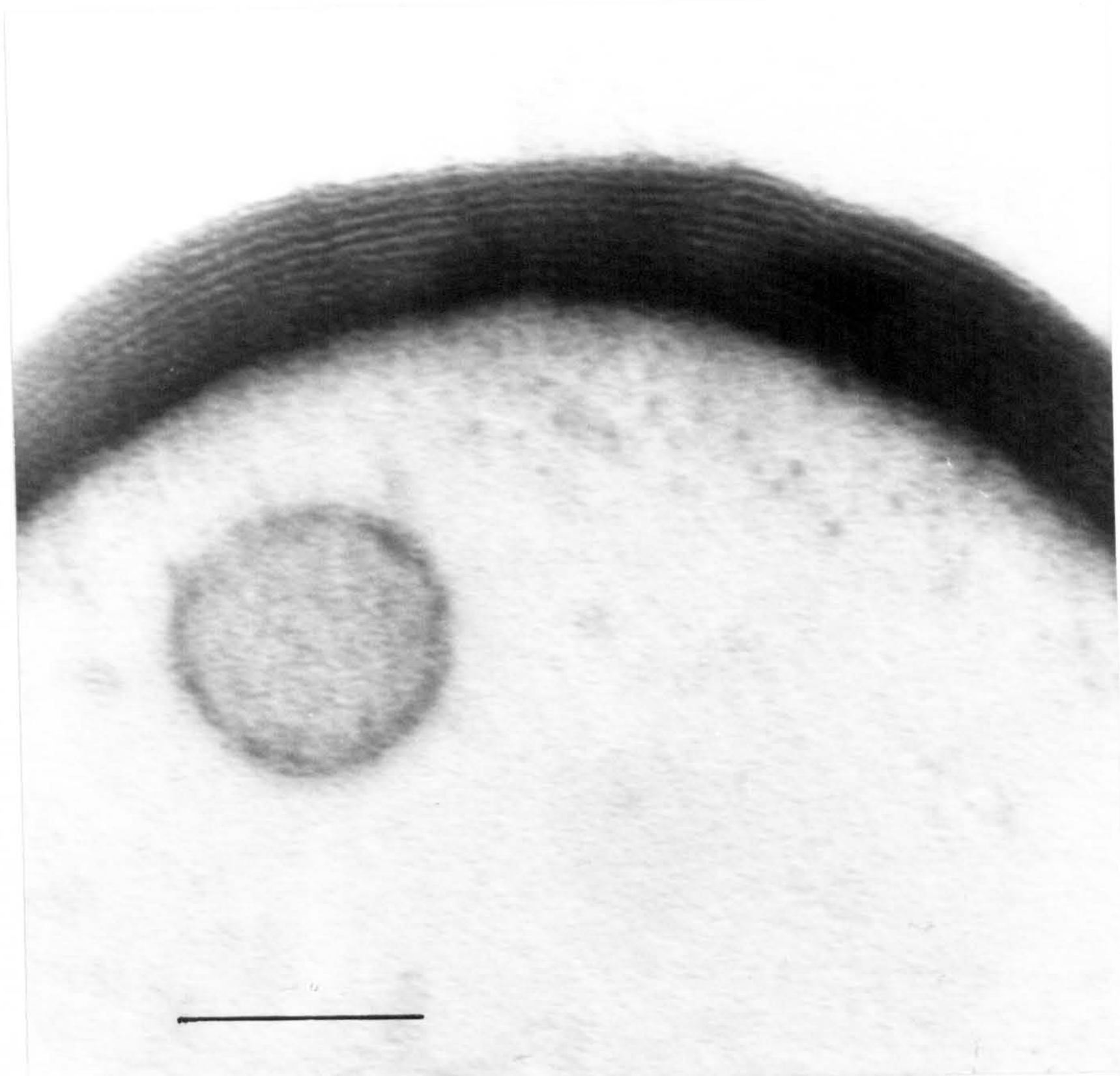
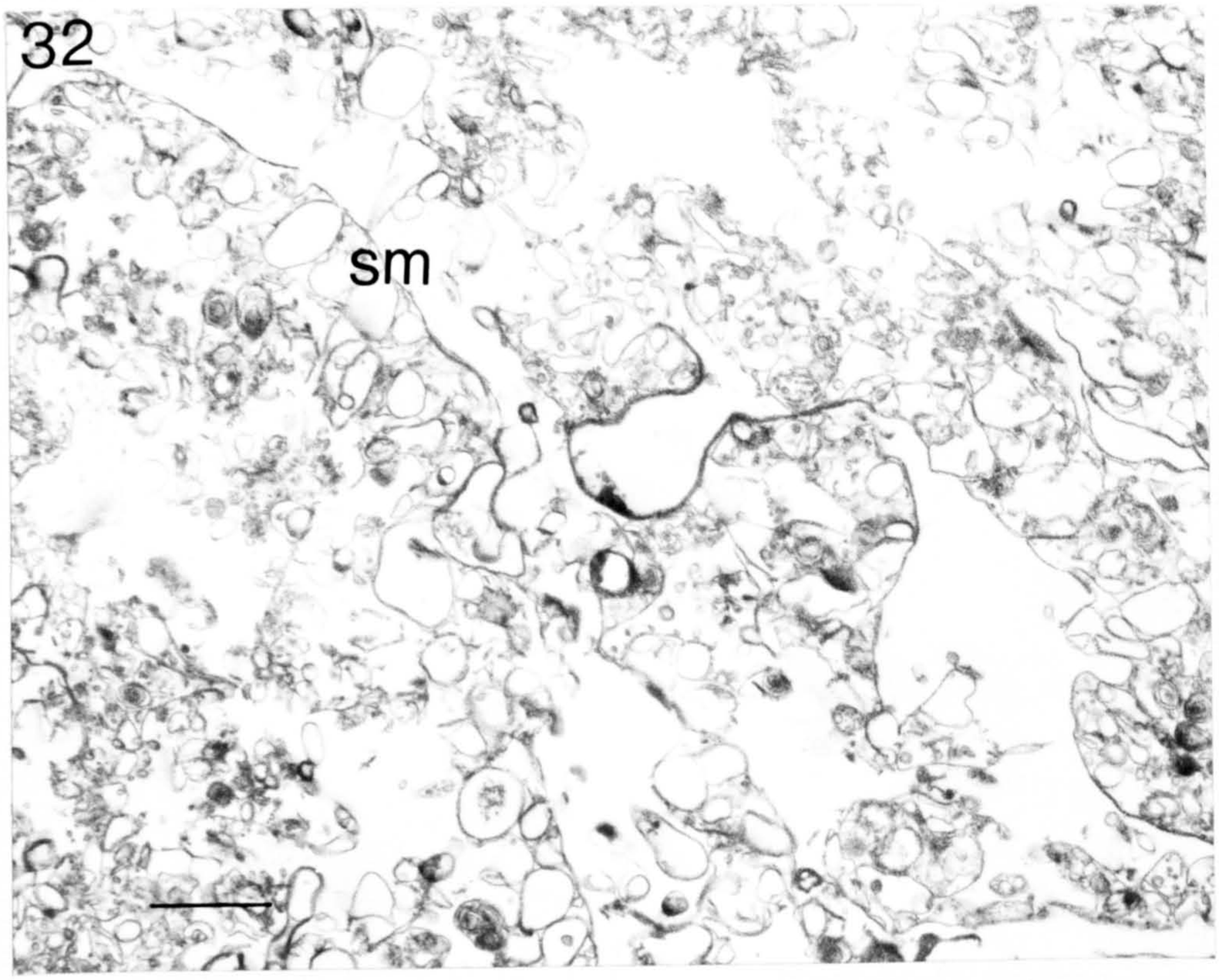
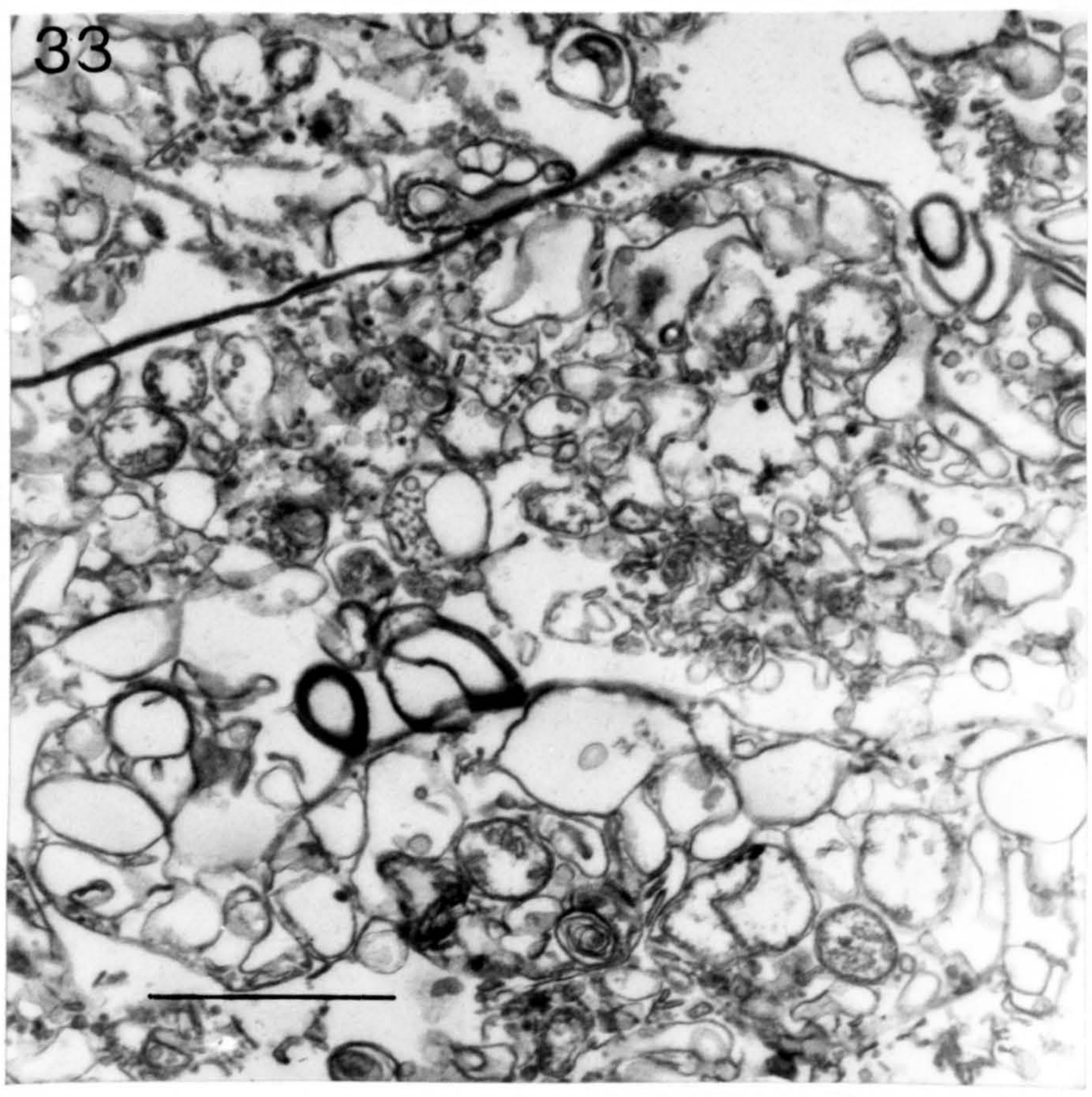


Figure 31. Enlargement of Figure 30 to show multilaminate membrane.

Bar = 0.1 μm.

Figure 32
and Figure 33. Material recovered from the alkaline phosphatase peak of a density gradient after centrifugation of the vortex supernatant pellet.
sm, surface membrane.
Figure 32 Bar = 2 μm.
Figure 33 Bar = 1 μm.


32
sm


33

It therefore seemed possible that SDH activity was soluble and not due to a mitochondrial enzyme. To test this possibility worms were subjected to the freeze-thaw and vortexing procedure outlined in 1.3.5. The freeze-thaw and vortex supernatants were sampled and the remainder of each supernatant was centrifuged at 73 500 x *g* for one hour. Whole mitochondria can be sedimented by a force of 10 000 x *g* for 25 minutes (Neville, 1976). The force used in this experiment was thus considerably greater than necessary to sediment whole mitochondria.

Table 4 shows the results of the SDH assay. Only a small proportion of SDH activity sedimented. Release of alkaline phosphatase was a little higher than usual, but otherwise this experiment gave fairly typical assay results. In both supernatants most of the protein was found not to sediment at the centrifugal force used.

A further experiment was conducted using a centrifugal force known to sediment fragmented mitochondria (submitochondrial particles). Racker (1962) used a force of 105 000 x *g* for 45 minutes for this purpose. In the present experiment a sedimentation field of 82 000 x *g* for 120 minutes was used. Again, this was in excess of the required force. The results (Table 5) indicated that most of the SDH activity remained in the supernatant. It was concluded that the activity attributed to succinate dehydrogenase was therefore not bound to mitochondrial membranes so SDH activity could not be considered a valid marker for this organelle. Once again, almost all of the protein in the supernatants remained there after centrifugation.

Table 4. Distribution of succinate dehydrogenase, alkaline phosphatase and protein in fractions produced by freeze-thaw and vortexing. Effect of centrifugation on succinate dehydrogenase activity of supernatants.

	succinate dehydrogenase		alkaline phosphatase		protein	
	units	%	units	%	mg	%
freeze-thaw supernatant (FTS)	0.606	13.2	0.419	7.0	4.830	14.9
vortex supernatant (VS)	0.576	12.6	1.981	33.0	5.354	16.5
denuded worm homogenate	3.396	74.2	3.602	60.0	22.294	68.6
total	4.578		6.002		32.478	
FTS 73 500 x *g* supernatant*	0.445	9.7	0.012	0.2	4.600	14.2
FTS 73 500 x *g* pellet*	0.012	0.3	0.220	3.7	0.132	0.4
VS 73 500 x *g* supernatant*	0.389	8.5	0.021	0.3	4.438	13.7
VS 73 500 x *g* pellet*	0.092	2.0	1.177	19.6	0.573	1.8

* centrifugation was at 73 500 x *g* for one hour

Table 5. Effect of centrifugation at forces known to sediment submitochondrial particles on succinate dehydrogenase activity of supernatants.

	succinate dehydrogenase		alkaline phosphatase		protein	
	units	%	units	%	mg	%
freeze-thaw supernatant (FTS)	1.548	13.3	0.112	2.0	4.556	15.2
vortex supernatant (VS)	1.218	10.5	1.971	34.4	5.319	17.8
denuded worm homogenate	8.862	76.2	3.652	63.7	20.062	67.0
total	11.628		5.735		29.937	
FTS 82 000 x *g* supernatant*	1.055	9.1	0.023	0.4	4.553	15.2
FTS 82 000 x *g* pellet*	0.122	1.0	0.055	1.0	0.102	0.3
VS 82 000 x *g* supernatant*	0.513	4.4	0.025	0.4	4.685	15.6
VS 82 000 x *g* pellet*	0.193	1.7	1.336	23.3	0.702	2.3

* centrifugation was at 82 000 x *g* for two hours

Alkaline phosphatase assay results showed that one third of the activity was released by freeze-thaw and vortexing and that almost all of this sedimented.

1.3.5.3. Enzyme characterization of the isolation method involving freeze-thaw and vortexing in HBSS followed by differential and density gradient centrifugation. Enzyme assays were performed on fractions obtained by the method of freeze-thaw in HBSS, vortexing and low speed centrifugation of released material. Rough endoplasmic reticulum had been detected in pellets. This is not found in the distal tegument but is present in the tegumental cell bodies and the gut epithelium. NADH cytochrome c reductase activity in fractions was determined in order to assess contamination by this organelle. In sonication experiments haematin was released from the gut and this led to a dark brown colouration in supernatants which persisted in the pellets. For such contamination to occur it seemed likely that worms had been severely damaged and that disruption of the gut had occured allowing extrusion of its contents. In order to assess damage to the gut in vortexing experiments, gut protease activity of fractions was determined.

The results of these assays are shown in Table 6 . It was found that 29 % of alkaline phosphatase was released from the worms during freeze-thaw and vortexing. This probably represents most of the alkaline phosphatase found in the surface membrane. In the vortex supernatant the enzyme was enriched 1.3 times compared to whole worms and this was increased to 17 times by centrifugation at 100 x *g*. Some 27 % of the worm protein was

Table 6. Distribution of protein, alkaline phosphatase, NADH cytochrome c reductase and protease in fractions produced using the finalised method of surface membrane isolation.

	protein	alkaline phosphatase[Δ]		
	%	%	specific[†] activity	relative[§] enrichment
freeze-thaw supernatant	5.1 ± 1.0[ε]	0.9 ± 0.3[ε]	0.029 ± 0.005	0.14 ± 0.03
vortex supernatant (VS)	22.0 ± 2.0	28.0 ± 1.6	0.274 ± 0.030	1.33 ± 0.13
denuded worm homogenate	72.9 ± 2.0	71.1 ± 1.9	0.203 ± 0.016	0.98 ± 0.03
total			0.208 ± 0.015	
VS 100 x *g* supernatant	20.0 ± 1.7	7.9 ± 1.3	0.083 ± 0.012	0.40 ± 0.06
VS 100 x *g* pellet	0.8 ± 0.1	12.8 ± 1.6	3.457 ± 0.250	16.64 ± 0.42

contd..

Table 6 contd.

	NADH cytochrome c reductase$^{\partial}$			protease$^{\Omega}$		
	%	specific† activity	relative§ enrichment	%	specific† activity	relative§ enrichment
freeze-thaw supernatant	6.7	0.175	0.37	34.4 $\pm$ 6.3$^{\varepsilon 1}$	1.062 $\pm$ 0.464	2.16 $\pm$ 0.26
vortex supernatant (VS)	5.5	0.143	0.30	24.4 $\pm$ 1.19	0.785 $\pm$ 0.388	1.45 $\pm$ 0.11
denuded worm homogenate	87.9	0.662	1.39	41.2 $\pm$ 4.40	0.339 $\pm$ 0.093	0.64 $\pm$ 0.05
total		0.478			0.520 $\pm$ 0.243	
VS 100 x *g* supernatant	5.1	0.161	0.34	ND$^{\gamma}$	ND	ND
VS 100 x *g* pellet	1.9	0.848	1.77	ND$^{\gamma}$	ND	ND

Δ hydrolysis of *p*-nitrophenylphosphate

∂ reduction of cytochrome c

Ω hydrolysis of Azocoll

$\dagger$ enzyme units / mg protein

$\S$ specific activity of fraction / total specific activity

ε standard error, n = 7; ε^1 standard error, n = 3

γ on one occasion the vortex supernatant was centrifuged at 73 500 x *g* for one hour. VS contained 25.4 % of the total activity and after sedimentation 21.5 % and 0.4 % of the enzyme activity were recovered in the supernatant and pellet respectively.

ND not determined

released by the surface membrane disruption procedure but less than 1 % of this sedimented with the surface membrane. Twelve per cent of the NADH cytochrome c reductase was released and 2 % was found in the pellet where the enzyme was enriched 1.8 times compared to whole worms. Of the protease activity, 59 % was released, with most of this appearing in the freeze-thaw supernatant. Protease activity of the 100 x *g* pellet was not determined but on one occasion (not tabulated) the vortex supernatant was sedimented at 73 500 x *g* for one hour. The vortex supernatant contained 25.4 % of the protease activity but only 0.4 % was found in the pellet and the enzyme was enriched in the pellet 0.02 times compared to whole worms.

1.4 DISCUSSION

1.4.1 The effect of tris buffer on ultrastructure.

Worms briefly incubated in hypotonic tris buffer on ice showed gross damage when morphological examination was performed. Internal cells were disrupted and the tegument was bleached and contained whorls of lamellate material. In contrast, HBSS had no apparent effect on internal structure. Some vacuolation of the tegument was seen but this may be similar to the transient vacuolation due to Eagle's medium described by Wilson and Barnes (1974b). These authors also showed that after 30 minutes incubation in HBSS, vacuolation of the tegument occurs.

Disruption of the muscle and parenchyma cells of worms incubated in hypotonic tris buffer was probably due to osmotic shock. The lamellate whorls in the tegument could not be attributed to osmotic shock as similar structures were seen in the pellet of released material described in 1.3.2. In that experiment the parasites were frozen in isotonic tris-buffered saline. The formation of the lamellate whorls was probably due to the use of tris buffer. The lamellate material resembles that found in lysosomal residual bodies. These are formed as a result of inadequate digestion of material sequestered in lysosomes (Pitt, 1975). Lamellate substances in lysosomal residual bodies are considered to correspond to the lamellar phase of polar lipids (Lüllmann-Rauch, 1979). Many cell types are known to exhibit this lysosomal storage as a result of drug treatment (Lüllmann-Rauch, 1979) and it is known to occur in the

schistosome tegument. Mutetwa (1978) found lysosomal storage in the tegument of *S mansoni* 4 days after treatment of infected mice with a schistosomicide (Ciba-Geigy C9333-GO/CGP4540). This gradually accumulated until, by 16 days after treatment, the tegument was filled with lysosomal residual bodies. Another drug (1,7 bis (*p*-amino-phenoxy) heptane) exerts a similar effect more rapidly. Residual lysosomes began to accumulate in the tegument only 13 hours after administering the drug to infected mice (Watts, Orpin and MacCormick, 1979).

Similar structures were recently reported to appear in the tegument after 10 minutes incubation in medium containing the plant lectin Concanavalin A (Simpson and McLaren, 1982). The structures found in the present study were discovered in worms recovered from mice by perfusion with Eagle's medium. The worms were in contact with this medium for about 30 minutes. The parasites were rinsed in TBS, then hypotonic tris buffer, spending about 2 minutes in each before being fixed for electron microscopy. It seems improbable that lysosomal storage could have occurred in the short time the worms were in contact with tris buffers. The buffers were ice cold and so metabolic functions would have been inhibited. An alternative proposition is that the buffer exerted a direct effect on tegumental lipids. Under certain conditions, lipids will form concentric whorls of electron dense material (Glauert and Lucy, 1968) so it may be that lipids in the schistosome tegument exhibit this effect in response to some action of, or reaction with, tris buffer.

It was not within the scope of this project to investigate

further the exact nature of the lamellate material. Its appearance adds further evidence to the observations of a number of workers that media used for incubation of schistosomes may not always be suited to that purpose. The transient vacuolation of the tegument found in worms incubated in Eagle's medium (Wilson and Barnes, 1974b) has also been detected following the use of Earle's salts plus lactalbumin hydrolysate (Simpson and McLaren, 1982). This may be due to a slight difference in the osmolarity of the media compared to that of worm cytoplasm or to localised salt uptake followed by a passive flow of water, as suggested by Wilson and Barnes (1974b). Much more severe and apparently permanent damage occurs in HBSS (Wilson and Barnes, 1974b), RPMI1640 (Simpson and McLaren, 1982) or phosphate buffered saline (Smith, Reynolds and von Lichtenberg, 1969; Simpson, Schryer, Cesari, Evans and Smithers, 1981). Indeed, a recent study of the effects on adult worm ultrastructure of a number of media concluded that few were suitable for maintenance of *S mansoni* (Carlisle, Weisberg and Bentley, 1983).

The reasons for the different effects on worm structure seen in different media are not known. It is clearly important, however, that any medium or buffer in which worms are to be held, even for short periods, should first be tested for its effect on worm ultrastructure. The medium used may be responsible for spurious results. Multilaminate vesicles bear a slight resemblance to lysosomal residual bodies but are smaller than the latter structures. McDiarmid, Podesta and Rahman, (1982) recently attempted to isolate multilaminate vesicles from total homogenates of *S mansoni*. An examination of their micrographs of isolated

multilaminate vesicles shows that some vesicles have a diameter of 500 nm, twice that reported for multilaminate vesicles (100-200 nm, Morris and Threadgold, 1968; 100-200 nm, Silk, Spence and Gear, 1969; 140-240 nm, Smith *et al*, 1969; 100-300 nm, Wilson and Barnes, 1974a). It is possible that the larger vesicles found by McDiarmid *et al* (1982) represent lysosomal residual bodies or the membrane whorls described here. These authors homogenised worms in tris buffer so formation of whorls may have occurred prior to homogenisation. Alternatively, the large "multilaminate vesicles" they described may represent true multilaminate vesicles which have become swollen because of some osmotic effect.

1.4.2 Succinate dehydrogenase as a mitochondrial marker in *S mansoni*.

In some experiments succinate dehydrogenase (SDH) activity was determined, as this is a well known mitochondrial marker enzyme. It was later shown, however, that the enzyme activity could not be sedimented by centrifugal forces known to sediment either whole mitochondria or submitochondrial particles. Thus the activity attributed to this enzyme was not apparently present in mitochondria. This opened up the possibility that SDH activity in *S mansoni* was soluble. However, it has been found that the activity was not due to succinate dehydrogenase at all. Vojvodic (personal communication) demonstrated that enzyme activity appeared considerable in the absence of substrate. Thus it seems likely that the apparent SDH activity was due to some purely chemical reaction.

A similar assay method to determine SDH activity in schistosome homogenates was used by Coles (1973) and Smith and Brown (1977). Coles measured activity in the absence of substrate but gave no indication that this was abnormally high. Thus, it seems that SDH activity is *S mansoni* may successfully have been determined by other workers. *S mansoni* has only low levels of tricarboxylic acid cycle enzymes (Coles, 1973; Smith and Brown, 1977) but true SDH activity may be even lower than was thought.

1.4.3 Methods of vibrating worms to release surface membranes.

Initial experiments using vortexing as the agitation method following freeze-thaw, were not successful in terms of the proportion of surface membranes liberated. Release of larger proportions of the enzyme could often be achieved with this method but vortexing was known to be of variable efficiency (Wells, personal communication). A more reproducible method of vibrating worms was therefore sought.

Twenty per cent of alkaline phosphatase activity was liberated using sonication. This probably represents two thirds of the alkaline phosphatase activity of the surface membranes and correlated well with SEM estimates of surface membrane denudation. It was obvious, however, that sonication damaged worms to a greater extent than vortexing. During sonication, supernatants turned brown, presumably due to the release of haematin from the gut. It was not known whether haematin escaped through the mouth or whether its release was due to

worms breaking open. Close inspection of a small number of worms in the scanning electron microscope suggested that the former route was more likely as there was no obvious damage to account for haematin release.

The presence of muscle in the pellet derived from the sonication supernatant was disturbing as this indicated that the basal membrane of the tegument had been breached and internal cells liberated. It was further evidence that sonication damaged worms much more than vortexing. Muscle had not been seen in released material when vortexing was employed. It also seemed that sonication caused the surface membranes to fragment much more than vortexing did and enrichment of alkaline phosphatase in the pellet was low when sonication was used. It is possible that the use of a lower centrifugal force might have increased enrichment but it is unlikely to have had a marked effect since the surface membrane was released as small vesicles. Density gradient centrifugation removed spines and muscle from the membrane and thus increased the purity of the preparation.

Transmission electron micrographs indicated that the basal membrane of the tegument remained intact following freeze-thaw and either vortexing or sonication. These results suggested that in neither case was contamination with internal components likely to be severe. However, transmission electron microscopy would only have shown large scale disruption of the basal membrane. If this membrane were breached, then contamination of released surface membrane with internal components might occur. In order to determine more accurately the state of worms after

surfaces were released, scanning electron microscopy was used. At no time did this reveal any damage to worms other than the denudation of the tegument. It did not show the damage which must have occurred to worms since muscle was released.

The vortexing technique was re-assessed in an experiment involving sequential vortexing of a tube of worms. It was found that there was a rapid rise in alkaline phosphatase activity of supernatants, with maximum activity reached after only 4 vortices. Release of 33 % of the enzyme occurred and this probably represents all of the surface membrane. The experiment showed that with practice it was possible to achieve maximum liberation of surface membranes using vortexing. It also showed that the use of more vortices than necessary for maximum alkaline phosphatase release did not result in increased release of protein. This suggested that vortexing caused little damage to worms other than to release surface membranes.

The greatest advantage of vortexing over sonication is the release of large sheets of surface membrane, as judged by morphological analysis. It was also found that surface membranes could be sedimented at 100 x *g* and that the pellet was enriched in alkaline phosphatase 17 times compared to whole worms. This represents a 3.5 fold improvement of the enrichment found using sonication and sedimentation at 7 200 x *g*. Yield was also apparently higher using vortexing. An average of 12.8 % of alkaline phosphatase activity was recovered in the vortex supernatant pellet compared to 5.1 % in the only sonication supernatant pellet.

1.4.4 Distribution of surface membrane denudation.

Sonicated worms were examined by scanning electron microscopy to discover the extent and distribution of surface stripping. Greater denudation can occur using vortexing but vortexed worms were not examined by SEM.

Females were more extensively denuded than males. The entire surface of female worms seemed to be equally affected. In males the dorsal surface exhibited greatest denudation. At the dorso-lateral margins of male worms the tegument was seen to be disrupted but only relatively small patches of surface membrane were missing. This may reflect the presence of many spines at the dorso-lateral margin, which could anchor the surface membrane as spines are attached to both the surface and basal membranes of the tegument. Examination of the gynaecophoric canal proved difficult as it was often obscured by the lateral margins of the worm. It seemed that denudation in the canal was patchy. This may be because the membrane lining the canal is protected from vibration against other worms by the presence of female worms or by the lateral margins of the male. The surface membranes lining the gynaecophoric canal contain little alkaline phosphatase (Wheater and Wilson, 1976) so optimum release of this enzyme could occur without release of surface membrane from the canal.

A similar apportionment of the effects of a surface membrane stripping procedure was seen when Oaks, Cain, Mower and Raj (1981) used Triton-X100 to disrupt *S mansoni* surfaces. In

that case the authors concluded that those surfaces not exposed to the detergent were least affected. It seems that freeze-thaw may be a more efficient method of disrupting the surface of the parasites as at least some of the membrane from the dorso-lateral margins and gynaecophoric canal was removed using freeze-thaw and sonication. The method of Oaks *et al* (1981) isolates only male dorsal and female surface membranes. The freeze-thaw method may therefore lead to an isolated membrane preparation which is more representative of the whole schistosome surface membrane.

1.4.5 Myelin figures.

Vesicles with concentric layers of closely apposed lamellae were frequently found in pellets regardless of the buffer or method of agitation used. These myelin figures were of various sizes and shapes and often contained as many as 12 or more stacked trilaminate membranes. Such structures are not found in schistosomes so must have arisen during the isolation procedure. They probably represent the same material as the "multilaminate stacks" reported to arise during isolation of multilaminate vesicles (McDiarmid *et al*, 1982).

The origin of the myelin figures is not known but some circumstantial evidence points to their being surface membranes. Figure 22 shows the tegument of the male dorsal surface following freeze-thaw and sonication. The extrapolation of dynamic processes from static micrographs has many pitfalls. However, a possible interpretation of Figure 22 is that the surface

membrane is in the process of coiling to form a myelin figure. Similarly, Figure 30 shows a sheet of membrane isolated by the freeze-thaw and vortexing method. The surface is multilaminate. Surface membranes with more than the usual double outer bilayer do sometimes occur *in vivo* but this is rare (Hockley and McLaren, 1973). A multilaminate appearance is common in the isolated membrane sheets. It is tempting to suggest that the outfolding of the multilaminate membrane in Figure 30 may have been fixed in the process of budding off to form a myelin figure. It is at least reasonable to assume that myelin figures and the lamellae aggregated with isolated surface membrane are of the same origin.

Further evidence of the nature of these myelin figures comes from microscopy of the material released by incubation in defined media containing cationised ferritin. Surface membrane sloughing occurs in such medium (Wilson and Barnes, 1977) and the shed membranes, representing the outer bilayer of the surface membrane, can be isolated by centrifugation of the medium.

Micrographs reveal that the isolated bilayers are in the form of multilayered stacks (Appendix 1). It therefore seems probable that myelin figures in the present experiments were derived from surface membrane. It may be that they represent both inner and outer bilayer since they were fixed for microscopy under conditions which did not stabilise outer bilayer in the experiments described in 1.3.1 to 1.3.3. The evidence therefore points to a surface membrane origin for myelin figures and stacked membrane sheets. McDiarmid *et al* (1982) suggested that

the membrane was derived from multilaminate vesicles. They were unable to substantiate this however, and the present evidence suggests it is more likely that myelin figures have a surface membrane origin.

The forces responsible for membrane stacking are not known. It may be that surface membranes have an affinity for themselves. Such an affinity may be the reason for the close apposition of the outer and inner bilayers *in vivo*. There must be some attraction between the bilayers, otherwise newly secreted membrane would be immediately lost to the medium.. The extra layers occasionally seen in surface membranes *in vivo* (eg Hockley and McLaren, 1973) have been interpreted as outer bilayer riding up over itself (Wilson and Barnes, 1977). Unless there exists some affinity of outer bilayer for itself, the membrane riding up would become detached. Insufficient information is available as to the properties of the schistosome surface membrane for definitive judgements to be advanced. It seems likely that this phenomenon is due to unusual properties of these membranes.

Myelin figures are not normally found in isolated membrane preparations although Zingales, Carniol, Abrahamson and Colli (1979) detected similar structures in plasma membrane isolates of *Trypanosoma cruzi*. The method of preparation of plasma membranes may be important in formation of myelin figures as they were not reported by other workers isolating surface membranes from *S mansoni* (Kusel, 1972; Bennett and Seed, 1977; Simpson *et al*, 1981; Oaks *et al*, 1981, 1983) or from trypanosomes (Hunt and Ellar, 1974; Voorheis, Gale, Owen and Edwards, 1979; Rovis and Baekkeskov, 1980).

1.4.6 The final method.

The method of isolation of surface membranes finally arrived at will be assessed in terms of results from electron microscopy and enzyme assays and then compared with other published methods.

1.4.6.1. Electron microscopy. Transmission electron micrographs of worms after freeze-thaw and vortexing show that muscle blocks approximately retained their normal position. This suggests that loss of internal components was minimal. Internal cells however, were much disrupted and organelles were intermixed. Figure 28 appears to show some large spaces in a tubercle. These spaces may simply be due to extraction of lipid; other electron translucent areas were found deeper within the body and the extraction of lipids during fixation could explain the apparent collapse of tubercles seen in SEM. The spaces could, however, indicate that internal components have been released from the tubercles. The basal lamellae of the tegument and the muscle layers may prevent large scale loss of internal organelles by maintaining their integrity and thereby containing internal components. At the tubercles, where the parenchyma extends above the muscle layers, any protection afforded by the muscles is lost. The tubercles may therefore be the most vulnerable areas of the tegument during freezing and thawing and they are thus likely routes of escape of non-tegumental components. No gaps in the basal membrane of the tegument were detected at the tubercles or elsewhere but there is reason to suspect some loss of internal components via the tubercles. A recent report (Oaks

et al, 1983) demonstrated disruption of the basal membrane as a result of freezing and thawing but the method used was substantially different to that employed here.

Since internal cell membranes were disrupted the cytoplasmic tubules which connect the apical tegument with the tegumental cell bodies would become open channels between the interior of worms and the medium. These tubules are therefore another possible route of escape for internal components.

The disruption of internal cells was probably due to freeze-thaw damage. Vortexing is most unlikely to have disrupted cells. Damage to cells during freezing is thought to be caused by formation of intracellular ice crystals or to the concentration gradients produced by freezing of extracellular solutes (Mazur, 1970; Meryman, 1974). In addition, ice crystals may grow during thawing and this further compounds damage (Mazur, 1966). Membranes are the chief target of freeze-thaw injury (Mazur, 1970) so this explains the disruption of cells and intermixing of their contents. Morphological examination of the pellet derived from the vortex supernatant appears to show that surface membranes are fairly pure. Non-tegumental components were rarely detected. Surface membranes were easily recognisable as long sheets with convolutions which presumably correspond to the membrane lining the tegumental pits. These sheets of membrane were also found after density gradient centrifugation. Other material present at this stage included myelin figures and many membrane-bound vesicles of various size, including multilaminate vesicles. It was not surprising to find that multilaminate

vesicles have a similar density to surface membranes in view of their supposed role. However, since they and the membrane-bound vesicles found sediment at 100 x *g*, they must aggregate with each other and with the surface membranes.

The origin of the membrane-bound vesicles is not known, but they may represent small fragments of surface membrane. There is some circumstantial evidence that many are discoid granules which have become swollen as a result of freeze-thaw (Chapter 3). These vesicles represent the major contaminant of the surface membranes, as judged by electron microscopy.

Aggregation of vesicles could be due to high concentrations of divalent cations (Kamath, Kummerow and Narayan, 1971) although aggregation was found in the experiment described in 1.3.1 where the buffer used contained no divalent cations. The addition of chelating agents might have prevented aggregation if it was due to the worms own divalent cations. However, considering the known sensitivity of the schistosome surface membrane, this may have caused more problems than it solved. Kusel (1972) pointed out that Ca^{++} was required for maintaining the integrity of the surface membrane sheets released by saponin. Removal of that ion with chelating agents might therefore lead to destabilisation of the surface membrane and its liberation as small fragments, which would be undesirable.

1.4.6.2. Enzymic analysis. Enzyme assays were used to assess contamination of isolated membranes. Results of NADH cyto-chrome c reductase assays show that endoplasmic reticulum and/or

mitochondria were released and sediment with the surface membranes. Occasionally some rough endoplasmic reticulum was seen in micrographs of the pellet from the vortex supernatant but there did not appear to be substantial contamination by this organelle. Both mitochondria and rough endoplasmic reticulum are found in tegumental cell bodies. Their release during surface membrane removal is therefore not surprising. Liberation of 12 % of this enzyme is unlikely to be accounted for simply by the release of organelles from tegumental cells. The distribution of the enzyme between these organelles in *S mansoni* is not known. The major source of mitochondria in the parasite is probably the muscle cells. The gut epithelium possesses much rough endoplasmic reticulum (Spence and Silk, 1970) and this may be the major source of that organelle. Thus, it is possible that mitochondria or endoplasmic reticulum from within the body of the worms was released.

Much of the gut protease is apparently released by freeze-thaw and vortexing. This enzyme may be present in membrane-bound vesicles as Triton X-100 enhances solubilisation of the enzyme (Dresden and Deelder, 1979). Such vesicles have been detected in the oesophagus by Spence and Silk (1970) and Bogitsh and Carter (1977) and these workers suggested they contained a proteolytic enzyme. It seems likely therefore, that the enzyme is soluble but held within vesicles until secreted into the lumen of the oesophagus. Its extraction during surface stripping might be due to release of the vesicles or of free enzyme. Only a very small proportion of the released protease was sedimented by centrifugation at 73 500 x *g* for one hour whereas

about one third of the enzyme activity can be sedimented from homogenates by centrifugation at 8 000 x *g* for 10 minutes (Dresden and Deelder, 1979). It seems therefore, that the protease activity released by freeze-thaw and vortexing was not membrane-bound. Release of soluble components during disruption procedures is much less important than release of particulate ones. Most of the protein in the freeze-thaw and the vortex supernatants is soluble so freeze-thaw may render the worms more permeable. The liberation of only small quantities of particulate components during membrane denudation suggests that the basal membrane and basal lamellae of the tegument and the muscle layers may together form a "sieve" preventing the release of a large proportion of internal organelles.

Release of proteolytic enzyme might be thought to affect proteins in the liberated surface membranes. However, the gut enzyme has a reported pH optimum of around ph4 (Timms and Bueding, 1959) or pH5 (Dresden, Ruttledge and Chappell, 1981) and exhibits little activity at neutral pH.

The enrichment of alkaline phosphatase in the vortex supernatant pellet was 16.6 (± 0.4) times that of total. This could be increased to 40 times after density gradient centrifugation (Roberts *et al*, 1983). It could be further increased if the resuspended pellet was subject to a second freeze-thaw and then washed by repeated centrifugation followed by density gradient centrifugation (Roberts *et al*, 1983). This second freeze-thaw was designed to detach from the surface membrane the small vesicles found in the pellets. Thus considerable

enrichment of surface membranes can be achieved using this method.

1.4.6.3. Comparison with other methods. Direct comparisons between the efficacy of this method and that of other workers are in most cases difficult to make. It is possible however, to make some comparisons.

The freeze-thaw method employed by Kusel (1972) is in many ways similar to that finally arrived at here. Kusel froze adult worms in HBSS by plunging them into liquid nitrogen. Surface membranes were washed off the thawed bodies which were suspended on a sieve. Although comparative data are not available this method of stripping the surface may not be as efficient as vortexing. Kusel (1972) sedimented surface membranes at 1 000 x *g* (10 minutes) and no further purification took place. He did not employ any surface membrane markers but morphological examination revealed that large sheets of surface membrane had been released and this is as expected from the present results. The force used to sediment the surface membranes was higher than in the present study and it is likely that enrichment of surface membranes in Kusel's final preparation was less than that achieved in the vortex supernatant pellet in the present study. As we have seen, much greater enrichment can be achieved using further centrifugation on a density gradient.

Oaks *et al* (1983) used freeze-thaw to disrupt the surface membrane. After vortexing the worms they centrifuged the supernatant at 2 500 x *g* (15 minutes). The pellet was enriched in alkaline phosphatase only 4 times compared to whole worms. This

is clearly inferior to the preparation reported here and may be due to their holding the worms in liquid nitrogen for fifteen minutes (as opposed to the same number of seconds in the present method), or to the high sedimentation rate.

Oaks *et al* (1983) used the calcium chloride surface membrane disruption method of Bennet and Seed (1977) and found their 2 500 x *g* pellet was enriched in alkaline phosphatase eight times and ATPase fifteen times compared to whole worms. This preparation contained large sheets of surface membrane similar to those seen using the present method. The surface membrane was removed from the parasites only in patches with much remaining, attached to the carcass. This method of surface membrane disruption is therefore not particularly efficient.

The technique of surface membrane denudation used by Simpson, Cesari, Evans and Smithers (1980) and Simpson *et al* (1981) makes use of the severe tegumental damage which occurs in certain media, leading to the appearance of surface proteins in the medium (Ruppel, 1978). Nuclei were found in the released material and presumably came from the tegumental cell bodies. Their escape was probably due to damage to muscle layers and to the membrane lining cytoplasmic connections. Whole nuclei are unlikely to have been major contaminants of the purified membrane preparation as they would have been separated from membranes by density gradient centrifugation. Their release does suggest that other plama membranes in the worm may also have been released.

Simpson *et al* (1980, 1981) collected the material released

into phosphate-buffered saline by centrifugation at 55 000 x *g* for one hour. The resulting pellet was then fractionated by discontinuous density gradient centrifugation. This technique produces small vesicles of surface membrane which are not likely to be easily separable from other released components (De Pierre and Karnovsky, 1973; Neville, 1975, 1976). Simpson *et al* (1980, 1981) used a number of surface membrane marker enzymes and utilized electron microscopy to characterise fractions. It is possible to deduce from the results given (Simpson *et al*, 1981) that enrichment of alkaline phosphatase in the fraction of highest enrichment was 14 times that of whole worms. Alkaline phosphodiesterase was enriched 27 times and Ca^{++}, Mg^{++}-ATPase 40 times. The figure for alkaline phosphatase suggests the final preparation of these workers is of similar enrichment to that of the pellet from the vortex supernatant in the present study. An enrichment of 40 times that of whole worms can be achieved after density gradient centrifugation with the present method, however.

Since alkaline phosphodiesterase and Ca^{++}, Mg^{++}ATPase are enriched to a greater extent than alkaline phosphatase they may be better surface membrane markers than alkaline phosphatase. It is estimated that one third of the alkaline phosphatase present in schistosomes is in the surface membrane (Roberts *et al*, 1983). This means that it may only be used as a surface membrane marker with care. The existance of an ATPase in the surface membrane preparation of Simpson *et al* (1980) with an enrichment three times that of alkaline phosphatase may mean that this ATPase is unique to the surface membrane and should therefore be an ideal marker. Recent evidence suggests that ATPases are found in the

surface membrane and the basal membrane of the tegument and that each membrane has a distinct ATPase (Podesta and McDiarmid, 1982).

Cesari, Torpier and Caproni (1983) used polycationic beads to isolate the surface membrane in a technique which took only 30 minutes. The preparation was enriched 11 times in alkaline phosphatase, 20 times in alkaline phosphodiesterase and 10 times in Ca^{++}-ATPase, relative to whole worms. It is likely that contamination of the preparation took place when the surface membrane was sheared, since we know much greater enrichment of alkaline phosphatase is possible. This contamination might be reduced by blocking free binding sites on the beads with anions after binding the beads to the surface membrane and before shearing the membrane (Jacobson, 1980). The potential of this technique remains unfulfilled but it may yet offer a method of surface membrane isolation which combines rapidity with a highly enriched preparation.

The use of saponin and calcium chloride by Kusel (1972) and Podesta and McDiarmid (1982) is difficult to compare with the present study. The latter authors used sequential incubation of worms in saponin plus calcium chloride to remove first the surface membrane and then the basal membrane of the tegument. They compared ATPases in the two preparations but gave no indication of enrichments. Some observations on saponin may be relevant at this stage. Saponin is irreversibly bound by membranes (Segal, Milo-Goldzweig and Seiffe, 1972) and probably forms complexes with cholesterol (Glauert, Dingle and Lucy, 1962) and proteins (Assa, Shany, Gestetner, Tencer, Birk and Bondi, 1973). This

may be the root of the difficulties mentioned by Knowles and Oaks (1979) in characterisation of *H diminuta* membranes released by saponin. Kusel (1972) noted some differences in components of membranes isolated using freeze-thaw and saponin, when separated by polyacrylamide gel electrophoresis and attributed these to the binding of saponin to membrane lipids. The difficulties in interpretation of results make the use of saponin in surface membrane isolation undesirable.

Sequential incubation of worms in digitonin apparently allowed extraction of first the outer then the inner bilayer of the surface membrane (McDiarmid, Dean and Podesta, 1983). The outer bilayer was labelled with ^{125}I and most of the radioactivity was removed from the worms during the first incubation. Alkaline phosphatase release was much greater during the second incubation than during the first, suggesting this enzyme is located in the inner bilayer. Unfortunately, relative enrichment of the enzyme in the inner bilayer preparation was not calculated.

Oaks *et al* (1981) used externally applied ^{3}H-Concanavalin A as the only quantifiable surface membrane marker. After treatment of worms with Triton X-100 and centrifugation of the medium they achieved an enrichment of Concanavalin A 116 times that of whole worms. This apparently suggests considerable enrichment in surface membranes. More recently Oaks *et al* (1983) used surface membrane enzyme markers to assess enrichment after isolation of the surface membrane using the Triton X-100 method. They found that alkaline phosphatase was enriched only four times

and ATPase only three times relative to whole worms. These figures compare very poorly with other methods for which enrichment data are available. The huge disparity between the enrichment in the surface membrane fraction of externally applied Concanavalin A and enzyme markers may be explained by the properties of lectins.

Specific lectin-binding to sugar molecules can be competitively inhibited by addition of appropriate saccharides. This property is exploited in the purification of lectins (Gordon, Blumberg, Lis and Sharon, 1972; Lotan, Skutelsky, Danon and Sharon, 1975) and in lectin affinity chromatography (Lotan and Nicolson, 1979). The schistosome tegument is believed to be rich in mucopolysaccharide (Wilson and Barnes, 1974a) and on rupturing the surface membranes a large source of saccharides would be released. This may account for the quantities of ^{3}H-concanavalin A which were not sedimented by centrifugation at 30 000 x *g* for one hour described as a "significant amount" (Oaks *et al*, 1981). If the label dissociates from the surface membrane it may reassociate with an appropriate exposed sugar. This is likely to include many membranes other than surface membranes. Since the length of incubation in Triton X-100 is necessarily geared to maximum release of marker it is possible that non-surface membranes will be labelled and liberated. These would then be isolated along with surface membranes. Dissociation and reassociation of radiolabelled Concanavalin A following surface membrane disruption has been reported by Roberts *et al* (1983) and McDiarmid *et al* (1983). Labelled lectins therefore appear to be poor markers for the schistosome surface membrane and results obtained using them

must be interpreted with care.

A possible drawback to the use of detergents in the disruption of worm surfaces is that selective solubilisation of worm components may occur. It is known for example, that Triton X-100 solubilises different proteins on the same membrane to different extents (Yu, Fischman and Steck, 1973; Critchley, Howell and Eichholz, 1975; Louvard, Marroux, Vannier and Desnuelle, 1975). Phospholipids are solubilised by Triton X-100 more readily than proteins (Sohn and Marrinetti, 1974). This could lead to severe difficulties of interpretation in the analysis of membrane components. It is possible that important constituents of the membrane would not be discovered because of their removal from the membrane. More likely, the partial extraction of particular proteins might lead to major components being classified as minor ones and *vice versa*. Analysis of membrane constituents would thus be difficult where this method of membrane denudation was used.

It must be pointed out that freezing and thawing also has deleterious effects on membranes. Membrane-bound antigens can be modified (Greiff, Strong and Seifert, 1971) and enzymes may be inactivated (Santarius and Heber, 1970) or stimulated (Takehara and Rowe, 1971).

No method of surface membrane disruption is likely to give ideal results. With freeze-thaw it is inevitable that the whole worm will be damaged to some extent. On the other hand, the use of detergents, calcium chloride or PBS appear to allow some control over the amount of damage. Worms need only remain in, for

example, detergent solutions long enough for the surface membranes to be damaged sufficiently to allow subsequent removal by agitation. In practice this may not be as simple as it appears as the surface membranes of different worms will inevitably be breached at different times and so underlying membranes may also be affected by detergents. From microscopical analysis it appears that freeze-thaw has a considerably greater effect on ultrastructure than have detergents or calcium chloride. Following removal of surface membranes with Triton X-100 or calcium chloride (Oaks *et al*, 1983) ultrastructure of the denuded worm body was apparently well preserved. As we have seen this is not the case with freezing and thawing. In spite of this, greater enrichment in surface membrane marker enzyme has been found using freeze-thaw in the present study than that found with any of the apparently less damaging methods of surface membrane disruption.

1.4.7 Conclusion

From the evidence available, it seems that the final method described for isolation of surface membranes from schistosomes provides a preparation which compares favourably with those obtained using other methods. Enrichment in the surface membrane marker enzyme, alkaline phosphatase, is high. The main constraint on further improvement in enrichment is probably damage caused by freezing and thawing worms. This excessively damages parasites as indicated by release of non-tegumental enzymes and may have led to contamination of the surface membrane preparation with membranes from within the worms. Greater purity might be achieved if the damage caused by freezing and thawing

worms could be reduced.

CHAPTER TWO

ALTERATION OF CONDITIONS OF FREEZING AND THAWING DURING ISOLATION OF SURFACE MEMBRANES

2.1 INTRODUCTION

The previous chapter gave details of attempts to isolate surface membranes from *Schistosoma mansoni* by using a method of disruption involving freezing and thawing of worms. The freeze-thaw process damaged worms considerably. Parenchymal cell membranes were broken and it seemed likely that sub-cellular organelles from within worms were released along with surface membranes. Many membrane-bound vesicles were found to co-purify with surface membranes and these may have been derived from cells within the worm body. Thus, as a result of freeze-thaw damage, surface membrane preparations were contaminated. Freeze-thaw was necessary in order to liberate the surface membranes but it damaged worms excessively.

Freeze-thaw damage to cells has been the subject of much research, particularly in relation to the storage of frozen cells and tissues. The cryopreservation of parasites has recently been reviewed by James (1980a). Storage of frozen trypanosomes (Lumsden, 1972) is now commonplace and recently, attempts have been made to cryopreserve juvenile schistosomes, newly transformed schistosomula (James and Farrant, 1977; Stirewalt, Lewis and Murrel, 1979; James, 1979, 1980b, 1981). In the most successful of these techniques, half of the infectivity of unfrozen schistosomula was achieved by injecting thawed parasites into mice (James, 1981).

There are therefore good reasons to believe that the excessive damage occuring to adult schistosomes as a result of

freezing and thawing may be reduced, or even prevented, by alteration of the conditions of freeze-thaw. Experiments designed to reduce freeze-thaw damage were conducted. Their object was to minimise release of markers for damage, while maintaining high alkaline phosphatase release. Damage was assessed by assaying protein, NADH cytochrome c reductase and protease release. Alkaline phosphatase was also assayed to show the extent of surface membrane denudation. Results in Table 6 were taken as a reference standard.

2.2 MATERIALS AND METHODS

Individual experiments are described in section 2.3, Experiments and Results. The present section contains details of experimental procedures commonly used.

2.2.1 Chemicals

Chemicals used were usually Fisons analytical reagents. Polyvinylpyrrolidone (M_r 44 000) was of laboratory reagent grade, obtained from BDH. Ethan-1,2-diol (ethylene glycol) was obtained from Fluka.

2.2.2 Containers used to hold adult schistosomes during freezing and thawing

Frequently, adult schistosomes were frozen in a container formed from either stainless steel mesh, or nylon mesh. Stainless steel containers were made from 4 cm x 4 cm squares of wire mesh (mesh size 200 holes/inch). The edges were folded under to keep sharp ends of wires away from the parasites. A small fold was made in two opposite sides of each boat to form a dip in the middle. Thus, stainless steel boats had shallow sloping sides and no corners.

Nylon boats were made from Nybolt 13N (Stannier) with an aperture size of 100 µm. Squares of Nybolt (3.5 cm x 3.5 cm) were made into trays with approximately 2 mm high sides, by pinching and applying heat at the corners.

2.2.3 Treatment of the Parasites

Worms frozen on boats were normally transferred to Hanks balanced salt solution (HBSS) on ice and allowed to thaw for 30 minutes. The container was then upturned over a funnel held over a plastic test tube and worms were gently washed into the tube using HBSS squirted from a Pasteur pipette. Worms were then rinsed in HBSS, vortexed 10 times and rinsed again. Bodies were then homogenised. On some occassions, the vortex supernatant was sedimented at 100 x *g*, as described in Chapter 1. Fractions were assayed for alkaline phosphatase, protein, NADH cytochrome c reductase and protease, as described in Chapter 1.

2.3 EXPERIMENTS AND RESULTS

2.3.1 The effect of increased cooling rate on release of markers

It was considered that the use of cryoprotective agents would probably be the only way to reduce significantly the freeze-thaw damage to worms. However, since the effects of these materials on membrane constituents were not known, it seemed prudent first to attempt to reduce damage by altering the rate at which worms were cooled.

Parasites cooled in a test-tube are insulated by the tube, by the buffer in which they are suspended and by other worms around them. The rate of cooling is therefore slow and non-uniform.

In order to increase and standardise the cooling rate, 250 worm pairs were spread over the surface of a stainless steel-mesh container and frozen by plunging the container into coolant. The worms were therefore in direct contact with coolant and were kept in coolant until bubbling ceased. The worms were thawed by placing the boat in 15 ml HBSS at $4^{0}C$ in a Petri dish on ice and leaving it there for 30 minutes. An aliquot of the medium was stored for assay. Worms were transferred to a test-tube and rinsed with HBSS by repeated careful addition and removal of HBSS until the removed supernatant had a volume of 10 ml. An aliquot of the supernatant was stored for assay. The volume of HBSS in the tube was taken to 3 ml and the tube was vortexed as described in Chapter 1. The vortex

supernatant was sampled for assay. The denuded worms were homogenised and the protein and alkaline phosphatase content of the homogenate was assayed.

Table 7 shows the results of protein and alkaline phosphatase assays of the samples for the two coolants used, liquid nitrogen (-196^{0}C) and partly thawed nitrogen (-210^{0}C). In neither case was release of protein or alkaline phosphatase reduced. In fact, release of both was higher than normally found when worms were frozen in tubes. This suggested even more damage had occurred in the present experiments than in experiments using conventional freeze-thaw in tubes. Small patches of haematin were observed during thawing and some worms were found to be broken transversally into pieces. Thus it was clear that these conditions were of no use in reducing damage to worms.

2.3.2 The effect of external cryoprotectant

Low molecular weight cryoprotective agents enter cells and some are known to affect cell physiology (Lyman, Preister and Papahadjopoulos, 1976; Travers, 1974). High molecular weight cryoprotectants have the advantage that they do not readily penetrate tissues. They should therefore have fewer side effects than penetrating cryoprotectants. Some experiments were conducted using the external cryoprotective agent, polyvinyl-pyrrolidone (PVP).

2.3.2.1. Worms in stainless steel mesh boats were coated with

Table 7. Effect of increased cooling rate on release of protein and alkaline phosphatase*.

	% protein		% alkaline phosphatase	
	liquid N_2	N_2-ice	liquid N_2	N_2-ice
thawing solution	20.6	14.5	16.5	2.9
rinsing solution	13.2	5.9	12.9	1.0
vortex supernatant	12.5	16.7	12.6	34.9
denuded worm homogenate	53.8	62.9	58.1	61.2

* Adult worms were layered on a stainless steel mesh which was plunged into either liquid nitrogen or partly thawed nitrogen.

25 % (w/v) or 50 % (w/v) PVP by dipping the boat into PVP solution and rapidly draining on tissues. The boat was then plunged into liquid nitrogen and worms were thereafter treated as described in Materials and Methods.

Some worms were found on thawing to have broken transversally into two or more pieces. Assay results are shown in Table 8 . Alkaline phosphatase release was at the expected maximum, assuming liberation of all surface membrane, with either concentration of PVP. Protein release was unusually high and the proportion of protein which appeared in the thawing medium was particularly high. Protein concentration had been determined using ninhydrin and a test showed that with this assay, polyvinylpyrrolidone reacted positively. Another test of PVP reactivity was carried out using the protein determination method of Lowry *et al* (1951), and it was found that quantities of PVP as low as 1 µg were detectable. This assay yields a blue colour with protein, but a green colour developed when PVP solutions were assayed.

2.3.2.2. Some further cryoprotection experiments were carried out using 50 % PVP and the protein assay of Lowry *et al* (1951). The results are shown in Table 9 . Overall release of protein was apparently 28 %, which was about the same as found when worms were frozen in tubes. Actual protein release may have been lower than this, however. Release of alkaline phosphatase appeared to be reduced by pre-treatment of worms with PVP.

Table 8. Effect of polyvinylpyrrolidone on release of protein and alkaline phosphatase during freeze-thaw and vortexing*.

	% protein		% alkaline phosphatase	
	25 % PVP	50 % PVP	25 % PVP	50 % PVP
thawing solution	29.8	37.0	3.0	1.8
rinsing solution	10.1	6.2	11.2	2.3
vortex supernatant	12.6	11.7	21.4	30.1
denuded worm homogenate	47.5	45.2	64.4	65.7

* Worms were coated with a solution of 25 % (w/v) or 50 % (w/v) polyvinylpyrrolidone then plunged into liquid nitrogen.

Table 9. Release of protein and alkaline phosphatase from worms treated with 50 % (w/v) polyvinylpyrrolidone before freeze-thaw and vortexing.

	% protein*	% alkaline phosphatase
thawing solution	9.7 ± 0.8†	1.1 ± 0.2
rinsing solution	5.8 ± 1.2	2.0 ± 1.0
vortex supernatant	13.0 ± 0.8	19.5 ± 0.9
denuded worm homogenate	71.6 ± 2.7	77.3 ± 0.8

* Protein concentration was determined by the method of Lowry *et al* (1951).

† standard error, n = 3.

2.3.3 The effect of internal cryoprotectants

The use of internal cryoprotectants requires incubation of tissues in medium containing the compound in order to allow it to penetrate. Before internal cryoprotectants were tested for their ability to reduce release of markers for damage, the effects of incubating worms in HBSS without additives were assessed.

2.3.3.1. Worms were maintained in HBSS on ice for 30 minutes, then frozen on a nylon mesh boat and thereafter treated as before. The incubation medium was sampled and the thawing and first rinsing buffer combined and sampled.

Results are shown in Table 10 . Some protein and gut protease were released during the incubation period. This could be due to regurgitation of gut contents. Total release of markers after freeze-thaw was about the same as found using conventional freeze-thaw in tubes (Chapter 1), except for cyto-chrome c reductase which was higher. As in previous experiments described in this chapter, some worms were found upon thawing to have broken into pieces.

2.3.3.2. Experiments were then conducted using penetrating cryoprotective agents. Worms were incubated in 20 % (v/v) ethanediol or 5 % (v/v) dimethylsulphoxide or 5 % (v/v) dimethyl-sulphoxide/7.5 % (w/v) sucrose in HBSS for 30 minutes. They were then frozen on nylon mesh boats and treated as before. In all cases some worms broke into pieces. Results of assays are shown in Table 11 . The cryoprotectants themselves may have influenced

Table 10. The effect of incubating worms in Hanks' balanced salt solution on release of markers during freeze-thaw and vortexing.

	% alkaline phosphatase	% protein	% cytochrome c reductase	% protease
incubation medium	0.0	0.6	0.0	4.3
freeze-thaw supernatant	2.2	17.9	26.6	25.4
vortex supernatant	24.4	15.7	14.1	30.5
denuded worm homogenate	73.4	66.3	59.2	39.8

Table 11. Effect of internal cryoprotectants on release of markers during freeze-thaw and vortexing.

	% alkaline phosphatase	% protein	% cytochrome c reductase	% protease
20 % (v/v) ethan-1,2-diol				
incubation medium	0.0	1.6	11.8	1.6
freeze-thaw supernatant	1.1	7.8	12.5	14.1
vortex supernatant	25.5	12.3	13.6	17.5
denuded worm homogenate	73.4	78.3	62.1	66.8
5 % (v/v) dimethyl sulphoxide				
incubation medium	0.0	0.6	5.9	0.4
freeze-thaw supernatant	3.0	12.7	13.8	26.0
vortex supernatant	20.6	8.9	2.5	18.7
denuded worm homogenate	76.5	77.8	77.8	54.9

contd....

Table 11 contd.

5 % (v/v) dimethylsulphoxide, 7 % (w/v) sucrose	% alkaline phosphatase	% protein	% cytochrome c reductase	% protease
incubation medium	0.0	0.5	1.2	1.4
freeze-thaw supernatant	3.2	16.8	9.5	37.1
vortex supernatant	21.4	6.8	4.6	21.2
denuded worm homogenate	75.5	75.9	84.7	40.3

assays since, for example, DMSO was found to depress alkaline phosphatase activity and stimulate protease, whilst ethanediol increased the apparent activity of NADH cytochrome c reductase. These effects would be greatest where concentration of cryoprotectant was highest, ie in the incubation medium.

None of the results was particularly good as there was little reduction in overall release of markers for damage compared to worms frozen in tubes without treatment with cryoprotectant. Cytochrome c reductase activity in the incubation medium was very high when ethanediol or 5 % DMSO was used as cryoprotectant and this suggested that worms were damaged by the conditions of incubation.

2.3.4 Two-step addition of ethan-1,2-diol to worms prior to freezing

Recent success by James (1981) in the cryopreservation of schistosomula, was due to the use of ethanediol in a two-step incubation. That author firstly incubated schistosomula in 10 % (v/v) ethanediol for 10 minutes at 37°C and 5 minutes at 0°C and secondly for 10 minutes in 35 % (v/v) ethanediol at 0°C. This regime aids penetration of ethanediol while circumventing its toxic effects at 37°C. It seemed prudent to use this technique to reduce freeze-thaw damage in adult schistosomes.

2.3.4.1. Until this point, cryoprotection experiments had always used direct contact between worms and coolant, using boats.

Boats were useful with external cryoprotectants but were not really necessary with internal ones, so in this experiment worms were frozen in tubes.

Worms were incubated in 5 % (w/v) ethanediol in Eagle's medium at room temperature for 30 minutes (initial experiments showed that 10 % ethanediol was toxic to adult schistosomes, as judged by motility, even though this was the concentration used by James (1981) on schistosomula). The worms were then transferred to a plastic test-tube on ice and 35 % ethanediol in HBSS was added. The parasites were frozen in 2 ml of this medium, then thawed. They were rinsed with HBSS and the worms were vibrated by vortexing 10 times.

Results of assays are shown in Table 12 . Release of all markers was much reduced compared to conventional freeze-thaw without cryoprotectant. Unfortunately, this included alkaline phosphatase release. Clearly, liberation of only 6 % of alkaline phosphatase is of no use in surface membrane isolation. This cryoprotective technique was too effective.

2.3.4.2. A method was needed which protected internal cells but still exposed the tegument to the full effects of freezing and thawing. Worms, infiltrated with cryoprotectant but frozen on boats, might represent a solution to this problem.

Worms were incubated in 5 % ethanediol in Eagle's medium for 30 minutes, then in 35 % ethanediol in HBSS on ice for one hour. The second incubation was prolonged in order to ensure

Table 12. Release of markers from worms treated with ethan-1,2-diol in a two-step process before freeze-thaw and vortexing.

	% alkaline phosphatase	% protein	% cytochrome c reductase	% protease
incubation medium	0.0	0.1	0.0	0.8
freeze-thaw supernatant	0.0	0.0	0.0	0.0
vortex supernatant	5.9	7.5	7.0	10.7
denuded worm homogenate	94.1	92.4	93.0	88.4

adequate penetration of cryoprotectant. Worms were then frozen on a nylon boat and fractions recovered as previously.

Breakage of worms again occurred and was estimated to affect about one quarter of the parasites. Results of assays are shown in Table 13 . Alkaline phosphatase release was high and release of other markers was low. Thus, despite worm breakage, this was a favourable result and so the technique was examined more fully.

The experiment was repeated and this time the vortex supernatant was sedimented by centrifugation at 100 x *g*. Results are shown in Table 14 . Release of markers for damage was low again and alkaline phosphatase release high. However, it was found that relative enrichment of alkaline phosphatase in the pellet was only 13.5 times that of whole worms. Thus, the result was no better than found with conventional freeze-thaw in tubes, without cryoprotective agents.

One explanation for this low enrichment might be that surface membranes were released in smaller fragments and were therefore less easily sedimented. This seemed to be borne out by the comparatively low proportion of alkaline phosphatase found in the pellet (with conventional freeze-thaw, 13 % of alkaline phosphatase was recovered in the pellet derived from the vortex supernatant, here it was only 5.4 %). Another possibility was that a previously unencountered source of easily sedimentable protein was released because of damage induced by the conditions of incubation. For example, nuclei or muscle

Table 13. Release of markers from worms treated with ethan-1,2-diol in a two-step process before freeze-thaw and vortexing. Worms frozen on nylon mesh.

	% alkaline phosphatase	% protein	% NADH cytochrome c reductase	% protease
incubation medium	0.4	1.0	0.0	1.2
freeze-thaw supernatant	6.0	4.3	2.7	7.4
vortex supernatant	25.6	3.3	2.4	0.0
denuded worm homogenate	68.0	91.4	94.9	91.3

Table 14. Surface membrane isolation by freeze-thaw and vortexing of worms treated by the two-step addition of ethanediol.

	alkaline phosphatase			protein	cytochrome c reductase		protease	
	%	specific* activity	relative† enrichment	%	%	specific* activity	%	specific* activity
incubation medium	0.0	-	-	1.3	3.3	0.927	0.5	0.190
freeze-thaw supernatant	3.2	0.101	0.82	3.9	3.3	0.327	14.5	1.884
vortex supernatant (VS)	23.0	0.498	4.05	5.7	1.4	0.092	14.1	1.241
denuded worm homogenate	73.8	0.102	0.83	89.1	92.0	0.390	70.9	0.398
total		0.123				0.387		0.500
VS 100 x *g* supernatant	11.1	0.233	1.89	5.9	2.3	0.109	15.1	1.289
VS 100 x *g* pellet	5.4	1.659	13.49	0.4	0.0	0.0	0.3	0.415

* enzyme units/mg protein

† specific activity in fraction / total specific activity

might have been released.

2.3.4.3. In order to test the theory that the incubation conditions might have damaged worms, some parasites were incubated in 5 % then 35 % ethanediol, as before. Then *without freezing* they were transferred to a plastic test-tube and vortexed 10 times.

Results (Table 15) indicated that either cryoprotectant or HBSS must have weakened worms, since markers were released. Liberation of 15 % of alkaline phosphatase suggested that the main site of damage, under these conditions, was the tegument.

2.3.4.4. It was not clear whether this pre-freezing damage was due to HBSS or ethanediol. Therefore, an experiment was conducted in which worms were incubated in 5 % ethanediol in Eagle's medium for 30 minutes at room temperature, then 35 % ethanediol in Eagle's medium (instead of HBSS). They were rapidly rinsed five times in HBSS alone, using a suction pump to remove medium and a Pasteur pipette to add fresh Hanks' balanced salt solution. There were two reasons for this rapid rinse in HBSS. Eagle's medium contains amino acids which would have interfered with the protein assay so it was desirable to dispose of this before freezing. In addition it was believed that this rapid wash might selectively remove ethanediol from the tegument, thereby rendering it more readily disruptable by freeze-thaw.

The results are shown in Table 16. Release of markers

Table 15. Effect of ethanediol in HBSS on worms in the absence of freezing.

	alkaline phosphatase			protein	cytochrome c reductase		protease	
	%	specific activity	relative enrichment	%	%	specific activity	%	specific activity
incubation medium	1.6	0.182	0.81	1.9	0.9	0.436	2.4	1.750
vortex supernatant (VS)	13.1	0.847	3.78	3.5	0.9	0.244	2.9	1.163
denuded worm homogenate	85.3	0.202	0.90	94.6	98.2	0.967	94.7	1.410
total		0.224				0.931		1.408
VS 100 x *g* supernatant	5.5	0.482	2.15	2.6	0.5	0.187	3.7	2.001
VS 100 x *g* pellet	3.4	2.304	10.29	0.3	0.1	0.267	0.0	0.161

Table 16. Distribution of markers in fractions produced by isolation of surface membranes from parasites incubated in ethanediol in Eagle's medium then rinsed in HBSS before freezing.

	alkaline phosphatase			protein	cytochrome c reductase		protease	
	%	specific activity	relative enrichment	%	%	specific activity	%	specific activity
freeze-thaw supernatant	4.9	0.045	0.32	15.4	11.1	0.179	37.7	1.209
vortex supernatant (VS)	21.9	0.382	2.71	8.1	0.0	-	12.6	0.765
denuded worm homogenate	73.2	0.135	0.96	76.6	88.9	0.287	49.7	0.319
total		0.141				0.247		0.492
VS 100 x *g* supernatant	13.5	0.299	2.12	6.4	0.0	-	9.8	0.752
VS 100 x *g* pellet	2.4	0.906	6.43	0.4	0.0	-	0.3	0.422

for damage was high. The brief rinse in HBSS prior to freezing may have done more harm than good. It was unclear whether or not replacement of HBSS with Eagle's medium had any effect on worms. The next experiment was designed to clarify this point.

2.3.4.5. Worms were incubated as before, in 5 % ethanediol in Eagle's medium at room temperature for 30 minutes, then in 35 % ethanediol in Eagle's medium for 60 minutes at 0^0C. They were transferred to a test-tube then rinsed in 35 % ethanediol in HBSS and vortexed 10 times. The supernatant was removed and the worms frozen on a nylon mesh boat, thawed and vortexed again. There was little release of markers during the first vortexing (Table 17) and this appeared to indicate that the previously encountered pre-freezing damage was due to Hanks' balanced salt solution rather than ethanediol. Release of markers for damage during the second vortexing step, was high but this may be explained by the fact that worms remained in HBSS plus 35 % ethanediol for 15 minutes whilst the first vortexing and removal of the first vortex supernatant took place. The damage caused by HBSS may have been responsible for the enrichment of alkaline phosphatase in the pellet being only 18 times that of whole worms.

2.3.4.6. In the next experiment, contact with HBSS was kept to a minimum. Worms were taken through the two-step incubation with ethanediol in Eagle's medium, then rapidly rinsed in 35 % ethanediol in HBSS and frozen on a nylon mesh boat. Exposure to HBSS was measured as 75 seconds. Fractions were recovered as previously and results are shown in Table 18 .

Table 17. Effect of ethanediol in Eagle's medium before and after freezing worms.

	alkaline phosphatase			protein	cytochrome c reductase		protease	
	%	specific activity	relative enrichment	%	%	specific activity	%	specific activity
incubation medium	0.2	ND	ND	ND	3.5	ND	0.6	ND
1st vortex supernatant (1VS)*	3.2	0.638	3.71	0.9	4.2	0.970	0.0	-
freeze-thaw supernatant	4.9	0.085	0.49	10.0	10.0	0.236	21.1	1.044
2nd vortex supernatant (2VS)	30.8	0.620	3.60	8.5	4.5	0.102	17.0	0.983
denuded worm homogenate	61.0	0.130	0.76	80.6	75.9	0.185	61.3	0.375
total		0.172				0.196		0.493
2VS 100 x *g* supernatant	14.1	0.312	1.81	7.8	3.8	0.096	12.6	0.790
2VS 100 x *g* pellet	10.8	3.140	18.26	0.6	0.2	0.059	0.2	0.183

* After centrifugation of the first vortex supernatant at 100 x *g* no pellet was obtained.

ND Not determined due to the presence of amino acids in Eagle's medium.

Table 18. Reduction of freeze-thaw damage to worms by ethanediol in Eagle's medium.

	alkaline phosphatase			protein	cytochrome c reductase		protease	
	%	specific activity	relative enrichment	%	%	specific activity	%	specific activity
incubation medium	0.7	ND	ND	ND	0.3	ND	1.3	ND
freeze-thaw supernatant	6.7	0.210	1.17	5.8	0.0	-	14.7	2.843
vortex supernatant (VS)	28.1	0.713	3.96	7.1	0.0	-	8.3	1.294
denuded worm homogenate	64.5	0.134	0.74	87.1	99.7	0.216	75.6	0.962
total		0.180				0.189		1.108
VS 100 x *g* supernatant	16.1	0.464	2.58	6.3	0.0	-	1.7	0.307
VS 100 x *g* pellet	5.5	1.903	10.57	0.5	0.0	-	1.4	0.774

ND Not determined due to the presence of amino acids in Eagle's medium.

In terms of marker release, results were good. Alkaline phosphatase release was high and release of other markers was low. The apparently very low release of cytochrome c reductase may have been due to the use of fewer worms than usual. Enrichment of alkaline phosphatase in the pellet was only 10.6 times that found in whole worms. It was clear that some protection was afforded to tissues but despite this, enrichment of alkaline phosphatase in pellets remained low.

2.3.4.6. A further modification of the technique was made in the hope of reducing damage to worms. Worms were treated exactly as in the previous experiment (two-step incubation in ethanediol in Eagle's medium, rapid rinse in ethanediol in HBSS, freeze on boats) except that after freezing the worms were transferred, on the nylon mesh, to HBSS at 37°C (instead of ice-cold) in order to increase the thawing rate. Rapid thawing is known to reduce the danger of ice crystals growing during re-warming (Mazur, 1970).

Results (Table 19 and 20) appeared to show that increasing the thawing rate had some effect in reducing release of markers for damage (compare to previous experiments) although on the two occasions the experiments were performed, alkaline phosphatase release into the thawing medium was high. On one occasion the thawing medium was centrifuged at 100 x *g* but most of the alkaline phosphatase activity remained in the supernatant (Table 20). Low protein content probably caused the apparently high enrichment of alkaline phosphatase in the pellet derived from the vortex supernatant, as there was too little protein

Table 19. Effect of rapid thawing after freezing ethanediol-treated worms.(I)

	alkaline phosphatase			protein	protease	
	%	specific activity	relative enrichment	%	%	specific activity
incubation medium	0.2	ND	ND	ND	0.0	ND
freeze-thaw supernatant	18.6	0.485	4.11	4.5	5.8	0.883
vortex supernatant (VS)	19.5	0.871	7.38	2.6	1.7	0.447
denuded worm homogenate	61.7	0.079	0.67	92.7	92.5	0.693
total		0.118				0.689
VS 100 x *g* supernatant	9.3	0.505	4.28	2.2	1.5	0.477
VS 100 x *g* pellet	4.2	1.255	10.64	0.4	0.0	-

ND Not determined due to the presence of amino acids in Eagle's medium.

Table 20. Effect of rapid thawing after freezing ethanediol-treated worms.(II)

	alkaline phosphatase			protein
	%	specific activity	relative enrichment	%
incubation medium	0.0	ND	ND	ND
freeze-thaw supernatant (FTS)	14.2	1.594	8.70	1.6
vortex supernatant (VS)	21.4	1.482	8.10	2.7
denuded worm homogenate	64.4	0.125	0.68	95.7
total		0.183		
FTS 100 x *g* supernatant	14.9	0.929	5.08	2.9
FTS 100 x *g* pellet	1.7	-		0.0
VS 100 x *g* supernatant	15.4	0.806	4.40	3.5
VS 100 x *g* pellet	5.2	4.538	24.80	0.2

ND Not determined due to the presence of amino acids in the medium.

present to measure it precisely. Yield of surface membranes in the pellet was low, so this was not a particularly useful method of isolating surface membranes.

2.4 DISCUSSION

2.4.1 Collation of enzyme assay results

Some interesting information can be gained by collating the results of enzyme assays. Figures for total release of markers from all of the experiments quoted in this chapter (except those involving PVP) were plotted against each other. Graphs of alkaline phosphatase release against release of other marker (Figures 34-36) showed no obvious relationship. Thus release of alkaline phosphatase was independant of release of markers for damage, and this was clearly desirable. A primary objective of the cryoprotection experiments was therefore satisfied. Release of markers for damage was reduced by cryoprotection whilst release of the surface membrane marker was largely unaffected.

Total release of protein and protease (Fig 38), protein and NADH cytochrome c reductase (Fig 37), and protease and cytochrome c reductase (Fig 39) showed a linear relationship and a regression line through each set of points was significantly different from horizontal.

Thus, it was clear that in general, if a freeze-thaw schedule reduced release of one marker for damage, then it reduced all of them. This suggests that each of these markers was equally valid as an indicator of damage to worms. Deviations from linearity may show that particular freeze-thaw regimes gave a greater or lesser degree of protection to particular tissues.

The graph of protein release versus protease release is striking because all points lie very close to the regression

Figure 34. Release of alkaline phosphatase compared to release of protein. Data taken from Tables 7 and 10 - 20. The regression line $y = 4.951 + 0.498\ (\pm 0.322)^{\dagger}x$ is not significantly different to the horizontal.

Figure 35. Release of alkaline phosphatase compared to release of NADH cytochrome c reductase. Data taken from Tables 10 - 18.
The regression line $y = 8.457 + 0.286\ (\pm 0.498)^{\dagger}x$ is not significantly different to the horizontal.

Figure 36. Release of alkaline phosphatase compared to release of protease. The data is taken from Tables 10 - 19. The regression line $y = 24.396 + 0.254\ (\pm 0.668)^{\dagger}x$ is not significantly different to the horizontal.

† standard error of the regression coefficient.

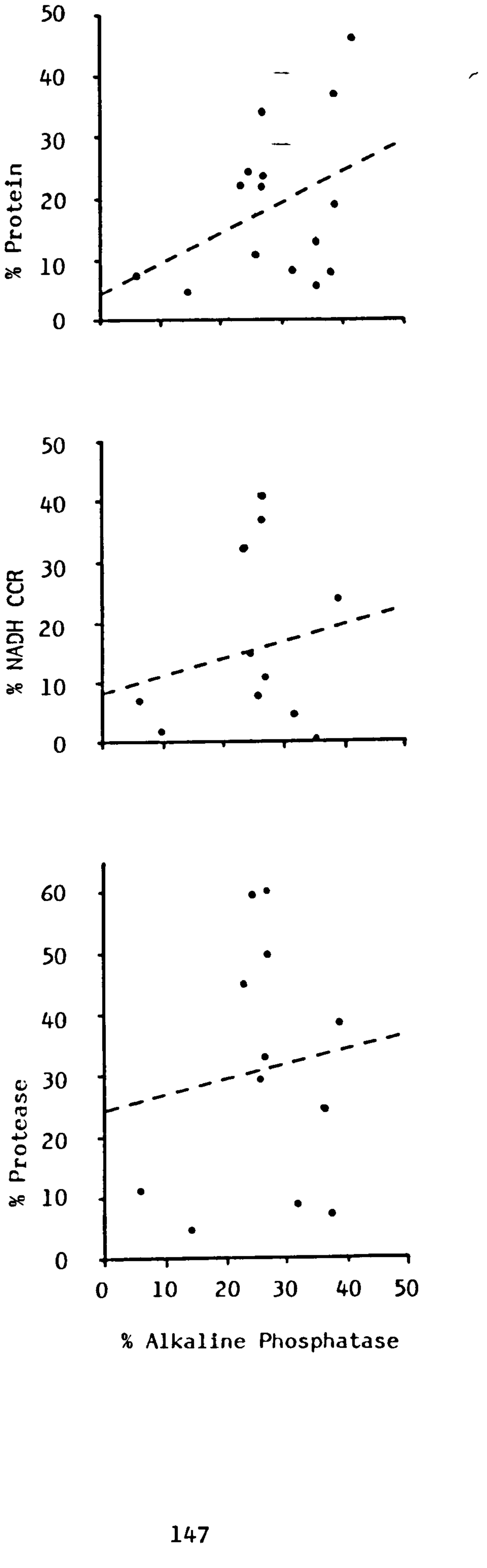
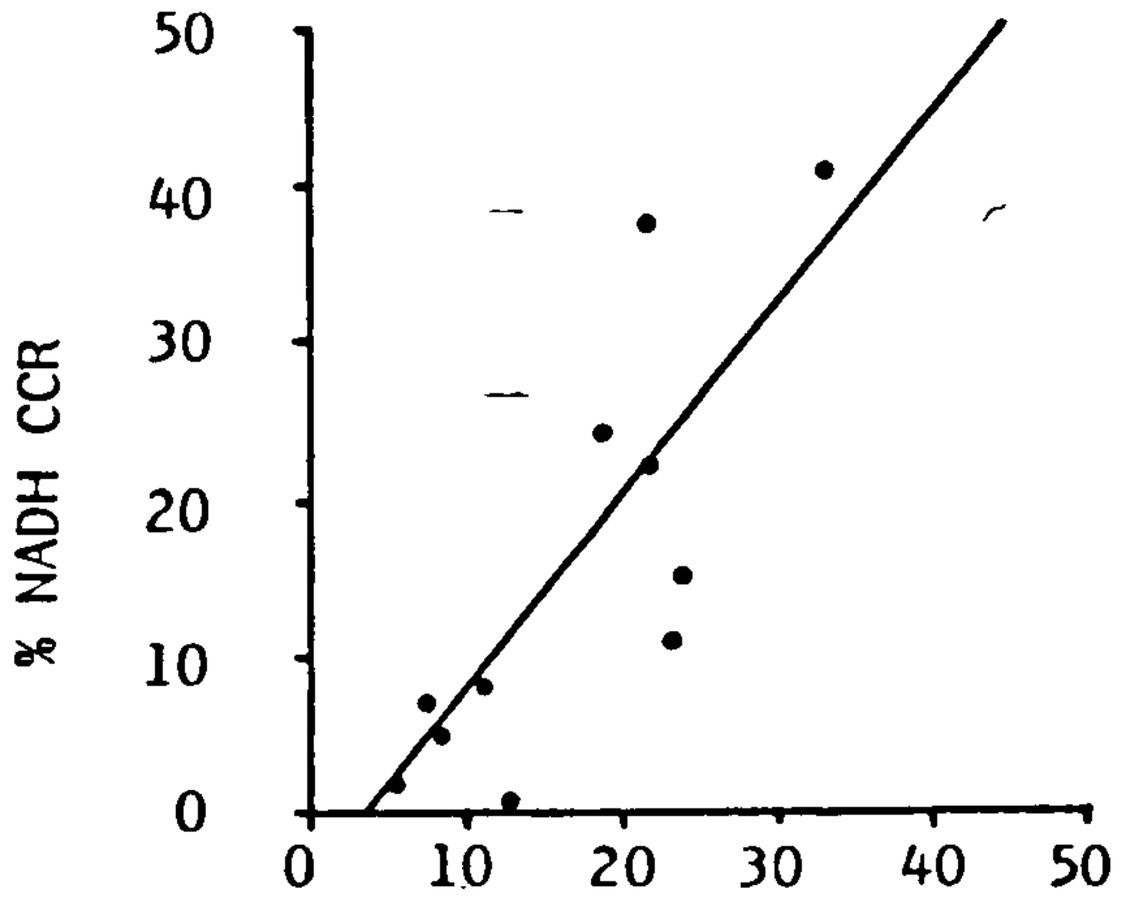

50
40
30
20
10
0
% Protein
50
40
30
20
10
0
% NADH CCR
60
50
40
30
20
10
0
% Protease
0
10
20
30
40
50
% Alkaline Phosphatase

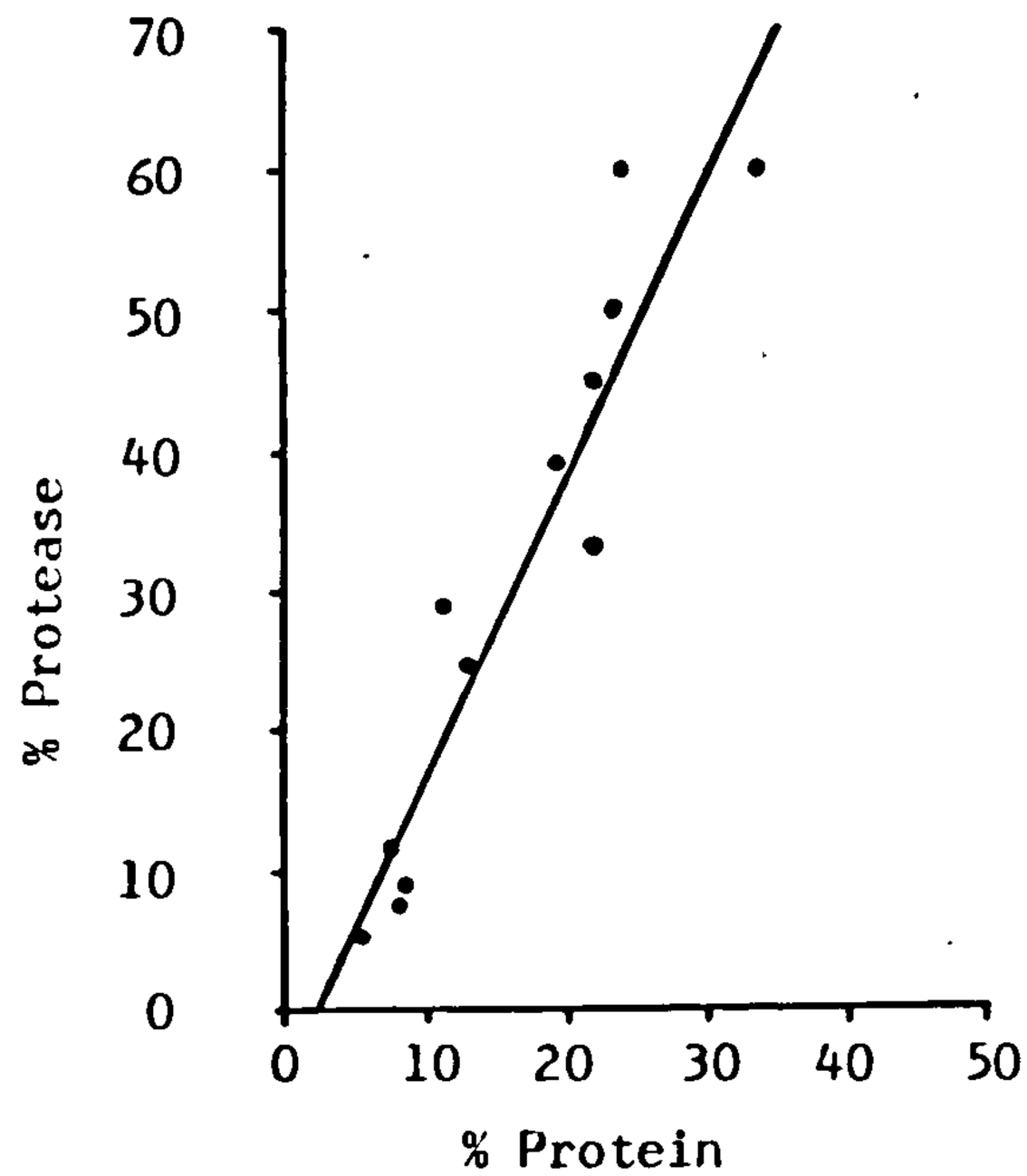
Figure 37. Release of protein compared to NADH cytochrome c reductase. The data is taken from Tables 10 - 18. The regression line $y = -4.435 + 1.205\ (\pm 0.311)^{\dagger}x$ is significantly different to the horizontal ($P<0.01$).

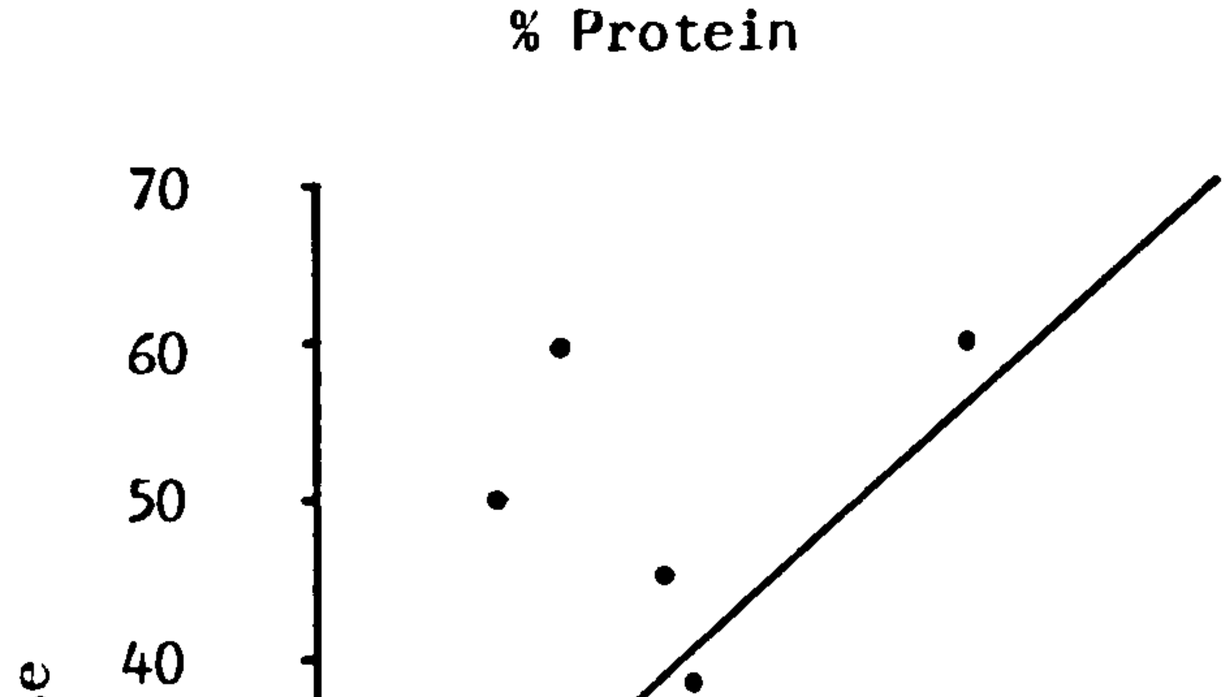
Figure 38. Release of protein compared to protease. The data is taken from Tables 10 - 19. The regression line $y = -4.491 + 2.160\ (\pm 0.239)^{\dagger}x$ is significantly different to the horizontal ($P<0.001$).

Figure 39. Release of NADH cytochrome c reductase compared to protease. The data is taken from Tables 10 - 18. The regression line $y = 19.314 + 0.886\ (\pm 0.365)^{\dagger}x$ is significantly different to the horizontal ($P<0.05$).

† standard error of the regression coefficient.


% NADH CCR
50
40
30
20
10
0
0 10 20 30 40 50
% Protein


% Protease
70
60
50
40
30
20
10
0
0 10 20 30 40 50
% Protein


% Protease
70
60
50
40
30
20
10
0
0 10 20 30 40 50
% NADH CCR

line. Since the gut protease is soluble and most of the protein released is soluble, protease may be typical of all soluble proteins in the worm. It seems that cryoprotection had a particularly marked effect on reducing the release of soluble components from worms.

2.4.2 Increased cooling rate

Increasing the cooling rate by freezing worms in direct contact with coolant appeared to cause more damage than caused by slower cooling in tubes. Although no attempt was made to measure cooling rate, it may be that it was too high. Rapidly cooled cells, before thawing, generally contain intracellular ice (Farrant, 1980) and on re-warming, ice crystals redistribute or grow to form larger crystals (Farrant, Walker, Lee and McGann, 1977; Asahina, 1965) which injure cells (Mazur, 1966). Rapid freezing may in some circumstances, be useful as a fixative method for electron microscopy, but this is probably because only small ice crystals are formed. These do little damage, so cell structure is preserved when freeze-substitution takes place. If adult schistosomes are frozen in direct contact with coolant at the temperature of liquid nitrogen, then freeze-substituted and viewed by transmission electron microscopy, ice-crystal damage can be seen (Wilson and Barnes, 1977) so cooling worms in this way was unlikely to reduce the damage caused by slower cooling.

2.4.3 External cryoprotectants

The high molecular weight cryoprotective agent, polyvinyl-

pyrrolidone (PVP) is known to suppress formation of ice crystals in a wide variety of frozen tissues, examined by electron microscopy, when it is used at concentrations of 25 % (w/v) and above (Skaer, Franks, Asquith and Echlin, 1977). It was therefore expected that the use of this compound at a concentration of 25 % (w/v) or 50 % would prevent damage to worms. Since PVP reacted with both of the protein assays used, its effects on release of protein was not clear. The effect of PVP on release of alkaline phosphatase was found to be variable but it appeared that release of this enzyme during surface membrane isolation fell when worms were treated with PVP prior to freezing.

James (1980b) believes that high molecular weight cryoprotectants, including PVP, "confer no significant protection to schistosomula" and that penetration of cryoprotectant is necessary in order to protect schistosomula from the deleterious effects of storage at low temperature. It therefore seemed prudent to attempt to reduce freeze-thaw damage using penetrating cryoprotectants..

2.4.4 Internal cryoprotectants

(The two-step addition of ethanediol is considered in the next part of the discussion.) The failure of 5 % dimethylsulphoxide (DMSO), 5 % DMSO plus 7.5 % sucrose and 20 % ethanediol to protect worms may be due to the use of inappropriate cooling and rewarming rates. Five per cent DMSO is a commonly used cryoprotectant but higher concentrations may have been necessary to protect schistosomes from freeze-thaw damage.

Ethanediol does not penetrate tissues well at low temperatures and this may explain the failure of this concentration of the compound to protect adult schistosomes from freeze-thaw damage.

2.4.5 Two-step addition of ethanediol

Good overall protection from the deleterious effects of freezing and thawing was achieved when worms, treated sequentially with 5 % and 35 % ethanediol, were frozen in a test-tube while suspended in 35 % ethanediol in HBSS. The results obtained suggested that damage was only about one quarter of that found using standard freeze-thaw in tubes, without treatment of worms with cryoprotectant. This method is based on a technique used for long term storage of schistosomula (James, 1981). No attempts to cryopreserve adult schistosomes are known, but this technique would clearly be an excellent starting point for such attempts. Worms were not examined ultrastructurally but it is anticipated that this technique would lead to considerably less morphological disruption of worm tissues than conventional freeze-thaw described in Chapter 1.

It is interesting that surface membranes were more readily removed from worms frozen on boats than worms frozen in tubes, even though both were treated with the same cryoprotectant. This difference was presumably due to the different freezing rates. Cryoprotectants are known to be more effective against injury caused by slow cooling than by rapid cooling (Farrant, 1980). Thus, worms frozen on boats would be protected less than those frozen more slowly, suspended in buffer and held

inside tubes. The apparent localisation in the tegument of damage to worms frozen on boats may be explained in the same way since internal cells will cool more slowly than thosein direct contact with the coolant. Freezing worms on boats, after incubation in cryoprotectant, therefore allows protection of internal cells while the tegument or surface membrane is damaged. That the damage does not extend far into worm tissues is evidenced by the low release of protein, NADH cytochrome c reductase and protease.

2.4.5.1. Worm breakage. In all experiments in which worms were frozen in direct contact with coolant, some parasites were found upon thawing, to have broken into pieces. No quantification of the extent of breakage was made but it was clear from observations of worms under the dissecting microscope that less breakage occurred when the two-step addition of ethanediol was used, than when worms were frozen on boats but without prior incubation in cryoprotectant solutions. This phenomenon was never recorded when worms were frozen and thawed in tubes without cryoprotectant (as in Chapter 1) but a few broken worms were detected when parasites were frozen and thawed in tubes after penetration of 35 % ethanediol.

The approximate series of extent of breakage was, from greatest to least, no cryoprotectant, boat > two-step ethanediol, boat > two-step ethanediol, tube > no cryoprotection, tube.

The reasons for this breakage are not known, but it was clearly influenced by freezing rate and cryoprotectant. That the higher freezing rate led to greater breakage suggests it was due

to formation of intracellular ice. It is not known why this would cause worms to break transversally rather than to simply fragment. Considerable breakage occurred at the higher freezing rate in the presence of cryoprotectant. This implies that either dehydration was not sufficient to prevent formation of intracellular ice or that the freezing rate was inappropriate. As we have seen, cryoprotectants are not very effective against damage caused by rapid cooling.

Slower freezing without cryoprotectant led to least breakage although release of markers for damage was high (Chapter 1), suggesting that the factors responsible for breakage were not the same as those responsible for release of markers for damage. It may be that ice formation, although generally reduced, was localised at some specific sites when cryoprotectant and/or rapid freezing was used and that breakage occurred at these sites. Thus, when worms were frozen on boats without prior treatment with cryoprotectant, both generalised and localised damage occurred.

The breakage seen in worms treated with ethanediol and frozen in a tube may be similarly accounted for. It seems that cryoprotectant actually increased breakage although release of markers for damage was reduced. It may have done so by confining ice formation to specific sites.

The nature of these sites and the reasons for localisation of ice formation there are not known. Once intracellular ice nucleation occurs, it will spread to surrounding extracellular spaces by breaking the cell membrane (Mazur, 1970). Ice in the extracellular fluid might be expected to damage surrounding cells

since solutes would be concentrated in the extracellular spaces. If this "solution effect" (Mazur, 1970) lysed cells, ice formation would spread in all directions and eventually worms might be broken into pieces.

2.4.5.2. Pre-freezing Damage. The damage which occurred when worms were treated with ethanediol and vibrated without firstfreezing and thawing, was probably due to prolonged incubation in Hanks' balanced salt solution, since damage was much less when HBSS was replaced by Eagle's medium. Release of markers in both cases suggested that the tegument was the site of damage. Some alkaline phosphatase was released by vortexing even when Eagle's medium was used and this suggests HBSS was not wholly responsible for pre-freezing damage.

It is possible that ethanediol damaged the tegument, since many conditions of incubation cause alterations in tegument ultrastructure (see Chapter 1). A further possibility is that worms were damaged by the sudden cooling from room temperature to ice temperature since many cell types are known to be injured by rapid cooling to $0^{o}C$ (Farrant, 1980). The prospect that surface membranes might be released simply by rapid cooling without freezing, followed by vortexing, is an intriguing one. However, since only 3 % of alkaline phosphatase was released by vortexing after storage in Eagle's medium, this is clearly not a route to isolation of large quantities of surface membrane.

2.4.5.3. Enrichment of alkaline phosphatase in the pellet derived from the vortex supernatant. By varying the condition in

which freezing and thawing of worms took place, it was possible to reduce substantially release of protein, cytochromec reductase and protease. It was impossible however, to improve greatly the enrichment of alkaline phosphatase in the pellet derived from the vortex supernatant relative to that obtained using freeze-thaw in the absence of cryoprotection. Some information on the reasons for this can be obtained by collating results from all of the experiments using two-step addition of ethanediol, regardless of the precise conditions, and comparing them with results from the experiments detailed at the end of Chapter 1.

These results are shown in Table 21 . Significantly less alkaline phosphatase was sedimented by centrifugation of the vortex supernatant at 100 x *g* when worms were frozen on boats after two-step treatment with ethanediol, than when frozen in tubes without any cryoprotection. This shows that yield of surface membranes in the pellet was lower when cryoprotection was employed. More of the surface membrane was released as small fragments with cryoprotection (this fragmentation of surface membranes was apparently exacerbated when rapid thawing of cryoprotectant-treated worms was employed). The reasons for the greater fragmentation of the surface membrane in cryoprotection experiments with ethanediol are not known, but it may be that the tegument was weakened by the conditions of incubation.

Relative enrichment of alkaline phosphatase in the vortex supernatant was significantly greater in experiments using the two-step addition of ethanediol, compared to the method finally adopted in the previous chapter. A comparison of relative

Table 21. Comparison of freezing worms in tubes without cryoprotection and freezing worms on nylon mesh with the two-step addition of ethanediol.

	% vortex supernatant alkaline phosphatase in VS pellet	% vortex supernatant protein in VS pellet	relative enrichment of alkaline phosphatase in vortex supernatant	relative enrichment of alkaline phosphatase in VS pellet
Tubes, no cryoprotection*	$45.7 \pm 4.4^{\varepsilon}$	3.6 ± 0.4	1.3 ± 0.1	16.6 ± 0.4
Nylon mesh ethanediol†	$23.0 \pm 2.7^{\varepsilon}$	8.3 ± 1.3	4.2 ± 1.0	13.5 ± 2.3
P∫	<0.001	<0.01	<0.02	NS

* data from Table 6

† date from Tables 14 - 20

ε standard error, n = 7

∫ Students t-test, probability of the two means being different

NS not significant

enrichments of this enzyme in the pellets derived from the vortex supernatants under these two conditions revealed no significant difference between them. This can be explained by the smaller proportion of protein which sedimented when the vortex supernatant was centrifuged at 100 x *g*.

It is clearly more difficult to protect adult worms from freeze-thaw damage than it is one-hour schistosomula and this is almost certainly due to the difference in size. Perhaps, by using different cryoprotectives and different freezing and thawing rates, it would have been possible to improve enrichment of alkaline phosphatase in the pellet derived from the vortex supernatant, over that found in the present experiments. The extensive experimentation described, yielded little information to support this view, however. It was therefore concluded that the final method described in the previous chapter could not be improved by cryoprotection.

CHAPTER THREE

ISOLATION OF TEGUMENTAL ORGANELLES

3.1 INTRODUCTION

Previous chapters have detailed the development of a method for isolation of schistosome surface membranes. The finalised method involved suspending the worms in a balanced salt solution, then freezing them in liquid nitrogen. The worms were then thawed and the bodies rinsed, vortexed, then rinsed again. The vortex supernatant was centrifuged at 100 x *g* and the resultant pellet was centrifuged on a linear sucrose gradient to yield a preparation enriched in surface membrane.

In this procedure, much of the starting material was discarded in the interest of increasing enrichment of the product. Little attention was paid to the discarded fractions but it might be possible to obtain valuable information by investigating the previously unused material. For example, a white flocculent deposit appeared at the base of the sucrose gradient. This was clearly of high density but otherwise its nature was unknown. This material has been investigated by means of electron microscopy and polyacrylamide gel electrophoresis.

The freeze-thaw supernatant and the supernatant remaining after sedimentation of the surface membrane at 100 x *g* might be expected to contain cytoplasmic proteins and tegumental organelles. If it were possible to isolate tegumental organelles it might then be feasible to elucidate the functions of the tegument. Little is known of the function of the discoid granules which are, by far, the most numerous tegumental

inclusion. It should be possible, by examining the above mentioned supernatants, to discover the fate of the discoid granules during surface membrane isolation and to develop a method for their isolation. Their relationship to the rest of the tegument might then be established by comparison of proteins and glycoproteins.

A marker for discoid granules was needed during isolation experiments, but nothing is known of their enzyme complement. It was therefore necessary to look for other distinctive molecules in these organelles. They are known to be rich in carbohydrate (Wilson and Barnes, 1974a) and although carbohydrate is too ubiquitous to be an ideal marker, current knowledge of discoid granules presented no other potential marker substances and a carbohydrate assay was therefore adopted as an aid to fraction enrichment.

3.2 MATERIALS AND METHODS

Methods used previously are not repeated here. This section contains technical details of procedures. Individual experiments are described in 3.3 Experiments and Results.

3.2.1 Examination of High Density Material and Supernatants.

Electron microscopy was used to determine the morphological nature of high density material present at the base of the sucrose gradient during surface membrane isolation and to examine the particulate material left behind in the 100 x *g* supernatant.

Approximately 4 000 worm pairs, previously frozen in batches of 250 worm pairs, were thawed. The worms were rinsed to give a freeze-thaw supernatant and then vortexed ten times and rinsed again to yield a vortex supernatant. The latter was centrifuged at 100 x *g* for 30 minutes, then the pellet was resuspended in distilled water and centrifuged again. This osmotic shock was intended to free the small membraneous vesicles adhering to the surface membrane (Figure 30). The pellet was then resuspended in tris-buffered saline (10 mM tris, 0.9 % (w/v) NaCl, pH 7.4 (TBS)) and centrifuged on a 20-50 % (w/v) linear gradient of sucrose buffered with 10 mM tris-HCl, pH 7.4, for 90 minutes at 115 000 x *g*. The white flocculent material at the base of the gradient was recovered, resuspended in TBS and sedimented in an Eppendorf 3200 centrifuge. The pellet was prepared for electron microscopy as described below.

The freeze-thaw supernatant and the supernatant remaining after the 100 x *g* centrifugation were each centrifuged at 140 000 x *g* for 45 minutes. The pellets were prepared for electron microscopy as described below.

3.2.2 Centrifugation.

Centrifuges and rotors used for each sedimentation rate are shown below unless given in 1.2.3. Average and maximum relative centrifugal force (RCF) are shown but elsewhere only maximum RCF is given.

RCF		Centrifuge	Rotor	rpm
x g_{av}	x g_{max}			
7 000	10 000	MSE HS25	16 x 15 ml outer ring	9 000
95 000	140 000	Beckman L2-65B	SW27.1	27 000
110 000	150 000	Beckman L2-65B	Type 65	41 000

3.2.3 Electron Microscopy

Fixation and preparation of pellets for transmission electron microscopy was by the second of the methods described previously (1.2.4). Briefly, samples were fixed in 4 % (v/v) glutaraldehyde in 0.1 M cacodylate buffer, post fixed in 1 % (w/v) osmium tetroxide in the same buffer then fixed in 1 % (w/v) uranyl acetate in aqueous solution containing 45 mg/ml sucrose.

3.2.4 SDS-PAGE

When necessary, supernatants were concentrated by ultrafiltration using Amicon and Minicon systems. Samples for electrophoresis were reduced by boiling for 2 minutes in 10 mM dithiothreitol in 62.5 mM tris, 2 % (w/v) sodium dodecylsulphate, 5 % (w/v) glycerol and 0.0025 % (w/v) bromophenol blue, pH 6.8. Samples were then applied to slab gels of various concentrations of acrylamide. Gels were prepared and electrophoresis carried out by the method of Laemmli (1970).

3.2.4.1. Protein staining. Immediately after electrophoresis, polypeptides were fixed by immersion of the gel in 10 % (w/v) trichloroacetic acid in 40 % (v/v) methanol, then stained with 0.25 % (w/v) Coomassie Brilliant Blue R250 (Bio-Rad) in 45 % (v/v) methanol, 10 % (v/v) glacial acetic acid and destained in methanol/acetic acid in aqueous solution.

3.2.4.2. Glycoprotein staining. Two methods of glycoprotein staining were used. The first involved fixation of polypeptides using trichloroacetic acid as above, then staining was carried out using periodic acid and freshly prepared Schiff's reagent by the method of Fairbanks *et al* (1971).

On one occassion, glycoproteins were visualised by overlaying the gel with radio-iodinated Concanavalin A (Con A) followed by autoradiography. Iodination of Concanavalin A essentially followed the method of Tsu and Merzenberg (1980). One milligram of Con A was reacted with 500 µCi, carrier-

free $Na^{125}I$ (Amersham) using chloramine T. The reaction was stopped after 2 minutes with sodium metabisulphite and free ^{125}I removed by gel filtration using a Pharmacia PD-10 column. The recovered label was found to be 93 % TCA precipitable.

The overlay method was modified from that of J G Lindsay (personal communication) and Gurd and Evans (1976). The gel was washed in five changes of 40 % (v/v) methanol, 7 % (v/v) acetic acid over 24 hours, then equilibrated with 5 mM tris-HCl, pH 7.6, $4^{0}C$, using four changes over a period of four hours. The gel was then incubated at $4^{0}C$ with 10 million counts per minute ^{125}I-Con A in the same buffer containing 2 mg/ml haemoglobin, for 24 hours. It was then washed for three days with three changes per day of 5 mM tris-HCl, pH 7.6, containing 2mg/ml haemoglobin and 0.5 % (w/v) polyoxyethylene sorbitan monolaurate (Tween 20) at $4^{0}C$. The gel was then dried and autoradiographed.

Autoradiographs and stained gels were scanned using a Joyce-Loebel Chromoscan 3.

3.2.5 Antisera Against High Density Pellet Polypeptides.

3.2.5.1. Preparation of antisera. High density material was isolated from about 1 000 worm pairs as described in 3.2.1. and the polypeptides separated by SDS-PAGE in four adjacent gel tracks. Theoutermost tracks together contained 10 % of the

total protein applied, the remainder being in the inner tracks. After electrophoresis the outer tracks were cut from the gel, fixed and stained with Coomassie blue. The inner tracks were stored at -20^{0}C without fixation. The portions of unfixed gel corresponding to the position of the two high density pellet polypeptides were then excised from the gel, homogenised in 0.9 % (w/v) saline and each polypeptide was injected intradermally into a naive New Zealand White rabbit. Forty-two days later the rabbits received a second similar dose of antigen and fourteen days after that blood was collected from the ear vein and the sera stored at -20^{0}C.

3.2.5.2. Immunodiffusion. A high density pellet derived from about 1 000 worm pairs was solubilized for immunodiffusion in 2 % Nonidet P-40 (Crumpton and Parkhouse, 1972) and placed in the centre well of a double immunodiffusion plate. Serial dilutions of antiserum were aliquoted into the outer wells of the plate (1 % agarose in barbitone buffer, pH 8.2, 2 mm diameter wells, placed 7 mm from the centre well). The plates were incubated for 3 hours at 37^{0}C in humid chambers.

3.2.5.3. Immunofluorescence. Adult schistosomes were recovered from infected mice by perfusion with Eagle's medium, fixed in 1 % (w/v) paraformaldehyde in 0.1M phosphate buffer, pH 7.2, 7.5 % (w/v) sucrose for 30 minutes at 4^{0}C, then washed in phosphate buffer. Worms were embedded in Bright Cryo-M-Bed and the blocks frozen using a freon spray. Sections 5 µm thick were cut on a Bright cryostat at -20^{0}C and placed on

0.8 mm acid washed microscope slides.

The sections were stained at room temperature in humid chambers. Slides were covered with 10 % (v/v) normal goat serum (NGS) in phosphate buffered saline (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 (PBS)) for 30 minutes, then drained and covered with antiserum of various dilutions in PBS plus 10 % (v/v) NGS for 30 minutes. Normal rabbit serum was used as a control. Slides were washed twice in PBS, then covered with 10 % NGS in PBS for 30 minutes and drained. The slides were then flooded with FITC-goat anti-rabbit serum (diluted 1 in 20 with PBS plus 10 % (v/v) NGS) for 30 minutes, then washed, mounted in 90 % glycerol, 10 % PBS and viewed in an Olympus epifluorescence microscope.

3.2.6 Carbohydrate Assay

The carbohydrate content of fractions was assayed by the phenol-sulphuric acid method of Dubois, Gilles, Hamilton, Rebers and Smith (1956), using a total volume of 1 ml. D-glucose was used as a standard.

3.3 EXPERIMENTS AND RESULTS

3.3.1 High Density Material

It was shown in Chapter 1 that spines are released from the schistosome tegument during surface membrane disruption by freeze-thaw and vortexing. It was also shown that spines were present in the pellet derived from the vortex supernatant but that they were absent from the membrane fraction recovered after centrifuging the pellet on a sucrose gradient. The white flocculent material routinely observed at the base of the sucrose gradient was clearly of high density (>1.232 g/ml) and it was considered that the tegumental constituent most likely to be of such high density, was spine.

The material at the base of the gradient was solubilized using sodium dodecyl sulphate and its constituent polypeptides separated by SDS-PAGE.

Figure 40 shows there were only two major polypeptide bands present. These were of molecular weight 45 000 and 68 000. No glycoproteins were detected by Con A overlay (see 3.3.3.4.).

Results of electron microscopy of the high density pellet are shown in Figures 41 and 42. The major component of the pellet appeared to be sheets of electron dense material of thickness 10 - 20 nm. Spines were also present in the pellet

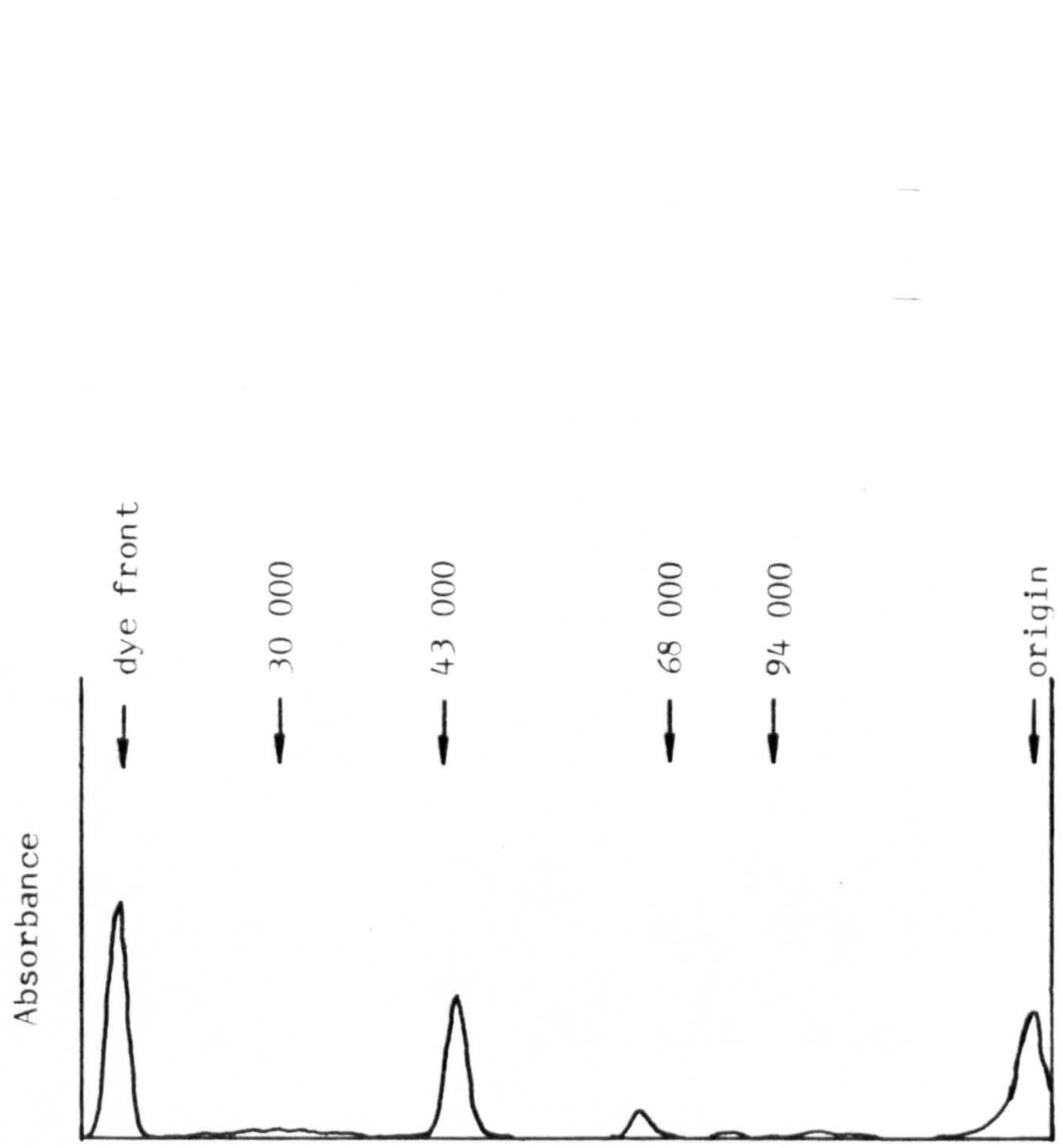
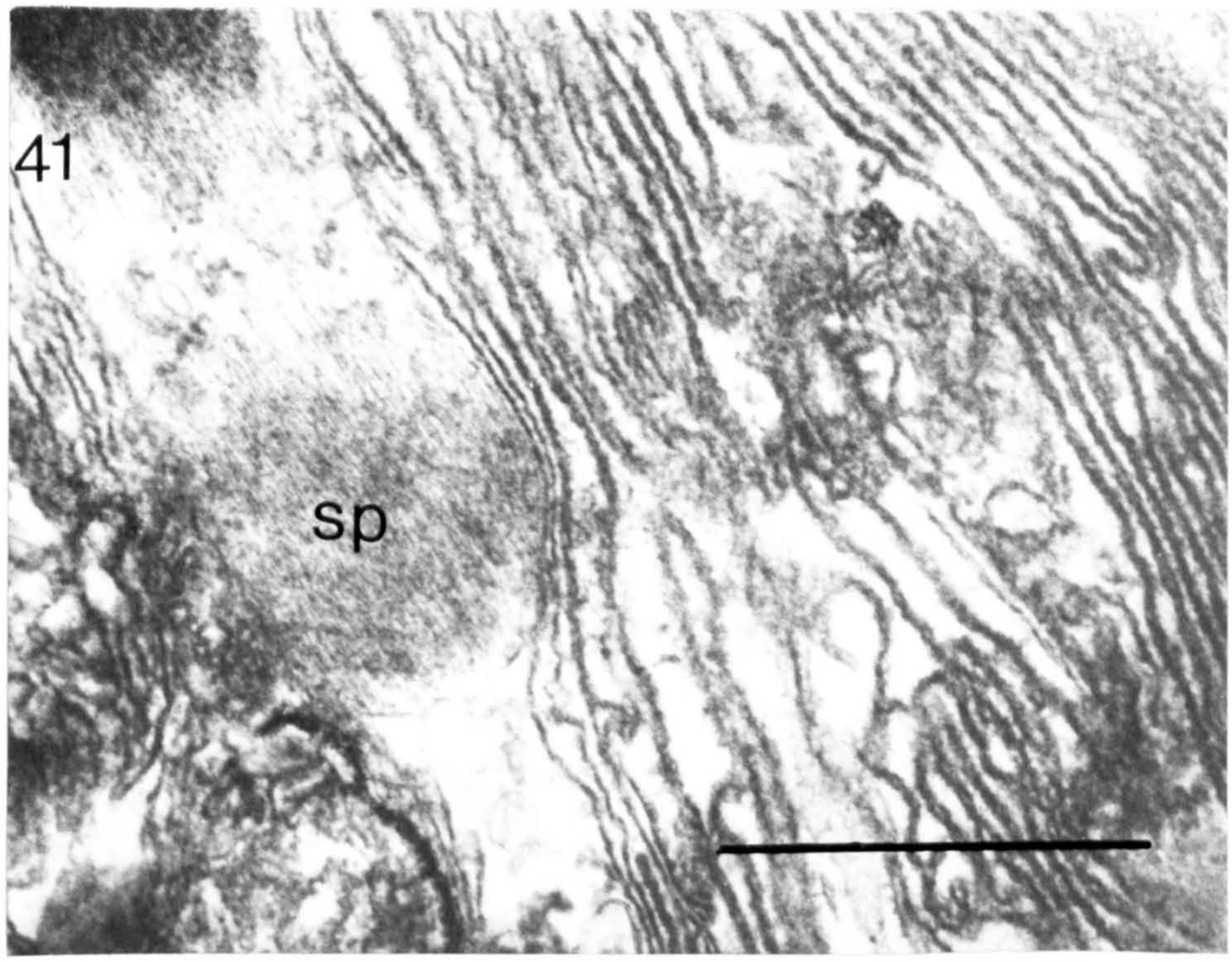
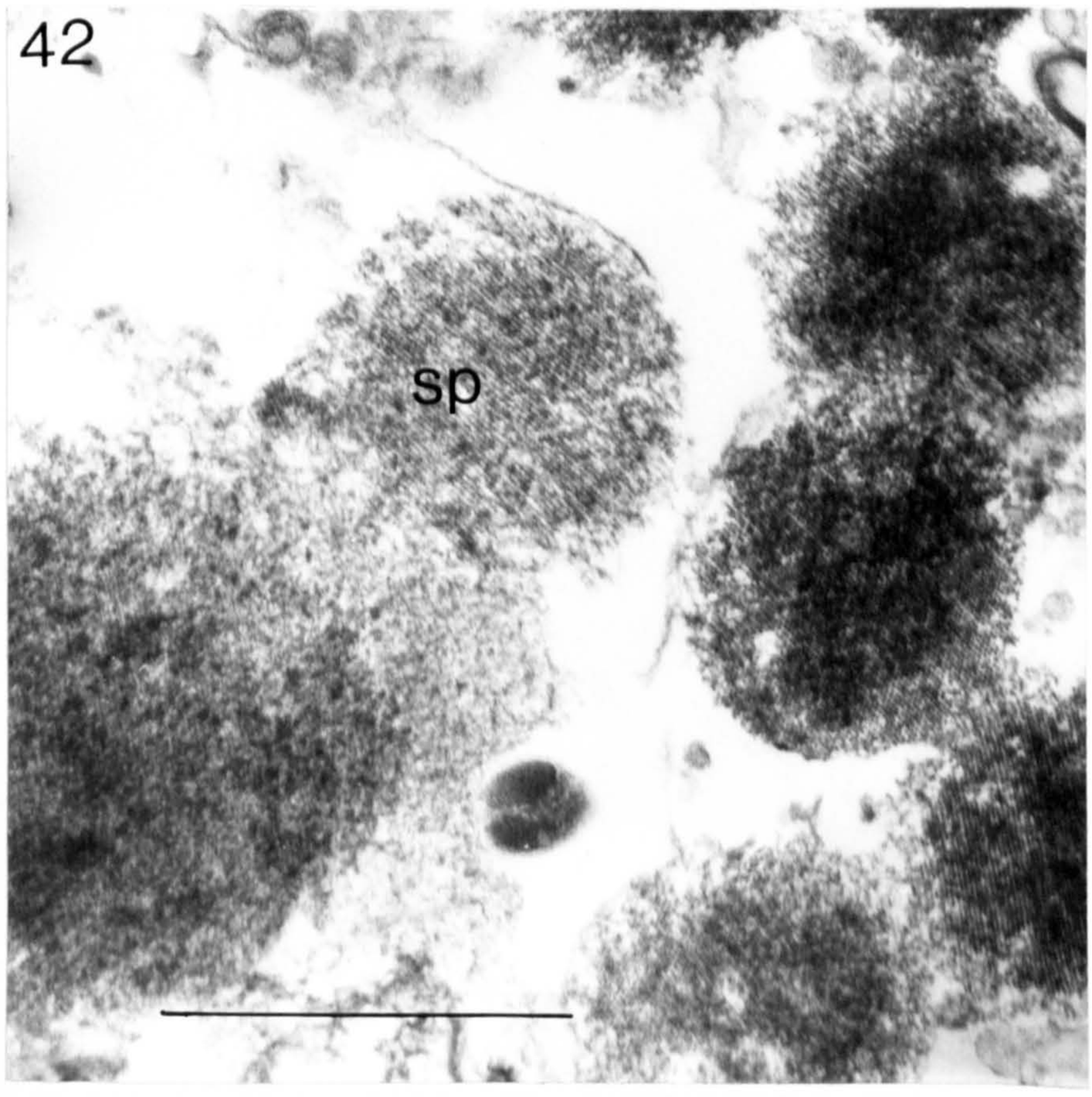


Figure 40. Densitometric scan of a Coomassie blue-stained gel after separation of the high density pellet polypeptides by SDS-PAGE.

Figure 41
and Figure 42. Transmission electron micrographs of the high density material recovered from the base of the sucrose gradient after surface membrane isolation.
sp, spine.
Bars = 1 μm.


41
sp


42
sp

and a few small membrane-bound vesicles and myelin figures were found. The periphery of the spines was less electron-dense than normal and some electron-lucid patches were seen in this area.

In order to confirm that the high density material was spine, an attempt was made to raise antibodies to each of the two polypeptides, as described in 3.2.5.1.

The antiserum to the 45 000 dalton polypeptide gave precipitin lines in immunodiffusion plates indicating that equivalence was at a serum dilution of 1 in 2 (Figure 43). Antiserum to the 68 000 dalton protein gave no precipitin lines.

The indirect fluorescent antibody technique failed to demonstrate any specific fluorescence in cryostat sections of schistosomes using either antiserum.

3.3.2 Freeze-thaw and Vortex Supernatants

When the freeze-thaw supernatant was subjected to centrifugation at 140 000 x *g* and the pellet examined by transmission electron microscopy, its contents were found to be heterogeneous (Figure 44). The main component was membrane-bound vesicles. These were of two types, small spheres with electron-dense contents and larger, empty vesicles. Long, multi-layered membrane sheets and myelin figures were present. A few discoid granules and multilaminate vesicles were seen but

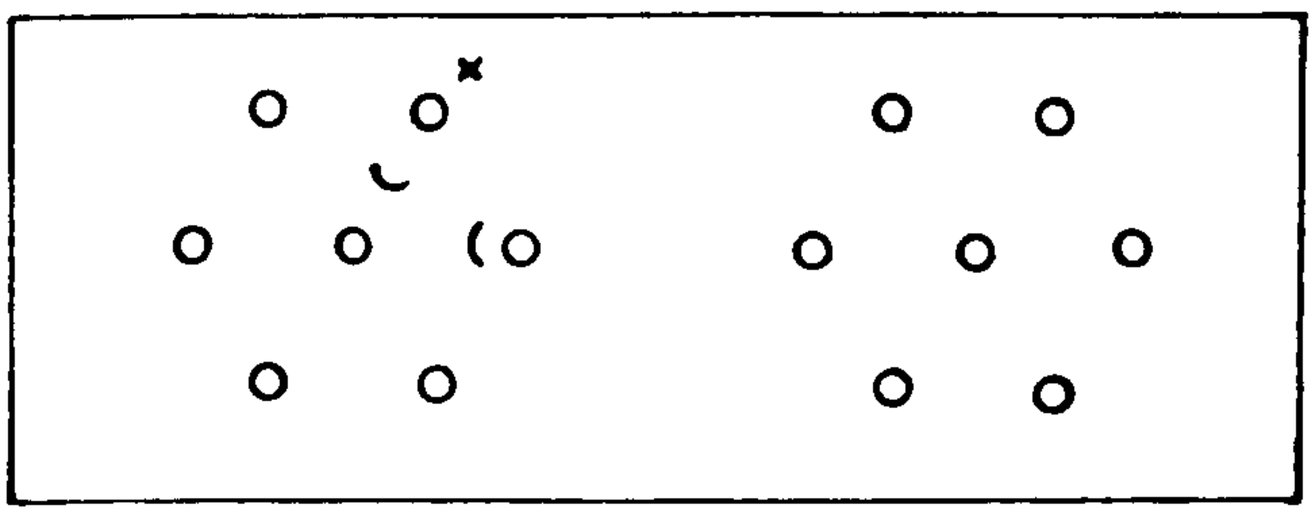


Figure 43. Immunodiffusion of antiserum raised against a 45 000 dalton polypeptide from the high density pellet. The centre wells contained high density material solubilized with Nonidet P-40 and the outer wells contained serial dilutions of antiserum beginning with 1:2 at the well marked 'x'.

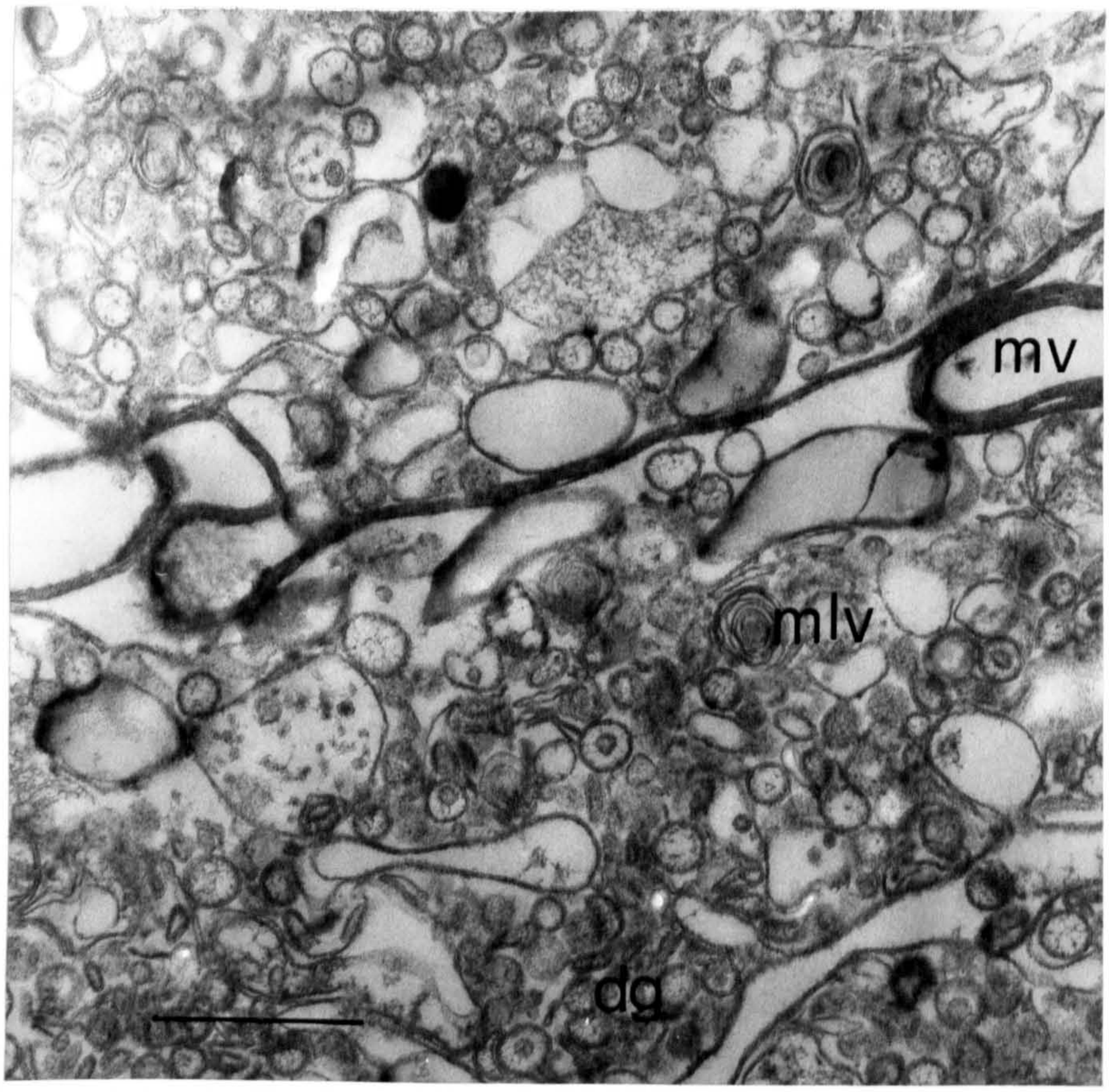


Figure 44. Pellet derived by centrifugation of the freeze-thaw supernatant at 140 000 x *g* for 45 minutes. dg, discoid granule; mlv, multilaminate vesicles; mv, myelin-like vesicle. Bar = 0.5 µm.

there were considerably fewer discoid granules present than might be expected, given their abundance in the tegument. Occasionally, some spines and muscle fragments were found.

After removal of much of the surface membrane by centrifugation at 100 x *g*, the remaining vortex supernatant was centrifuged at 140 000 x *g*. This pellet, which was much larger than the above described pellet, was similar to it in content. Membrane-bound vesicles were the major constituent (Figure 45).

The most striking feature of these results was the paucity of discoid granules in the two pellets. There are many small spherical vesicles with electron-dense contents however, and it may be that discoid granules have lost their characteristic shape and become spherical. A comparison of the volumes of discoid granules and these microvesicles may add credence to this hypothesis. Discoid granules are oblate spheroids of dimensions 160 nm x 30 nm (Wilson and Barnes, 1974a). The volume of a discoid granule (found from the formula $4/3\ \Pi a^2 b$, where a and b are the radii of the major and minor axes respectively) is 400 000 nm^3. The average diameter of the microvesicles is about 100 nm which gives a volume of 525 000 nm^3 per vesicle. This difference can be accounted for by only a minor error in measurement (10 %).

The relative absence of discoid granules and the presence of vesicles of similar volume in these pellets suggested discoid granules

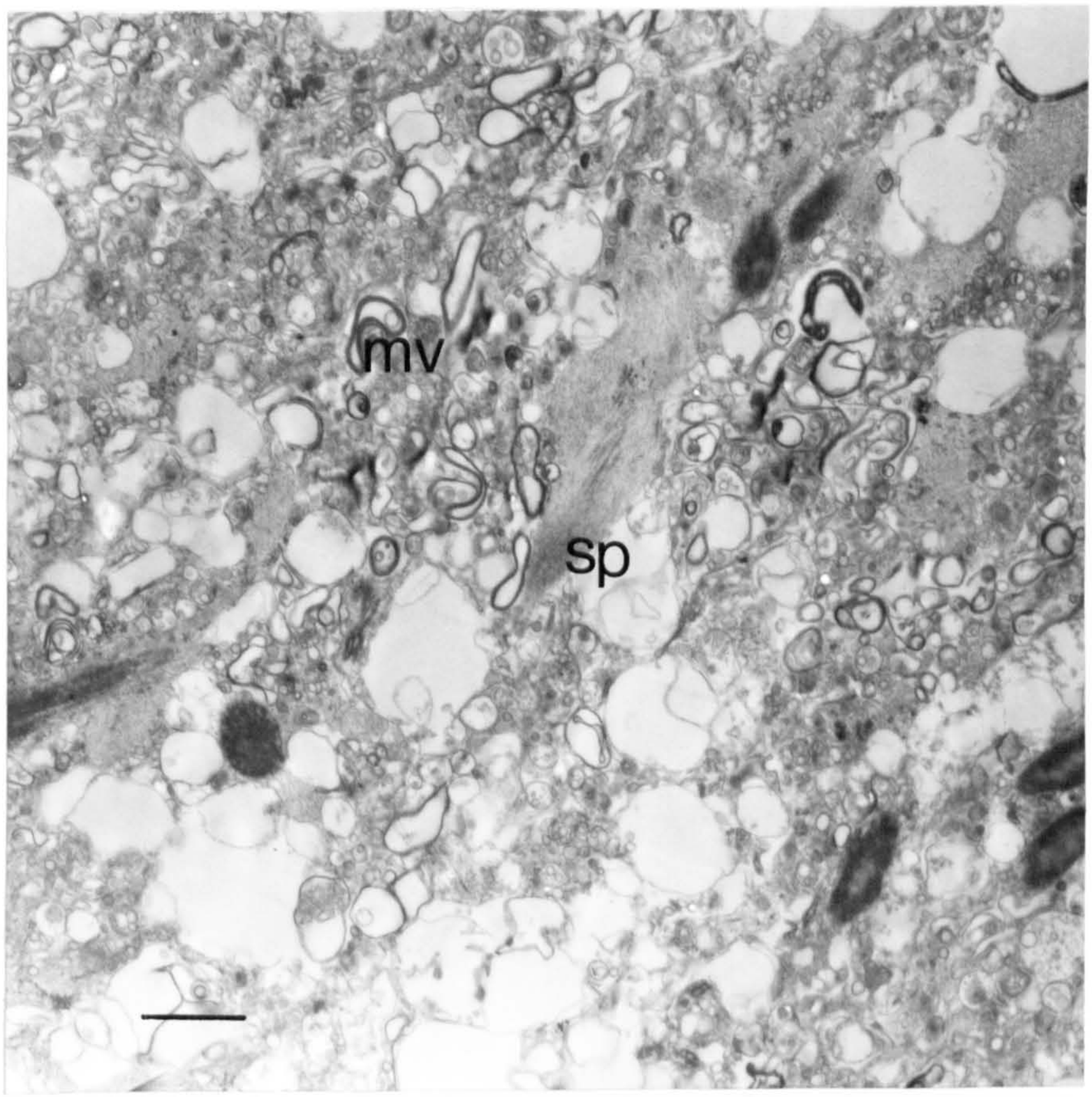


Figure 45. Pellet derived by centrifugation of the vortex supernatant at 140 000 x *g* for 45 minutes after removal of the surface membrane material by prior centrifugation at 100 x *g* for 30 minutes.

mv, myelin-like vesicle; sp, spine.

Bar = 1 µm.

might be altered during surface membrane isolation.

3.3.3 Isolation of Microvesicles

An experimental scheme was devised to isolate the microvesicles in order that more might be discovered concerning the nature, and perhaps thereby the role, of discoid granules.

3.3.3.1. Isolation method and biochemical content of fractions. Carbohydrate was used as a marker for discoid granules on the assumption that a high specific content of carbohydrate might indicate the presence of discoid granules. To prevent interference with the assay, glucose was omitted from the Hanks' balanced salt solution.

About 250 worm pairs, suspended in glucose-free Hanks' balanced salt solution (GFH), were frozen and thawed as described in Chapter 1. They were rinsed in GFH and vortexed. The freeze-thaw and vortex supernatants were then individually centrifuged at 100 x *g* for 30 minutes, then at 10 000 x *g* (30 minutes) and finally at 150 000 x *g* (45 minutes). After each centrifugation step an aliquot of the supernatant was stored for assay and each pellet was resuspended in GFH and stored for assay.

Results of protein, alkaline phosphatase and carbohydrate assays are summarised in Table 22. Only very small pellets could be derived from the freeze-thaw supernatant and the

Table 22. Distribution of protein, alkaline phosphatase and carbohydrate in fractions produced by freeze-thaw and vortexing of worms and by differential centrifugation of the vortex supernatant.

	protein[χ]	alkaline phosphatase[Δ]			carbohydrate[τ]		
	%	%	specific* activity	relative[†] enrichment	%	specific content[ϕ]	relative[ǂ] enrichment
freeze-thaw supernatant	8.5	2.7	0.054	0.310	5.7	0.181	0.67
vortex supernatant (VS)	19.5	34.4	0.310	1.780	8.0	0.112	0.41
denuded worm homogenate	72.0	62.6	0.151	0.868	86.3	0.325	1.20
total			0.174			0.272	
VS 100 x *g* pellet	0.9	14.9	2.767	15.900	0.7	0.217	0.80
VS 100 x *g* supernatant	18.2	13.1	0.125	0.720	9.2	0.138	0.51
VS 10 000 x *g* pellet	1.0	8.2	1.073	6.170	0.4	0.157	0.58
VS 10 000 x *g* supernatant	16.0	4.9	0.053	0.310	9.2	0.156	0.57
VS 150 000 x *g* pellet	0.4	1.8	0.846	4.860	0.7	0.769	2.83
VS 150 000 x *g* supernatant	15.8	1.1	0.012	0.070	8.2	0.140	0.52

χ determined using Folin-phenol reagent

Δ hydrolysis of *p*-nitrophenylphosphate

τ determined using phenol-sulphuric acid

* enzyme units/mg protein

† specific activity of fraction/total specific activity

ϕ mg carbohydrate/mg protein

ǂ specific content of fraction/total specific content

quantities of protein, alkaline phosphatase and carbohydrate detected were below the limits of accuracy of the assays used. The results shown therefore refer to the vortex supernatant only. When protein and alkaline phosphatase assays were applied to the previously described fractions, the results obtained fell within the expected range. Some 14 % of total worm carbohydrate was released by freeze-thaw and vortexing. The fraction with the highest specific content of carbohydrate (ie carbohydrate/ protein) was the 150 000 x *g* pellet and of the three pellets, this one had the lowest specific activity of alkaline phosphatase, suggesting the pellet was enriched in discoid granules and contained little contaminating surface membrane.

The figures for carbohydrate content of the fractions cannot give a true reflection of the glycoprotein content so, to increase the specificity of the assay, low molecular weight carbohydrates were removed from the final supernatant and denuded worm homogenate by dialysis. The carbohydrate concentrations of these fractions was measured and used to recalculate the carbohydrate content of worms. Dialysable carbohydrate accounted for 13 % of the previous total. Relative enrichment of non-dialysable carbohydrate in the 150 000 x *g* pellet was 3.3.

3.3.3.2 Removal of glycogen from fractions. Dialysis was an inadequate method for removal of non-discoid granule carbohydrate from fractions. This was partly because of the reserves of glycogen held in the parenchyma (Reissig, 1970). To increase

the specificity of the carbohydrate assay, it was decided to treat supernatants and the denuded worm homogenate with α-amylase to hydrolyse glycogen to low molecular weight oligosaccharides which could then be extracted by dialysis.

Microvesicles were prepared as described in the preceding section using 1 500 - 2 500 worm pairs and the fractions assayed for protein, alkaline phosphatase and carbohydrate. The freeze-thaw supernatant, denuded worm homogenate and final supernatant were then warmed to 20°C and α-amylase added. The number of enzyme units used was based on the results of the carbohydrate assay and was at least 10 times in excess of the suppliers estimated requirement for complete hydrolysis of all the carbohydrate present. The reaction was allowed to proceed for 5 minutes, then the reaction mixtures were placed on ice. Dialysis was carried out for 24 hours against 4 - 5 changes of 0.1 M phosphate buffer, pH7.4 at 4°C. The carbohydrate content of the dialysed fractions was then determined and the results used to calculate specific content and relative enrichment.

The results of the protein, alkaline phosphatase and first carbohydrate assays are shown in Table 23 . Table 24 shows the results of carbohydrate assays after amylase hydrolysis and dialysis of supernatants and the homogenate. Protein and alkaline phosphatase determinations confirmed the results of the previous section, as did the first carbohydrate assay.

Table 23. Distribution of protein, alkaline phosphatase and carbohydrate during isolation of microvesicles.

protein distribution	%
freeze-thaw supernatant	3.1 ± 0.4*
vortex supernatant (VS)	25.3 ± 2.5
denuded worm homogenate	71.6 ± 2.4
VS 100 x *g* pellet	0.8 ± 0.3
VS 1 000 x *g* pellet	0.7 ± 0.1
VS 150 000 x *g* pellet	0.3 ± 0.1
VS 150 000 x *g* supernatant	22.0 ± 2.4

contd...

Table 23 contd.

alkaline phosphatase distribution

	%	specific activity*	relative enrichment†
freeze-thaw supernatant	0.3 ± 0.03ε	0.022 ± 0.004	0.10 ± 0.02
vortex supernatant (VS)	28.3 ± 2.10	0.246 ± 0.026	1.13 ± 0.03
denuded worm homogenate	71.4 ± 2.00	0.219 ± 0.030	0.99 ± 0.00
total		0.220 ± 0.028	
VS 100 x *g* pellet	13.0 ± 4.00	3.773 ± 0.328	17.35 ± 0.72
VS 10 000 x *g* pellet	6.3 ± 1.20	2.174 ± 0.527	9.68 ± 1.39
VS 150 000 x *g* pellet	0.4 ± 0.60	0.275 ± 0.044	1.24 ± 0.11
VS 150 000 x *g* supernatant	0.4 ± 0.30	0.004 ± 0.001	0.18 ± 0.00

contd...

Table 23 contd.

carbohydrate distribution

	%	specific content$^{\phi}$	relative enrichment‡
freeze-thaw supernatant	2.4 ± 0.6$^{\varepsilon}$	0.311 ± 0.724	0.77 ± 0.19
vortex supernatant (VS)	14.2 ± 3.6	0.240 ± 0.086	0.57 ± 0.17
denuded worm homogenate	83.4 ± 4.1	0.469 ± 0.006	1.17 ± 0.06
total		0.405 ± 0.027	
VS 100 x *g* pellet	0.7 ± 0.1	0.376 ± 0.061	0.93 ± 0.14
VS 10 000 x *g* pellet	0.5 ± 0.2	0.307 ± 0.088	0.76 ± 0.23
VS 150 000 x *g* pellet	1.0 ± 0.3	1.200 ± 0.249	3.05 ± 0.74
VS 150 000 x *g* supernatant	12.7 ± 2.9	0.252 ± 0.098	0.60 ± 0.18

ε standard error, n = 3

ϕ mg carbohydrate / mg protein

* enzyme units / mg protein

$\ddagger$ specific content of fraction / total specific content

† specific activity of fraction / total specific activity

Table 24. Carbohydrate distribution after removal of glycogen* from supernatants and homogenates.

	%	carbohydrate specific[ϕ] content	relative[§] enrichment
freeze-thaw supernatant	2.4 ± 0.5[ε]	0.139 ± 0.018	0.78 ± 0.19
vortex supernatant 100 x *g* pellet	1.5 ± 0.5	0.376 ± 0.061	2.03 ± 0.30
VS 10 000 x *g* pellet	1.3 ± 0.7	0.307 ± 0.088	1.90 ± 0.82
VS 150 000 x *g* pellet	2.3 ± 1.1	1.200 ± 0.249	6.99 ± 2.48
VS 150 000 x *g* supernatant	7.8 ± 3.0	0.058 ± 0.007	0.34 ± 0.10
denuded worm homogenate	84.6 ± 5.8	0.228 ± 0.052	1.18 ± 0.04
total		0.191 ± 0.037	

contd...

Table 24 contd.

	carbohydrate : alkaline phosphatase ratio[0]	relative enrichment (carbohydrate : alkaline phosphatase)[α]
freeze-thaw supernatant	6.51 ± 0.30	8.42 ± 2.54
vortex supernatant 100 x *g* pellet	0.10 ± 0.02	0.12 ± 0.02
VS 10 000 x *g* pellet	0.14 ± 0.01	0.184 ± 0.06
VS 150 000 x *g* pellet	4.59 ± 1.07	5.68 ± 2.04
VS 150 000 x *g* supernatant	14.16 ± 0.71	17.70 ± 4.69
denuded worm homogenate	1.14 ± 0.38	1.18 ± 0.05
total	0.94 ± 0.28	

* glycogen was removed by α-amylase treatment and dialysis

ϕ mg carbohydrate / mg protein

§ specific content of fraction / total specific content

0 mg carbohydrate / units alkaline phosphatase

∝ carbohydrate : alkaline phosphatase ratio of fraction / total carbohydrate : alkaline phosphatase ratio.

ε standard error, n = 3

Hydrolysis and dialysis reduced the total carbobydrate content of worms by an average of 51.9 %. Results of the second carbohydrate assay show that the glycogen hydrolysis/ dialysis treatment increased the relative enrichment of carbohydrate in the final pellet from 3 times to 7 times that of whole worms. To measure the contamination of the microvesicular pellet with surface membranes, specific content and relative enrichment of carbohydrate against alkaline phosphatase were calculated in exactly the manner they are normally calculated against protein. The results (Table 24) show that the carbohydrate enrichment is 5.7 times that of whole worms.

3.3.3.3. Electron microscopy of microvesicular fraction and the 10 000 x *g* pellet. To discover the morphological nature of the microvesicular fraction and the 10 000 x *g* pellet, these fractions were isolated as previously described. Assay results fell within the expected range. The 100 x *g*, 10 000 x *g* and 150 000 x *g* pellets were processed for transmission electron microscopy.

The 100 x *g* pellet was composed of sheets of surface membrane as previously described. The 10 000 x *g* pellet (Figure 46) was quite heterogeneous, containing spines, membrane sheets and membrane-bound vesicles which varied in size. The 150 000 x *g* pellet (Figures 47 and 48) was mostly composed of small membrane-bound vesicles, some of which had electron-dense contents. Some multilaminate vesicles were found and occasionally a spine was seen.

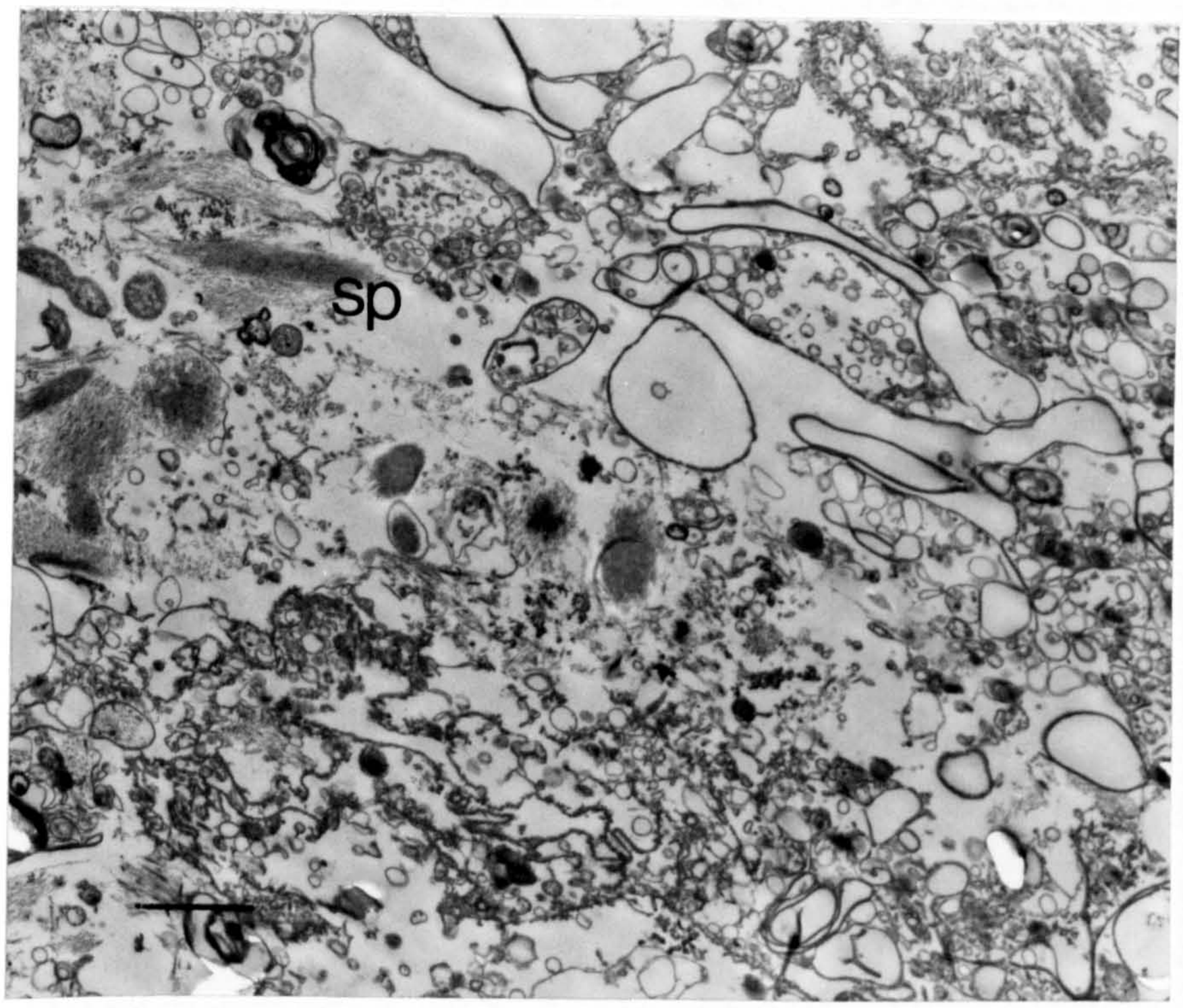


Figure 46. Pellet derived by centrifugation of the vortex supernatant at 10 000 x *g* for 30 minutes after removal of the surface membrane material by prior centrifugation at 100 x *g* for 30 minutes.

sp, spine.

Bar = 1 µm.

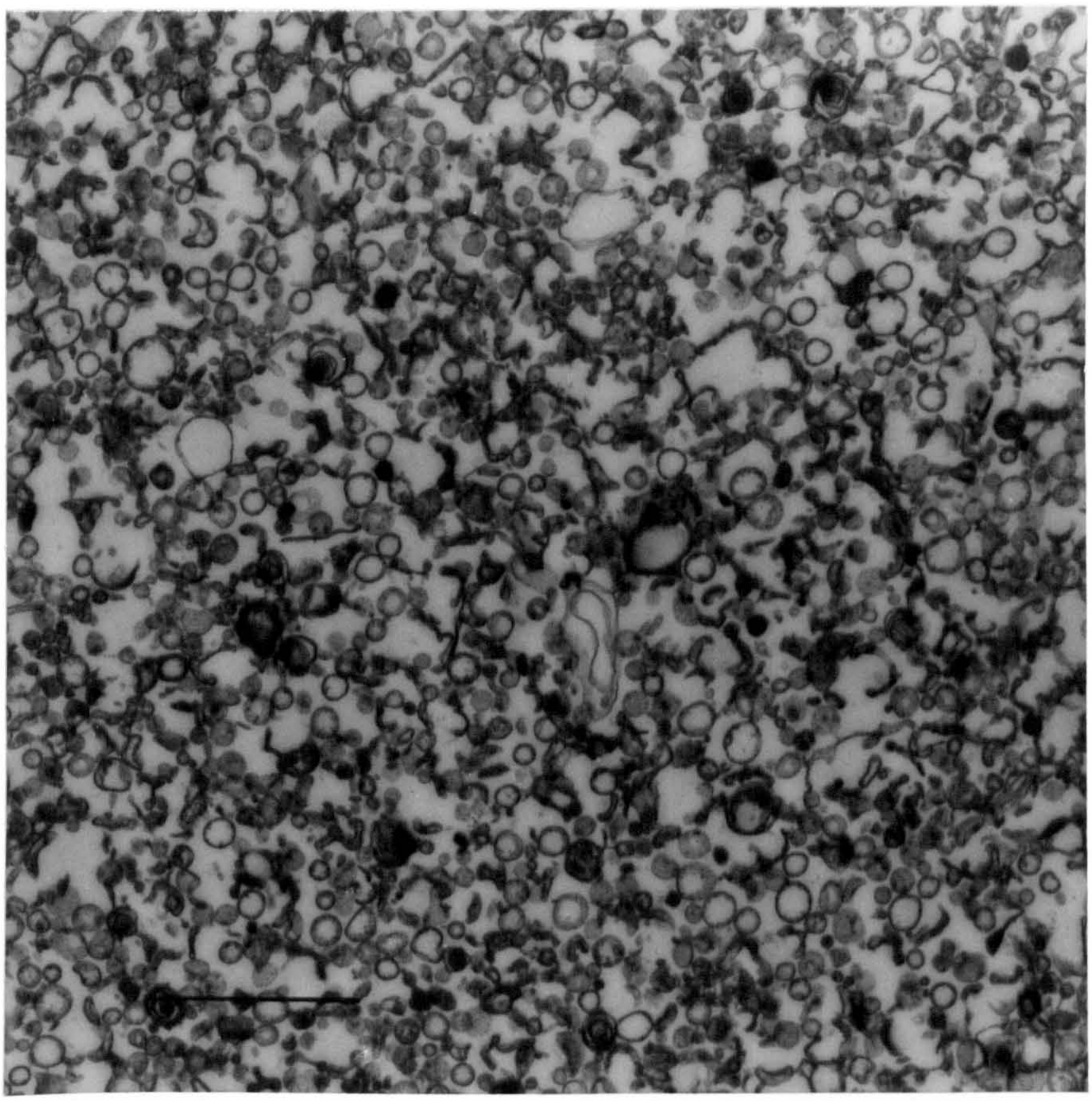


Figure 47. The 150 000 x *g* pellet derived by differential centrifugation of the vortex supernatant. Bar = 0.5 μm.

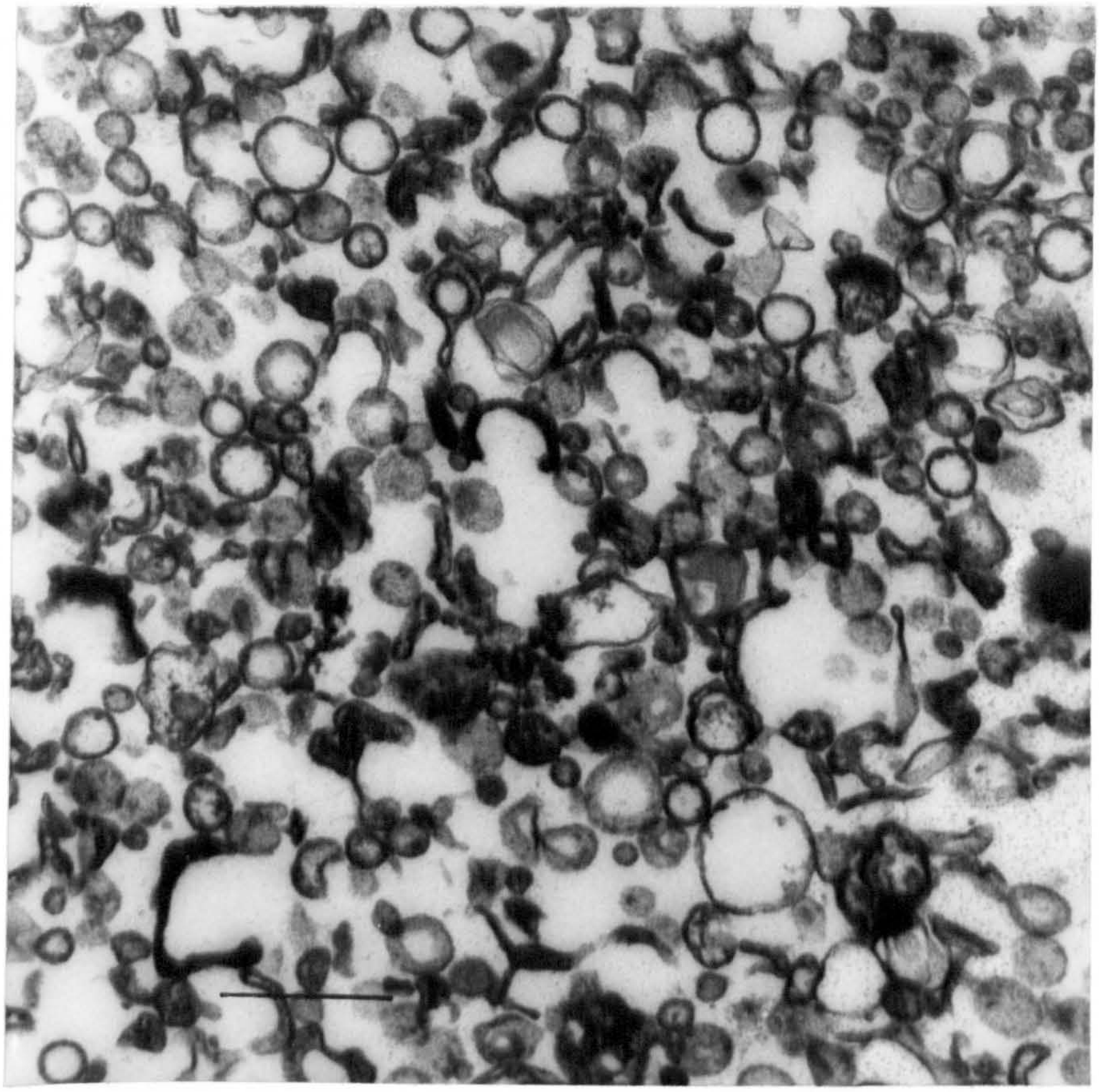


Figure 48. Higher magnification of the material in Figure 47.

Bar = 1 μm.

The isolation technique therefore apparently achieved its objective, that of isolating the microvesicles described in 3.3.2.

3.3.3.4. Proteins and glycoproteins of the microvesicular and other fractions. SDS-PAGE was used to compare the proteins and glycoproteins of the multivesicular fraction with other fractions using samples prepared as described in 3.3.3.2. Prior to electrophoresis, the final supernatant and the freeze-thaw supernatant were concentrated to a small volume. The final supernatant contains soluble proteins released by vortexing and is probably principally composed of soluble cytoplasmic proteins from the tegument and other parts of the worm.

Initially, 7.5 % polyacrylamide gels were used and these were stained only for glycoproteins using the periodic acid-Schiff reagent method. The result is shown in Figure 49 . The surface membrane fraction contained a broad band of rapidly migrating glycoprotein, another band of slowly migrating glycoprotein and a discrete doublet of intermediate mobility. The 10 000 x *g* pellet gave almost identical bands with the rapidly migrating band perhaps staining slightly more intensely than in the 100 x *g* pellet. The microvesicular fraction contained the rapidly migrating glycoprotein and a little of the slowly migrating glycoprotein, but the doublet was not detected. The soluble fraction contained the slow migrating glycoprotein and two others not present in the pellets. Samples of denuded worm homogenate showed little other than the slow migrating band

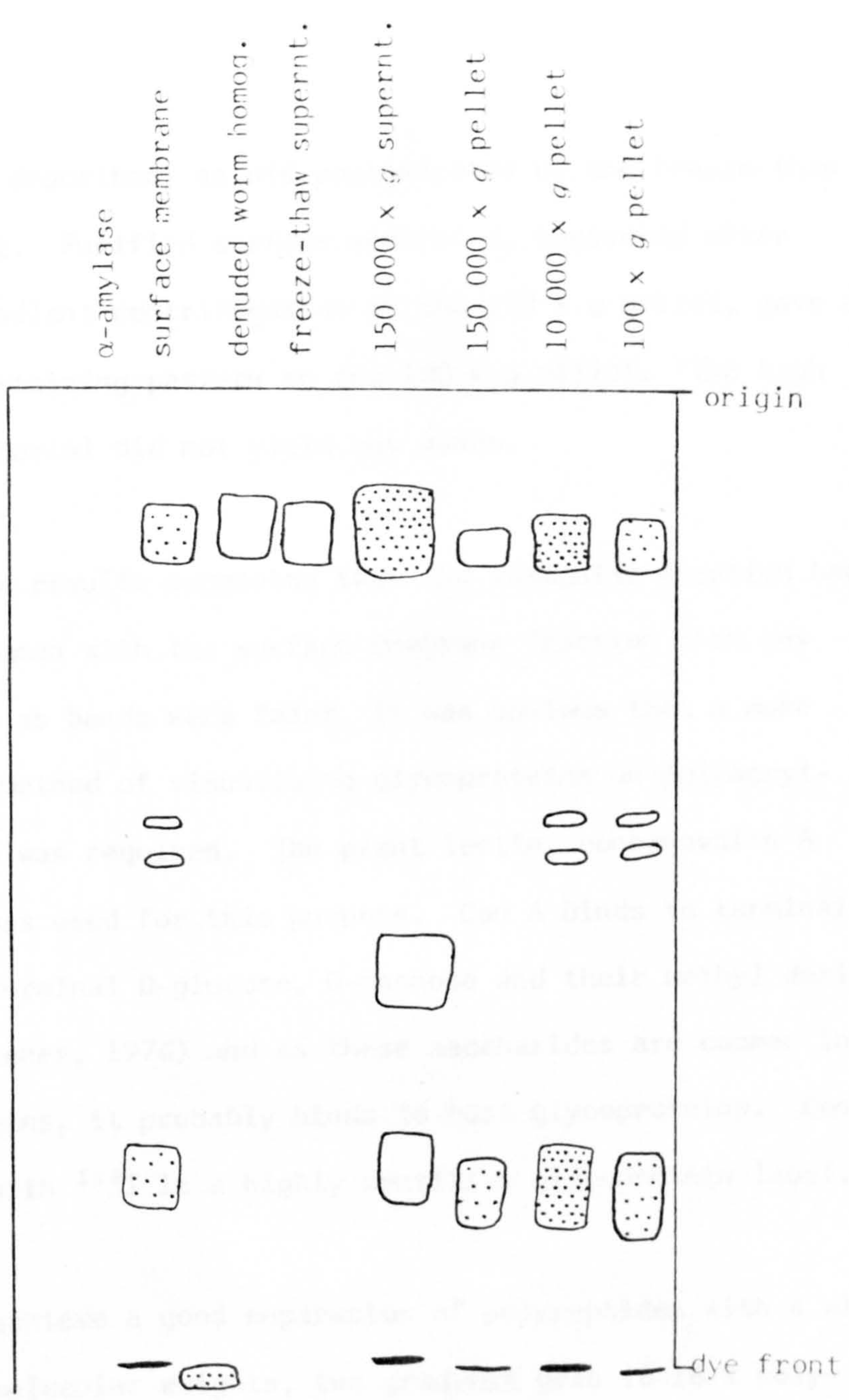


Figure 49. Diagram of a periodic acid-Schiff stained gel showing the major glycoproteins in fractions from the microvesicle isolation procedure together with α-amylase and surface membrane prepared as described in 1.3.5.

previously described, as did concentrates of the freeze-thaw supernatant. Purified surface membranes, recovered after density gradient centrifugation of the 100 x *g* pellet, gave an identical staining pattern to the 100 x *g* pellet. The high density material did not yield any bands.

These results suggested that the vesicular fraction had more in common with the surface membrane fraction than any other, but as bands were faint, it was obvious that a more sensitive method of visualising glycoproteins on polyacrylamide gels was required. The plant lectin, concanavalin A (Con A), was used for this purpose. Con A binds to terminal and non- terminal D-glucose, D-mannose and their methyl derivatives (Liener, 1976) and as these saccharides are common in glycoproteins, it probably binds to most glycoproteins. Con A, labelled with ^{125}I is a highly sensitive glycoprotein label.

To achieve a good separation of polypeptides with a wide range of molecular weights, two gradient gels (6-16 % polyacrylamide) were used. One of these was stained with Coomassie blue and the other with ^{125}I-Con A.

Results of Coomassie blue staining and results of autoradiography after ^{125}I-Con A overlay are shown in Figures 50 to 61. Table 25 shows the approximate molecular weights of the polypeptides.

Six glycoproteins were found in the vesicular fraction

Figures 50 - 61. Densitometric scans of SDS-PAGE gels showing the major polypeptides and glycoproteins in fractions from microvesicle isolation. FTS, freeze-thaw supernatant; DWH, denuded worm homogenate. Arrows show the position of α-amylase.

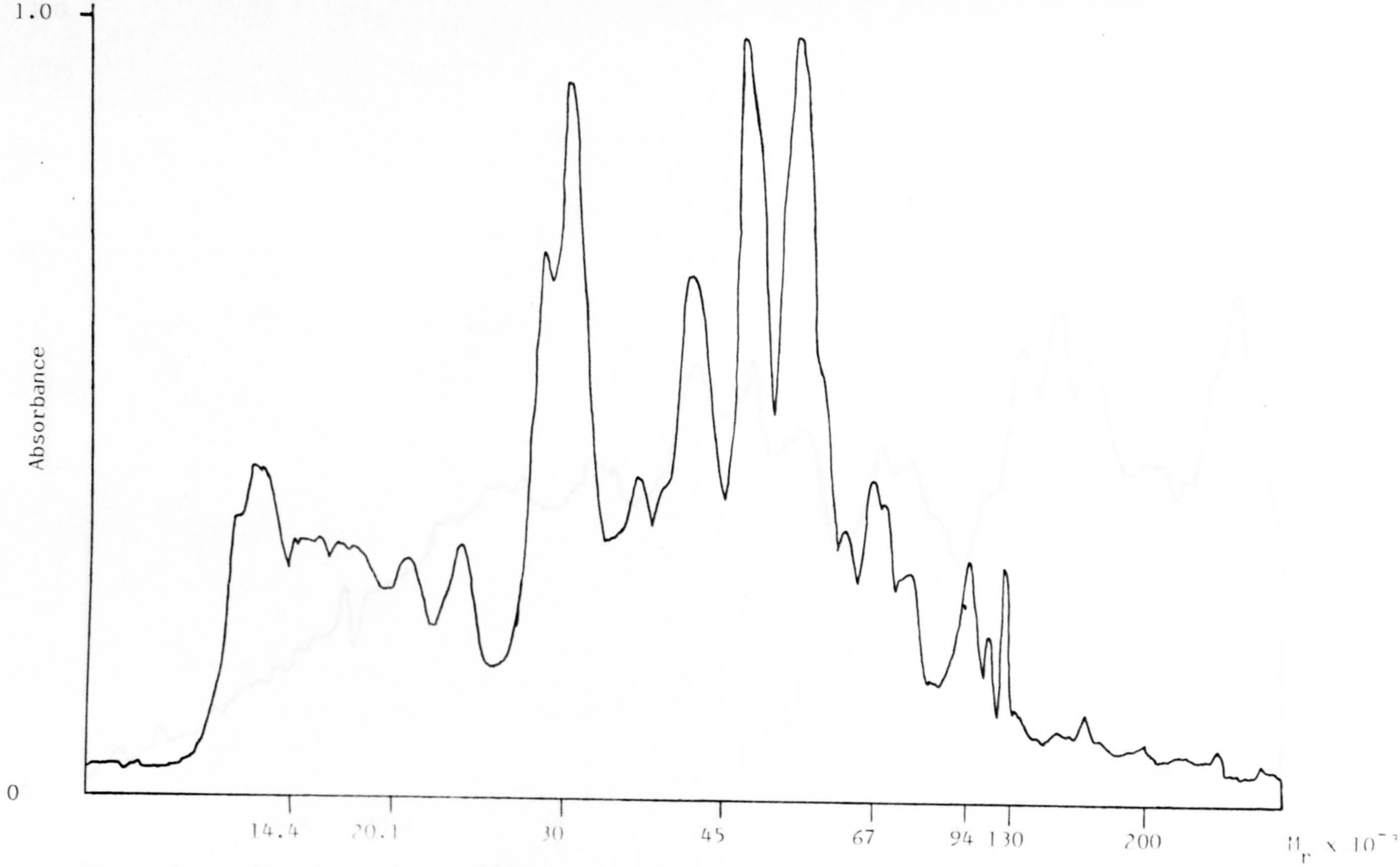


Figure 50. FTS polypeptides. Coomassie blue stain.

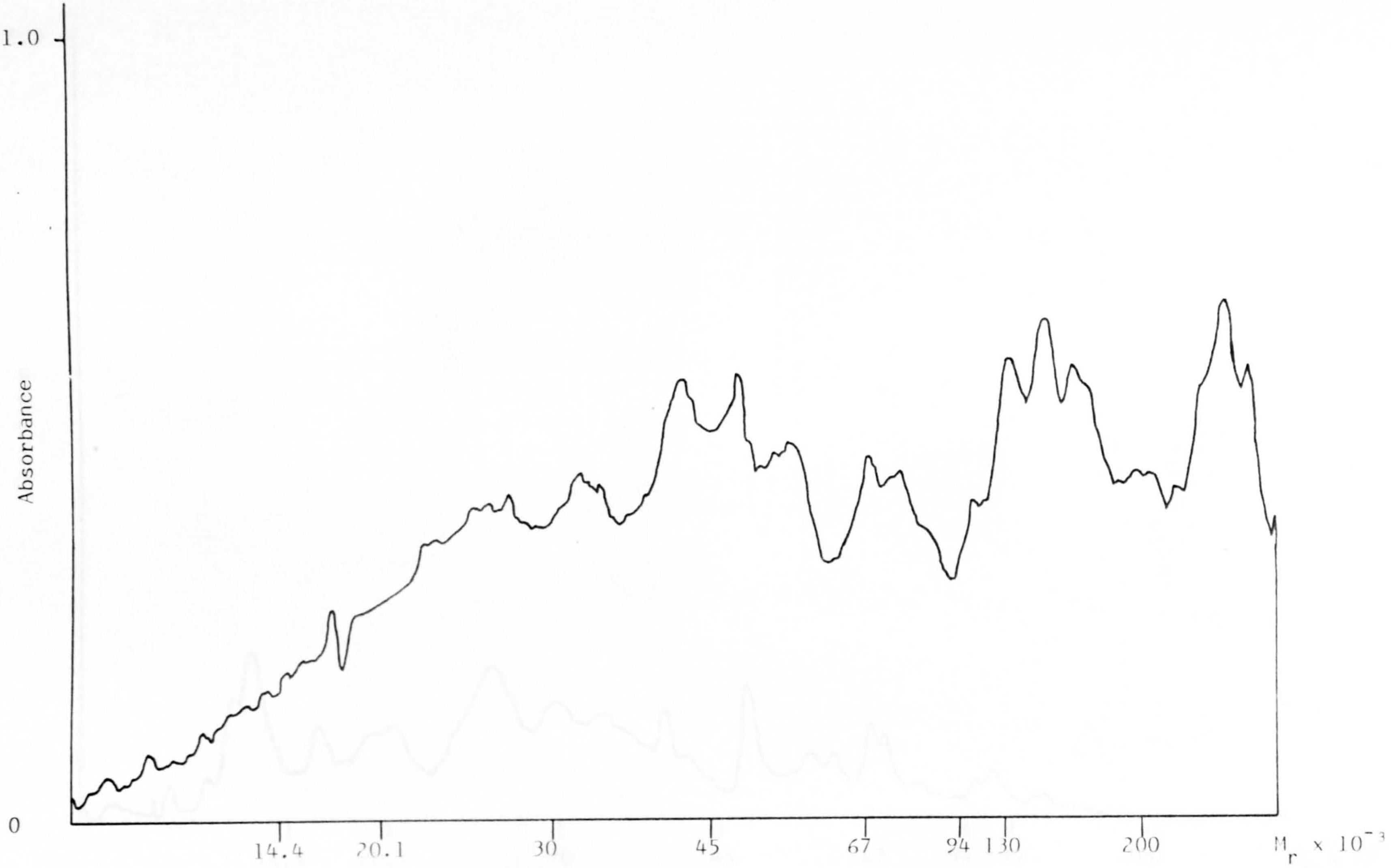


Figure 51. FTS polypeptides. ^{125}I-Con A overlay.

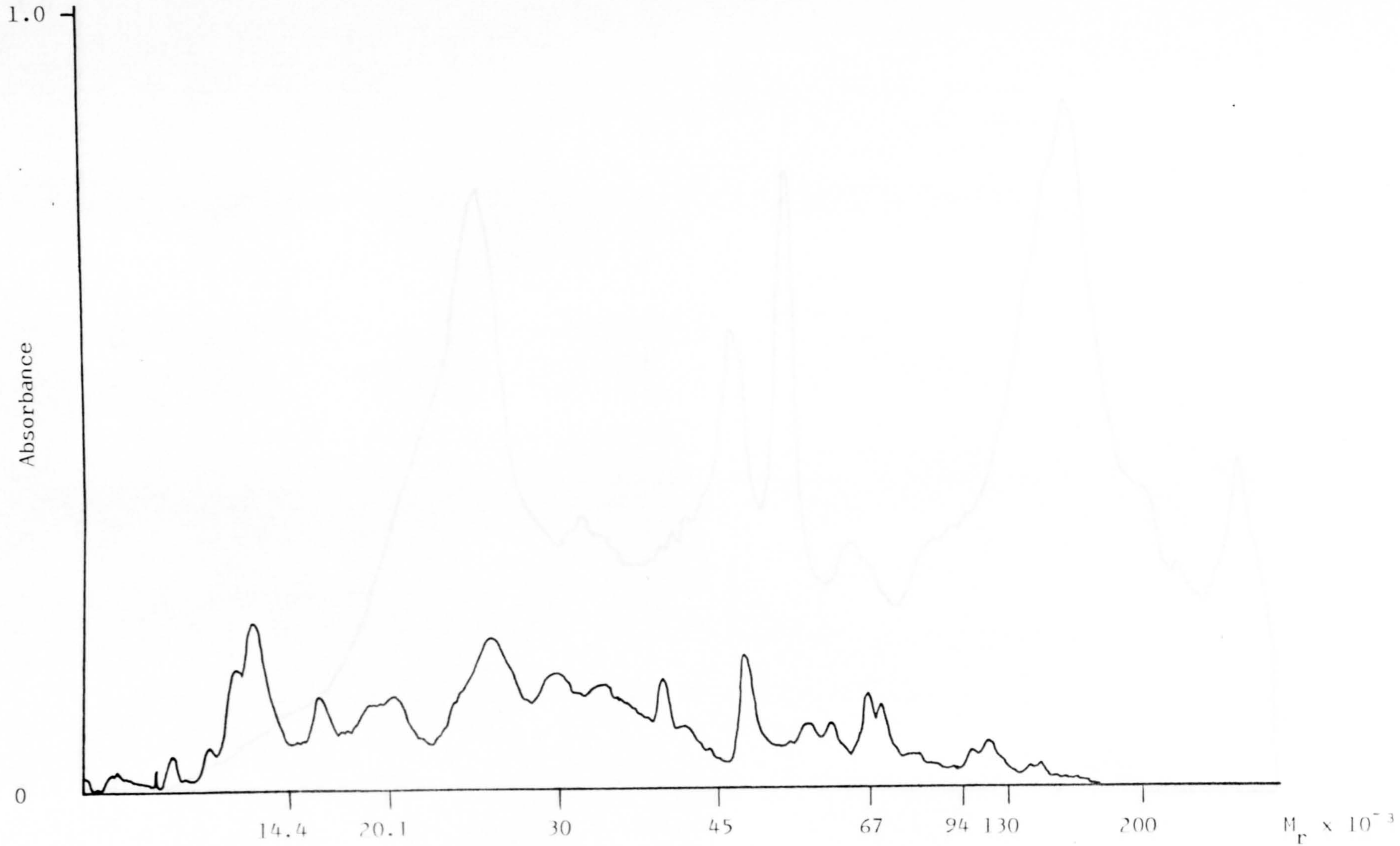


Figure 52. 100 x *g* Pellet. Coomassie blue stain.

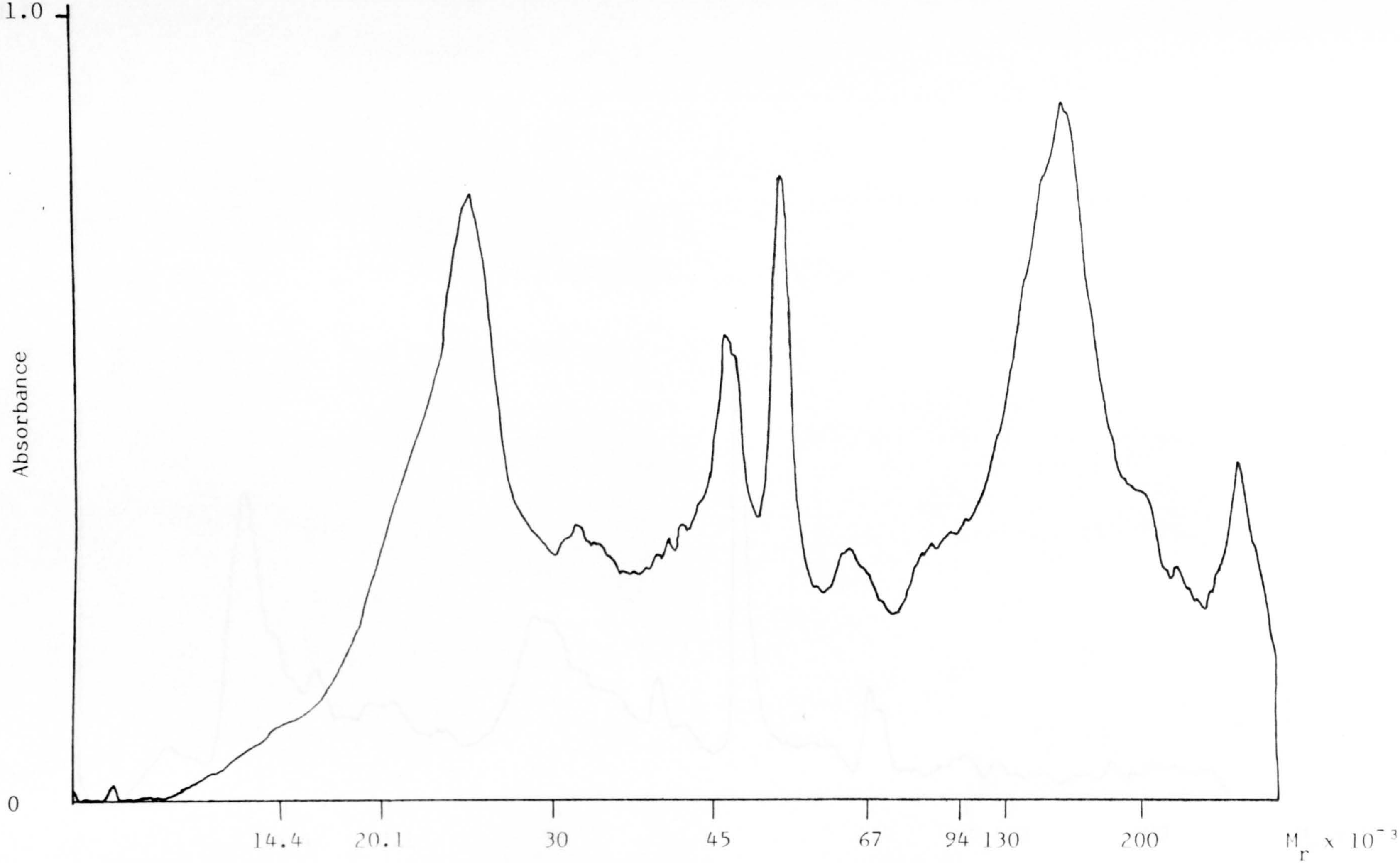


Figure 53. 100 x *g* Pellet. ^{125}I-Con A overlay.

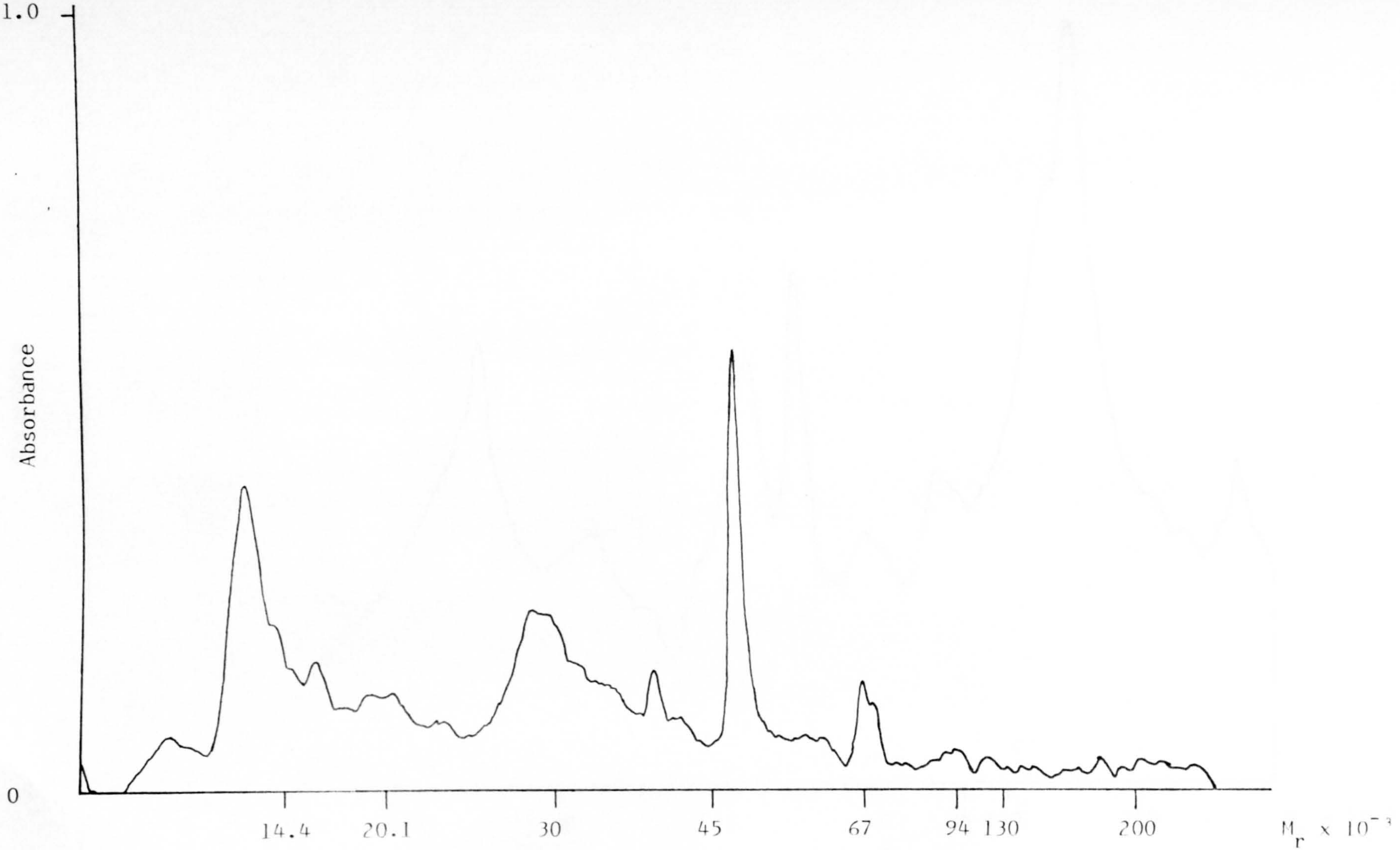


Figure 54. 10 000 x *g* Pellet. Coomassie blue stain.

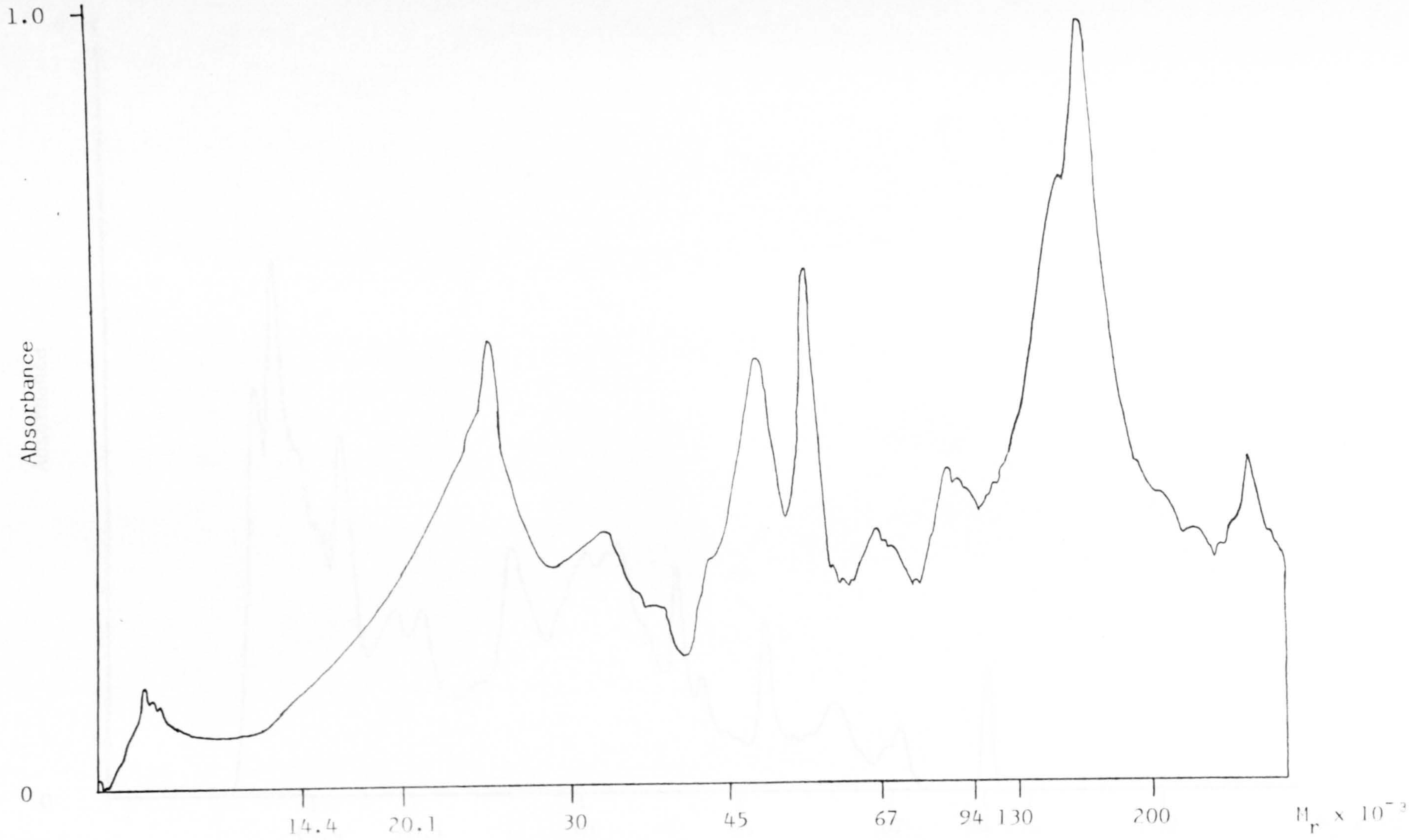


Figure 55. 10 000 × *g* Pellet. ^{125}I-Con A overlay.

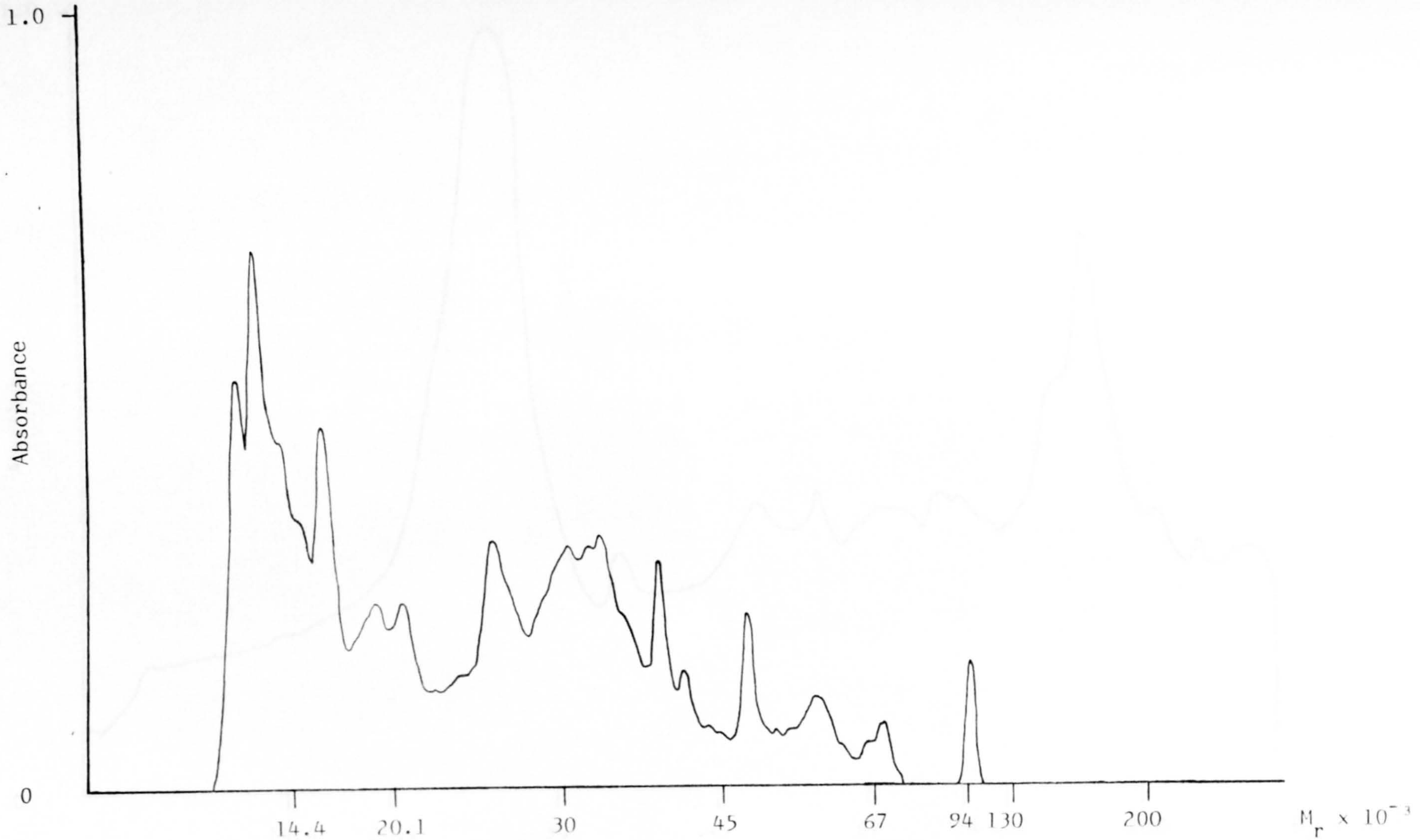


Figure 56. 150 000 x *g* Pellet. Coomassie blue stain.

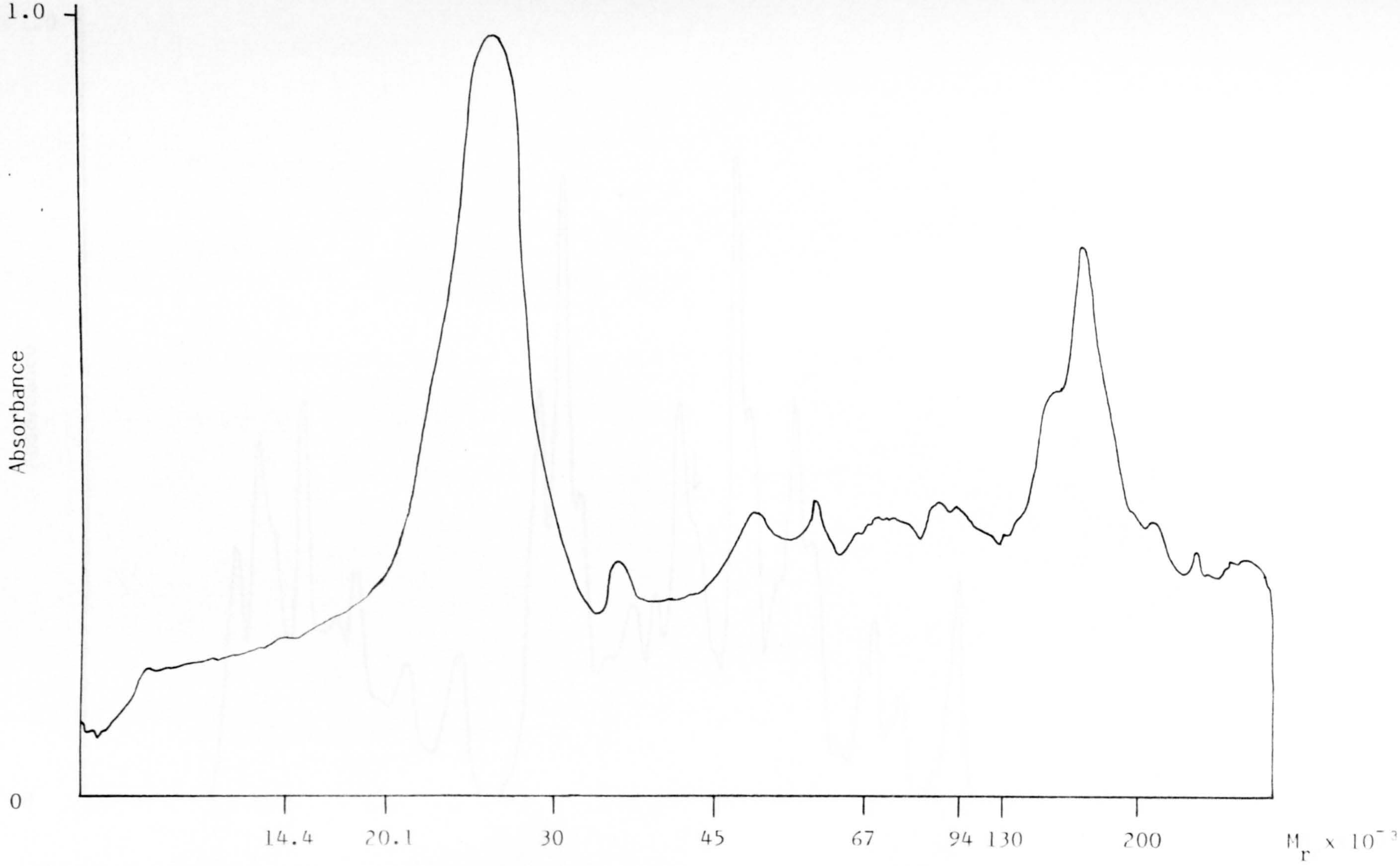


Figure 57. 150 000 x *g* Pellet. ^{125}I-Con A overlay.

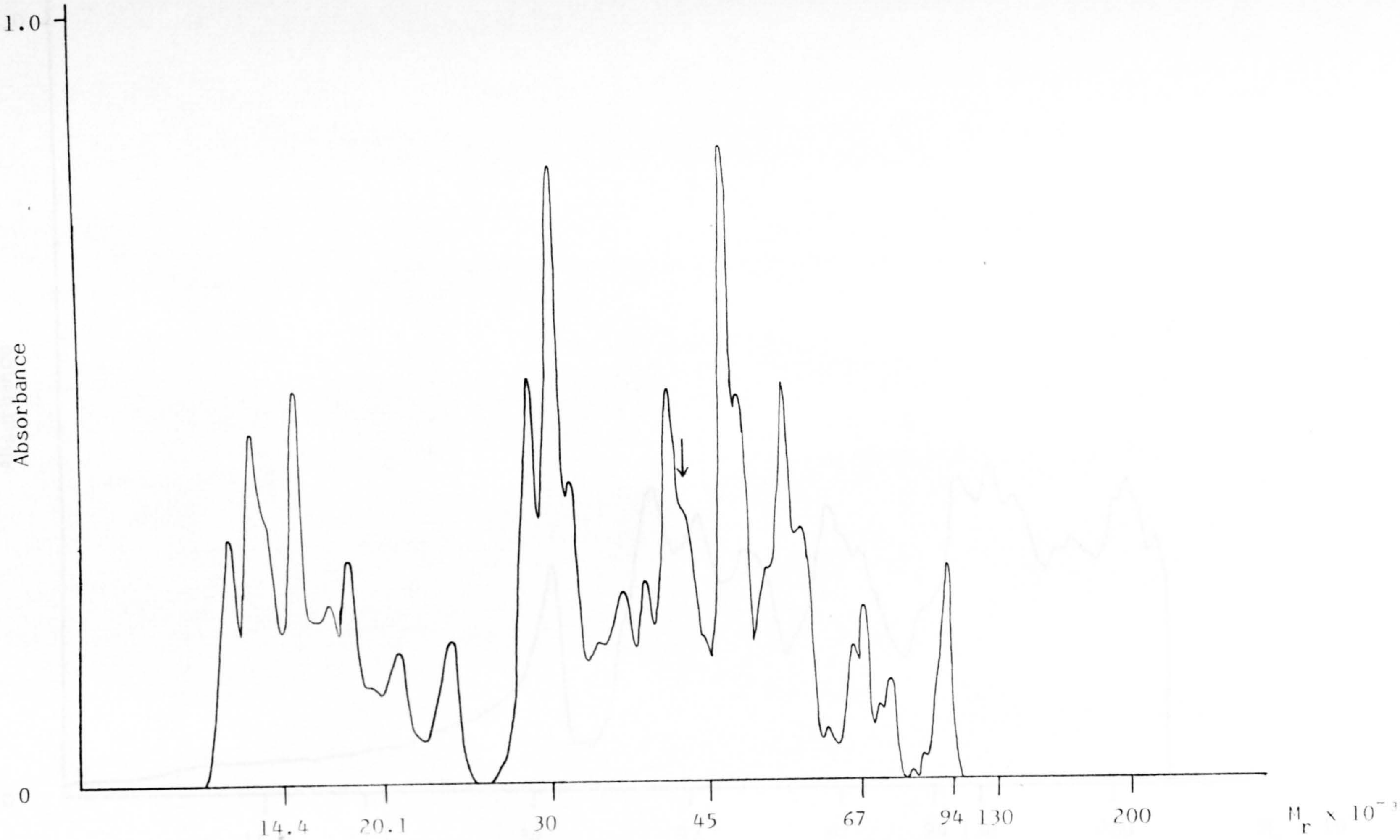


Figure 58. 150 000 x *g* Supernatant. Coomassie blue stain.

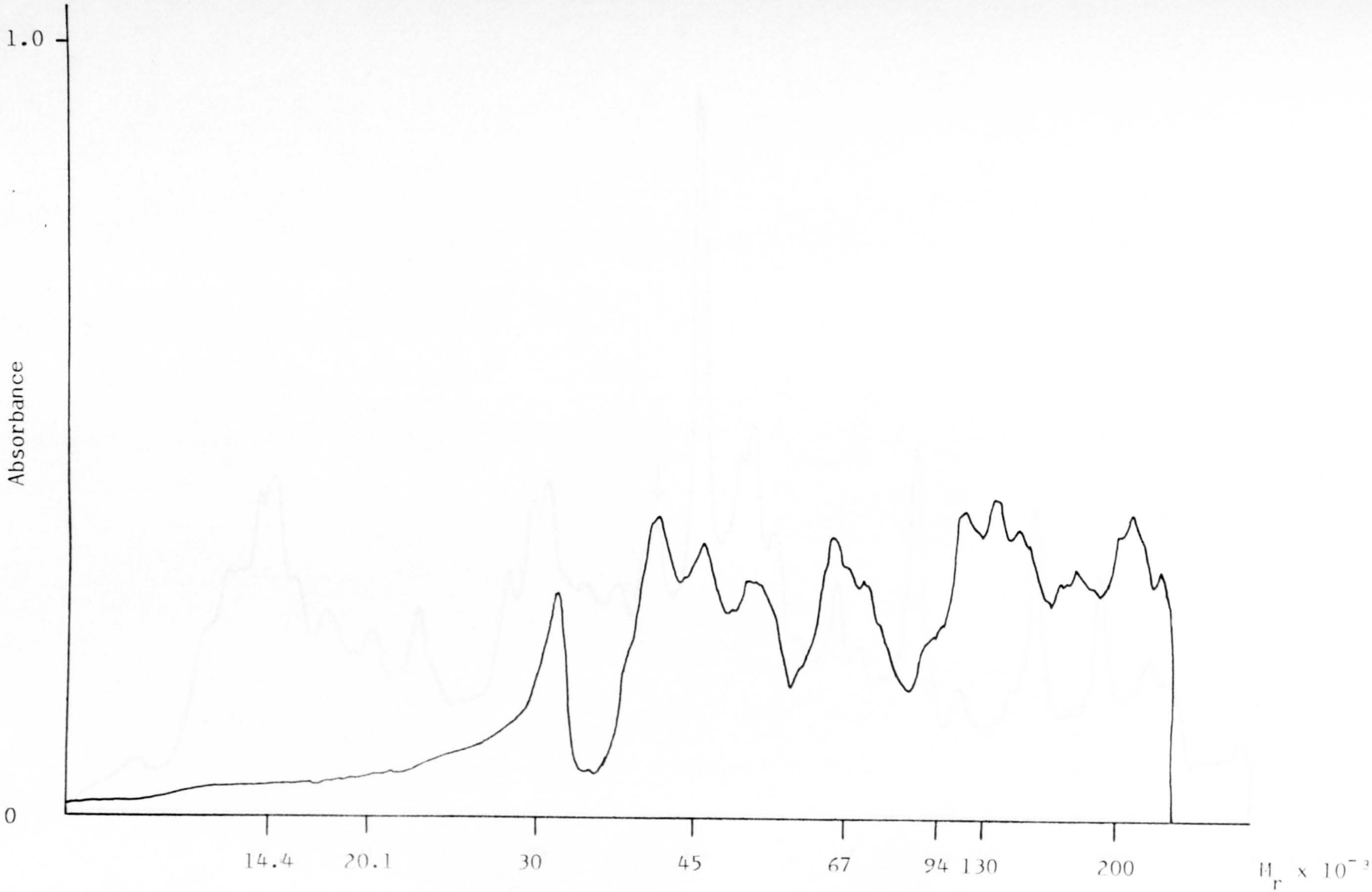


Figure 59. 150 000 x *g* Supernatant. ^{125}I-Con A overlay.

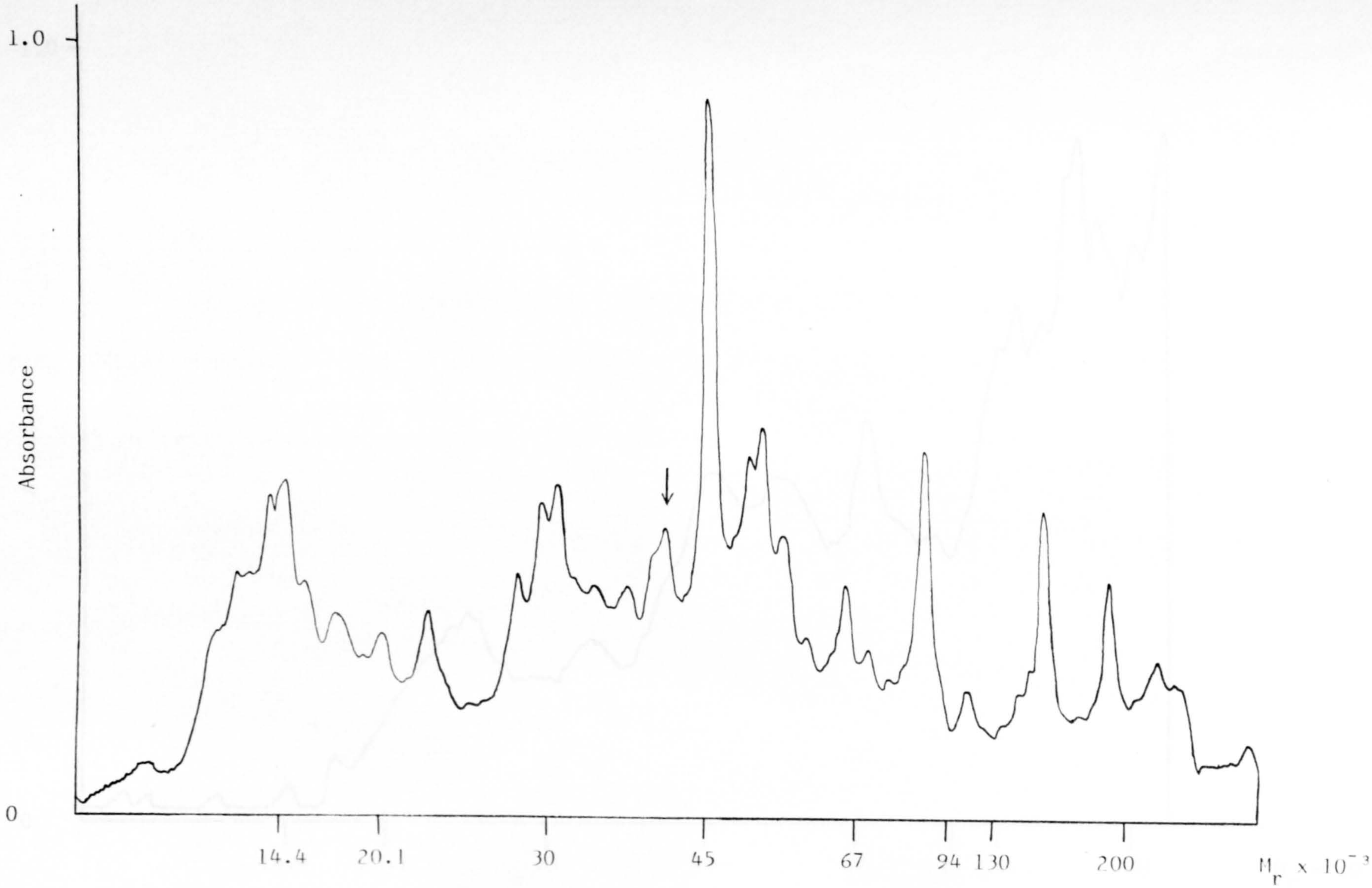


Figure 60. DWH. Coomassie blue stain.

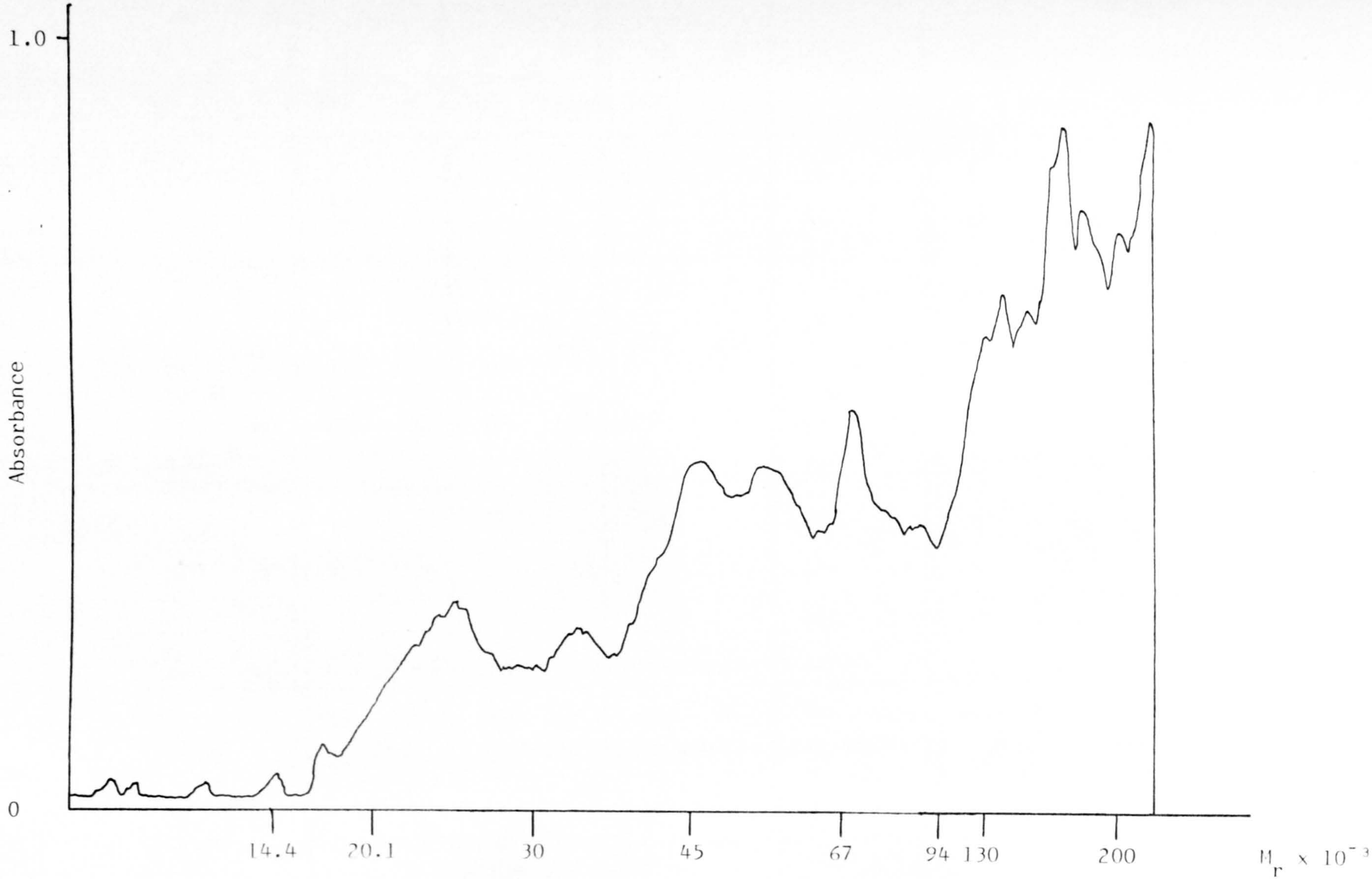


Figure 61. DWH. ^{125}I-Con A overlay.

Table 25. Polypeptides and glycoproteins in fraction.

FTS		100gP		10 000gP		150 000gP		150 000gS		DWH	
P	GP	P	GP	P	GP	P	GP	P	GP	P	GP
	A								A		
	B		B		B				B	B	B
	C								C	C	C
										D	D
	E								E		E
				F							
190	190		190		190		190		190	190	190
	145								145	145	145
		133									
		125									
111										111	
105		105		105							
95		95		95	95	95	95	95		95	

contd....

Table 25 contd.

FTS		100gP		10 000gP		150 000gP		150 000gS		DWH	
P	GP	P	GP	P	GP	P	GP	P	GP	P	GP
								86			
								80		80	
76	76							76	76	76	
								73			
70		70		70				70			
68	68	68	68	68	68	68	68	68	68	68	68
58								58		58	
		56		56		56		56		56	
52	52	52	52	52	52		52	52	52	52	52
								49		49	
45	45	45	45	45	45	45		45	45	45	45
39	39	39		39		39		39	39	39	
		37		37		37		37		37	
35								35			
										34	34

contd...

Table 25 contd.

FTS		100gP		10 000gP		150 000gP		150 000gS		DWH	
P	GP	P	GP	P	GP	P	GP	P	GP	P	GP
	32	32	32		32	32	32	32	32	32	32
						31		31		31	31
29		29		29		29		29		29	
27								27		27	
	25	25	25		25	25	25				25
22				22		22		22		22	
19.5		19.5		19.5		19.5		19.5		19.5	
		18		18		18					
								17		17	
16		16		16		16		16		16	
14.5								14.5		14.5	
										14	
12.5		12.5		12.5		12.5		12.5		12.5	
12		12				12					

contd...

Table 25 contd.

FTS		100gP		10 000gP		150 000gP		150 000gS		DWH	
P	GP	P	GP	P	GP	P	GP	P	GP	P	GP
								11.5			
		11									
		8.5		8.5							

FTS is freeze-thaw supernatant.

100gP, 10 000gP and 150 000gP are the pellets derived from sequential centrifugation of the vortex supernatant at 100 x *g*, 10 000 x *g* and 150 000 x *g*.

150 000gS is the supernatant remaining after such centrifugation.

DWH is denuded worm homogenate.

P = polypeptides, GP = glycoproteins.

Macromolecules of apparent molecular weight greater than 200 000 daltons are designated a letter only.

and two of these represented the major bands seen in PAS-stained gels. All of the glycoproteins were virtually ubiquitous except one, the major band in the vesicular fraction, with an apparent molecular weight around 25 000. This polypeptide was found in all fractions containing particulate matter but was not present in the soluble fraction, confirming the result of PAS-stained gels. In addition, this was the predominant glycoprotein in the surface membrane fraction and the vesicular fraction, all other fractions having somewhat less of this material.

A minor glycoprotein of 95 000 daltons appeared in the vesicular and 10 000 x *g* pellets and may also be present in the surface membrane fraction. Glycoproteins of 32 000 and 68 000 daltons are found predominantly in the soluble fraction and may be cytoplasmic as is the 39 000 dalton glycoprotein in the freeze-thaw supernatant and soluble fraction. Glycoproteins of apparent molecular weight 45 000, 52 000 and 190 000 are most concentrated in the surface membrane fraction and the 10 000 x *g* pellet and are probably associated with surface membrane.

Many of the Coomassie blue staining bands were found in all fractions examined, but two minor bands (molecular weights 22 000 and 31 000) were found in the vesicular fraction and soluble fraction, but not in the surface membrane fraction or the 10 000 x *g* pellet. The source of these proteins is

unclear. Major bands at 27 000 and 52 000 daltons in the freeze-thaw supernatant and soluble fraction were minor bands or absent altogether in all other fractions, suggesting they represent cytoplasmic proteins.

A major band of 95 000 daltons is present in the freeze-thaw supernatant, soluble and vesicular fractions and denuded worm homogenate but is less concentrated in other fractions and may be cytoplasmic. Bands at 12 000 and 12 500 daltons were very important in the vesicular fraction but were major bands in all fractions. A 16 000 dalton polypeptide was found in all fractions but was a major band in the vesicular fraction and may be a discoid granule polypeptide. A polypeptide of 45 000 daltons is a major band in all fractions other than the vesicular fraction. This is believed to be a spine protein.

All of the glycoproteins were stained with Coomassie blue but some were visualised more readily using ^{125}I-Con A than the protein stain. Into this category fall the polypeptides with apparent molecular weight 25 000, 32 000 and 190 000 and all of those with molecular weights greater than 200 000. Some glycoproteins were more readily visualised using Coomassie blue than ^{125}I-Con A, suggesting they have few saccharide residues to which this lectin can bind. These include the glycoproteins with apparent molecular weights of 39 000 and 45 000.

3.4 DISCUSSION

3.4.1 High Density Material

The failure of the antisera raised against the high density pellet polypeptides to bind to spines in worm sections does not prove that the two antigens were not of spine origin. This negative result may have been due to low specific antibody concentration, as suggested by the results of immunodiffusion where only the most concentrated solutions of one of the antisera precipitated antigen. This in turn was probably due to the small quantities of antigen available for each immunization. The argument that the two polypeptides in the high density material are from spines, is not refuted by this result since the antisera did not bind to any worm component.

Actin is a structural component of most cells and the presence of a 45 000 dalton polypeptide in the high density pellet suggested that spines contained actin. This has recently been confirmed by measurement made from micrographs of negatively stained spines (Cohen, Reinhardt, Castellani, Norton and Stirewalt, 1982) and by the discovery that the actin-specific fluorescent stain NBD-phallacidin binds to spines (Kusel *et al*, 1984). It now seems likely that spines are composed of hexagonally packed actin filaments and that cross-bridges link the filaments (Cohen *et al*, 1982).

Similar, hexagonally packed, arrays of actin filaments have

been found in the acrosomal process of horse shoe crab sperm and in sea urchin eggs (De Rosier, Mandelkow, Sillman, Tilney and Kane, 1977). The filament bundle from horse shoe crab sperm contains two other proteins, one of which (molecular weight 55 000) is present in equimolar ratio to actin, there being somewhat less of the third (95 000 dalton) protein (Tilney, 1975). Two proteins are found in combination with actin from sea urchin eggs and these have molecular weights of 58 000 and 220 000 (Kane, 1975a). It is believed that the proteins of 55 000 daltons from horse shoe crab sperm and 58 000 daltons from sea urchin eggs are responsible for cross-linking the actin filaments (De Rosier *et al*, 1977).

Of the two polypeptides in the high density pellet, that of 45 000 daltons probably corresponds to actin and the other is likely to be the cross-linking protein. Since only two polypeptides were found it is probable that the sheets present in the high density pellet were derived from spines by degeneration of the paracrystalline lattice. The still recognisable spines in the high density pellet showed signs of disintegration around their periphery. This may have been caused by osmotic stress. Indeed, Kane (1975a, b) has demonstrated the unpacking and repacking of actin filaments from sea urchin eggs in response to changes in salt concentration which alters the binding of the cross-linking protein.

It is interesting that an antiserum was raised to actin although this is a highly conserved protein. It seems probable,

therefore, that schistosome actin is sufficiently different to rabbit actin to allow its recognition as foreign material by the rabbit.

3.4.2 Discoid Granules

3.4.2.1 Purity of the preparation. The microvesicular fraction is a much cleaner preparation than the 140 000 x *g* pellet prepared from the post-100 x *g* centrifugation supernatant. The additional centrifugation at 10 000 x *g* removed spines and large sheets of membrane, large membrane-bound vesicles as well as dense cytoplasm.

The microvesicular fraction is believed to be enriched in discoid granules for the following reasons :

a) the microvesicles present are of similar size to discoid granules ;

b) the fraction is rich in carbohydrate, a known constituent of discoid granules (Wilson and Barnes, 1974a) ;

c) it contains little alkaline phosphatase. (If the vesicles had been formed from surface membrane, this enzyme might be expected to be present at a higher specific activity.) ;

d) the ratio of carbohydrate to alkaline phosphatase is much higher than in whole worms, again suggesting a high ratio of discoid granules to surface membrane.

It is, clearly, unfortunate that discoid granules did not maintain their characteristic morphology during isolation

procedures but the above factors do suggest that the microvesicular fraction is enriched in discoid granule. It is not known why the organelles altered their shape but it was probably due to the changes in osmotic pressure which occur during freezing and thawing (Meryman, 1974). Modification of discoid granule shape was reported by Wilson and Barnes (1974a) when, after treatment of adult schistosomes with permanganate fixative, the discoid granules became spherical. Further evidence that freeze-thaw caused the swelling of the organelles comes from the surface membrane isolation of Oaks *et al* (1981), using Triton X-100. Discoid granules maintain their integrity under the conditions used by those authors since they are clearly present in the material sedimented by high speed centrifugation. It seems, therefore, that the method of Oaks *et al* for removal of surface membrane, may be a useful starting point for isolation of discoid granules without altering their morphology.

Carbohydrate is not an ideal marker for discoid granules since carbohydrates will be present throughout the worm. Glycogen could be eliminated by hydrolysis of supernatants and worm homogenates with α-amylase and subsequent dialysis. The action of α-amylase on glycogen has been characterised by Whelan and Roberts (1952). The enzyme hydrolyses α-1,4 glucoside bonds releasing maltose and maltotriose. It does not hydrolyse α-1,6 glucoside bonds which are present at the branch points of glycogen. Limit dextrins remain after extensive hydrolysis, the largest of which is an octosaccharide. Maltose, maltotriose

and the limit dextrins will be withdrawn by dialysis, thus removing this major source of carbohydrates.

None of the pellets was treated with α-amylase since it was considered unlikely they would contain significant amounts of glycogen. The centrifugal force used to sediment glycogen (Bueding and Orrell, 1964) is ten times that used here to sediment microvesicles. Glycogen is easy to recognise in electron micrographs and none was ever detected in pellets.

3.4.2.2. Supposed fate of discoid granules. There are three main possibilities as to the fate of discoid granules *in vivo*.

a) that they contribute to the spines. This was hinted at by Smith *et al* (1969) who found that both discoid granules and spines could be stained with a carbohydrate stain, phosphotungstic acid, and observed discoid granules in close proximity to spines. The phosphotungstic acid-staining of spines could not be repeated by Wilson and Barnes (1974a) and they could not stain spines with any of the carbohydrate stains employed. Further circumstantial evidence for the contribution of discoid granules to spines comes from the observation by McLaren (1980) that the absence of discoid granules in the developing schistosomulum, 2 to 3 weeks after infection, coincides with the reappearance of spines.

b) that they contribute to the ground substance of the tegument. Wilson and Barnes (1974a) observed dense "blobs" in the cytoplasm of the tegument of adult worms. The frequency of "blobs" was found to be inversely proportional to that of discoid

granules. This suggested that the discoid granules lost their bounding membrane and broke down to mix with the cytoplasm. Moreover it was found that discoid granules stained strongly for carbohydrate, the blobs stained lightly and the ground substance of the tegument stained more diffusely, again suggesting a link between the three. It is not at all clear why it should be necessary to package substances into vesicles simply to transport them around the tegument but perhaps this would provide a more rapid delivery of substances should it be necessary to effect repairs, than the apparently simpler mechanism of mass production in, and diffusion from, the tegumental cells.

c) that they fuse with the surface membrane. This was suggested by McLaren (1980) who observed the apparent fusion of discoid granules with surface membrane lining the pits in the tegument of 4-week old worms. This phenomenon was seen only rarely in younger or older worms. It was suggested that discoid granules may help maintain the integrity of the surface membrane.

3.4.2.3. Discoid granule-like structures in other trematodes. Some assistance in establishing the fate of discoid granules may be gained from a study of similar organelles in other trematodes. Electron-dense bodies are found within the tegument of most digenetic trematodes. They vary in shape but all are of similar size. In almost all cases little is known about these bodies and there is little evidence to point to their function. On the basis of electron microscope observations, the electron-dense tegumental inclusion bodies of *Gorgoderina* (Burton, 1964) and *Haematoloechus* (Burton, 1966) are believed to contribute to

the tegumental cytoplasm, whereas those of *Cyathocotyle* apparently contribute to the glycocalyx on the parasite surface (Erasmus, 1967).

In the case of *Gorgoderina*, the electron-dense body is crystalline and has no limiting membrane. Thus it seems most unlikely to fuse with the surface membrane and contribution of the crystalline material to the cytoplasm seems probable. There is therefore a precedent for transport of material from tegumental cells to the distal tegument without that material being in solution in the cytoplasm. This makes the packaging of substances into membrane-bound vesicles, solely for transport to another area of the tegument, more plausible. If the tegumental cytoplasm is very viscous, diffusion of solutes may be slow and distribution in organelles may be more effective.

In one digenean there is good experimental evidence that the electron-dense inclusion bodies contribute their contents to the surface of the parasite. Hanna (1980a) has shown by autoradiography that the Tl bodies of *Fasciola hepatica* take up ^{3}H-labelled leucine while in the tegumental cells, then travel to the base of the tegument. Although Tl bodies were not observed near the surface membrane, label appeared on the surface suggesting rapid movement to, and discharge onto, the surface by the bodies. Tl bodies are believed to be responsible for replacement of the surface glycocalyx of juvenile flukes if this becomes complexed with host antibody (Hanna 1980b, c).

From electron microscope observations, schistosomula and adult schistosomes do not appear to possess a glycocalyx at the parasite surface (McLaren, 1980). If the discoid granule contributes to the surface there must be some reason for it doing so, other than replenishment of glycocalyx.

3.4.2.4. Comparison of polypeptides in the vesicular fraction, surface membrane, soluble fraction and high density material. The major glycoprotein in the vesicular fraction had an apparent molecular weight of 25 000 although it spread from 20 000 to 30 000.. From the gel scans it seems this was a major peak in all three sequentially derived pellets, particularly the vesicular fraction and the surface membrane fraction, while all other glycoproteins in the vesicular fraction were present as more prominent peaks in other fractions. These factors lead to the conclusion that the 25 000 dalton polypeptide is a discoid granule glycoprotein. Its presence in the surface membrane may be due to contamination of that fraction with discoid granules or, more likely, may be indicative of a link between discoid granules and surface membrane.

Since the major band in the vesicular fraction was absent from the soluble fraction, the results presented here suggest there is no link between discoid granules and soluble cytoplasmic components. The presence of the 25 000 dalton glycoprotein in the freeze-thaw supernatant is probably due to release of some surface membrane during freeze-thaw. It may be due to release of soluble components of the tegument cytoplasm but the complete

absence of the glycoprotein from the soluble fraction renders this unlikely. This absence cannot be explained by α-amylase digestion or adsorption to the concentration membrane since the polypeptide is present in the freeze-thaw supernatant and denuded worm homogenate which were similarly treated.

The other possibility, that discoid granules contribute to spines, seems improbable since of the two spine polypeptides, only one (45 000 daltons) was found in the vesicular fraction and it was not a major component of that fraction. No glycoproteins were found in the high density pellet using Concanavalin A overlay.

Of the three possible fates of discoid granules, the one best supported by these results is contribution to the surface membrane.

3.4.2.5. The presumed role of the discoid granule. If discoid granules are secretory bodies then it is not unreasonable to expect their function to be affected by agents such as ouabain and cytochalasin B which disrupt secretory processes. Wilson and Barnes (1974b) assessed the effects of these agents on secretory processes in the tegument. Oubain appeared to have little effect on discoid granules but it is possible that cytochalasin B affected discoid granule function. One effect of cytochalasin B was the appearance in surface channels of small pockets of membrane. These pockets might be interpreted as discoid granules fusing with the surface membrane. The

micrograph of discoid granules fusing with surface membrane shown by McLaren (1980) bears a striking resemblance to the pockets of membrane shown by Wilson and Barnes (1974b) and they may well show identical events.

Cytochalasin B is believed to disrupt the microfilament web which lies just under the plasma membrane of many cells (Malaisse, Malaisse-Lague, van Obberghan, Somers, Devis, Ravazzolaand Orci, 1975) and disruption of the microfilaments is thought to be necessary for fusion to take place between secretory vesicles and plasma membrane. For this reason the secretion of insulin by rat pancreatic β cells is enhanced by treatment of the cells with cytochalasin B (Malaisse *et al*, 1975). Cytochalasin B thus accelerates secretory processes.

If discoid granules fuse with the surface membrane they presumably do so by fusion of their limiting membrane with the inner bilayer of the surface membrane complex. Fusion of a single-membrane-bound organelle with a double plasma membrane is not likely to lead to extrusion of contents onto the surface. It is more likely that their contents would be released between the outer and inner bilayers. The electron micrograph which apparently shows discoid granules fusing with the surface membrane (McLaren, 1980) does not permit any interpretation of the method of fusion.

Assuming discoid granules contribute their contents to the surface membrane, the reason for doing so remains obscure. As

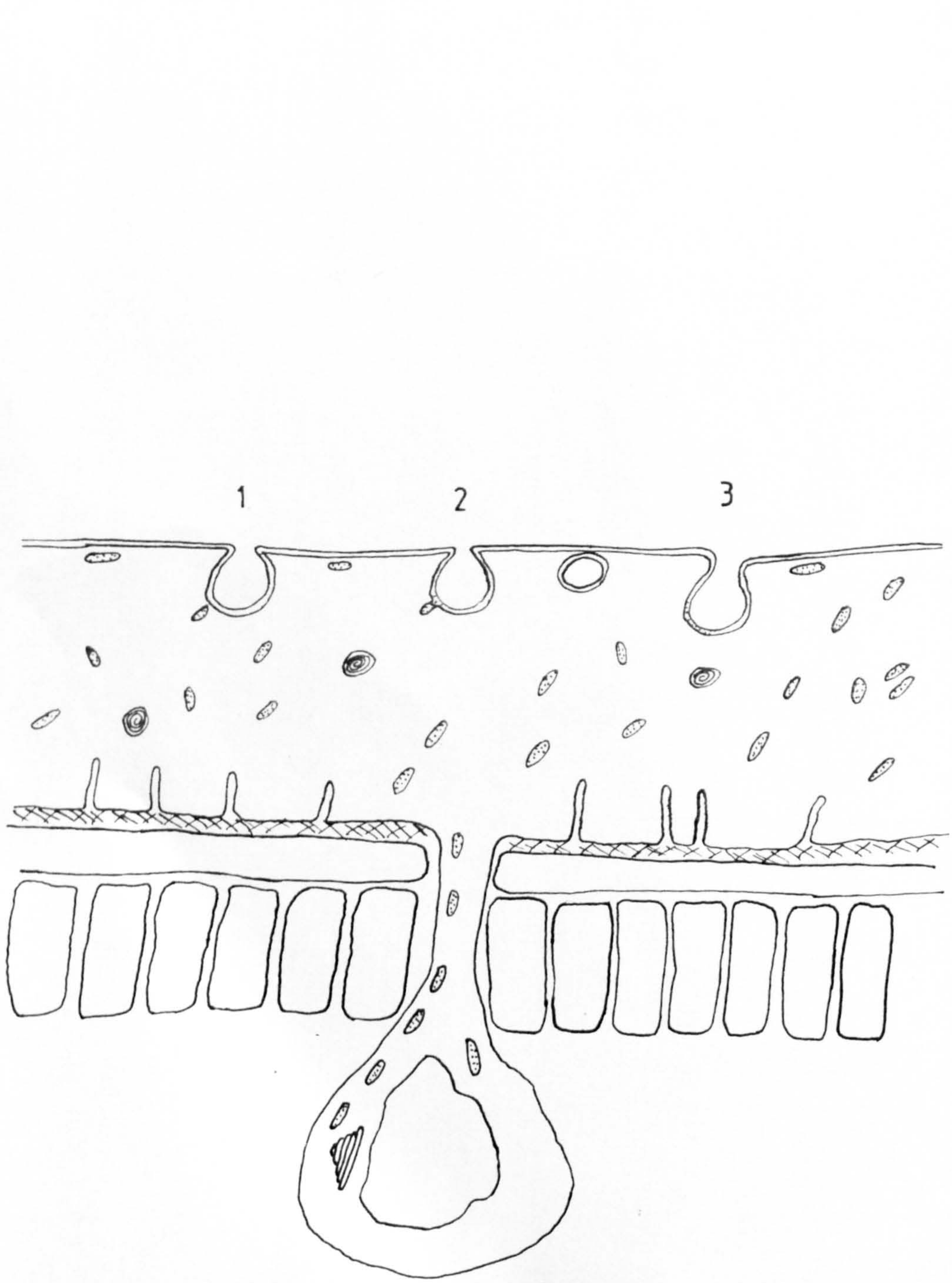
previously mentioned, the Tl bodies in the tegument of *Fasciola hepatica* are involved in replacement of glycocalyx following immune attack. The equivalent function in schistosomes, that of replacement of the outer bilayer of the tegument, is fulfilled by the multilaminate vesicles (Wilson and Barnes, 1977). It is clear from lectin binding studies (Simpson and Smithers, 1980; Linder and Huldt, 1982) and experiments involving electron microscopy after colloidal iron (Stein and Lumsden, 1973) and ruthenium red (McDiarmid and Podesta, 1984) staining that carbohydrates are exposed at the surface of adult *S mansoni*. However, a glycocalyx, as seen in schistosome cercariae or in *F hepatica* and at the surface of many other cell types, does not seem to be present, although this is currently a matter of some dispute (McDiarmid and Podesta, 1984). The main reason for supposing discoid granule contents are not extruded onto the surface, however, is the problem of penetrating the double bilayer.

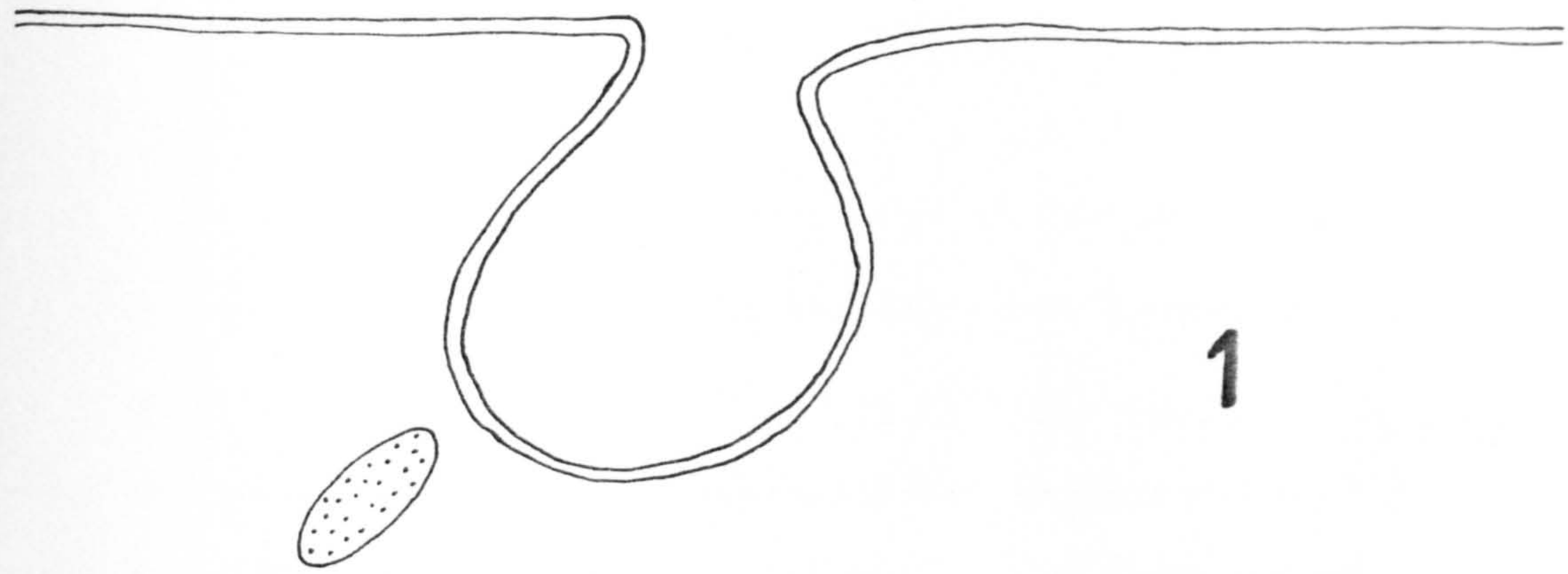
An intercalary secretion might act as an adhesive or even a lubricant between the outer and inner bilayers. Some forces must keep the outer and inner bilayers closely apposed while allowing the outer to slide over the inner bilayer during turnover (Wilson and Barnes, 1977). A thin glutinous layer between the bilayers could serve both purposes.

The following seems a plausible working model for the course of events in secretion of discoid granule contents. The discoid granules are manufactured in the tegumental cells by the endoplasmic reticulum and Golgi apparatus in the same manner as

secretory vesicles in other systems (Wilson and Barnes, 1974a). They pass up the cytoplasmic connections into the tegument where they remain until required. In response to some unknown stimulus, a cytoskeletal system below the surface membrane is disrupted and discoid granules close to the surface membrane fuse with the inner bilayer and extrude their contents between the two membranes. This process of fusion and discharge must be rapid since it is seldom detected by electron microscopy. Figure 62 summarises this model.

Figure 62. A model for the fate of discoid granules. They are produced in the tegumental cells, are moved to the apical tegument and fuse with the inner bilayer of the surface membrane, extruding their contents between the bilayers.





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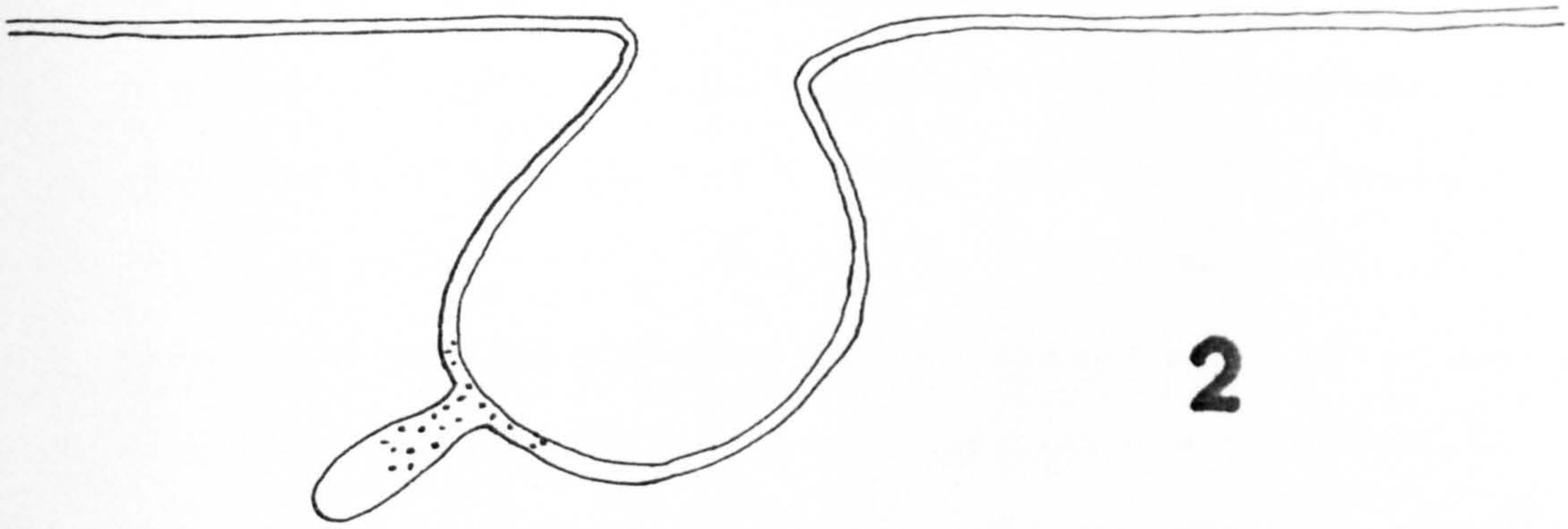

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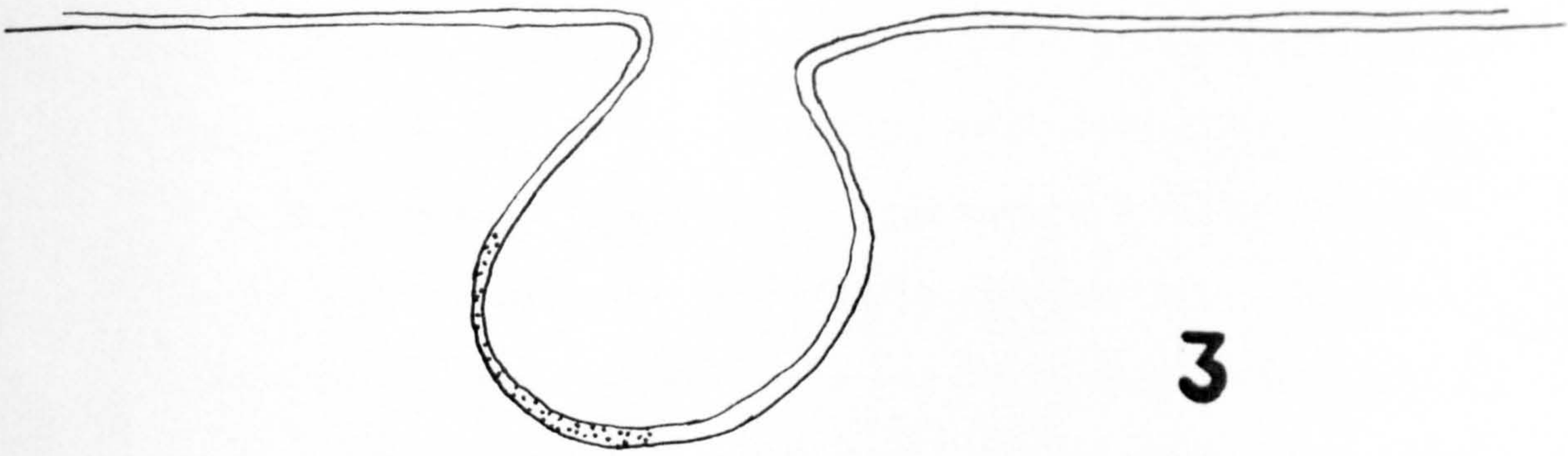

3

GENERAL DISCUSSION

GENERAL DISCUSSION

The final method for isolation of schistosome surface membranes involved freezing and thawing worms suspended in Hanks' balanced salt solution. The worms were washed, vortexed, then washed again. The second washings were pooled and centrifuged to yield a pellet enriched 17 times in the surface membrane marker enzyme. If this crude preparation was further fractionated by density gradient centrifugation, spines sedimented at the bottom of the tube and surface membranes could be recovered from a band in the gradient. Enrichment of alkaline phosphatase in this band was 40 times that of whole worms (Roberts, MacGregor, Vojvodic, Wells, Crabtree and Wilson, 1983). Where direct comparisons with other published methods are possible, enrichment of alkaline phosphatase in the crude preparation was at least as high as the final preparation using other methods. Enrichment of this enzyme in the material from the density gradient is considerably higher than reported using any other method for isolation of the surface membrane of *S mansoni*.

Although only one third of the total alkaline phosphatase is present on the surface (Roberts *et al*, 1983) it was nevertheless assumed that it was an adequate marker for the surface membrane since only one third of the total alkaline phosphatase could be released by freeze-thaw and vortexing. Indeed, if it is assumed that all of the released alkaline phosphatase originated from the surface membrane, then the actual enrichment of surface membrane may be three times the enrichment of the enzyme, ie 120 times that of whole worms.

From the evidence of alkaline phosphatase enrichment alone it is clear that the method described here represents an advancement in surface membrane isolation. In addition however, it was found that levels of gut protease and NADH-cytochrome c reductase in the membrane preparations were low. No other study of schistosome surface membrane isolation has quantified contamination. Electron microscopy revealed the final preparation to be largely composed of surface membrane.

It is difficult to define an acceptable level of purity in a surface membrane preparation. Neville (1976) compared isolates of plasma membrane in over one hundred reports covering mammalian tissue types or cell lines and found enrichment of the plasma membrane marker enzyme varied from 4 times to 47 times that of the tissue homogenates. Most were in the range 4 to 25 times. At first sight it appears that purity of the schistosome surface membrane preparation described here is very high. The maximum possible enrichment varies from cell type to cell type in mammalian cells and is found from the ratio of cell protein to plasma membrane protein (Neville, 1975). The amount of protein present in the schistosome surface membrane is unknown (and may bear little relation to mammalian values) so maximum possible enrichment cannot be calculated. In any case, an acceptable level of purity will vary with the use of the preparation. For example, a whole worm homogenate is useless for demonstrating surface membrane proteins by SDS-PAGE while a crude preparation may show the major proteins just as well as a more refined preparation. The isolation method given here provides the user with a choice of two preparations of varying purity.

When this work began, alkaline phosphatase was the only enzyme shown to be located in the surface membrane. Other workers have recently used adenosine triphosphatase and phosphodiesterase as additional surface membrane markers although the localization of these enzymes in the surface membrane has not been confirmed cytochemically. It seems that the ion requirements of the ATPase have not yet been fully elucidated since it is variously reported as Ca^{++}-ATPase (Cesari, Simpson and Evans, 1981), Na^{+}, Mg^{++}-ATPase (Podesta and McDiarmid, 1982), Ca^{++}, Mg^{++}-ATPase (Simpson, Cesari, Evans and Smithers, 1980) and Na^{+}, K^{+}, Mg^{++}-ATPase (Oaks, Cain, Mower and Raj, 1983). Measurement of ATPase activity requires assay of released phosphate. It would have been undesirable to freeze worms in any medium other than Hanks' balanced salt solution (which contains phosphate) and cost precluded the use of radiolabelled ATP so at no point was ATPase assayed.

Electron micrographs illustrated the internal damage which occurred when worms were frozen and thawed. For freeze-thaw to be of use in surface membrane disruption, it was only necessary to damage the outer portion of the worms. Internal damage probably contributed to contamination of membrane fractions so it seemed prudent to attempt to reduce this damage. Release of markers for soluble and particulate internal components could be reduced using cryoprotectants and this reduction did not prevent release of surface membrane. It appeared that the surface membrane was liberated in smaller, less easily sedimented, fragments however, and this meant that enrichment of alkaline phosphatase in surface membrane preparations was no higher when cryoprotect-

ant was used than when worms were frozen in the absence of cryo-protectant. Although a number of freeze-thaw regimes were used without improving alkaline phosphatase enrichment there is scope for extending this study, particularly with regard to freezing and thawing rates. Fragmentation of the surface membrane appeared to depend to some extent on the rate of thawing and perhaps the use of a lower thawing rate would have circumvented this problem.

It appears that tegumental spines are easily isolated and consist of two polypeptides. One of these has been identified elsewhere as actin and the other almost certainly cross-links the actin. It is to be envisaged that actin together with a cross-linking protein will be identified in spines of other trematodes. In the present study, the isolated spines were apparently disrupted by osmotic shock. For confirmation that spines were isolated, the electron microscopy should ideally be repeated on material prepared in the absence of osmotic shock and further attempts should be made to raise antisera to the polypeptides in the high density pellet using greater quantities of antigen and also using adjuvant. The antisera could then be used to confirm that both proteins are from spines.

It is extraordinary that when so much attention is paid to the properties of the surface membrane, so little is known about the discoid granules which are the major inclusion body of the tegument. A better understanding of the function of these organelles must surely lead to a fuller knowledge of tegumental properties. Attempts were made here to isolate discoid granules from adult worms and, although the preparation was perhaps some-

what crude, it was possible to gain some information about the contents of the discoid granules. It was clear from comparisons of glycoproteins present in the surface membrane, tegumental cytoplasm, spines and discoid granules that contribution of discoid granules to surface membrane was their most probable fate. This evidence alone is not sufficient to prove the role of these organelles, although there is some corroboration from electron microscope observations (McLaren, 1980). The glycoprotein believed to form a major part of the contents of discoid granules (that of apparent molecular weight 25 000) is known to possess binding sites for soybean agglutinin (MacGregor, Stott and Kusel, 1985). It is interesting that binding sites for this lectin are not present on the surface of schistosomula up to and including three weeks post infection (Murrell, Taylor, Vannier and Dean, 1978; Simpson, Correa-Oliveira, Smithers and Sher, 1983) while they are present in adult worms (Simpson and Smithers, 1980; MacGregor *et al*, 1985). It may be significant that discoid granules have been seen in fusion with the surface membrane at four weeks post infection (McLaren, 1980).

Antibodies raised against the 25 000 dalton glycoprotein and conjugated with gold particles, could be used to demonstrate the location of the glycoprotein by electron microscopy of tegument thin sections. This would show whether the glycoprotein is common to the discoid granules and the surface membrane. Pulse-chase experiments with radiolabelled molecules followed by autoradiography of worm sections might provide conclusive proof of the fate of discoid granules. Such experiments have been conducted (Wilson and Barnes, 1979) but discoid granules did not become

labelled, perhaps because the tracer molecules used, glucosamine and leucine are not present in these organelles. Different amino acids or sugars may give more conclusive results.

If discoid granule contents provide lubrication between the outer and inner bilayers of the surface membrane complex it is probable that the discoid granule glycoprotein is rich in sialic acid. This would make individual molecules highly charged and give the mutual repulsion and high viscosity which are necessary for lubrication. The intercalary lubricant theory might be tested *in vitro* using the antibiotic tunicamycin. This inhibits glycosylation of proteins (Duskin and Mahoney, 1982) and should remove any lubricant properties of the discoid granule contents. If non-glycosylated protein is secreted between the two bilayers, turnover would be prevented. A second method of testing the lubricant theory would be to purify the discoid granule glycoprotein by lectin affinity and gel filtration chromatography. It would then be possible to determine whether the saccharide composition was consistent with that of a lubricating glycoprotein.

There is much yet to be discovered about discoid granules. The method of isolation of these organelles described here may provide one starting point for such studies.

APPENDIX ONE

MORPHOLOGICAL ANALYSIS OF MATERIAL SECRETED IN RESPONSE TO THE BINDING OF CATIONIZED FERRITIN

A.1 INTRODUCTION

It is generally accepted that the outer bilayer or membranocalyx of the schistosome surface membrane turns over by shedding and is replaced by extrusion of the lamellate material within multilaminate vesicles (Hockley and McLaren, 1973; Wilson and Barnes, 1974a, 1977; Kusel, McKenzie and McLaren, 1975). Collection of secreted material from incubation media should equate to isolation of outer bilayer. Murrell, Vannier and Ahmed (1974) collected material released into defined culture medium after 36 - 48 hours of incubation. However, since Wilson and Barnes (1974b) showed that after four hours in defined medium worms exhibited some ultrastructural deterioration, such a technique is undesirable. It is possible to maintain worms for some weeks in medium containing undefined supplements (Clegg, 1965; Basch, 1981a, b). Unfortunately serum proteins present in these supplements greatly outweigh the small amounts of material released by the worms.

The rate at which turnover takes place may be dependant on the conditions of the parasite environment. In the presence of anti-host antibody, turnover is rapid (Perez and Terry, 1973). Similarly, rapid turnover occurs when worms are cultured in medium containing cationized ferritin, which binds to the surface, and under these conditions, the outer bilayer has a half-life of 2 - 3 hours (Wilson and Barnes, 1977).

The enhancement by cationized ferritin of outer bilayer shedding may permit isolation of secreted material following

short incubations in defined media and thus provide a route to the characterization of the outer bilayer.

This appendix describes the isolation of material secreted in response to cationized ferritin binding and the morphology of the released material.

A.2 MATERIALS AND METHODS

About five hundred worm pairs, recovered from infected mice by perfusion with Eagle's medium, were incubated in 30 ml Eagle's medium containing 0.7 mg ml^{-1} cationized ferritin at 37°C for 30 minutes in an atmosphere of 95 % air, 5 % CO_2. The worms were then washed five times in Eagle's medium and incubated for a further four hours in 100 ml Eagle's medium alone at 37°C in an atmosphere of 95 % air, 5 % CO_2. Worms were then removed from the medium by sieving and the secreted material sedimented by centrifugation at 100 000 x *g*. The resultant pellet was fixed and processed for electron microscopy by the second of the methods described in Chapter 1, that is, 4 % (w/v) glutaraldehyde and 1 % (w/v) osmium tetroxide in cacodylate buffer, were used as primary and secondary fixatives respectively. The pellet was then fixed in uranyl acetate/sucrose in aqueous solution, dehydrated in a series of ethanols, cleared in propylene oxide and embedded in an Epon-Araldite mixture. Sections were cut on a Reichert Om U3 ultramicrotome and micrographs were taken using a Kratos Corinth 500 transmission electron microscope.

A.3 RESULTS

The pellet of secreted material was found to be largely composed of membranous material (Figures 63 and 64). This took the form of long multilamellar sheets which were highly folded. The membranous material was very electron dense, appearing quite black in micrographs due to the ferritin which seemed to be present on most of the membranes. At low magnification the stacking of the membranes and the electron opacity of the ferritin combined to give the multilamellar sheets the appearance of thick black lines and these could only be resolved into lamellae at high magnification (Figure 64). Organisation of the multilamellar sheets appeared quite haphazard with the number of lamellae varying along the length of the sheet where some lamellae left or rejoined the sheet. Amorphous electron dense material was present in the pellet and may have been ferritin. Occasionally a spine was seen.

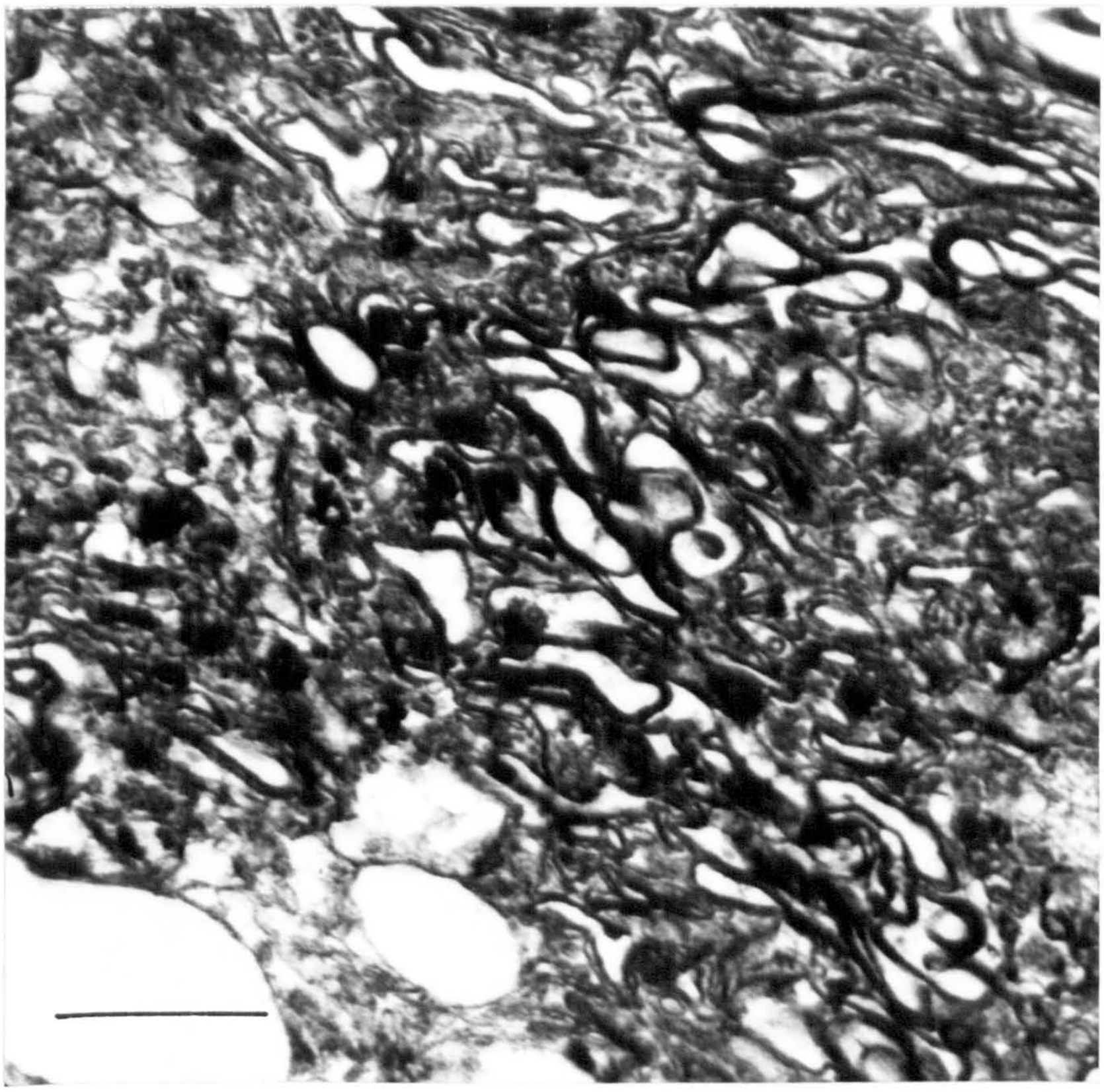

Figure 63. Material secreted by worms after treatment with cationized ferritin.

Bar = 0.5 µm.

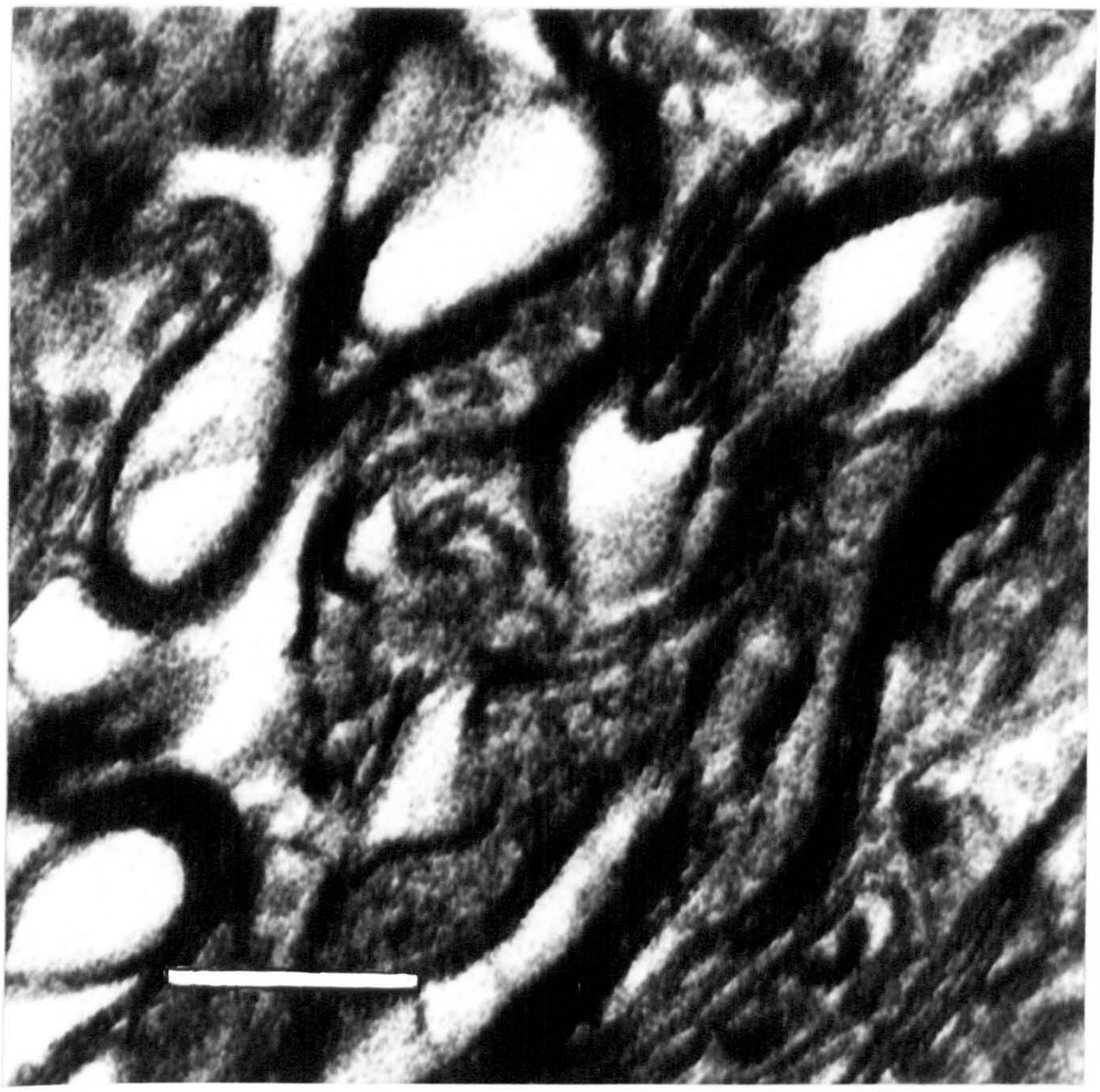

Figure 64. Higher magnification of material secreted after treatment with cationized ferritin.

Bar = 0.1 µm.

A.4 DISCUSSION

The membranous material in the pellet was identifiable as outer bilayer of the surface membrane owing to the presence of ferritin. Little else was present in the pellet which was remarkably homogeneous. The discovery of a few spines suggests some worms were damaged but from the number of spines found it was clear that this damage was not extensive. Wilson and Barnes (1977) examined schistosomes labelled with cationised ferritin and found no ultrastructural damage. It seems that cationized ferritin may indeed be a useful tool in isolation of the outer bilayer. The presence of this protein may hinder the biochemical characterization of the outer bilayer however.

Digitonin releases the outer bilayer from schistosomes (McDiarmid, Dean and Podesta, 1983) but may also lead to problems in characterization because of differential solubilization of membrane components (Helenius and Simons, 1975). McDiarmid *et al* (1983) quantified outer bilayer release by covalently labelling the worm surface with ^{125}I. Digitonin released 90 % of the bound label. On the basis of alkaline phosphatase assay (they believe this enzyme to be in the inner bilayer) their outer bilayer preparation contained 20 % of the inner bilayer as well. No direct comparison can be made with the present method but contamination of the outer bilayer preparation with inner bilayer is likely to be less when cationized ferritin is used to accelerate a natural secretory process than when detergents are used to disrupt membranes.

Stacking of membranes was seen previously (Chapter 1) and may be a property of the outer bilayer. Alternatively, it is possible that in the present case, stacking is due to cross-linking by the cationized ferritin. Membrane stacking was not reported by McDiarmid *et al* (1983) who used (non-cationized) ferritin-labelled Concanavalin A as an outer bilayer marker. The cationized ferritin used in the present study is polycationic (Danon, Goldstein, Marikovsky and Skutelsky, 1972) and so cross-linking of membranes with a negative charge may be possible using this molecule.

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TEGUMENT SURFACE MEMBRANES OF ADULT *SCHISTOSOMA MANSONI*: DEVELOPMENT OF A METHOD FOR THEIR ISOLATION

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Several approaches to surface membrane stripping have been applied to the adult schistosome. Membrane removal was evaluated by the use of different extrinsic and intrinsic markers of which alkaline phosphatase proved to be the most reliable. After initial studies employing incubation of worms in buffer alone, Triton X-100 or freeze/thaw, the last method was chosen for development. The final method applies a single freeze/thaw step to adult worms in balanced salt solution followed by short bursts of agitation on a vortex mixer to release the tegument. Differential and density gradient steps subsequently yield a final membrane pellet enriched over 130 times in surface alkaline phosphatase. The method has been characterized during its development using electron microscopy and enzyme markers for contaminant worm fractions.

Key words: *Schistosoma mansoni*; Freeze/thaw; Tegument surface membranes; Alkaline phosphatase

INTRODUCTION

The schistosome surface is the major host/parasite interface and is therefore assumed to be a prime target for host immune attack. However, schistosome infections in the mammal are of long duration and it is clear that adult worms are able to evade the host immune response. The presence of an unusual double outer membrane at the tegument surface [1] undoubtedly plays a significant role in the mechanisms of evasion [2]. The double membrane has been interpreted as an inner plasma membrane overlain by an outer, secreted bilayer [3]. Adult and juvenile worms are able to slough

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Abbreviations: MEM, minimal Eagle's medium; Con A, concanavalin A; WGA, wheat germ agglutinin; HBSS, Hanks balanced salts solution; TCA, trichloracetic acid; TBS, Tris-buffered saline; PMSF, phenylmethylsulphonyl fluoride; TPCK, 1-1-tosylamide-2-phenyl-ethylchloromethyl ketone; TLCK, tosyl-L-lysine chloromethyl ketone; IU, international unit. *Membrane stripping fractions:* S1, thaw supernatant; S2, vortex supernatant; H, denuded worm homogenate; TOT, total worm homogenate (S1 + S2 + H); S2P, S2 pellet; S2S, S2 supernatant; S2P f/t, S2P after freeze/thaw treatment; S2S f/t, supernatant after repelleting of S2P f/t; GP, membrane pellet recovered from density gradient.

this outer bilayer in response to irritants such as cationised ferritin [4], lectins [5,6] and anti-host antibody [7]. Host molecules on the surface of the adult worm may occlude parasite antigens and therefore offer protection to the worm [8–13].

To achieve a greater understanding of the properties of the tegument surface, it is necessary to isolate the tegument surface membranes to a high degree of purity and yield. Kusel [14] removed the adult schistosome tegument using both freeze/thaw and detergent extraction techniques. However, no further purification of released material was carried out and only electron microscopy used to assess fraction purity. More recent approaches to the isolation of tegument membranes have involved a single incubation in phosphate-buffered saline [15], 0.2% Triton X-100 [16] and sequential incubations in digitonin [17].

In the present paper we describe a technique for isolating the schistosome adult surface membranes which we believe offers certain advantages over the above methods. The preparation has been rigorously examined using electron microscopy in addition to marker and contaminating enzyme activities. The schistosome surface membranes can be prepared to a high level of yield and purity by a straightforward and convenient freeze/thaw method.

MATERIALS AND METHODS

The methods used throughout the study are detailed in this section. Their application to the problem of membrane isolation is described in the Results section.

Parasite maintenance and worm recovery. This was as described previously [3]. Mature parasites were obtained from LACA mice by perfusion via the aorta. Approximately 40 ml minimal Eagle's medium (MEM) (Wellcome Laboratories) containing 4 units ml^{-1} heparin (Sigma) were used for each mouse. The worms were washed several times with MEM minus heparin to remove serum and erythrocytes. Worms perfused by this method, incubated for 30 min in MEM, then reinjected into the hepatic portal vein of naive mice and perfused 24 h later, were 100% viable. No problems were encountered with erythrocytes adhering to worms.

Surface markers. Several extrinsic and intrinsic tegument surface markers were evaluated to provide information about the degree of surface enrichment obtained during membrane isolation.

(*a*) *Alkaline phosphatase*. Non-specific alkaline phosphatase has been located on the surface of adult worms histochemically and enzymatically [18–23]. The tegument enzyme activity is localised in the pits and is absent from the male ventral surface [20–22]. However, the enzyme is not exclusive to the worm surface (Wheater, personal communication). Experiments were performed to determine the percentage total enzyme activity associated with the tegument. The surface activity was measured using the approach of Levy and Read [24], and the assay procedure of Ernst [22].

omitting potassium chloride from the buffer. Live worms were incubated for 5 min at room temperature or 37°C with *p*-nitrophenol phosphate (Sigma) and the release of *p*-nitrophenol compared with that of the total worm homogenate under identical conditions.

The alkaline buffer conditions are likely to be harmful to the tegument and consequently incubation times were kept to a minimum and worms were transferred to the buffer immediately before the incubation.

(*b*) *Lectins*. Concanavalin A and wheat germ agglutinin (Con A and WGA) are known to bind to the adult schistosome [25]. These lectins (Sigma) were labelled with ^{125}I using Chloramine T (Sigma) by the method of Hunter and Greenwood [26]. 30 worm pairs in 1.5 ml Hanks balanced salt solution (HBSS) (made up with Fisons chemicals) were incubated in the presence of Con A (0.4 μg, 1.42×10^4 cpm) or WGA (0.4 μg, 1.68×10^4 cpm) for 30 min. 0.1 mM Mn^{2+} was added for the Con A labelling. The appropriate competing sugar was included in the incubation mixture for control labellings.

(*c*) *Iodination of surface proteins*. Lactoperoxidase-catalysed iodination was selected as it is believed to label only surface proteins (reviewed by Hubbard and Cohn [27]). The details of the method used are described in Roberts et al. [28]. Worms were iodinated and then transferred to MEM and incubated at 37°C for 90 min to remove free iodide prior to membrane isolation.

Assessment of fraction homogeneity. Particulate and supernatant fractions obtained during membrane stripping methods were monitored for protein, alkaline phosphatase and contaminant enzyme markers.

(*a*) *Protein content*. 0.1 ml suitably diluted sample was assayed in a final volume of 1 ml by the method of Lowry et al. [29]. The absorbance (*A*) was measured at 750 nm together with a standard curve of 0–0.1 mg bovine serum albumin (Sigma).

(*b*) *Alkaline phosphatase*. 0.1 ml 20 mM *p*-nitrophenyl phosphate in 0.1 M glycine /NaOH buffer, pH 10.5, containing 2 mM $MgCl_2$ and 1% Triton X-100 (Koch-Light) was added to 0.1 ml of appropriately diluted sample. Incubation was for up to 15 min at 37°C and the reaction stopped by the addition of 0.8 ml 0.5 M NaOH. The absorbance was read at 420 nm against appropriate blanks together with a standard curve of 0–0.1 mM *p*-nitrophenol (Sigma).

(*c*) *Contaminant enzyme markers*. (*i*) *Gut contamination*. Gut protease activity was determined using the method for acid proteinase [30] at pH 3.9 with haemaglobin (Sigma) as substrate. The concentration of released peptides was monitored after trichloroacetic acid (TCA) precipitation by a modification of the Lowry method. Solution A of Lowry et al. [29] was amended to 1 M NaOH in 10% Na_2CO_3 and 0.35 ml of this solution was added to 0.4 ml sample supernatant. After 10 min 0.3 ml diluted Folin-Ciocalteau reagent (Sigma) was added with agitation. After a further 20 min the tubes were read at 750 nm against appropriate enzyme and substrate blanks, together with a standard curve of 0–10 μg tyrosine in 0.4 ml 1.2% TCA, treated as above.

(*ii*) *Mitochondrial contamination.* Cytochrome oxidase, present in schistosomes [31], was used as a mitochondrial marker [32–34]. Samples in 0.1 M potassium phosphate buffer, pH 7.2, 2% Triton X-100 were monitored over several minutes at 550 nm. Occasionally samples contained a reducing factor which masked the cytochrome oxidase activity. 1.5 mM cyanide was used to inhibit the cytochrome oxidase activity allowing this reducing activity to be measured and a correction to be made. (*iii*) *Cytoplasmic contamination.* Lactate dehydrogenase was assayed by the method of Bergmeyer and Bernt [35] using 0.1 ml sample in a final volume of 1 ml. The absorbance change at 340 nm was followed for several minutes at room temperature. (*iv*) *Endoplasmic reticulum (ER) and outer mitochondrial membrane contamination.* NADH cytochrome *c* reductase activity was monitored by the method of Beaufay et al. [36]. The reaction was followed for 2–3 min at 37°C in a final volume of 1 ml.

(*d*) *Electron microscopy. (i) Scanning Electron Microscopy (SEM).* Worm bodies after freeze/thaw (f/t) and tegument stripping were fixed in 4% glutaraldehyde (Taab), 0.1 M phosphate buffer, pH 7.4, for 4 h at 4°C. They were washed in double-distilled water, dehydrated in acetone, critical point dried using liquid carbon dioxide, sputter coated with gold and examined using a Cambridge Stereoscan 600 or ISI 100A machine. (*ii*) *Transmission electron microscopy (TEM).* Pellets of parasite surface membranes were examined at various stages of isolation and purification using TEM. Treated worms were also examined. Preparations of samples for EM were modified from Hockley and McClaren [1]. Specimens were fixed in 4% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4, for 4 h at 4°C. After washing in 0.1 M cacodylate, the samples were post-fixed firstly in 1% osmium tetroxide, 0.1 M cacodylate buffer for 2 h at 4°C, and secondly, following washing in double-distilled water, in 0.5% aqueous uranyl acetate containing 45 mg ml^{-1} sucrose for 1½ h at 4°C. The specimens were washed in distilled water, dehydrated in ethanol and embedded in Epon Araldite. Sections were cut on a Reichert OM U3 ultramicrotome, stained with uranyl acetate and lead citrate and examined in a Kratos Corinth 500 or Hitachi HU 12A Electron Microscope.

RESULTS

The tegument surface membranes of the adult schistosome constitute a very small proportion of the total plasma membrane within the organism. Therefore, prior to the use of traditional techniques for membrane purification, the tegument needs to be detached from the parasite with minimal contamination from other plasma membranes. This having been accomplished, contaminating organelles can be removed. The development of a surface membrane isolation procedure has therefore been divided into two parts, evaluation of techniques for membrane stripping and purification of the surface membranes from this crude preparation.

Validation of alkaline phosphatase as a surface marker. When live, adult schistosomes were incubated with alkaline phosphatase substrate, 33.6% ± 5.5% (mean ± S.E., n = 5 separate experiments of 2–5 replicates) of the activity of the total worm homogenate was detected. This figure was taken as a guideline to the amount of enzyme available for release by subsequent tegument stripping procedures.

Evaluation of methods for tegument stripping. A variety of techniques were examined for their potential to release worm surface alkaline phosphatase. Initially detergent extraction versus f/t was studied and compared with incubation of worms in buffer alone. Batches of 20 worm pairs were washed quickly in ice-cold TBS (10 mM Tris/HCl, Sigma, 0.84% NaCl, Fisons, pH 7.4) and incubated in 1 ml of treatment buffer for 5 min on ice. The supernatants were removed (S1) and replaced with a further 1 ml of treatment buffer. A shearing step (if used) of 5 × 1 second mixes on a vortex mixer followed to give supernatant 2 (S2). The remaining worm bodies were washed and homogenised in 2 ml of TBS (H). Alkaline phosphatase activity was assayed on the three fractions. Three treatments were examined: (*1*) Incubation in TBS. (*2*) f/t in 10 mM Tris/HCl, pH 7.4. (*3*) Incubation in 0.1% Triton X-100 in 10 mM Tris/HCl, pH 7.4. The results are summarised in Table I.

The test treatments alone did not release significant alkaline phosphatase activity (S1, 0.3%–4.0%); physical shearing was always required (S2). Significant release of alkaline phosphatase was not obtained by incubation of worms in TBS (0.3%) or incubation plus shearing (1.6%). Treatment (1) was therefore discarded and treatments (2) and (3) taken forward for further study.

We next examined whether the alkaline phosphatase activity released by vortexing

TABLE I

Release of alkaline phosphatase from worm pairs following freeze/thaw + vortex or incubation in Triton X-100 detergent

Treatment	% alkaline phosphatase distribution		
	S1	S2	H
1. TBS incubation	0.28 ±0.13(9)	1.29 ±0.46(9)	98.4 ±0.52(9)
2. f/t 10 mM Tris/HCl	4.03 ±0.76(10)	23.1 ±2.34(10)	72.8 ±2.76(10)
3. 0.1% Triton in 10 mM Tris/HCl	2.05 ±1.18(6)	17.0 ±1.89(6)	80.9 ±2.06(6)

Key: S1, supernatant 1 (after incubation or f/t); S2, supernatant 2 (after shearing by vortex mixer); H, remaining worm body homogenate. Results are expressed as mean ± S.E.(n).

was particulate. The vortex supernatant plus washings (S2) were centrifuged at 74 000 × *g* for 60 min (MSE High Speed 25 centrifuge) and the resulting S2 pellet and supernatant (S2P and S2S) assayed for alkaline phosphatase activity and protein (Table II). Treatment of worms with 0.1% Triton X-100 released substantially less protein than the f/t treatment (6% and 18%, respectively). However, after shearing in Triton, only 30% of released alkaline phosphatase pelleted at 74 000 × *g* (96% for f/t plus vortex in Tris buffer). Solubilisation of the enzyme from the membranes was clearly occurring which was undesirable and hence only the f/t method was taken forward for further development.

Following the incubation of worms with labelled lectins or their lactoperoxidase-catalysed iodination, the partioning of ^{125}I counts into worm fractions was compared with the distribution of protein and alkaline phosphatase during membrane stripping (Table III). Neither Con A or WGA were found to partition with the alkaline phosphatase activity. Rather, a large percentage of lectin was released into the thaw supernatant S1 (29%) compared to alkaline phosphatase (3%). 50–58% of the lectin remained with the worm bodies after tegument stripping whereas a large proportion of the surface alkaline phosphatase appeared to be removed (16–24% of total alkaline phosphatase activity). It was concluded that the lectin-surface carbohydrate bond was cleaved and reassociation of lectin occurred as soon as new carbohydrate sites were presented, (either soluble carbohydrate released into S1 or denuded worm body carbohydrate). Labelling of surface membranes with ^{125}I also proved unsatisfactory. 55% of the ^{125}I counts remained with the worm body after tegument removal. This suggested that the ^{125}I labelling was not exclusive to the surface of the parasite, a conclusion validated in subsequent work (Roberts et al. [28]).

Handling of worms. This section describes steps investigated during the development of a method for membrane isolation. The effect of Tris buffer upon live worms was examined by TEM. Two methods of physical shearing were considered, sonication and the use of a vortex mixer. SEM and TEM were applied to worms after f/t and f/t plus vortex to study the effect of these treatments upon the worms. The release of protein and alkaline phosphatase from the worm as a response to increasing physical shearing was measured. Finally, precautions to suppress any hydrolytic enzyme activity during freezing, storage, thawing and physical shearing were considered.

(*a*) *Tris buffer damage to worms: examination by TEM.* Parasites incubated on ice for 5 min in TBS show slight leakage of protein and significant release of carbohydrate as indicated by release of bound lectin (Table III). Suspected tegument damage was checked by washing worms in 10 mM Tris buffer and examination by TEM. Fig. 1 illustrates that a brief (2–3 min) incubation in Tris buffer results in the disappearance of normal tegumental inclusions and the appearance of large whirls of unrecognisable material. The use of hypotonic Tris buffer in washing and freezing parasites was therefore discarded and HBSS used in its place. Worms can be incubated in this solution for 30 min before structural changes are apparent [37].

TABLE II

f/t and detergent treatment of worms; is released alkaline phosphatase pelletable?

Treatment	% alkaline phosphatase				% protein			
	S1	S2	H	% S2 pelletable	S1	S2	H	% S2 pelletable
f/t in 10 mM Tris/HCl	2.83 ±0.97(4)	19.9 ±4.1(4)	77.3 ±4.6(4)	96.1 ±1.37(4)	18.1 ±3.6(4)	11.5 ±1.8(4)	70.4 ±4.7(4)	17.9 ±3.2(4)
0.1% Triton in Tris/HCl	2.40 ±2.10(2)	17.3 ±6.9(2)	80.4 ±9.0(2)	30.4 ±5.4(2)	6.05 ±3.05(2)	8.60 ±0.6(2)	85.4 ±2.5(2)	13.1 ±0.1(2)

Centrifugation of S2 was at 74,000 × *g* for 60 min.

TABLE III

Partitioning of ^{125}I-labelled lectin; protein and alkaline phosphatase during f/t + vortex

	Protein (% distribution)			Alkaline phosphatase (% distribution)			^{125}I counts (% distribution)		
	S1	S2	H	S1	S2	H	S1	S2	H
Con A Control (TBS)	0.9	1.9	97.2	0.0	0.8	99.2	17.8	20.6	61.6
Test (f/t)	15.1	4.3	80.6	2.5	15.7	81.8	29.0	11.2	58.8
WGA Control (TBS)	0.7	1.4	97.9	0.0	0.3	99.6	6.1	15.8	78.1
Test (f/t)	15.6	6.7	77.7	2.8	24.0	73.2	29.1	20.7	50.2
^{125}I Control (TBS)	1.3	1.7	97.1	0.5	0.7	98.8	2.9	3.8	93.3
Test (f/t)	29.9	11.4	58.7	6.9	30.9	62.2	20.6	24.9	54.5

Worms iodinated directly using lactoperoxidase (see text).

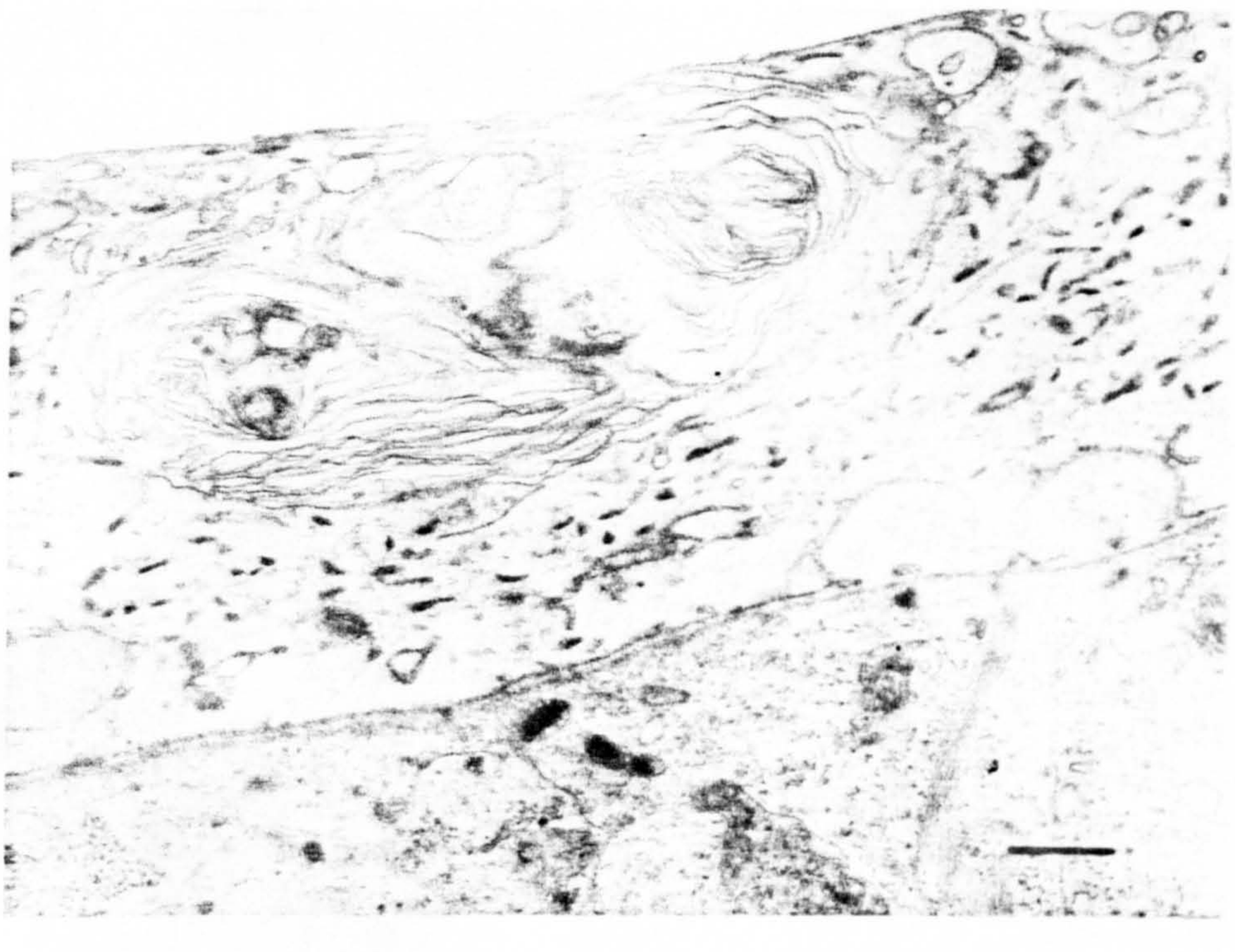


Fig. 1. TEM micrograph through the dorsal tegument of an adult male schistosome after 2–3 min incubation in 10 mM Tris/HCl, pH 7.4, at 4°C. This brief treatment in hypotonic buffer causes widespread damage to the tegument. Bar represents 1 μm.

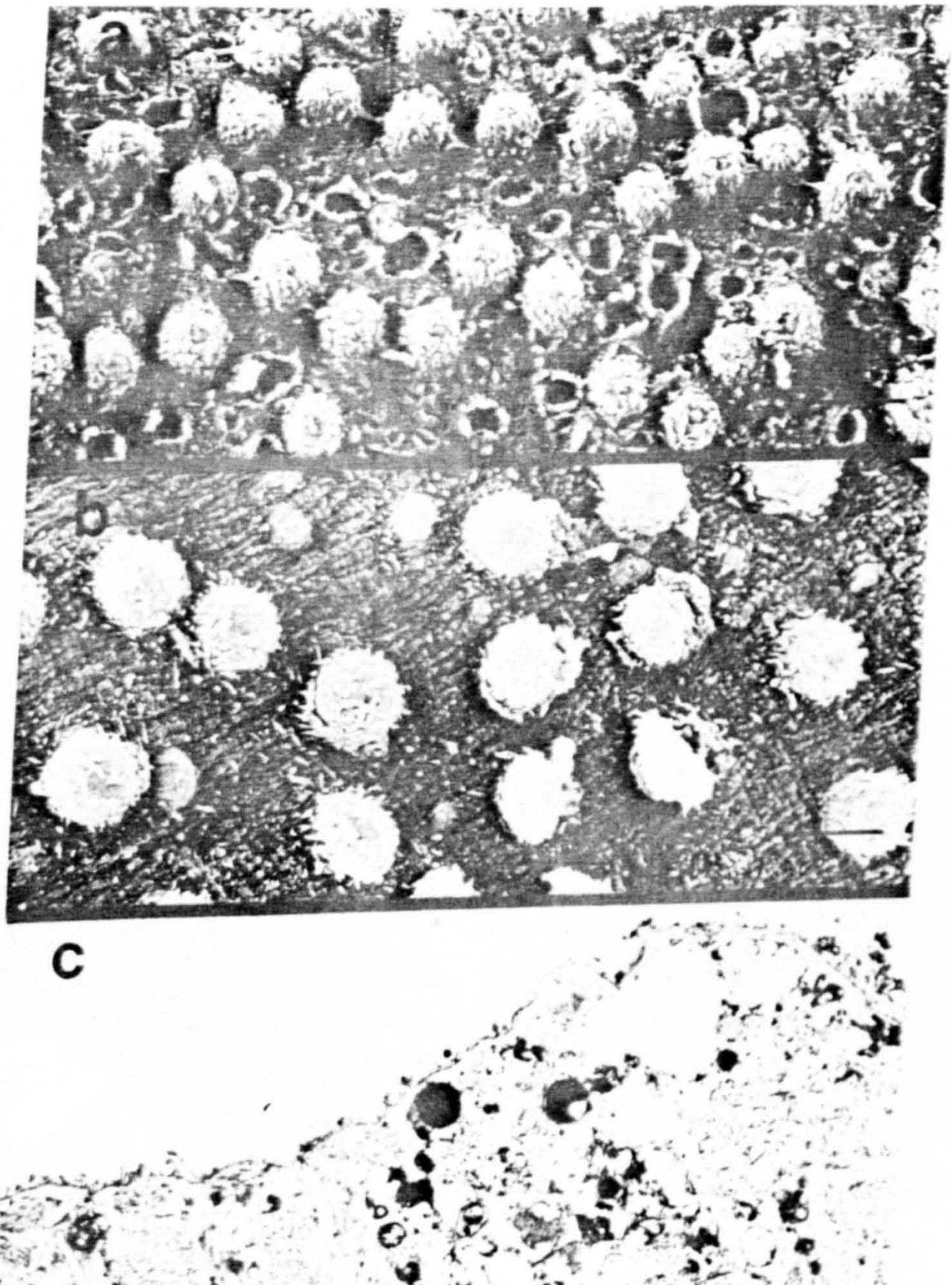
(*b*) *Vortex mixing versus sonication as a means of tegument shearing*. Mild sonication. (MSE 100 watt Ultrasonic Disintegrator, amplitude of 4 μm for 20 s), provided a consistent, reproducible method for shearing of worm surfaces after f/t. However, substantial amounts of worm gut pigment were released, indicating an unacceptable level of worm damage was occurring. Shearing using a whirlimixer (Fisons) was substituted. The tube of resuspended worms was pressed firmly against the cup of the whirlimixer and as soon as a vortex was created, the tube was withdrawn (approx. 1 second). No release of haematin was detectable and the vortex supernatant was white and opaque with released membrane material.

(*c*) *Effect of f/t plus vortexing on worms: examination using SEM and TEM*. Freezing and thawing of worms causes holes to appear in the male dorsal surface and a generalised tegument loosening (Fig. 2a). Some tegument is lost during this action, especially by the female worms. After f/t plus vortex, (Fig. 2b) all the tegument has been removed from the male dorsal surface and the female worm surface, but small portions of tegument remain on the male ventral surface. Some tubercles appear to be damaged and this was verified by TEM (Fig. 2c). The basement membrane of the tegument remained, similarly, muscle had not been released. However, occasional dorsal tubercles had lost some of their contents, presumably due to fracturing of the tegument basement membrane. A small amount of parenchyma therefore probably contaminated the vortex supernatant (S2) and provided the source of mitochondrial marker enzyme found there (see later).

(*d*) *Protein and alkaline phosphatase release during f/t plus vortex*. The majority of the protein and alkaline phosphatase appeared in the supernatant during the first three vortices (Fig. 3) and reached a plateau between 4 and 8 vortices. 10 vortices were used in subsequent work to allow for variation between users of the mixer and to ensure maximum tegument release. During surface membrane stripping the percentage release of alkaline phosphatase in the thaw supernatant (S1) was 3.7 ± 0.3% corresponding figures for the vortex supernatant (S2) and denuded worm bodies (H) were 30.2 ± 0.8% and 66.1 ± 0.7% respectively (means ± S.E., $n = 49$). Hence the mean percentage release of total worm alkaline phosphatase upon f/t plus vortex was 33.9%.

(*e*) *Suppression of hydrolytic enzyme activity*. Precautions were taken to suppress any hydrolytic enzyme activity that might be released during f/t plus vortexing. Immediately upon thawing, the HBSS thaw supernatant was removed and the worms gently washed twice in TBS + phenylmethylsulphonyl fluoride (PMSF), (1 mM final concentration, Sigma). The replacement of HBSS by TBS removed the divalent cation activators of phospholipase activity. The damaging effect of Tris buffer upon live worms was not considered a problem once the worms had undergone freeze and thaw.

Worms stored at −20°C for longer than three months yielded a diffuse membrane band upon density gradient centrifugation. Subsequently worms were stored at −70°C and PMSF was included in the final HBSS wash immediately before freezing. Recently TPCK (20 μg ml^{-1}) and TLCK (5 μg ml^{-1}) (Sigma) have been included with PMSF as protease inhibitors.


a
b
c

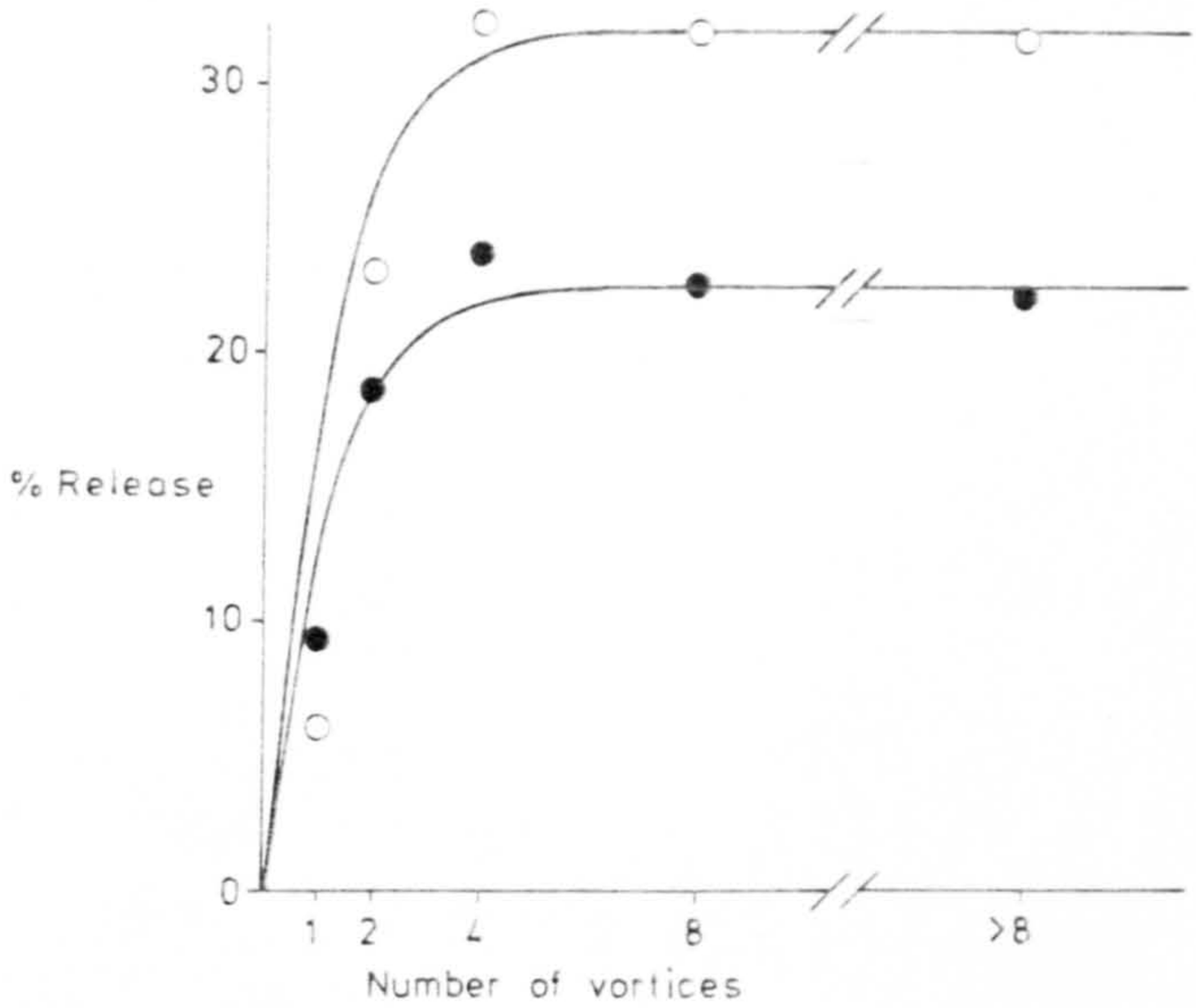


Fig. 3. Percentage release of protein (●) and alkaline phosphatase (○) from worms subjected to sequential vortexing performed by a 1 second contact with a whirlimixer. Percentage release is expressed as percentage of total homogenate. Worms were treated in HBSS throughout.

TABLE IV

Differential centrifugation of S2 and treatment of S2 pellet.

(a)	Alkaline phosphatase (AP)		
	% S2 enzyme pelleted	Relative* enrichment S2P	No. determinations
100 × *g*/30 min	58.9 ± 2.7	21.4 ± 4.0	7
5 000 × *g*/30 min	69.0 ± 2.4	15.1 ± 1.9	9
(b) after centrifugation of S2 at 100 × *g*			
No treatment or wash in buffer only	56.8 ± 2.8	18.4 ± 2.9	11
Osmotic shock	57.4 ± 2.5	20.7 ± 1.0	25
Freeze/thaw	38.0 ± 4.1	23.7 ± 4.5	7

Results are expressed as mean ±S.E.

* Relative enrichment specific activity of AP in S2P/specific activity of AP in whole worm homogenate

Fig. 2. SEM micrograph of adult male schistosome dorsal surface following f/t (a) and f/t plus 10 × 1 second vortices (b). The tegument is completely removed in (b). A TEM micrograph through a single dorsal tubercle (c) illustrates that some tubercles burst and hence release parenchyma into the medium after f/t plus vortexing. Bars represent 10 μm (a and b), and 1 μm (c).

Purification of surface membranes from released tegument. F/t plus vortexing of worms loosens and removes the tegument. The surface membranes are therefore contaminated with other tegúmental components such as spines, cytoplasmic inclusions and tegument ground substance. Differential and density gradient centrifugation have been used to purify the crude surface membrane preparation. Additional procedures of osmotic shock and a second f/t have also been applied to the crude membrane pellet.

(*a*) *Differential centrifugation.* The vortex supernatant (S2) was centrifuged at either 100 or 5000 × *g* and the pellet of highest relative enrichment in alkaline phosphatase determined. Forces above this are expected to pellet mitochondria [37] and tegumental inclusions and were therefore not used.

59% of the alkaline phosphatase activity released into S2 was pelleted by centrifugation at 100 × *g* for 30 min (Table IVa). Slightly more enzyme activity pelleted by centrifugation at 5000 × *g* for 30 min (69%), but the relative enrichment of enzyme in the pellet was lower (15-fold compared to 21-fold). Any small amount of haematin released from the worms by f/t plus vortexing also pelleted at this higher *g* force. Accordingly, 100 × *g* for 30 min was subsequently used to prepare crude surface membrane pellets (S2P) from the vortex supernatant (S2).

Two S2P preparations were compared by TEM (Fig. 4). The replacement of HBSS after f/t by TBS (and hence decrease in divalent cation concentration) did not affect the release and pelleting of long pitted sheets of tegument. However, both crude surface membrane pellets were contaminated with spines (Fig. 4a) and apparent tegument ground substance adhered to the membranes.

(*b*) *Application of osmotic shock and f/t to purify the S2P preparation.* These procedures were tried with the aim of removing adherent or trapped tegument cytoplasm and increasing the S2P alkaline phosphatase enrichment. The S2P pellet was resuspended in 5 ml distilled water, pH 7.2, and left on ice for 10 min before centrifuging at 100 × *g* for 30 min. Alternatively the S2P pellet was resuspended in 2 ml TBS and PMSF and frozen by plunging and holding in liquid nitrogen. The suspension was thawed at room temperature initially and finally on ice before repelleting at 100 × *g* for 30 min.

A slight improvement in alkaline phosphatase relative enrichment was achieved (20.7 and 23.7 compared to 18.4, Table IVb). No reduction in the overall amount of enzyme recovered occurs after osmotic shock of the S2P pellet although a significant loss is observed with freeze/thaw (38% compared to 58%, Table IVb).

(*c*) *Density gradient centrifugation and fractionation.* The crude membrane pellet, S2P, after osmotic shock or freeze/thaw treatment (S2P/Os or S2P/f/t) was applied

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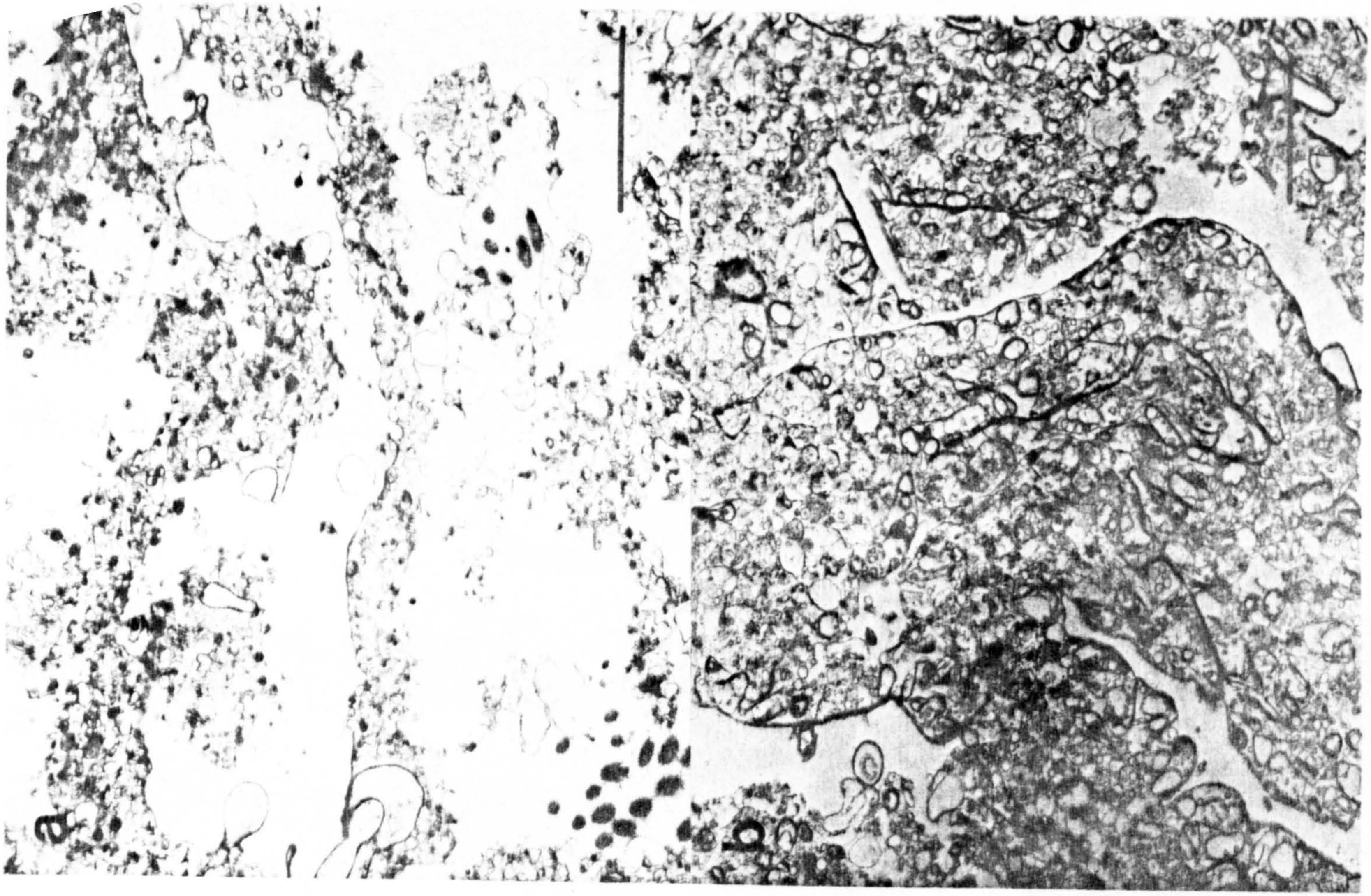
Fig. 4 TEM micrographs of preliminary membrane pellets (S2P) prepared using HBSS throughout the procedure (a) or replacing HBSS with TBS immediately after worm thawing (b). Bars represent 5 μm (a) and 2 μm (b)



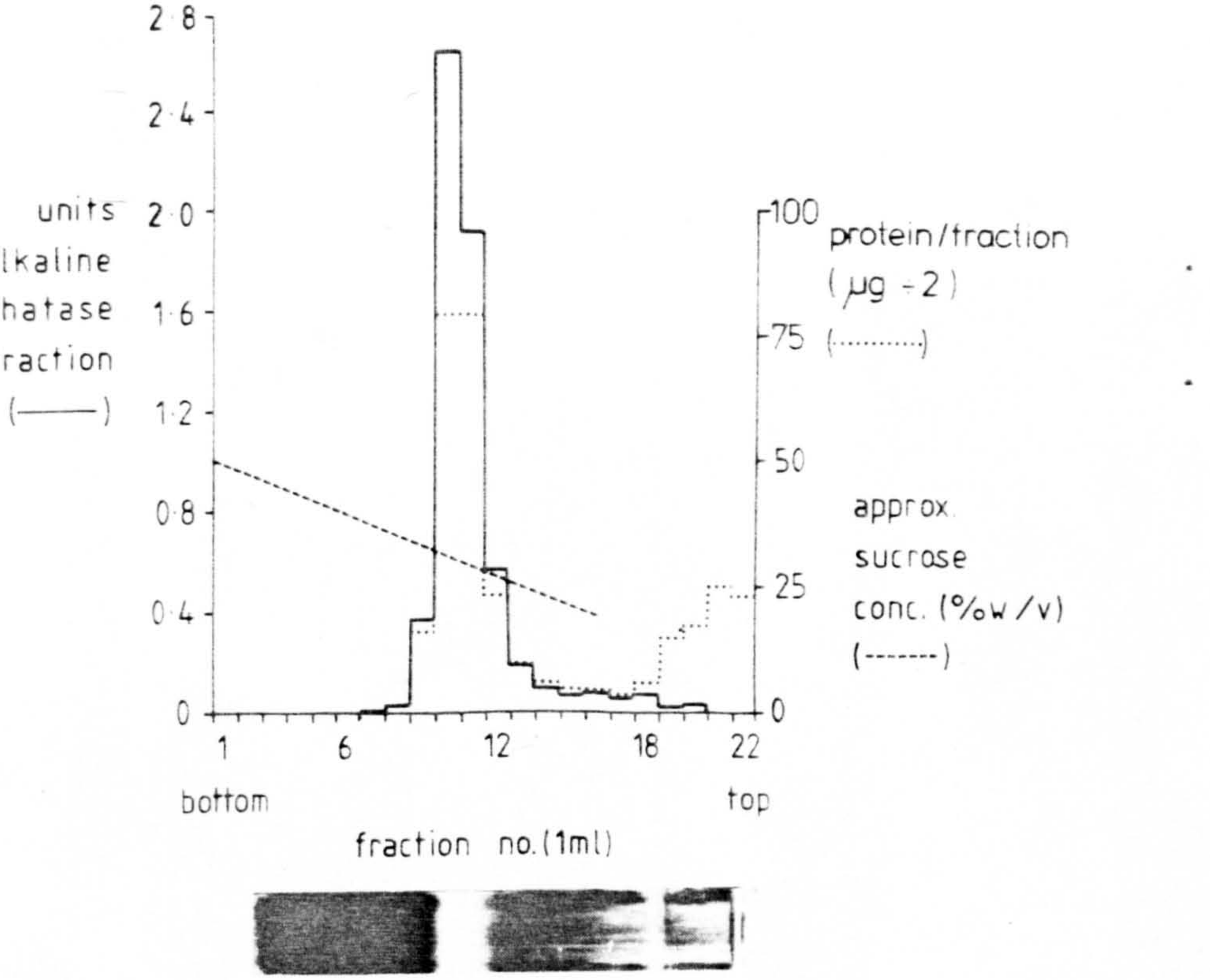
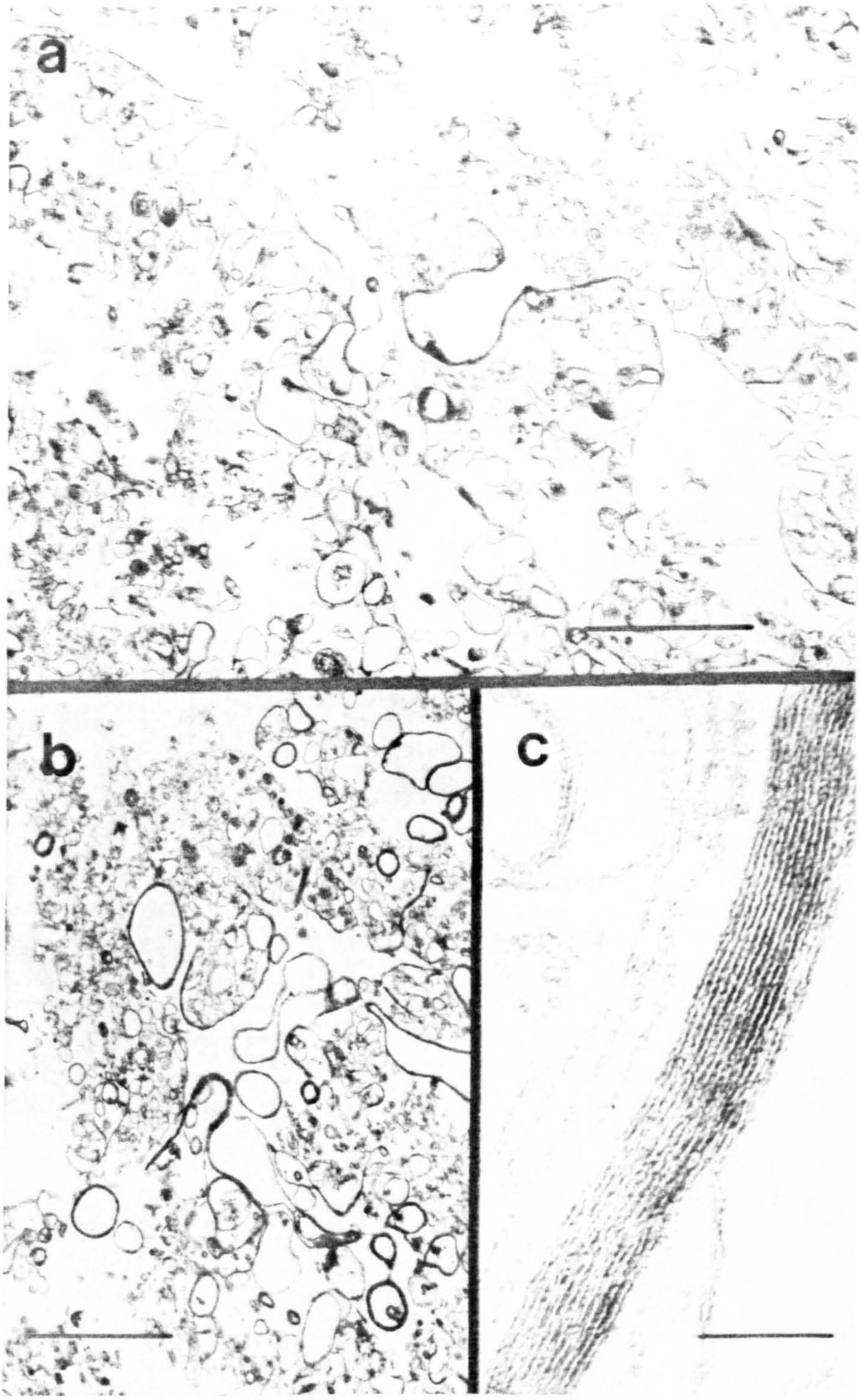


Fig. 5. Fractionation of the S2P suspension on a continuous sucrose density gradient, 20–50% sucrose in 10 mM Tris/HCl, pH 7.4. The distribution of protein and alkaline phosphatase activity is illustrated, together with a photograph of the gradient after centrifugation.

to the top of a 20–50% continuous sucrose gradient (sucrose in 10 mM Tris/HCl, pH 7.4). Centrifugation was for 1½ h at 112 000 × *g* in a Beckmann L2-6B ultracentrifuge (SW27 rotor, 17 ml cellulose nitrate tubes). A photograph and profile of alkaline phosphatase and protein distribution on the gradient is illustrated in Fig. 5. A single band of material collects at a density approximating to 1.18 g cm^{-3}. The two or three aliquots richest in alkaline phosphatase were pooled and centrifuged for 40 min at 74 000 × *g* to give a final membrane pellet.

TEM micrographs of two gradient pellets are shown in Fig. 6. The differences in their preparation are explained in the figure legend. Both membrane pellets are similar

Fig. 6. TEM micrographs of final membrane pellets (GP) showing long sheets of tegument surface membrane isolated from S2P osmotic shock (a). Large membrane vesicles predominate in the gradient pellet isolated from S2P freeze/thaw (b). Both preparations contain multilamellar aggregates of membrane (c). Bars represent 0.5 μm (a), 2 μm (b) and 0.1 μm (c).


a
b
c

in that the contaminating spines and a large proportion of the tegument ground substance have been removed. However, after an earlier osmotic shock treatment, long sheets of tegument surface membrane are still obtained (Fig. 6a) whereas some vesiculation of membranes occurs after f/t of S2P (Fig. 6b). Both preparations contain multilamellar membrane aggregates (Fig. 6c) which could represent accumulation of tegument outer bilayer.

Final method for surface membrane isolation. Batches of 12–13 mice are perfused (see methods) and the worms washed free of debris with fresh MEM. Undamaged worms are transferred to plastic test tubes, the medium washed out with five changes of HBSS and parasites in a volume of 2–3 ml HBSS are plunged into liquid nitrogen until freezing takes place. Tubes are stored at –70°C until required and four are routinely used for one membrane isolation. Two or six tubes have also been used depending upon requirements, with corresponding alteration of buffer volumes. Worms were allowed to thaw initially at room temperature, finally on ice and the parasite tegument surface membranes removed and purified as described earlier in this section. Volumes used in the preparation together with dilutions made for protein and alkaline phosphatase assays are shown in Table V. These assays are carried out on every membrane preparation, a typical result is shown in Table VI.

Using the mean of all the membrane strippings carried out to date, 217 ± 21 µg gradient pellet protein (mean ± S.E., $n = 29$) was obtained from four tubes of worms, (1810 ± 82 worm pairs, 91.9 ± 4.1 mg protein, mean ± S.E., $n = 25$). From replicate protein assays on numbers of worm pairs, the mean protein content of 1 worm pair

TABLE V

Final membrane stripping method – volumes and dilutions used for protein and alkaline phosphatase assays

Fraction	Total volume (ml)	Dilution	Volume + buffer (ml)	AP (µl)	Protein (µl)
S1	25	× 1.6	(1.0 + 0.6)	100	100
S2	25	× 1.6	(0.5 + 0.3)	50	100
H	8	× 20	(0.1 + 1.9)	100	100
S2S	25	× 1.6	(1.0 + 0.6)	100	100
S2P	2.6	× 7.5	(0.1 + 0.65)	50	100
Gradient fractions				20 or 10 at peak	

was found to be 0.051 mg. Therefore, expressed as amount of protein per 1000 worm pairs, the yield of gradient pellet was 132 ± 9 μg (mean ± S.E., $n = 29$).

Characterization of final method. The specific activity and relative enrichment of alkaline phosphatase increases throughout the membrane isolation method (Fig. 7).

TABLE VI

Results table from a typical surface membrane stripping experiment

	Protein		Alkaline phosphatase			
	Total mg in fraction	% of total	Total IU in fraction	% of total	Specific activity IU[a] mg^{-1} protein	Relative enrichment
S1	9.38	11.9	0.496	2.43	0.053	0.205
S2	12.9	16.4	5.56	27.2	0.431	1.66
H	56.6	71.7	14.4	70.4	0.254	0.981
Total	78.9	-	20.4	-	0.259	1.00
S2S	12.5		2.28		0.182	0.703
S2S f/t	0.261		0.217		0.831	3.21
S2P f/t	0.475		2.14		4.51	17.4
GP	0.203		2.08		10.3	39.6

[a] IU, μmol substrate released min^{-1} at 37°C.

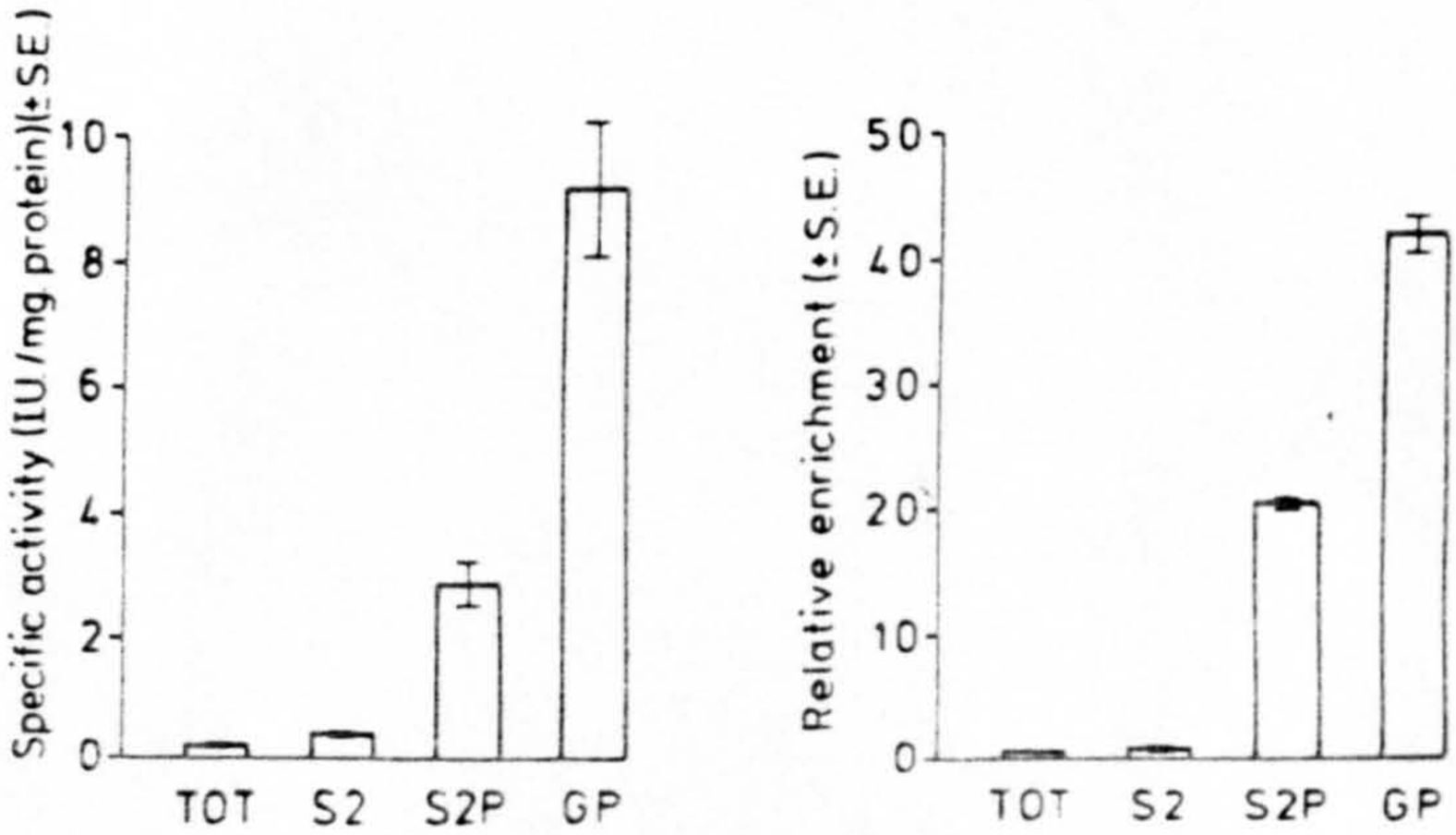


Fig. 7. Enrichment of surface membrane marker, alkaline phosphatase during the membrane isolation method. Relative enrichment is expressed as the specific activity of alkaline phosphatase in the fraction divided by the specific activity of this enzyme in the total worm homogenate.

The final pellet recovered from the gradient is enriched over forty times in surface marker enzyme when compared to the whole worm homogenate. When allowance is taken for the percentage surface location of the enzyme, this enrichment figure is over 130 fold.

Fig. 8 shows the characterization of four contaminant enzyme markers during membrane isolation. Lactate dehydrogenase (cytoplasmic marker) is released from the worms during f/t plus vortex, but is absent from the S2P. Similarly, small amounts of haemoglobinase (gut enzyme) and NADH cytochrome *c* reductase (ER and mitochondrial marker) released during f/t plus vortex do not contaminate the S2P. In these three enzyme assays the final gradient pellet was not therefore examined. The small amount of cytochrome oxidase (mitochondrial inner membrane marker) present in the S2P was removed by fractionation on the sucrose gradient.

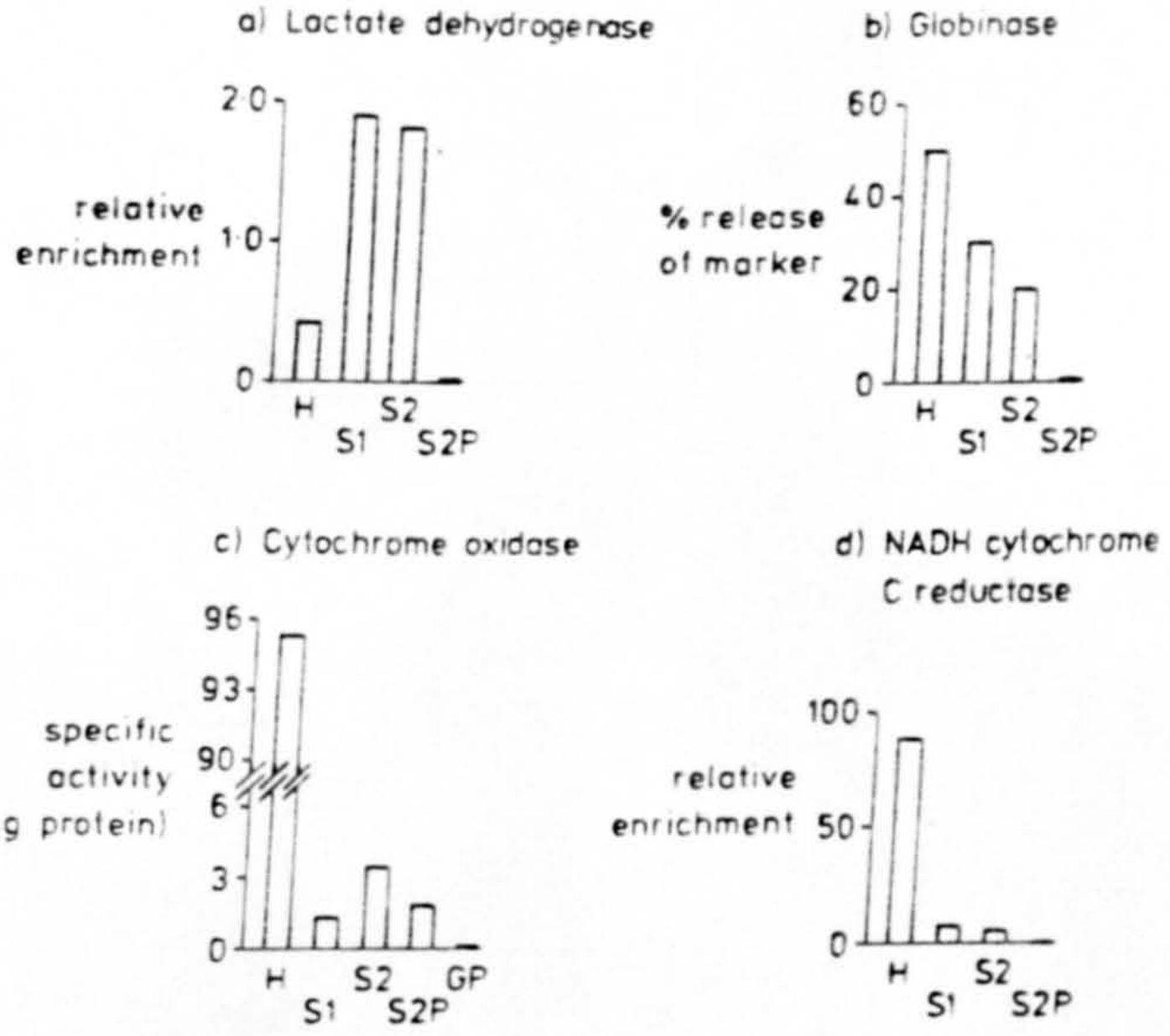


Fig. 8. Activity of contaminating marker enzymes during tegument surface membrane isolation.

DISCUSSION

The use of alkaline phosphatase as a marker for the schistosome surface membrane has played a central role in our acceptance or rejection of different tegument stripping methods and of alternative routes in membrane purification. After an extensive histochemical study Wheater and Wilson [23] suggested that alkaline phosphatase could be used as a marker for the tegument membranes. However, the enzyme is not confined to the worm surface and occurs in some inner body membranes, especially the female vittelaria (Wheater, personal communication). A 34% surface location for

this enzyme was concluded after a comparison was made of the alkaline phosphatase activity of intact live worms and worm homogenate. Several other experimental results supported this figure. The alkaline phosphatase activity released by f/t plus an increasing number of vortices reached a plateau at 32% of that of the total worm homogenate. The mean release of enzyme into thaw and vortex supernatants (S1 + S2) over 49 experiments was 34%. SEM of worms after f/t plus vortex confirm that this action removes the total worm tegument surface membranes.

Simpson et al. [15] estimated that 20% of worm alkaline phosphatase was located at the surface. However the figure is provisional as it is based upon WGA tegument binding and some problems of lectin dissociation and reassociation were recognised. Cesari [39] estimated that 75% of the enzyme was at the worm surface. Specific activities were used for this calculation, i.e. activity per mg protein. No account was taken of the wide variation in protein content between the membrane pellet (very low) and the denuded body (relatively high) which resulted in a correspondingly high calculated percentage surface location.

Kusel [14] first removed the worm tegument by f/t or incubation in a $CaCl_2$/saponin mixture (also used in [39 and 40]). Parasites from either treatment were washed on a fine gauze and released surface fragments centrifuged at 1000 × *g* for 10 min. This preparation is comparable to our S2P and electron micrographs showing long, pitted sheets of surface membranes are common to both preparations. No estimations of surface enrichment or yield exist to compare with the present work.

An apparently much gentler technique has been developed by Simpson et al. [15]. The adult surface membranes are released by incubation in chemically defined media, pelleted at 55 000 × *g* for 1 h and applied to a three-step discontinuous sucrose gradient. Four fractions were separated, two were shown to be qualitatively similar by polyacrylamide gel electrophoresis and were enriched in 5'-adenosine monophosphatase (subsequently identified as non-specific alkaline phosphatase, [41]). Electron micrographs illustrate that membranes released by the above method formed vesicles, long pitted sheets observed in the present study were absent, although multilammellar fragments of membrane were evident in both preparations. The different approaches of the two methods are revealed when the release of alkaline phosphatase is compared. f/t plus vortexing releases 34% of the total worm alkaline phosphatase whereas only 8% is released by incubation of worms in phosphate-buffered saline medium. Our method is designed to remove as much of the worm surface as possible, but 40% of the released enzyme activity is subsequently lost upon centrifugation at 100 × *g*. This step selects for the large sheets of surface membrane which pellet at a low *g* force due to their size, plus the accompanying dense spines. Many small membrane vesicles, difficult to identify as surface or inner-tegument membranes, remain in the supernatant. The method of Simpson et al. releases much less of the available surface membranes but then pellets the majority of the released material (82%) by centrifugation at 55 000 × *g*. However, after density gradient centrifugation both yield (144 µg membrane protein from 100 mg total worm protein) and total alkaline phosphatase

relative enrichment (11.9) are lower than those obtained in the present study (236 μg protein and 43.9, respectively). (Data taken after pooling gradient fractions 1 and 2 of Simpson et al. [42].)

Various detergent solutions have been used to remove the schistosome surface membranes. Oaks et al. have applied their method for releasing tapeworm tegument in 0.2% Triton X-100 [43] to adult schistosomes [16]. McDiarmid et al. [17] have used sequential incubations in 0.1% digitonin to obtain inner and outer surface membranes separately. All of these workers, Simpson et al. [15] and ourselves have attempted to use lectin binding to trace the surface membranes during removal and fractionation. Only Oaks et al. [16] reported no problems with dissociation and reassociation during fractionation, a fact they attribute to lectin bond stabilisation in non-ionic detergent [44]. However, elsewhere [15], the use of Con A is rejected as it is known to damage the schistosome adult tegument [45]. In our early work, very little alkaline phosphatase was released from the worms in Triton X-100 unless a vortex step was introduced. More importantly, the released alkaline phosphatase could not be pelleted at 74 000 × *g* and we concluded that components were being solubilised from the membrane. A recent report [46] uses 1% Triton X-100 to solubilise enzymes including alkaline phosphatase from the tapeworm tegument. No enzymes were assayed in the study of Oaks et al. [16] so this problem was not mentioned.

McDiarmid et al. [17] obtained inner and outer bilayer preparations, monitoring alkaline phosphatase and the binding of diazotised [^{125}I]iodosufanilic acid as respective membrane markers. Estimations of contamination of one preparation with another were based upon the assumption that the total tegument surface membranes were removed during the two incubations, also that the markers were associated exclusively with one membrane or the other. The surface/body distribution of alkaline phosphatase was not investigated so no comparative figures of relative enrichment can be given. Yields of surface membrane are not reported either.

In conclusion we feel that the surface isolation method described here is the only one that has been rigorously characterised for purity using a three-fold investigation of electron microscopy, surface marker enzyme and soluble and particulate contaminating enzyme markers. A major advantage of the method is that the first step of freezing allows tubes of worms to be stored and thus the method can be scaled up to give a substantial yield of highly enriched tegument surface membranes.

However two factors need to be mentioned. Ourselves and other workers (Payares and Evans, personal communication, [17]) believe the alkaline phosphatase to be positioned at the inner of the two surface membrane bilayers, although the parasite can hydrolyse externally added phosphomonoesterase substrates (present work and [24]). Monitoring this enzyme alone will not reveal whether outer bilayer is being lost during the purification process. Work is continuing to identify a suitable marker for the outer bilayer to clarify this point. For the present we must assume from EM evidence that outer bilayer is recovered along with the inner plasma membrane in the final gradient pellet. Secondly, the final gradient pellet contains adherent tegument

ground substance and techniques of osmotic shock and a second f/t have not completely removed this contamination. Multiple washes of the S2P in buffer before it is applied to the gradient may prove to be a more successful method of removal. However, this is the only detected contamination found in the final membrane preparation and an alkaline phosphatase relative enrichment of over 130-fold suggests that a substantial level of purity has been achieved [47].

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