A study of heat shock protein 70 (HSP70) in the parasite *Schistosoma mansoni*.

A thesis presented for the degree of

DOCTOR OF PHILOSOPHY

by

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ABBREVIATIONS

The abbreviations used in this thesis are those recomended in the Instructions to Authors of the Biochemical Society (1986), with the following additions:

BSA	bovine serum albumin
DMS	dimethyl suberimidate
DMSO	dimethylsulphoxide
DTBP	dimethyl 3,3'-dithiobispropionimidate
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GMEM	Glasgow's modification of Eagle's minimal essential medium
Hb-PBS	PBS containing bovine haemoglobin
HRP	horse radish peroxidase
HSP90	heat shock protein of molecular weight 90000
HSP70	heat shock protein of molecular weight 70000
HSP60	heat shock protein of molecular weight 60000
IgG	immunoglobulin G
LAH	lactalbumin hydrolysate
MEM w/o methionine	minimal essential medium without methionine
MHC	major histocompatibility complex
M_r	relative molecular weight
NBCS	newborn calf serum
NMS	normal mouse serum
NRS	normal rabbit serum
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline

PMSF	phenylmethylsulphonylfluoride
PZQ	praziquantel
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEA	triethanolamine
TEMED	N,N,N',N',-tetramethy-1,2-diaminoethane
TLCK	N-a-P-tosyl-L-lysine chloromethyl ketone
TPCK	L-1-tosylamide-2-phenylethylchloromethyl ketone

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Summary

It seems that the function of HSP70 in the parasite is likely to be similar to that in other organisms and that is in their ability to bind to several polypeptides and protein structures. HSP70 was found to bind to several proteins in the parasite, the binding being transient with some and stable with the other proteins. The identity of these proteins or polypeptides has yet to be determined.

Studying localization of HSP70 revealed that it was not an exposed surface molecule but soluble, intraparasitic protein. HSP70 was, however, present in the membrane fraction of the parasite where it seemed to be mostly a peripheral membrane protein although a small fraction of it partitioned in the hydrophobic fraction which suggested its association with an integral membrane protein.

The chemotherapeutic drug, praziquantel, which is used effectively to treat schistosomiasis, caused an induction of HSP70 synthesis and, very interestingly, caused a prominent increase in its secretion from the parasite. The mechanisms by which praziquantel causes this and what effect that this might have on the parasite needs further studies.

Chapter One
General Introduction

1.1 Schistosomiasis, the disease.

1.1.1 Prevalence

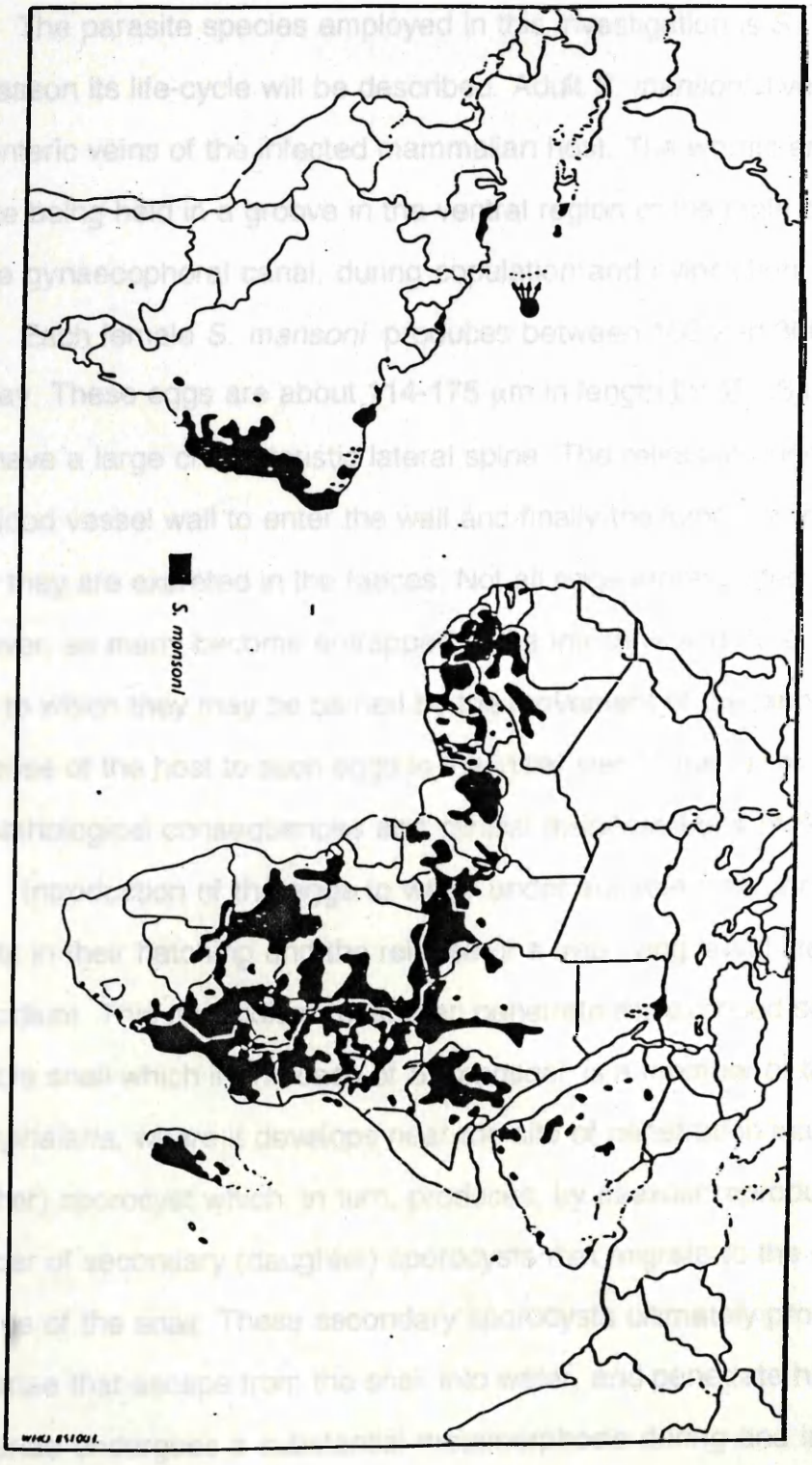
Schistosomiasis is a chronic debilitating disease caused by helminths of the genus *Schistosoma*, which live within the blood vessels of the hepatic portal system around the gastrointestinal tract (*S. mansoni* and *S. japonicum*), or of the vesical plexus around the urogenital system (*S. haematobium*). The life-cycles of the three species are basically similar, and involve fresh water snails as intermediate hosts. It has been estimated that over one thousand million people living in tropical and subtropical countries are exposed to Schistosomiasis, and that 200 million people are actually infected (Iarotski and Davis, 1981).

1.1.2 Geographical distribution

The distribution of schistosomiasis is determined by that of its intermediate host - freshwater gastropod snails. For the main schistosomes of man, *Biomphalaria* species are the molluscan hosts of *S. mansoni*, *Oncomelania hupensis* of *S. japonicum*, and *Bulinus* of *S. haematobium*.

S. mansoni is the most extensively studied schistosome, and forms the subject of this project. This pathogen causes intestinal schistosomiasis, being unique among the schistosomes in that schistosomiasis mansoni is prevalent in both the New and Old worlds. Rollinson and Southgate (1987) describe the geographical distribution of this species in detail : "The parasite occurs in Libya, Oman, Saudi Arabia, Yemen, People's Democratic Republic of Yemen, and is distributed discontinuously over the greater part of Africa, south of the Sahara and Madagascar. In South America, intestinal schistosomiasis exists in Brazil, Surinam and Venezuela. In the Caribbean, it is endemic in Puerto Rico, St. Lucia, Guadeloupe, Martinique, Dominican Republic, Antigua and Montserrat." Figure 1.1 illustrates the global distribution of *S. mansoni*.

Figure 1.1 The global distribution of *Schistosoma mansoni*.



1.2 The parasite

1.2.1 The life-cycle of *S. mansoni*

(See figure 1.2).

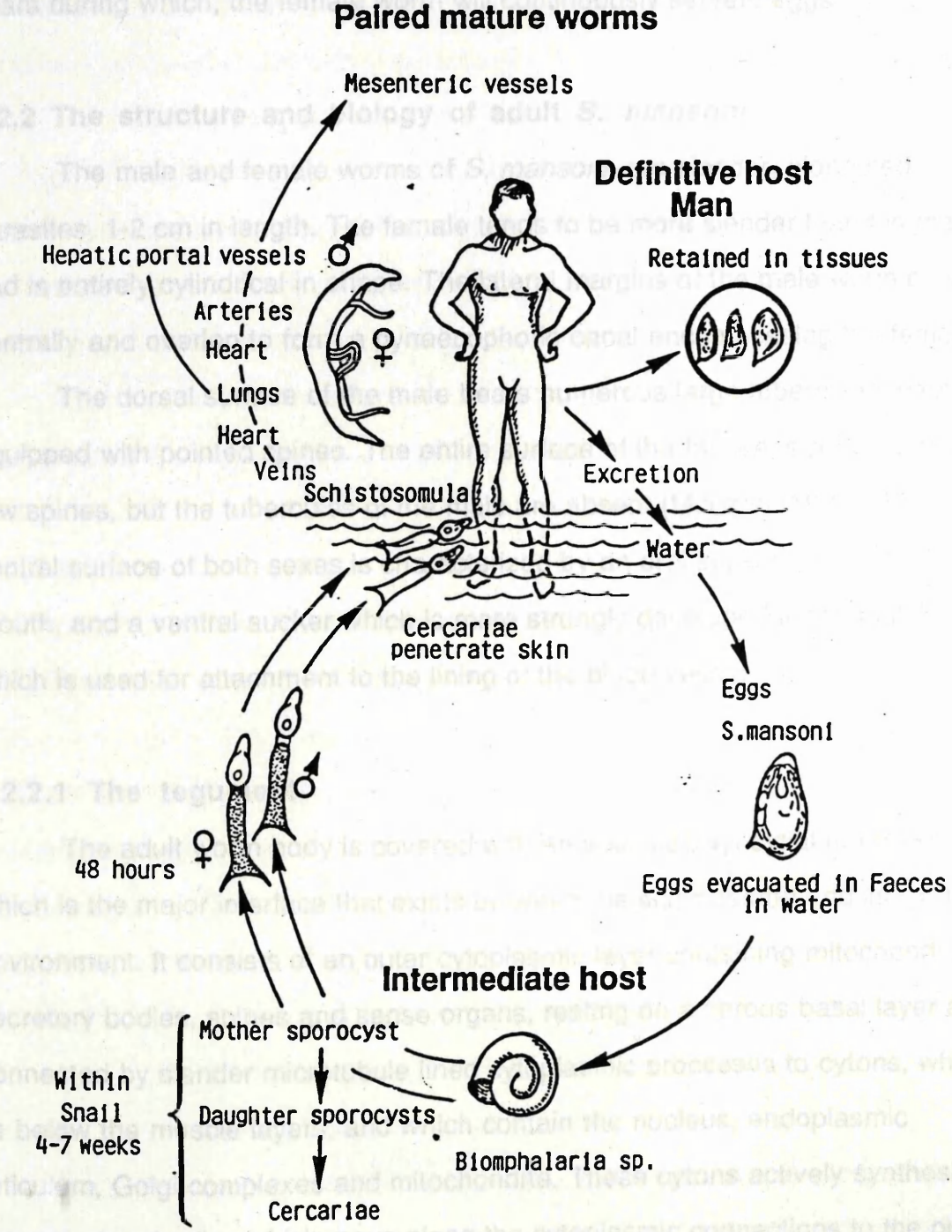
The parasite species employed in this investigation is *S. mansoni* and for this reason its life-cycle will be described. Adult *S. mansoni* live in the mesenteric veins of the infected mammalian host. The worms are dioecious, the female being held in a groove in the ventral region of the male body, referred to as the gynaecophoral canal, during copulation and oviposition.

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

Introduction of the eggs to water under suitable environmental conditions results in their hatching and the release of a free living larval stage known as a miracidium. This miracidium must then penetrate the exposed soft parts of a suitable snail which in the case of *S. mansoni* is a member of the genus *Biomphalaria*, where it develops near the site of penetration into a primary (mother) sporocyst which, in turn, produces, by asexual reproduction, a variable number of secondary (daughter) sporocysts that migrate to the digestive gland or gonads of the snail. These secondary sporocysts ultimately produce numerous cercariae that escape from the snail into water, and penetrate human skin. The cercariae undergoes a substantial metamorphosis during and immediately after penetration to become a schistosomulum.

Schistosomula remain in the skin for one or several days before migrating

Figure 1.2 The Life-cycle of *Schistosoma mansoni*.



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

1.2.2 The structure and biology of adult *S. mansoni*

The male and female worms of *S. mansoni* are slender, elongated parasites, 1-2 cm in length. The female tends to be more slender than the male, and is entirely cylindrical in shape. The lateral margins of the male worm curve ventrally and overlap to form a gynaecophoric canal encompassing the female.

The dorsal surface of the male bears numerous large tubercules, each equipped with pointed spines. The entire surface of the female is pitted, with a few spines, but the tubercules of the male are absent (McLaren,1980). The ventral surface of both sexes is characterized by an oral sucker, perforated by the mouth, and a ventral sucker which is more strongly developed in the male and which is used for attachment to the lining of the blood vessel.

1.2.2.1 The tegument

The adult worm body is covered with an acellular, syncytial tegument which is the major interface that exists between the schistosome and its external environment. It consists of an outer cytoplasmic layer containing mitochondria, secretory bodies, spines and sense organs, resting on a fibrous basal layer and connected by slender microtubule lined cytoplasmic processes to cytons, which lie below the muscle layers, and which contain the nucleus, endoplasmic reticulum, Golgi complexes and mitochondria. These cytons actively synthesize the secretory bodies which move along the cytoplasmic connections to the outer cytoplasmic layer. For *S. mansoni*, it has been suggested by Kohn *et al.* (1979) that the fibrous basement layer on which the outer tegumental region rests is composed of oxytalin fibres, and Cohen *et al.* (1982) have proposed that the

spines are composed of actin filaments. The outer plasma membrane consists of two lipid bilayers (McLaren and Hockley, 1977). This unusual structural feature has been shown to have considerable significance with regard to the ability of the flukes to survive attacks by the immunological defences of the host. The tegument of the adult parasite is derived directly from that of the penetrating infective cercarial stage, and during the development and maturation of the adult schistosome undergoes many morphological and functional changes (Erasmus, 1987). Biochemical studies indicate that the schistosome tegument displays not only absorptive and secretory functions, but also protects the parasite against host immune attack (reviewed by McLaren, 1980). The tegumental outer membrane seems to turnover rapidly, replacement and repair being affected by membranous bodies within the tegument.

1.2.2.2 Uptake of solutes

Glucose, amino acids, purine and pyrimidines from host blood can be absorbed transtegumentally across the exposed dorsal surface of male worms (Levy and Read, 1975; Podesta, 1983; Rumjanek, 1987). Facilitated and simple diffusion are known to occur across the tegument, and simple diffusion is also assumed to function at the level of the gut lining. The male appears to boost female nutrition by transferring glucose, iron, cholesterol and other nutrients to her across the ventral surface of his tegument (Haseeb *et al.*, 1985; Silveira *et al.*, 1986; Smyth and Halton, 1983).

1.2.2.3 Membrane turnover

As schistosomula develop from the skin to the lung stage, either *in vivo* or *in vitro*, their surface becomes less antigenic, as assessed by antibody binding and *in vitro* antibody- or complement-dependent killing assays. Some of the reduction in surface reactivity seems to be attributable to turnover and shedding of surface components.

Membrane turnover has been a difficult parameter to measure in schistosomes - particularly in adult worms, due to the extreme fragility of the tegumental structures during *in vitro* incubation. Experiments attempting to measure membrane turnover indicated that in adult worms, the membranocalyx had a half-life of 2-3 hours (Wilson and Barnes, 1977). Other results measuring the release of incorporated radioactive leucine showed that there was a rapid loss of this amino acid, attaining half of its original concentration after 3 hours of incubation (Wilson and Barnes, 1979). Using another approach, employing a double labelling technique, Kusel and Mackenzie (1975) have found similar results for protein turnover, but in these experiments it materialized that whole membrane fragments were becoming detached from the parasites. These data imply that the membrane of adult worms was replaced as a whole. On the other hand, it is also probable that *in vitro* incubation promoted tegument release which, no doubt, would mask a physiological membrane turnover. That *in vitro* incubation of adult worms compromises the integrity of the tegument was shown by Simpson *et al.* (1981), who took advantage of the damaging influence of specific media for obtaining purified preparations of surface membranes. In contrast, schistosomula appear to have a more stable membrane, at least during the first two or three days after transformation. The reason for this may lie in the fact that, *in vitro*, there is little biosynthetic activity at this stage and growth of the parasite, with membrane manufacture, ensues later. This idea is substantiated by the observation that schistosomula are not very sensitive to several inhibitors of protein synthesis and that they do not display cells undergoing mitosis.

1.2.2.4 Internal structure

Circular and longitudinal muscles, together with a network of nerve fibres, underlie the tegument, allowing body contractions and movement.

The digestive system consists of a short oesophagus, leading from the oral sucker to the intestine which divides in front of the ventral sucker to form two

lateral gut caecae that reunite behind the reproductive organs to form the posterior gut caeca.

The reproductive organs are located between the two lateral gut caeca. The males have 6-9 testes located behind the ventral sucker. The testes lead to a seminal vesicle which is connected to the gonopore by a vas deferens. The female has a single ovary which lies in front of a seminal receptacle, and a pair of vitelline glands near the posterior union of the lateral gut caecae. The oviduct and vitelline ducts open into the uterus, which usually contains one or two eggs.

1.3 Control of schistosomiasis

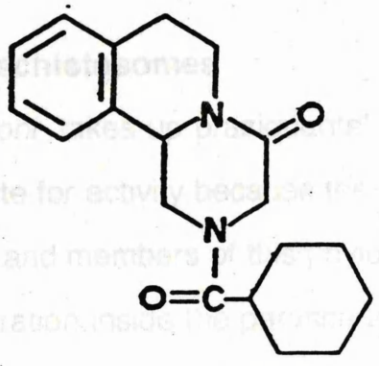
Chemotherapy plays a leading role in the control of schistosomiasis as in all other helminthic diseases. Praziquantel is currently the drug of choice for the chemotherapy of human schistosomiasis (Andrews, 1985). It is effective against a variety of trematodes as well as adults and some larval cestodes in man and other animals. The broad spectrum activity of this drug was first reported by Gonnert and Andrews (1977).

Praziquantel, 2-cyclohexylcarbonyl (1,2,3,6,7,11b) hexahydro-4H-pyrazin (2,1-a) isoquinolin-4-one, ($C_{19}H_{24}N_2O_2$; MW 312.42) (figure 1.3), possesses an asymmetric center. The antihelmintic activity is mainly concentrated in the (-)-isomers, which has R-configuration (Andrews *et al.*, 1983). Praziquantel is effective against all five species of schistosomes pathogenic in man. The cure rates often exceeds 90% (Andrews, 1985).

1.3.1 Pharmacokinetics

Praziquantel is metabolized within 5 minutes and is very rapidly distributed throughout all organs, with the highest concentration occurring in the liver and kidneys (Harder, Andrews and Thomas, 1987 a). Praziquantel itself is the active molecule requiring no metabolic alteration in order to become effective (Andrews *et al.*, 1983; Wang *et al.*, 1983). Metabolism of praziquantel is

Figure 1.3 Structure of praziquantel.



$C_{19}H_{24}N_2O_2$

stereoselective (Staudt *et al.*, 1992). The major human oxidative metabolite of praziquantel, trans-4-hydroxy praziquantel exerts roughly similar pharmacological activity.

The antischistosomal effect of praziquantel is not related to the absolute height of the maximal plasma concentrations, but rather to the length of exposure to the drug, i.e., praziquantel follows a threshold / time model rather than a peak height model. Studies *in vivo* and *in vitro* indicate that the threshold concentration is reached in man after application of curative drug doses.

1.3.2 Drug uptake by schistosomes

Schistosoma mansoni takes up praziquantel rapidly. Surface penetration appears to be a prerequisite for activity because the thick cuticle of nematodes prevents entry of the drug and members of this phylum are unaffected by praziquantel. The concentration inside the parasite equals that in the medium within 2 minutes and increases further with time. Drug uptake is reversible and not dependent on the calcium concentration of the external medium. Praziquantel appears to be distributed equally throughout the parasite tissues and there is no formation of metabolites in the parasite.

1.3.3 The antischistomicidal action of praziquantel

The two striking phenomena observed in schistosomes exposed to praziquantel are : an almost instantaneous contraction and spastic paralysis of the parasite musculature and a rapid structural damage of the syncytial tegument. These two effects are generally regarded as the two primary effects of praziquantel.

The praziquantel-induced contraction of schistosomes depends on the flux of divalent cations - particularly calcium - which follow drug-induced increases in membrane permeability (Andrews, 1985). Calcium influx into *S. mansoni* proceeds within 1 minute in the presence of 1μM praziquantel (Pax *et*

al., 1978; Harder, Andrews and Thomas, 1987 a). Characterization of praziquantel-stimulated muscle contraction in a medium with an elevated Mg^{2+}:Ca^{2+} ratio suggested that praziquantel was altering the activity of Ca^{2+} channels in the tegument and muscle of the schistosome.This altered channel activity leads to increased levels of cytoplasmic calcium and thus the muscle contraction and tegumental blebbing (Blair, Bennett and Pax, 1992).

Time course studies revealed that the onset of tegumental damage, observed as surface blebbing and vacuolization is extremely rapid (Becker *et al.*, 1980; Mehlhorn *et al.*, 1983; Bricker *et al.*, 1983). The vacuolization arises from the foldings of the basal tegumental membrane, while the blebbing results from pinching off evaginations of the outer tegumental membrane (Shaw and Erasmus, 1983 a). The molecular mechanisms underlying the rapid vacuole formation and the equally rapid blebbing, which is almost instantaneously induced by contact with praziquantel, is not known. It is possible that changes in the permeability to ions, especially calcium, are responsible.

The other effects of praziquantel are often referred to as 'secondary' because they are considered to be related to, or a consequence of, the primary effects. Some processes can be related to the primary effects of the drug on the tegument (antigen exposure, interference with the uptake of glucose and ATP, interference with the ouabin receptor, reduction in ATPase content), while others can be related to the sustained contraction (decrease in glycogen and increase in lactate excretion) (Andrews, 1985; Harder, Andrews and Thomas 1987 b; Harder *et al.*, 1987).

The chemotherapeutic action of praziquantel seems to be dependent upon the immune status of the host. Such reports have come from studies on drug-treated chronically infected mice (Sabah *et al.*, 1985; Brindley and Sher, 1987); naive mice treated concominantly with praziquantel and immune serum (Doenhoff *et al.*, 1987; Modha *et al.*, 1990) and mice vaccinated with radiation-attenuated cercariae and then treated with praziquantel on day 1 or day 6 post

challenge (Flisser, Delgado and McLaren, 1990; Piper *et al.*, 1991). Treatment of adult schistosomes with the drug is believed to erode the parasite surface and expose previously hidden antigens (Harnett and Kusel, 1986, Brindley and Sher, 1987; Flisser and McLaren, 1989), which are targets of immune effector mechanisms that mediate parasite death. Two of the exposed antigens have been characterized as 27 kDa enzyme with esterolytic activity on the parasite surface (Doenhoff *et al.*, 1988), and a 200 kDa tubercle antigen of *S. mansoni* (Brindley *et al.*, 1989). Studies by Modha *et al.* (1990) have revealed that the combination of praziquantel with a monospecific antiserum raised towards the 27 kDa enzyme results in enhanced damage to the tubercles of male worms. The 200 kDa antigen was recognized as a target for a monoclonal antibody that was synergistically active with praziquantel *in vivo* (Brindley *et al.*, 1989).

A typical feature of worms obtained from praziquantel-treated infected animals is loss of surface spines at the tubercles (Modha *et al.*, 1990; Linder and Thors, 1992). Spine distruction and disintegration *in vivo* was associated with binding of host anti-actin autoantibodies and gelsolin to actin, which is the major constituent of spines (Linder and Thors, 1992).

The broad-spectrum anthelmintic activity of praziquantel is based not on a universal mechanism but on a number of effects to which the nature and location of the parasite, and the host immune system may contribute (Harnett, 1988). It is possible that more than one effect operates against any one parasite population. Although the majority of schistosomes may be eliminated by an antibody-mediated mechanism, a small percentage may succumb to one or a combination of other factors such as those related to tegument damage.

1.4 Profile of the schistosome antigens presented during a chronic infection

During a normal infection, the schistosome presents a complex array of antigens to its host, with major antigenic stimuli believed to come both from the

adult worm and from eggs. The complex antigenic mixture includes secreted antigens, particularly degraded surface antigens and possibly cytosolic antigens released during membrane shedding. A study by Lewis and Strand (1991), showed that essentially all the proteins secreted from the worm were immunogenic, whereas most of the major structural membrane and tegumental proteins were not reactive with immune sera from humans infected with *S. mansoni*. However, The major source of released proteins appeared to be the tegument.

Norden and Strand (1984 a) performed detailed analysis of adult worm antigens recognized by sera from patients infected with *S. mansoni, S. haematobium* or *S. japonicum*. These analyses concentrated on antigens present in the glycoprotein fraction, prepared by affinity chromatography using immobilized concanavalin A, since previous work (Strand, McMillan and Pan, 1982) had shown that this fraction was the most antigenic. Proteins were labelled biosynthetically and glycoprotein antigens were analyzed by one- and two-dimensional gel electrophoresis. Of approximately 50 major polypeptides labelled in each species of schistosome (Aronstein and Strand, 1983), some 20-30 were found to be antigenic as determined by immunoprecipitation with the homologous infection sera. Extensive cross-reaction was observed; thus, sera from patients infected with *S. haematobium* precipitated all but three of the antigens recognized by *S. mansoni* sera amongst the glycoproteins derived from adult worms of *S. mansoni*. A slightly lower degree of cross-reaction was observed using sera from patients infected with *S. japonicum*. Similar findings were described by Kelly *et al.* (1987), who compared antigens immunoprecipitated from cell-free translation products synthesized from adult worm mRNA of *S. mansoni* and *S. haematobium*.

Norden and Strand (1984 b) also compared glycoprotein antigens prepared from eggs of each of the three species of schistosome and labelled by radioiodination. In each species, approximately 20 antigens were

immunoprecipitated using human infection sera, although the patterns observed following two-dimensional gel analyses showed marked differences between the species. Nevertheless, extensive cross-reaction was again observed; sera from patients infected with *S. mansoni* or *S. haematobium* could not be distinguished on the basis of their reactivity, although there were some differences in the immune responses of patients infected with *S. japonicum.*

The significance of these studies is that they give an indication of the antigenic complexity of the schistosome. Of the three major species which infect humans, it appears that *S. mansoni* and *S. haematobium* may be more closely related antigenically to each other than to *S. japonicum*, although a relatively small proportion of the polypeptide antigens is specific to any one species.

Newport, Culpepper and Agabian (1988) showed that although the human immune response to *Schistosoma mansoni* was erratic, virtually all infected individuals reacted to two schistosome proteins, one of 70 kDa and the other of 38 kDa. Antibodies to these schistosome proteins were not detectable in uninfected individuals or in persons infected with other parasitic helminths. The 70 kDa schistosome antigen was identified as a homologue of heat shock protein 70 (HSP70).

1.5 The heat shock response

The exposure of cells to from a wide variety of species to an increase in temperature results in the enhanced synthesis of several proteins, which have been referred to as heat shock proteins. This phenomenon has been called the heat shock response even though most of the heat shock proteins are induced by other stress agents and during normal development of an organism.

1.5.1 Regulation of the response

The molecular response to temperature increase has been extensively studied in both prokaryotic and eukaryotic organisms. Cells respond to elevated

temperature by increasing either the amount or the activity of a transcriptional factor that is specific for the heat shock genes. The result is increased transcripition of the heat shock genes, which leads to an increase in the concentration of heat shock proteins in the cell. The magnitude of the response and its duration depend on the severity of the temperature increase. The maximal response is obtained after a shift to a lethal temperature, in which case, non-heat shock protein synthesis is shut off and cells make heat shock proteins at maximal rates as long as protein synthesis continues. In addition to heat, other stresses such as ethanol,virus infection, amino acid analogues and DNA damage also function by increasing the amount or activity of the heat shock transcription factor. A converse response, that is a selective reduction in heat shock protein synthesis, is seen when cells are shifted down in temperature. In eukaryotes, temperature activation is mediated by a *cis* - element, or heat shock element (HSE), located 80-150 nucleotides upstream of the start site of RNA transcription of all heat-inducible genes (Pelham, 1985). This element (nnTCnnGAAnnTCnnnGAAnnTTCnnGAAn) consists of a highly conserved nucleotide sequence (Pelham, 1982) that is specific for heat shock DNA-binding proteins, called the heat shock factor (HSF). Specific heat shock factors have been identified in *Drosophila* (Wu *et al.*, 1987), in HeLa cells (Kingston, Schuetz and Larin, 1987), in *Saccharomyces cerevisiae* (Wiederrecht *et al.*, 1987) and in other organisms. Heat shock factor binds to heat shock element to initiate transcription of heat shock genes (Pelham, 1982). This factor is a protein of which the size varies from organism to organism. It is present in normal quantities in the cell cytoplasm and these quantities remain unchanged during heat shock. At high temperature, however, this factor binds more efficiently to heat shock element (HSE) (due to some structural changes in heat shock factor) and thus enhances the transcription of heat shock genes (Larson *et al.*, 1988). Similar transcriptional enhancement by heat shock factor (HSF) also occurs during low pH, treatment with calcium ions (Mosser *et al.*, 1990) or exposure to

anti-heat shock factor antibodies (Zimarino *et al.*, 1990).

1.5.2 Structure and function of heat shock proteins

Most heat shock proteins have been defined by their apparent molecular weight (Craig, 1985; Lindquist and Craig, 1988). The heat shock proteins are grouped into families of different size (Table1.1). Members of a given family share several features in addition to size, and similar groups of families have been described in different species. What is perhaps most surprising is the extreme conservation of sequence and function throughout phylogeny among the members of a given heat shock protein family. Most heat shock proteins or their homologues are expressed in the absence of stress, often in a tissue-specific manner and at particular stages of development.

It should be noted that the numbers assigned to the heat shock protein families do not represent precise molecular weights for these proteins because members of each family have slightly different molecular weights as they come from different species. For example, the heat shock protein 83 (HSP83) from *Drosophila* is a member of the HSP90 group.

1.5.2.1 Heat shock protein 90 (HSP90) family

In the eukaryote HSP90 is abundant in normal cells, is highly phosphorylated on serines and threonines, and is localized to the cytoplasmic compartment of the cell. A small fraction of it translocates to the nucleus after heat shock. HSP90 complexes with a variety of normal cellular proteins (Table1.1). The most thoroughly studied are the glucocorticoid receptors that are maintained in an inactive conformation bound to HSP90 until activated by hormone. Several kinases are transiently complexed with this heat shock protein; most notable are several of the tyrosine kinases encoded by oncogenes. Another kinase, one that phosphorylates the eukaryotic translocation-initiation factor, eIF-2a subunit, is activated by HSP90. The cytoskeletal proteins, actin and

Table 1.1 The heat shock protein families and their important functions.

Family	Major members	Important physiological functions	Possible role in the immune response	Proteins complexed with
HSP90	HSP90, HSP83	Prevention of steroid receptor binding to DNA. Tyrosine kinase phosphorylation.	Tumor resistance, autoimmunity	Glucocorticoid receptors Tyrosine kinase eIF-2α Kinase Yeast protein kinase C Tubulin, Actin
HSP70	HSP70, BiP,dnaK HSC70, grp78.	Protein translocation Protein folding and unfolding Dissociation of protein complexes in the presence of ATP Assembly of multimeric complexes	Immunoglobulin assembly Class II antigen processing Antigen of many pathogens autoimmunity	Clathrin-coated vesicles Prepro-α factor Nuclear proteins IgG heavy chains p53 tumor antigen DNA replication/ initiation complex Calmodulin SV40 T-antigen Microtubules
HSP60	HSP65, GroEl	Protein folding and unfolding Assembly of multimeric complexes ATPase activity	Antigens of many pathogens Autoimmunity	Rubisco Cytochrome C F1-ATPase Phage collars Temperature sensitive mutants
Small heat shock proteins	HSP18,HSP22, HSP23, HSP26, HSP27, HSP34, HSP40	Might be important in development Organization or protection of the cytoskeleton Thermoresistance of mammalian cells	Antigens of pathogens	
Ubiquitin	Ubiquitin	Protein degradation	Class I antigen processing Lymphocyte homing autoimmunity	

tubulin, are associated non-covalently with HSP90. Thus, HSP90 functions as a chaperone which prevents premature and senseless interactions between steroid receptors and DNA. How the other interactions are related to the role of HSP90 in heat shock remains a puzzle although the chaperone concept is a likely candidate. Microfilaments and microtubules are not unusually sensitive to stress although a prolonged and severe heat shock modifies both structures. In contrast, the intermediate filament network is very thermal sensitive. Possibly, HSP90 protects and aids in the recovery of these cytoskeletal systems.

1.5.2.2 Heat shock 60 (HSP60) family

In the eukaryote, members of the HSP60 family are localized to the cytoplasmic organelles such as the mitochondrion and the chloroplast (Hemmingsen *et al.*, 1988). The HSP60 homologue in the chloroplast is the ribulose-P_2 Carboxylase/ Oxygenase (Rubisco) heavy chain binding protein that is required for assembly of the hexadecameric enzyme complex (Cheng *et al.*, 1989). The yeast HSP60 protein is encoded by the MIF4 gene in which mutations give rise to phenotypes defective in mitochondrial function (Reading, Hallberg and Meyers, 1989).

The bacterial homologue of HSP60 is the GroEl protein of *Escherichia coli*, a major protein in this cell and the best-characterized heat shock protein at the functional level. This protein works in association with GroES, another polypeptide encoded in the same operon, and uses ATP as an energy source. GroEl is formed of two stacked rings of seven subunits of 60-65 kDa molecular weight. GroES is a seven subunit ring (7x10 kDa mol. wt.).

The work of Bochkareva and colleagues (1988), showed GroEl to be loosely associated with nascent proteins. GroEl maintains these proteins in a partially unfolded conformation probably similar to the previously described molten globule structure (Martin *et al.*, 1991), an unfolded conformation with native-like secondary structures. By using different forms of Rubisco as folding

substrates, the role of the GroEl-GroES complex has been clarified (Goloubinoff *et al.*, 1989). It does not drive the folding of proteins but it increases its efficiency by preventing aggregation of nascent, unfolded polypeptides. Different studies with β-lactamase (Laminet *et al.*, 1990) or citrate synthase (Buchner *et al.*, 1991) have confirmed this folding function of the GroEl-GroES complex. It also participates in the assembly of macromolecular structures. It facilitates the transfer of proteins across the intracellular membranes by maintaining them in a conformation competent for translocation. Based on these functions, these proteins have been referred to as chaperonins.

1.5.2.3 Small heat shock proteins

These are small molecular weight stress proteins that vaguely resemble each other in hydropathy profiles but not sequence except for a short region that is homologous to a sequence in the mammalian lens α-crystallin. These low molecular mass stress proteins (15-40 kDa) are most abundant in stressed plants where some are found as RNA nucleoproteins and others are translocated to the chloroplasts and to the mitochondria. In all eukaryotic cells, they can form huge granular arrays (Arrigo, 1987). The small heat shock proteins are found during various stages of embryonic development and, in yeast, during sporulation (Craig, 1985). The function of small heat shock proteins is unknown. It has recently been reported that a related, heat-inducible, 25 kDa turkey protein acts as an inhibitor of actin polymerization (Miron *et al.*, 1991).

1.5.2.4 Ubiquitin

All eukaryotic cells employ a small, very highly conserved (even more so than the heat shock proteins described above) polypeptide called ubiquitin to mark a protein for degradation. There are three kinds of genes encoding a ubiquitin sequence, and one contains multiple ubiquitin sequences contiguously arrayed as a polyubiquitin. This polyubiquitin gene has heat shock regulatory

elements. Levels of ubiquitin rise 5-7-fold after the stress. In the ubiquitin degradation pathway there are three kinds of ubiquitin-specific enzymes, called E1, E2 and E3. Free ubiquitin is activated in the presence of ATP and E1 and then transferred via an E2 to form covalent linkages with target proteins that have been bound by an E3. It is the E3 that recognizes proteins destined for degradation. One isomer of E3 is postulated to bind to unfolded proteins. Two of the yeast E2 enzymes that transfer ubiquitin to an E3 have been cloned and are heat shock proteins (Seufert and Jentsch, 1990). The product of E1, E2 and E3 activities is a polyubiquinated protein that is degraded by a large multicomponent organelle containing within it the proteosome (Matthews *et al.*, 1989), a complex of about eight subunits with protease activity. Polyubiquinated protein complexes increase about 2-fold in heat-shocked cells. There is no ubiquitin in the prokaryote, but one of the heat shock genes in *E. coli* encodes an ATP-dependent protease (Schlesinger, 1990).

1.5.2.5 Heat shock protein 70 (HSP70) family

The various members of the heat shock protein 70 (HSP70) family belong to the most conserved proteins in the cell. In addition to HSP70 species strongly inducible by heat shock or other forms of cellular stress, several constitutively expressed HSP70 proteins, sometimes termed heat shock cognate 70 (HSC70s), are known. These components have essential functions even under apparently non-stressful conditions. Different HSP70 species fulfill related functions probably based on a common mechanism of action (reviewed by Gething and Sambrook, 1992).

In most organisms, HSP70 is the most abundant heat shock protein. In *E. coli*, there is only a single HSP70-related protein, the product of the dnaK gene, which is essential for growth (Lindquist and Craig, 1988). However, in most, if not all eukaryotes, there are multiple genes encoding a set of related HSP70 proteins, some of which are present under optimal growth conditions, while

others are expressed after a stress. These proteins are found in several compartments of the cell. The major inducible member is usually found predominantly in the nucleus during and immediately after stress, moving to the cytoplasm upon recovery. Two constitutively expressed proteins are present in organelles, one (BiP/ grp78) in the endoplasmic reticulum (ER) and the other (mHSP70) in the mitochondrion. Genetic analysis in yeast has shown that these three classes of HSP70 perform essential functions in their respective compartments (Craig, Kang and Boorstein, 1990).

Structure

The HSP70 proteins are highly conserved, showing 60-78% identity among eukaryotic proteins and 40-60% identity between the *E. coli* HSP70, DnaK and the eukaryotic proteins (Lindquist, 1986).

The protein has two major domains : a highly conserved compact amino-terminal portion containing an ATP-binding site, weak ATPase activity and a calmodulin-binding site (Stevenson and Calderwood, 1990) connected by a protease-sensitive region to a more diverse carboxyl domain containing sites for localization to the nucleus (Milarski and Morimoto, 1989). The ATP-binding domain and the ATPase activity are associated with the 44 kDa terminal fragment (Chappell *et al.*, 1987). This domain has been crystallized and shown to be similar to actin (Flaherty, Flaherty and McKay, 1990). The peptide-binding domain shows some sequence homology with the Human Leukocyte Antigen (Class I) (Rippman *et al.*, 1991). HSP70 does not bind to a specific sequence (Flynn, Chappell and Rothman, 1989), but probably has a higher affinity for hydrophobic aliphatic residues (as shown recently for BiP) (Flynn *et al.*, 1991).

The native molecule is a dimer (Schlesinger, 1990) but forms higher oligomeric complexes with many structures in the cell (see Table1).

Function

Biochemical characterization of HSP70 proteins has uncovered a number of shared properties. All HSP70 proteins examined bind ATP with high affinity and possess a weak ATPase activity. *In vitro* studies with mammalian proteins have shown that HSP70 proteins can interact with a variety of peptides, and that their release is dependent upon the hydrolysis of ATP (Flynn, Chappell and Rothman, 1989). HSP70 proteins have been shown to participate in a number of specific protein-protein interactions. In the current, unifying view, HSP70 proteins bind to protein substrates, altering or maintaining conformations or interactions with other proteins; release from such substrates is ATP-dependent.

The vast majority of data concerning eukaryotic HSP70 function also support the idea that these proteins interact with a variety of cellular proteins, and are essential for the movement of proteins within cells, from the time they are synthesized until they have reached their final destination, be it transport through the secretory pathway (Vogel, Misra and Rose, 1990; Mierynk *et al.*, 1992), translocation into mitochondria (Kang *et al.*, 1990) or degradation in the lysosome under some starvation conditions (Chiang *et al.*, 1989). The cytoplasmic HSP70 proteins that have been implicated in the regulation of the heat shock response have been shown to be involved in a variety of cellular processes. The first HSP70 to be associated with a biochemical activity was the proteins purified because of its ability to release clathrin from coated vesicles that mediate endocytosis. The uncoating enzyme, later shown to be an HSP70, hydrolysed ATP in a clathrin-dependent manner, driving disaasembly of the clathrin coat (Chappell, 1987). Genetic and biochemical evidence demonstrated a role in protein translocation for HSP70 proteins localized to the cytoplasm. It is likely that HSP70 is required to maintain precursors in a translocation-competent conformation. In addition, cytoplasmic HSP70 appears to interact with many newly synthesized proteins, since many pulse-labelled proteins of human cells coimmunoprecipitate with HSP70 antibody (Beckmann, Mizzen and Welch,

1990; Nelson *et al.*, 1992).

The HSP70 homologue of *E. coli*, DnaK, cooperates functionally with two additional heat shock proteins DnaJ and GrpE (Craig and Gross, 1991). Recently, it has been shown that DnaK, DnaJ and GroEl (HSP60) act successively in the protein folding pathway (Langer *et al.*, 1992). DnaK recognizes the folding polypeptide as an extended chain and cooperates with DnaJ in stabilizing an intermediate conformational state lacking ordered tertiary structure. Dependent on GrpE and ATP hydrolysis, the protein is then transferred to GroEl which acts catalitically in the production of the native state.

A functional cooperation of HSP70 proteins with DnaJ and GrpE-like proteins is likely to exist also in eukaryotes. This view is supported by the recent identification of various eukaryotic DnaJ homologues in different cellular locations (Luke, Sutton and Arndt, 1991). A candidate protein for an HSP60-like function in the eukaryotic cytosol has been proposed (North, 1991).

1.5.3 Heat shock proteins and the immune response

The major heat shock protein families, their biological functions and their possible role in the immune response are listed in Table 1.1.

1.5.3.1 Antigen processing

Many characteristic functions of heat shock proteins, namely the unfolding, translocation and disintegration of proteins, are reminiscent of the steps underlying antigen processing. Indeed work by Pierce and coworkers (1989) has provided evidence for a more general involvement of HSP70 cognate in the MHC-class II processing and presentation of certain antigenic peptides (VanBuskirk, Crump and Pierce, 1989 ; De Nagel and Pierce, 1991). This feature possibly relates to the peptide scavenging activity of heat shock proteins at the sites of intracellular antigen processing and presentation.

1.5.3.2 Antigenicity

Heat shock proteins as antigens of infectious agents

Several heat shock proteins, in particular, the chaperones of the HSP70 and HSP60 families represent major antigens of many pathogens. This holds true for antibody or T-cell responses or both. Although it is not fully understood why heat shock proteins are so often major antigens, the most straightforward explanation would be that these proteins are produced in large quantities by pathogens following exposure to various host defence mechanisms. Antibodies against HSP70 cognates have been described in infections with malaria, Chagas' disease, leishmaniasis, schistosomiasis, lymphatic filariasis, onchocerciasis, leprosy and tuberculosis (Kaufmann, 1990 a). HSP65 (which belongs to the HSP60 family) is a major antigen of a variety of bacterial infections including leprosy, tuberculosis, Q-fever, syphilis, Legionnaires' disease, lyme disease and trachoma.

Heat shock proteins and autoimmunity

In different autoimmune diseases, increased antibody levels to HSP70 cognates have been observed : these include antibodies to HSP65 in rhematoid arthritis , to HSP90 in ankylosing spondylitis, and to ubiquitin, HSP70 and HSP90 in systemic lupus erythmatosus. Despite the prevalence of these antibodies in different autoimmune diseases, their significance is not fully understood. Although in normal cells, heat shock proteins are hidden within the intracellular compartments, some cells when they are stressed express heat shock proteins on their surface (Karlsson-Parra, 1990). Such stressed cells might become targets of anti-heat shock protein antibodies and could trigger other autoimmune reactions.

1.5.4 Heat shock response in parasites

During their life-cycles, parasites are exposed to major temperature differences when moving from invertebrate vectors to homeothermic mammalian hosts. Most parasites undergo a classical heat shock response after temperature shifts or exposure to other types of stress such as metabolic poisons (Lawrence and Robert-Gero, 1985). This response is associated with the transcripitional activation of heat shock genes and the inhibition of normal protein synthesis (Newport, Culpepper and Agabian, 1988). Despite the increase in the synthesis of heat shock proteins upon transformation, basal production of heat shock proteins occurs in poikilothermic vectors, except in malaria parasites. Malarial sporozoites do not synthesize HSP70 although all blood stages do (Bianco *et al.*, 1986). Promastigotes of *Leishmania major* in the sandfly constitutively express most heat shock proteins at low temperatures and in *L. donovani*, a HSC70 gene coding for an antigen related to the HSP70 family is constitutively expressed during all stages of the life-cycle (Maresca and Carratu', 1992).

It has been suggested that the stimulus for parasite heat shock protein induction is not necessarily an abrupt change in temperature, but rather a change in osmolarity or the presence of some unidentified cue provided by a change in environment. For example, the response can be elicited in leishmanial promastigotes or schistosome schistosomula maintained at room temperature, by placing the organisms in a medium more closely resembling conditions offered by the mammalian host (Van der Ploegh *et al.*, 1985; Blanton, Ioula and Parker, 1987). Production of HSP70 in microfilariae is increased by a shift in temperature from 37°C to 42°C (Selkirk *et al.*, 1987) indicating that constitutive synthesis and responsiveness to heat may be independent manifestations.

1.5.4.1 Parasite heat shock genes

Parasite heat shock proteins are encoded by multigene families and are arranged in a head-to-tail tandem array (Newport, Culpepper and Agabian,

1988). *Plasmodium falciparum* is an exception; it possesses at least five different HSP70 genes located on four different chromosomes (Sharma, 1992).

Most of the studies on heat shock genes from pathogens have been at a structural level and not at a transcriptional level, so that very little is known about the mechanisms of heat shock gene regulation in parasites. In *T. brucei*, transcripts of HSP70 and HSP83 were 25-100-fold more abundant in trypomastigotes at 37^{0}C than in insect stages (Van der Ploeg *et al.*, 1985). In *L. major*, four HSP70 genes have been found arranged in tandem with intragenic regions of about 380 nucleotides (Lee *et al.*, 1988). These genes contain miniexons that are trans-spliced under heat shock conditions at 37^{0}C.

1.5.4.2 Functions of the heat shock proteins in the parasite

Besides the universal phenomenon of increased synthesis of heat shock proteins in response to a rise in temperature, parasite heat shock proteins appear to play unique roles in differentiation. Hunter *et al.* (1984) first suggested that heat shock was involved in the transformation of promastigotes to amastigotes. Van der Ploeg *et al.* (1985) then established that a temperature shift can induce differentiation of *L.major in vitro*. Evidence has now accumulated supporting a role for heat shock in differentiation of many parasites, including *S. mansoni* and *Naegleria gruberi*, although modification of growth or environmental conditions other than heat shock can cause a heat shock response and also induce differentiation (Newport, Culpepper and Agabian, 1988).

Other interesting functions of heat shock proteins examined in the parasites concern mtp70, a mitochondrial member of the HSP70 family isolated in *T. cruzi* and ubiquitin. Mtp70 is located in the trypanosome mitochondrion in the region where DNA replication occurs and it has been suggested that mtp70 participates in this replication (Engman *et al.*, 1989). *T. cruzi* also possess over 100 ubiquitin genes organized into a single cluster that contains both

polyubiquitin and ubiquitin fusion genes (Swindle *et al.*, 1988). Ubiquitin may either sensitize proteins to proteases or act by itself as a protease. It has been suggested that parasite ubiquitin could be secreted into the cytoplasm of the host so that sufficient amounts of small peptides or amino acid derived from the host's proteins are provided for nourishment and survival of the parasite (Swindle *et al.*, 1988).

1.5.4.3 Immunogenicity of parasite heat shock proteins, and specificity of the host response

Heat shock proteins are the most commonly documented immunogenic proteins of infectious agents. The response to the antigens has been described on the basis of humoral responses and at the T-cell level (reviewed by Mollenhauer and Schulmeister, 1992; Kaufmann, 1992). The immunogenicity of parasite heat shock proteins is not necessarily surprising as, despite their much mentioned phylogenetic conservation, they display significant dissimilarities at the amino acid sequence level and are highly abundant (Kaufmann, 1990; Lindquist, 1986). Immunization of animals with denatured heat shock proteins generally results in an antibody response that cross-reacts with respective denatured antigens from a broad spectrum of species (Kelly and Schlesinger, 1982). Immune responses to heat shock proteins presented by pathogens during the course of infection on the other hand are more varied, occasionally as a result of subtle differences in amino acid sequence. At one extreme, responses to bacterial HSP60 proteins are so broadly cross-reactive that the antigen is known as 'common antigen' (Hansen *et al.*, 1988). Antibodies against schistosome and malarial HSP70 proteins appear to be directed at a small number of epitopes located somewhere at the carboxy-terminus of the molecules. The responses can serve to differentiate between species of the two genera (Hedstrom *et al.*, 1987; Kumar *et al.*, 1990), although that against the schistosome antigen occasionally cross-reacts with homologues from other

genera (Moser, Doumbo and Klinkert, 1990), in analogy to the case for the Brugia protein (Selkirk *et al.*, 1989).

1.5.5 Heat shock proteins in *Schistosoma mansoni*

Schistosoma mansoni is a complex multicellular organism whose life-cycle alternates between a fresh water molluscan host and a warm-blooded, mammalian host (see figure 1.2). Cercariae, the infective larvae of *S. mansoni*, are released in fresh water and must penetrate the skin of the human host within several hours in order to continue the life-cycle of the parasite. Transformation to the next developmental stage, the schistosomulum is accompanied by many morphologic and biochemical changes : loss of tail, doubling of the surface membrane, loss of glycocalyx, change from heterochromatic to euchromatic nuclei, water intolerance (Stirewalt, Cousin and Dorsey, 1983), and reduced pyruvate metabolism (Mahmoud, 1984). Many methods have been devised to induce this transformation *in vitro*, but the minimum requirements are an isotonic medium and an increase in temperature (Cousin, Stirewalt and Dorsey, 1986).

In this complex life-cycle of schistosomes, cercariae are released from snails into fresh water of which the temperature can range from 15-30^{0}C and penetrate the host where they encounter the temperature of 37^{0}C. The organisms are thus exposed to a sharp temperature rise. In addition, they face an osmotic pressure that is much greater than that of fresh water and twice as great as that found inside the snail host. This sudden change in temperature could be expected to stress the intruder, as it should dramatically alter the rates of metabolic reactions and of denaturation of proteins (Barret, 1986). This induces the synthesis of several heat shock proteins (Yuckenberg, Poupin and Mansour, 1987; Blanton, Loula and Parker, 1987; Hedstrom *et al.*,1987) which are homologous to those of the host. Production of these proteins, whose function is thought to be related to development and / or turnover of structural proteins, is constitutive during the parasite's sojourn in the mammalian host.

Heat shock protein 70 (HSP70) is the major protein synthesized by newly transformed schistosomula (Yuckenberg, Poupin and Mansour, 1987; Blanton, Loula and Parker, 1987) and may be necessary for the events occurring at the time of cercarial penetration of the skin and shortly thereafter. Studies on the transformation of cercariae support the notion that the induction of heat shock protein synthesis in parasites is not necessarily a response to heat, but an exploitation of a primitive and universal pathway toward adaptation for survival in a poikilothermic environment. As mentioned before, certain heat shock proteins appear to play an unknown role in mediating the development of a variety of cell types (Lindquist, 1986). Larval and adult schistosomes constitutively synthesize HSP70 (Hedstrom *et al.*, 1987; Newport, Culpepper and Agabian, 1988), but transforming schistosomula express dramatically higher levels at a period when synthesis of nearly every other protein is repressed (Yuckenberg, Poupin and Mansour, 1987; Blanton, Loula and Parker, 1987; Blanton and Licate ,1992); synthesis of other proteins commences 16 hours later-eventually reaching a steady-state plateau (Yuckenberg, Poupin and Mansour, 1987). This response can be reproduced by placing cercariae in 23^{0}C mammalian cell culture media, but not by incubating them in 37^{0}C water (Blanton, Loula and Parker, 1987). Temperatures above 35^{0}C are lethal to cercariae swimming in water (Lawson and Wilson, 1980), although organisms subjected to this form of stress respond by increasing the synthesis of 58 kDa and 60 kDa proteins whose relationship, if any, to classic heat shock proteins or thermotolerance is unclear.

1.5.5.1 Heat shock protein 70 (HSP70)

As mentioned earlier, one of the major antigens recognized in humans and animals infected under experimental conditions with *S. mansoni,* is a 70 kDa molecular weight protein. sequence comparison of cDNA indicated 80% identity at the amino acid level with human HSP70 and 71% with *Drosophila.* Regions of sequence divergence between the polypeptides occur predominantly near the 3'

terminus of the molecule (Hedstrom *et al.*, 1987).

Another species of Schistosoma, *S. japonicum*, also elicits a similar response in infected animals (Scallon, Bogitsh and Carter, 1987) and in infected humans (Hedstrom *et al.*, 1988).

The anti-HSP70 antibodies elicited do not cross-react with host HSP70, despite the striking similarity and conserved nature of these proteins. Moreover, antibodies to schistosome HSP70 are not detected in uninfected individuals (Hedstrom *et al.*, 1988). Thus, it is thought that structural differences between the host and parasite homologues are responsible for inducing specific antibodies during the course of infection and that, within the context of infection, parasites present HSP70 antigen in a manner which on the one hand provokes an immunodominant response, and, on the other hand, avoids a host antibody response directed against the conserved regions of the molecule. This response is directed towards the carboxy terminus of the protein, the least (but nevertheless strongly) conserved portion of the molecule (Hedstrom *et al.*,1988; Moser, Doumbo and Klinkert, 1990).

Antibodies induced to the HSP70 during *S. japonicum* and *S. mansoni* infections do not cross-react even though the antigens are highly conserved in secondary sequence (Hedstrom *et al.*, 1988). The antibody response to these proteins thus appears to be species-specific and directed to minor differences in the structure of the two antigens. It appears also, that such limited diversity is sufficient to discriminate between schistosome species. On the other hand, antibodies binding to the *S. mansoni* HSP70 are observed in some individuals with Schistosomiasis haematobium (Hedstrom *et al.*, 1987) and with filariasis and malaria (Moser, Doumbo and Klinkert, 1990), which indicates that even though antibody responses can discriminate between HSP70 of different species, the hypervariable C-terminal domain of the molecule contains antigenic sites that are shared with the HSP70 proteins of *S. haematobium* and other parasites.

Antibodies to HSP70 are generated after a considerable duration of infection. a detectable antibody titre to HSP70 is observed only 5-6 weeks post-infection (Hedstrom *et al.*, 1987; Moser, Doumbo and Klinkert, 1990). The delayed response to HSP70 is consistent with the finding that chronically infected patients recognize HSP70 whereas individuals with acute Schistosomiasis do not (Moser, Doumbo and Klinkert, 1990). the immunodominant nature of HSP70 may be a reflection of the relatively high abundance of this protein. It is estimated that HSP70 constitutes over 1% of the protein synthesized by adult schistosomes (Newport, Culpepper and Agabian, 1988).

The HSP70 gene in many eukaryotic organisms has been shown to exist in multiple copies that are usually dispersed in the genome. The *S. mansoni* gene, however, is arranged in the genome as tandemly repeated copies of identical or closely related genes (Hedstrom *et al.*, 1987).

1.5.5.2 Heat shock proteins 86 and 40 (HSP86 and HSP40)

Two more heat shock proteins recognized by human infection sera are of M_r 86 (Johnsone *et al.*, 1989), and 40 kDa (Nene *et al.*, 1986) proteins. Antibodies to HSP86 are detected in roughly 50-60% of uninfected individuals. The 40,000 protein is one of the major proteins of eggs and miracidia and elicits a strong immune response in over 90% of patients. The antigen consists of a family of at least four near identical proteins, probably encoded by a multi-gene family and expression of this polypeptide is differentially regulated around the parasite's life-cycle. This M_r 40,000 protein shares a block of sequence homology with a-crystallins and *Drosophila* small heat shock proteins.

1.5.5.3 *Schistosoma mansoni* responses to heat

Heat shock protein 70 (HSP70) is not observed in schistosomula until at 8 hours after transformation and by 24 hours, synthesis of HSP70 declines. When

the schistosomula are heat-shocked, new HSP70 synthesis is observed in the 24 hour-old schistosomula and not in earlier stages (Blanton and Licate, 1992). As with HSP70 synthesis, the level HSP70 mRNA was maximal in schistosomula at 8 hours, but declined by 24 hours. Heat shock at this time will again induce mRNA levels of HSP70. PAGE analysis of *in vitro* translation products of cercarial RNA revealed that the major protein induced by heat shock was HSP70. RNA for HSP70 was present in untreated cercariae, but heat shock increased the amount of this protein produced by *in vitro* translation by approximately 5 fold. In agreement with the above, Northern blots of cercarial RNA revealed that with heat shock, message levels of HSP70 rose dramatically in cercariae. *In vivo* synthesis of HSP70 has not been observed in cercariae (Yuckenberg, Poupin and Mansour, 1987; Blanton, Loula and Parker, 1987). Thus, the *in vivo* translation of HSP70 discoordinates with the transcription of its mRNA.

Throughout the first 24 hours the regulation of HSP70 transcription appears to respond appropriately to heat shock. Peak levels of HSP70 mRNA are present in newly transformed schistosomula since a further increase in temperature to 42^{0}C fails to induce further expression of the message. After 24 hours at 37^{0}C a new temperature set point is reached for schistosomula, and HSP70 transcription is no longer maximal. At this time raising the temperature will result in elevated levels of mRNA and new protein synthesis. Induction of HSP70 transcription with a rise in temperature is a universal pattern in all other organisms studied. Failure of cercariae and schistosomula at early stages of development to produce HSP70 despite the availability of the corresponding mRNA suggests that there is a post-transcriptional block to translation in these life-cycle stages and that the block is reversed over time to allow expression of the available mRNA. Failure to produce HSP70 is a common finding in the early developmental stages of several organisms (Lindquist, 1986).

Blanton reported that in adult worms a rise in temperature from 37^{0}C to 42^{0}C induced the synthesis of heat shock proteins of Mr 100,000, 84,000,

70,000, 43,000 and 34,500. The 70,000 protein was very prominent both before and after a rise in temperature. Johnsone *et al.*, (1989) also showed that adult worms respond to heat shock by increasing the synthesis of polypeptides of 70 and 86 kDa, in both metabolically labelled samples and *in vitro* translation products which suggests a specific effect of heat on the transcription of the gene encoding the 70 and 86 kDa polypeptides. The 86 kDa protein was found to be highly homologous to the large heat shock proteins of *Saccharomyces cerevisiae* (HSP90) and *Drosophila melanogaster* (HSP83).

1.6 Aims of the project

The aim of this project was to define the role that HSP70 plays in the parasite *S. mansoni*. The inhibition of the synthesis of HSP70 after irradiation of the parasite (Wales, Kusel and Jones, 1992) and the immunity that these irradiated parasites confer upon infection led us to think that HSP70 played an important role in the schistosome .

In search for the functions of HSP70, obvious questions concern their localization in the parasite and their interactions with other molecules and particular cellular structures. Therefore I aimed, at first, at localization of HSP70 in the parasite. The prominent immune response to it made it a good candidate for surface location. This will be studied by the method of indirect immunofluorescence.

HSP70 is found to interact with a variety of proteins or polypeptides in prokaryotes and eukaryotes. Thus, studying its association with proteins of the parasite, identification of these proteins and studying their interactions in more detail, is hoped to give an indication to how HSP70 functions in the parasite.

Given the nature of the interactions of HSP70 with different molecules and the speculation on its role in renaturing denatured schistosomular proteins which result upon transformation and after irradiation of the parasite, it was interesting to understand its role in drug-treated parasites. Praziquantel is the drug of choice

in the treatment of schistosomiasis. The exact mechanism of action of this drug, although widely studied, is not known yet. The drug causes severe erosion of the surface of the parasite and antigen exposure. The effect that praziquantel has on the synthesis and expression of HSP70 will be studied and how this heat shock protein might participate in repairing the damage caused by praziquantel will be discussed.

Chapter Two
Materials & Methods

2.1 Life-cycle maintenance

2.1.1 Parasite strain

The life-cycle of a Puerto Rican strain of *Schistosoma mansoni,* described by Smithers and Terry (1965), is routinely maintained in our laboratory, department of Biochemistry, University of Glasgow. This *S. mansoni* strain, and the intermediate host, *Biomphalaraia glabrata,* were originally obtained from stocks at the NIMR, Mill Hill, London. Some snails were also obtained from the Department of Biological sciences, University of York.

2.1.2 Infection of snails

2.1.2.1 Preparation of miracidia

Materials

Sorenson's buffer : Solution A : 50 mM KH_2PO_4

Solution B : 60 mM Na_2HPO_4

Sorenson's buffer consists of 5% by volume solution A, and 95% by volume solution B.

Trypsin

Method

Guts from mice infected 8 weeks previously were dissected and washed with 15 mM NaCl and then homogenised in Sorenson's buffer. The homogenate was transferred to a conical flask, trypsin was added at a concentration of 1mg / gut, and the suspension was incubated at 37^{0}C for 2 hours with gentle shaking. After trypsin digestion the homogenate was strained through 2 layers of muslin cloth and the filtrate was washed and centrifuged (800g for 10 minutes) twice. The sediment of packed eggs was recovered and added to a conical flask containing 250 ml / gut of aquarium water. The eggs were then allowed to hatch under a light source at room temperature.

2.1.2.2 Infection of snails

A stock of snails was maintained in plastic aquaria and fed regularly on dried lettuce. Snails were exposed in 4 x 2 cm glass bottles to a batch of 10 - 15 freshly hatched miracidia. An aliquot of water containing the miracidia was added to snails individually placed in a bottle and the snails were then left at 25^{0}C for 24 hours. Following this, snails were pooled and transferred to a plastic aquarium. All snails were kept in the dark for 6 weeks at 25^{0}C before being subjected to a bright light source in order to induce shedding of cercariae.

2.1.3 Routine infection of mice

Materials

Mice : BALB/c bred and housed in the Biochemistry / Physiology departments, University of Glasgow.

Anaesthetic : Sagatal May & Baker Ltd.

Iodine stain for cercariae : Iodine (Analar grade; BDH chemicals) made up in ethanol and water.

Infection procedure

Mice were infected percutaneously, using the ring exposure technique descried by Smithers and Terry (1965).

Mice were anaesthetised with Sagatal : H_2O : ethanol, 1:10:1 by volume, at a dose of 0.2 ml for a mouse of approximately 20 grams.

The anaesthetised mice were shaved ventrally and laid on their backs. Metal rings of 300 µl capacity were placed on the shaved abdomens of the mice.

Several 50 µl aliquots of a suspension of newly-emerged cercariae were fixed and stained with a drop of iodine solution then counted under the light microscope. Aliquots containing approximately 150 cercariae were

introduced into the metal rings placed on the mice. The mice were left undisturbed for 20 to 30 minutes, to allow cercarial penetration.

2.1.4 Perfusion of mice for recovery of adult worms

Materials

Citrate saline : 0.05 M trisodium citrate	Farmachem
0.145 M sodium chloride	Farmachem
Sagatal	May & Baker Ltd.

Procedure

Adapted from Smithers and Terry (1965).

Mice were perfused 8 weeks after infection. A lethal dose of undiluted Sagatal (1ml / 100g body weight) was administered intraperitoneally, and the abdominal and thoracic cavities of the dead animal were opened. Rubber bands were used to secure the mouse to a perspex sheet, supported in the vertical position by a metal clamp. An incision was made in the hepatic portal vein, and a 50 ml plastic syringe filled with citrate saline was inserted into the heart. By exerting gentle pressure on the syringe, the saline was flushed through the circulation, and worms from the opened hepatic portal vein were washed onto a piece of muslin placed onto a wire gauze positioned directly below the dead mouse. The worms were transferred to a petri dish containing warm medium and then to a universal bottle in which they were washed 3 times with warm GMEM and prepared for culturing.

2.1.5 Culture of parasites

2.1.5.1 Plasticware & Glassware

All plasticware was from Sterilin.

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

Microscope slides and coverslips were from Chance Proper Ltd.

2.1.5.2 Culture media

(i) GMEM (Glasgow's modification of Eagle's Minimal Essential Medium). Table 2.1 describes how GMEM was prepared. Table 2.2 lists the final composition of GMEM.

(ii) Minimum essential medium without methionine

(MEM w / o methionine) Flow.

Glutamine, penicillin, and streptomycin were added to give final concentrations of 2 mM, 100 I.U. / ml and 100 I.U. / ml, respectively.

2.1.5.3 Foetal Calf Serum (FCS)

Obtained from Northumbria Biologicals Ltd.

FCS was depleted of complement activity by incubation at 56^0 C for 30 minutes. Aliquots of the heat-inactivated serum was stored at -20^0 C.

2.1.6 Preparation of schistosomula

Cercariae were transformed to schistosomula by one of the two methods.

2.1.6.1 Mechanical transformation

Method was adapted from Colley and Wikel (1974), with some modification.

Snails (*Biomphalaria glabrata*) infected with a Puerto Rican strain of *S.mansoni* were induced to shed cercariae by exposure to bright light in fresh spring water. The cercariae were then concentrated by gravity sedimentation on ice for 45 minutes. The supernatant, consisting of aquarium water, was decanted, and the sedimented cercariae gently resuspended in 10 ml of GMEM medium warmed to 37^0 C. The cercariae were passaged ten times through a 21G1 1/2 gauge needle attached to a 10 ml syringe. This shearing

Table 2.1 Preparation of 500 ml of GMEM (Glasgow's modification of Eagle's medium)

Component	stock concentration	add stock (mls)	working concentration
BHK-21	10x	50	1x
Glutamine	200 mM	5	2 mM
Sodium bicarbonate	7.5% (w / v)	15	0.225% (w / v)
Penicillin / streptomycin	10000 I.U./ ml	5	100 I.U./ ml
Sodium hydroxide	5M	to pH 7.4	
Distilled, deionised water		to 500 ml	

All stock solutions were obtained from Gibco.

Table 2.2 Composition of GMEM

Ingredients	mg / ml
$CaCl : H_2O$	264
$Fe(NO_3)_3 : 9\ H_2O$	0.1
KCl	400
$MgSO_4 : 7\ H_2O$	200
NaCl	6400
$NaHCO_3$	2750
$NaH_2PO_4 : H_2O$	124
Glucose	4500
Phenol red	15
L-Arginine : HCl	42
L-Cystine	24
L-Glutamine	292
L-Histidine HCl : H_2O	21
L-Isoleucine	52.4
L-Leucine	52.4
L-Lysine HCl	73.1
L-Methionine	15
L-Phenylalanine	33
L-Threonine	47.6
L-Tryptophan	8
L-Tyrosine	36.2
L-Valine	46.8
D-Ca Pantothenate	2
Choline Chloride	2
Folic acid	2

j-inositol	3.6
Nicotinamide	2
Pyridoxal HCl	2
Riboflavin	0.2
Thiamine HCl	2
Penicillin	100 I.U./ ml
Streptomycin	100 I.U./ ml

pH was adjusted to 7.4 with 5M NaOH

stress resulted in separation of cercarial bodies and tails.

The newly transformed schistosomula were washed three times by slow centrifugation (1000 rpm for 2 minutes) in warm medium. The tail-rich supernatant was decanted, and the parasite bodies were cultured for 3, 24, 48, 72, and 96 hours. For incubations lasting overnight or longer, the culture medium was supplemented with 10% heat-inactivated FCS.

2.1.6.2 Transformation by intra-peritoneal infection of mice with cercariae.

Cercariae were obtained, counted, and sedimented as above. The sedimented cercariae were then resuspended in aquarium water at a concentration of 6000 cercariae per 1 ml. 0.5 ml aliquots of the cercarial suspension were injected intraperitoneally into 10 mice. At 3 days, 4 days, and 8 days after injection of the cercariae, the peritonia of the mice were cut open and the schistosomula were collected in warm medium supplemented with heparin. The schistosomula were washed 3 times with warm GMEM by resuspension in GMEM and allowing them to sediment to the bottom of the tube. The schistosomula were then prepared for culture.

2.2 Metabolic labelling with ^{35}S-methionine.

2.2.1 Radioisotope

L - [^{35} S] - methionine (in 20 mM potassium acetate solution , containing 0.1 % β-mercaptoethanol) in different batches from 1030 to 1476 Ci (38.1 - 54.6 T Bq) / mmol was used.

2.2.2 Radiolabelling procedure

Parasites were labelled with ^{35}S-methionine GMEM w / o methionine. Labelling time and the final concentration of the label will be shown for each experiment.

2.2.3 Quantifying uptake of free radioisotope and incorporation into protein : trichloroacetic acid (TCA) precipitation.

50 μl of parasite extracts were removed to ice. 2 ml of ice-cold 10% (w / v) TCA, and 10 μl of FCS to act as a protein carrier in precipitation, were added. The precipitates were spun down for 10 minutes at 2000 rpm in a bench centrifuge.

The resulting supernatants should now contain the free ^{35}S-methionine taken up by the parasites, but not incorporated into protein. 0.5 ml from these supernatants were transferred to scintillation vials. 4.5 ml Ecoscint were added, and mixed well.

The pellets were washed by resuspension in 2 ml 10% (w / v) TCA, and centrifugation as before. The final pellets were dissolved in 100 μl 90% formic acid. 400 μl of saline were added to the dissolved pellets and the total 500 μl were transferred to scintillation vials. 4.5 ml Ecoscint were added, and mixed well. The samples were counted on a scintillation counter. Each sample was counted for either 1, or, 3 minutes.

2.3 Chemical cross-linking

2.3.1 Cross-linkers

Dimethyl 3,3'-dithiobispropionimidate dihydrochloride (DTBP)

Dimethyl suberimidate dihydrochloride (DMS)

Both reagents were obtained from Pierce Chemical Co.

Both are homobifunctional imidoesters reactive towards primary amines. DMS is non-cleavable whereas DTBP is cleavable by thiols at the disulphide bond that it contains.

2.3.2 Buffer

Triethanolamine (TEA) buffer:

50 mM TEA-HCl (Sigma) pH 8.0 with 10 M KOH,

80 mM KCl,

5 mM MgAcetate.

2.3.3 Cross-Linking Procedure

2.3.3.1 Testing the function of the cross-linkers on a standard protein

Method adapted from Lumsden and Coggins (1978).

Aldolase (Sigma), was used as a standard protein to test the reactivity of the cross-linkers. Aldolase was made up at a final concentration of 1mg / ml (estimated by reading the absorbance at 280 nm ; A_{280} of 1mg / ml aldolase = 1.00).

DTBP and DMS were dissolved in ice-cold 1M TEA-buffer to give fresh stock solutions of 50 mg / ml and the pH was adjusted to 8.0 with 10 M KOH. Aliquots of these fresh stock solutions were added quickly to separate 50 µl aliquots of the aldolase solution to give final concentrations of 1, 2, and 3 mg of the cross-linker per ml of aldolase solution. Cross-linking was carried out at 0^0C or room temperature for either 30 minutes or 1 hour. The reaction was quenched by addition of 100 µl of 1M ammonium chloride (BDH) per ml of aldolase solution and incubation for 10 minutes at room temperature. Samples were then analyzed on reducing and non-reducing SDS gels.

2.3.3.2 Cross-linking adult worm proteins

Method adapted from Wang and Richards (1974).

^{35}S- methionine-labelled or unlabeled worms were washed 3 times with ice-cold TEA buffer and resuspended in 1 ml of the same buffer. DTBP and DMS were freshly prepared as 20 mg / ml stocks in ice-cold TEA buffer and the pH adjusted to 8.0 with 10 M KOH. The chemical cross-linkers were added quickly to the adult worms at a final concentration of 1 mg / ml, and the worms were incubated at room temperature for 1 hour. Following that, the worms were

washed several times with TEA buffer to remove the cross-linker, solubilized as in section 2.4, and prepared for either immunoprecipitation or immunoblotting.

2.4 Solubilization of adult worms

Materials

PBS : 0.14 M Sodium chloride — Fisons,
2.7 mM Potassium chloride — Koch-light Ltd.,
1.5 mM Potassium dihydrogen phosphate — BDH,
8.1 mM disodium hydrogen orthophosphate — BDH.

Sodium azide (Sigma) was added to a final concentration of 0.02% (w / v).

Or,

TEA buffer : see section 2.3.2.

Nonidet P40 (Sigma) : 1% in PBS containing 1mM EGTA,
or 1% in TEA buffer

PMSF (Sigma) : 0.1M solution in acetone

Procedure

Adult worms were washed in cold PBS, or TEA buffer 3 times by resuspending and allowing them to sediment to the bottom of the homogenizer tube. PBS, or TEA buffer and 1% Nonidet P40 (both cold) were added to a final concentration of 0.5% Nonidet P40. 1µl of PMSF in acetone was also added and the worms homogenized for 2 minutes. The homogenate was incubated on ice for 30 minutes after which it was centrifuged for 10 minutes in a Beckman Eppendorf bench centrifuge on 'high' at 4^{0}C. The supernatants and the pellets were retained at -20^{0}C to be analyzed later.

2.5 Immunoprecipitation

2.5.1 Materials

Adult worms (numbers differed according to experiment), ^{35}S-methionine-labelled, and solubilized in 0.5% Nonidet P40.

Fixed and heat killed *Staphylococcus aureus* bacteria, Cowan I strain (Sigma),10% suspension, washed and resuspended in Nonidet P40 solution,

Or,

Protein A-Sepharose (Sigma), swelled, washed, and made up to 20% suspension in Nonidet P40 solution.

10% (w / v) bovine serum albumin

0.5% (w / v) Nonidet P40 in 10 mM Tris-HCl pH 7.3 containing 0.5 M NaCl.

0.05% SDS in Nonidet P40 buffer

10 mM Tris-HCl pH 7.3.

PBS

2.5.2 Antisera

Rabbit anti-MS2-HSP70 antiserum : This antiserum was a very generous gift from Dr. Mo-Quen Klinkert, Centre of Molecular Biology, University of Heidelberg, and it was used throughout the project to study HSP70. The antiserum was raised in this way :

The *Schistosoma mansoni* cDNA library was screened with a pool of human infection sera. A clone was selected and lysogenized into *Escherichia coli* Y1089 and induced for the synthesis of β-galactosidase fusion protein. From the human serum pool, antibodies were affinity purified using the β - galactosidase fusion protein immobilized on nitrocellulose filter. Antibodies which were eluted from nitrocellulose-bound proteins recognized a 70 kDa heat-shock protein present in *Schistosoma mansoni* adult worms by Western blotting. The cDNA was sequenced and shown to have extensive homology

with HSP70 proteins from a variety of organisms.

The cDNA insert was subcloned into a plasmid expression vector pEx34b. The construct gave rise to a fusion protein which was solubilized by subsequent extraction with Triton X-100, 1 M and 7 M urea, and further purified by preparative gel electrophoresis and electroelution. Antibodies to this gel-purified recombinant protein were raised in rabbits and were found to recognize a 70 kDa heat shock protein in *Schistosoma mansoni* extracts.

Pre-immune rabbit serum, or, irrelevant monoclonal antibody.

Rabbit anti-partially purified M_r 32 kDa glycoprotein from adult worm membranes : Payares *et al* (1985).

Monoclonal anti-72 kDa heat shock protein : Amersham

Monoclonals anti-32-38 kDa antigens from schistosomula : Mill Hill, London.

Rabbit anti-(mouse immunoglobulin)

2.5.3 Procedure

Method adapted from Johnsone & Thorpe (1987).

Immunoprecipitation using antibodies which bind Protein A

1/10 of a volume of the washed bacterial or sepharose suspension was added to the adult worm extract (in Eppendorf tubes if bacteria was used, or in 13.5 ml polypropylene centrifuge tubes if Protein A-Sepharose was used) and mixed gently on the rotator for 30 minutes at 4^0 C. The samples were centrifuged for 4 minutes in a Beckman, Eppendorf bench centrifuge on 'high' at 4^0C, or in a bench top centrifuge at 3500 rpm. The supernatants were transferred to Eppendorf or 13.5 ml polypropylene centrifuge tubes and Nonidet P40 solution was added to a final volume of 950 µl. 10 µl of albumin solution were also added to each sample. The pellets were discarded.

5µl of the two rabbit sera were added to separate samples and they were mixed and incubated at room temperature for 30 minutes.

50 µl of the washed bacterial or sepharose suspension were added to each sample and mixed on the rotator for 1 hour at 4^0 C.

The samples were centrifuged for 2 minutes as above. The supernatants were discarded and each pellet was washed by resuspending and vortexing in 1 ml of the Nonidet P40 solution, SDS solution, and 10 mM Tris buffer, respectively, and centrifugation as above.

The pellets were analyzed by resuspending in 30 µl sample buffer, with or without β-mercaptoethanol, according to experiment, boiled for 5 minutes and applied to SDS polyacrylamide gels followed by fluorography.

Immunoprecipitation using antibodies which do not bind Protein A

1 ml of the 10% suspension of Protein A-Sepharose was centrifuged for 2 minutes in a bench centrifuge at 4^0C. The supernatant was discarded and the pellet was resuspended in 1 ml PBS. 100 µl of rabbit anti-(mouse immunoglobulin) were added and mixed on the rotator for 1 hour at 4^0C.

The suspension was centrifuged as above and washed twice with PBS. The final pellet was resuspended in 1 ml PBS, 10 µl of albumin solution were added, and the preparation was kept on ice.

2 µl of the irrelevant monoclonal antibody were added to the adult worm extract in a polypropylene tube and incubated for 30 minutes at 4^0C.

100µl of the antibody-Protein A suspension were added to the extract and the incubation was continued for a further 30 minutes at 4^0C.

The extract was centriufged for 1 minute as above and the supernatant was transferred to a fresh tube. A second 100 µl of antibody-Protein A were added and the mixture was incubated and centrifuged as above.

300 µl of the supernatant were transferred to separate polypropylene tubes and 5 µl of the albumin solution were added to each tube. The pellet was discarded.

1 µl of the monoclonal antisera were added to separate samples,

mixed and incubated on ice for 1 hour.

50 µl of antibody-Protein A were added to each sample. The samples were mixed on the rotator for 2 hours at 4^0C.

The samples were centrifuged, washed and analyzed as for the rabbit sera.

2.6 Pulse - Chase experiments

2.6.1 *in vitro*

Freshly perfused adult worms were washed with GMEM w / o methionine , divided up into groups of 10 worms , and then resuspended in 2 ml GMEM w / o methionine to which ^{35} S-methionine was added to a final concentration of 23 µCi / ml. The parasites were labelled for 30 minutes at 37^0C / 5 % CO_2 , after which the labelled medium was taken off. Parasites were washed and resuspended in GMEM and incubated at 37^0C / 5% CO_2. Samples were removed to ice at 0 , 30 minutes , 1, 2 , 3 , 4, 6, 21 and 25 hours. The worms were washed with ice-cold PBS and prepared for immunoprecipitation.

2.6.2 *in vivo*

8 week-infected mice were injected subcutaneously with 100µCi , 500µCi, or , 1mCi of ^{35}S - methionine in a total volume of 2 ml GMEM. Adult worms were obtained by perfusion of the hepatic portal vein 2 , 4 , 6 , 8 , 21 and 24 hours after injection of the label. The worms were washed quickly in GMEM , removed to ice , and prepared for SDS-PAGE.

A piece of liver was taken from each mouse and hand homogenised in a total of 1ml. The homogenate supernatant was then used for TCA-precipitation and SDS-PAGE analysis.

1ml of blood was also withdrawn from the heart of each mouse before perfusing, allowed to clot at room temperature, and then kept overnight in the

fridge. Blood was then centrifuged at 6500 rpm in a Beckman, Eppendorf bench centrifuge at 4^{0}C for 3 minutes. The serum was collected, spun again as above for 5 minutes, and the supernatants were analyzed for, incorporation of ^{35}S-methionine into serum proteins by TCA-precipitation, serum protein concentration, and then they were analyzed by SDS-PAGE.

2.7 Separation of proteins : SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Both 16 cm slab gels and mini-gels were used.

A discontinuous buffer system was used, based on the method described by Laemmli (1970).

2.7.1 Gel preparation

Materials

Acrylamide (Electrophoresis grade)	From Fisons plc.
N,N'-methylene bisacrylamide (Electrophoresis grade)	
Sodium dodecyl sulphate (SDS)	
Ammonium persulphate	From BDH chemicals
N,N,N',N'-tetramethyl-1,2-diaminoethane (TEMED)	From Sigma
Tris	From Boehringer Mannheim Gmbh.

Stock solutions:

Solution A : 30% (w / v) Acrylamide

0.8% (w / v) N,N-methylene bisacrylamide.

Solution B : 3 M Tris-HCl buffer, pH 8.8.

Solution C : 0.5 M Tris-HCl buffer, pH 6.8.

Solution D : 10% (w / v) SDS.

Solution E : 1.5%, or 10% (w / v) Ammonium persulphate (made up fresh for each gel)

Gel composition

10% and 7.5% (w / v) acrylamide resolving gels were used. A 3% (w/v) acrylamide stacking gel was used with both. Table 1.3 gives the composition of the different gels.

Table 2.3 Composition of SDS-polyacrylamide gels.

Solution	Slab gels		Mini gels		
	R 10%	S 3%	R 7.5%	R 10%	S3%
A	13.3 ml	2.5 ml	1.56 ml	2.1ml	0.25 ml
B	5.0	-	0.775 ml	0.775 ml	-
C	-	5.0 ml	-	-	0.69 ml
D	0.4	0.2	62.5µl	62.5µl	25 µl
E	2.0	1.0	12.5µl	12.5µl	10 µl
distilled water	19.3	11.3	3.85	3.32	1.5 ml
Mix gently ; then add					
TEMED	50µl	15µl	10 ml	10 ml	10 ml

Preparing the gels

1. <u>16 cm slab gels</u>

<u>Materials</u>

2 glass plates for electrophoresis tank model V16 (Bethesda Research Labs.), small plate : 7.8" x 6.3" ; large plate 7.8" x 7.5".

Spacer set of 3, 1.5 mm width

20-well comb, 1.5 mm width

These materials were all obtained from Bethesda Research Labs.

The glass plates were cleaned with laboratory detergent, rinsed thoroughly with distilled water and then with ethanol. The three spacers were assembled on the larger glass plate, and the smaller glass plate placed over the spacers, with the neoprene blocks fitting closely against the edge of the small plate. The plate assembly was clamped together with strong metal clips positioned to press on the outer sides of the plates just over the spacer positions. The assembly was checked for leakage using a small volume of water.

The resolving gel mixture was poured into the clamped plate assembly as soon as the TEMED had been added, avoiding production of air bubbles during pouring. A space of about 3.5 cm was left above the resolving gel. The top of the resolving gel was gently overlaid with 70% (v / v) ethanol to give a smooth surface. After polymerization (20 to 30 minutes), the ethanol overlay was poured off, and the surface of the resolving gel was rinsed with stacking gel buffer.

The stacking gel was poured into the remaining space and the comb was carefully inserted. The assembly was left undisturbed while the stacking gel polymerized (up to 1 hour).

After polymerization, the comb was carefully removed to expose the sample wells. The wells were filled with electrophoresis buffer (see below) until the samples were ready for application.

2. Mini gels:

The Mini-PROTEAN II dual slab cell (Bio-Rad) allows rapid analysis of protein samples in miniature polyacrylamide gels. The cell allows analysis to be completed two to three times faster than is possible with conventional 16 cm plates, while maintaining comparable resolution.

Materials

Mini-PROTEAN II slab cell, lower buffer chamber, and lid

Casting stand

Inner cooling core : houses both the upper and lower electrodes and the connecting jacks

Sandwich clamp assemblies

Glass plates, one short (inner) plate and one long (outer) plate

0.75 mm thick spacers

0.75 mm thick combs

The longer glass plate was laid down first, then the two spacers were placed along the short edges of the plate. The shorter glass plate was placed on top of the spacers so that the bottom end of the spacers and the glass plates were aligned.

The glass plate sandwich was gently slid into the clamp assembly and the four screws on it were tightened. The clamp assembly was transferred to one of the casting slots in the casting stand. The gels were casted as before except that very little amounts of the stock solutions were used as shown in Table 2.3.

2.7.2 Sample preparation

2.7.2.1 Protein estimation

Protein estimation was carried out by the method of Winterborne (1986). It is a dye-binding assay that measures the amount of dye that binds to protein dried onto filter paper.

Materials

Standard protein ; BSA from Sigma

0.4g Coomassie Brilliant Blue R (Sigma) dissolved in 250 ml ethanol and 630 ml distilled water

Glacial acetic acid from Fisons

Destain solution : 10% (v/v) ethanol, 5% (v/v) acetic acid.

Desorbing solution : 1M potassium acetate in 70% (v/v) ethanol.

Whatman 3MM paper

Procedure

1. Make a 1 mg / ml solution of BSA and calculate the exact concentration from its absorbance at 280 nm.
2. Mark a grid of 1 cm squares in pencil on the paper.
3. In separate squares spot 1,2,5, and 8 µl of the standard and up to 8 µl of the test protein solutions. Allow to dry.
4. Mix 6 ml of acetic acid with 44 ml of Coomassie solution and filter. immerse the paper in the mixture and agitate gently for 1 hour.
5. Transfer the paper to destain solution, agitate gently for a few minutes, discard the liquid and repeat until a clear background is obtained.
6. Dry the paper at room temperature or in an oven.
7. Cut out the squares (including one with no protein) and place in separate test tubes. Add 1 ml of desorbing solution to each, mix, leave for 1 hour and read the absorbance of the liquid at 590 nm.
8. Plot the absorbance against protein content for the standard and from this read off the amount of protein in the test solution.

2.7.2.2 Sample preparation

Sample buffer

0.1M Tris-HCl, pH 6.8

2% (w / v) SDS	from Fisons
10% (w / v) sucrose	from Koch-Light Labs
0.001% (w / v) bromophenol blue	from BDH chemicals

β–mercaptoethanol was added to sample buffer at a final concentration of 5% (v / v) if the samples were to be reduced.

Protein samples were dissolved in sample buffer, and placed in a boiling water bath for 10 minutes. After cooling to room temperature, samples were ready for electrophoresis.

2.7.3 Electrophoresis

Electrophoresis tank: model v-16 from Bethesda Research Labs.

Electrophoresis buffer : 0.025 M Tris,
0.192 M glycine (BDH chemicals),
0.1% (w / v) SDS.
pH was 8.3.

Electrophoresis was carried out at 45 mA, constant current, for approximately 3 hours, or overnight at 4 mA per gel.

For mini-gels, electrophoresis was carried out at 200 V, constant voltage, for approximately 45 minutes.

2.7.4 Fixing and staining the gels

Gels were fixed and stained for 1 hour in 0.1% (w / v) Kenacid blue (Coomassie blue) stain (Sigma) in 50%(v / v) methanol, 5%(v / v) acetic acid. Destain: 25% (v / v) methanol, 10% (v / v) acetic acid. Gels were destained until a clear background was obtained.

2.7.5 Molecular weight calibration of SDS-PAGE

Molecular weight estimation of polypeptides resolved by SDS-PAGE were determined by calibrating gels with a set of molecular weight standards (Pharmacia). These were :

Molecular weight standard	Molecular weight
Phosphorylase b	94000
Albumin	67000
Ovalbumin	43000
Carbonic anhydrase	30000
Trypsin inhibitor	20100
α-Lactalbumin	14400

Rainbow™ protein molecular weight markers (Amersham) : contained the same cocktail of proteins as above plus :

Myosin	200000

This is a mixture of individually coloured and purified proteins, combined to give bands of approximately equal intensity when electrophoresed on a polyacrylamide gel.

This mixture was used because when transferred to a nitrocellulose paper, the markers were coloured and so staining with amido black was not required.

2.8 Identification of radiolabelled proteins: Fluorography.

Method adapted from Wales (1989).

Materials

Sodium salicylate	Sigma
X-Omat X-ray film (18 cm x 24 cm)	Technical Photo systems
Film cassettes (18 cm x 24 cm) with intensifying screens	Sky plastics Ltd.
Fixer and Developer	Kodak

Method

Stained and destained gels were washed with several changes of distilled water to remove acetic acid. Each gel was immersed in 1M sodium salicylate, covered, and stored in the dark at room temperature for 30 minutes. After this incubation the gel was dried onto Whatman 3MM filter paper under vacuum. The gel was removed to a dark room and placed in contact with **X**-ray film in a cassette backed by an intensifying screen. The film was exposed at -70^0 C until the bands developed.

2.9 Indirect immunofluorescence studies

2.9.1 Antisera

Rabbit anti-schistosome recombinant HSP70

Pre-immune rabbit serum

These antisera were a kind gift from Dr. Mo-Quen Klinkert.

FITC conjugated donkey anti rabbit IgG SAPU

2.9.2 Different treatments of adult worms to reveal the location of HSP70.

2.9.2.1 Intact adult worms

After fluorescent labelling (section 2.9.3) and washing were complete, adult worms were immobilized with carbamyl choline ("Carbachol" ; obtained from Sigma), at a concentration of 1mg / ml made up in GMEM.

2.9.2.2 Fixation with formaldehyde

Formaldehyde fixation was carried out before labelling began. Adult worms were resuspended in 1ml GMEM. Formaldehyde was added to give a final concentration of 0.1%. Adult worms were incubated in the presence of formaldehyde for 15 minutes at 37^0C / 5% CO_2. They were then washed 3 times with cold GMEM containing 0.1% (w / v) BSA, and resuspended in the antibody solution.

2.9.2.3 Treatment with Triton X-100

Adult worms resuspended in 0.1% (w / v) Triton X-100 (Sigma) made up in GMEM, and incubated for 15 minutes at 37^0C / 5% CO_2. They were then washed and treated as above.

2.9.2.4 Separation of the schistosome bodies from the surfaces by freeze-thawing

Adult worms resuspended in 1ml GMEM or PBS were frozen on solid CO_2 and methanol, thawed, and vortexed for 3 times. The bodies of the worms were allowed to sediment and the supernatant was retained. The bodies were then prepared for either, fluorescent labelling, or, SDS-PAGE. The supernatants containing the membranes were spun at 'high' in Beckman, Eppendorf bench centrifuge for 10 minutes and the membrane pellet was resuspended in sample buffer containing β-mercaptoethanol, boiled, and analyzed on SDS-gels.

2.9.2.5 Preparation of frozen sections of the adult worms with the cryostat

Materials

Cork sheets, cut to circles of 15 mm in diameter to fit inside the cryostat machine.

Tissue Tek II O.C.T. compound : embedding medium for frozen tissue speciments (from Lab-Tek products).

Egg albumin coated slides.

Cryostat sectioning machine : Temperature inside -25^{0}C.

Method

Adult worms were washed and resuspended in GMEM. Each worm was transferred with a 21G1 1/2 needle to a cork circle overlaid with embedding medium. The piece of cork containing the worm was lowered in a flask containing liquid nitrogen by using forceps, and hence allowing the medium and the worm to freeze. The cork circle was then held in place vertically in the cryostat sectioning machine by means of the embedding compound. The required width of the section was adjusted to 12 µm, and the

worm was cut into thin sections using a very fine blade. The sections were transferred to a slide coated with egg albumin and prepared for fluorescent labelling.

2.9.3 Immunofluorescence

Adult worms were resuspended in 500 µl GMEM containing 0.1% (w/v) BSA. Antiserum or pre-immune serum was added to a final dilution of 1/20 and the worms were kept for 30 minutes at room temperature. After that, the medium was aspirated and frozen to be used again, and the worms were washed 3 times with cold GMEM containing 0.1% BSA and resuspended in 500 µl of the same medium. FITC- conjugated anti-rabbit IgG was added to a final dilution of 1/10 and the worms were kept at room temperature in the dark for 30 minutes. After the 30 minute incubation, the worms were again washed 3 times with cold GMEM containing 0.1% BSA and resuspended in the same medium. 1mg / ml Carbachol was added when required, and the worms were mounted on microscope slides under coverslips supported on small ridges of silicone grease.

For adult worm sections and cytocentrifuged schistosomula, slides were washed in PBS. The sections were either fixed in 100% methanol for 10 minutes at -20^{0}C, or left unfixed. Slides were air dried and then rehydrated in PBS. Non-specific binding was blocked by treating with new born calf serum (NBCS) for 20 minutes at room temperature. Excess serum was drained and slides were incubated with antiserum or pre-immune serum at a dilution of 1/20 in PBS, containing 1% (w/v) BSA, and 0.1% (w/v) sodium azide, for 1 hour at room temperature. The slides were rinsed with several changes of PBS. They were then incubated with FITC-conjugated anti-rabbit IgG, diluted 1/10 in PBS for 1 hour at room temperature. Slides were again rinsed with several changes of PBS and were mounted in Citfluor (glycerol based mountant).The slides were viewed with a Leitz Ortholux microscope, and the

intensity of fluorescence quantitated using a Leitz MPV compact attachment. Filter block N2 of the Leitz pleompak filter system was used to measure FITC fluorescence. The average background reading from each slide was subtracted from the readings taken from the worms.

2.9.4 Statistical analysis of fluorescence results

Students two-sample t-test and the Mann Whitney U-test were used to compare the mean fluorescence measurements, and the median values of different groups, respectively, obtained for pre-immune and anti-HSP70 treated worms.

In all cases, both tests gave the same significance levels.

2.10 Solubilization of membrane proteins

Materials

10 mM Tris-HCl pH 7.3

0.5% Triton X-100 (Sigma) in 50 mM Tris-HCl pH 8.0 to which sodium vanadate and 0.2 µl of the protease inhibitors PMSF and TPCK (Sigma) were added.

Adult worm membranes obtained by the method of freeze-thawing as described in section 2.9.2.4.

Method

Membranes were collected and washed in 10 mM Tris-HCl pH 7.3. They were solubilized in 20 µl 0.5% Triton X-100 buffer on ice for 30 minutes. After that they were centrifuged in a Beckman Eppendorf bench centrifuge on 'high' for 20 minutes at 4^{0}C. The supernatant was retained and the pellet was washed with Triton X-100 buffer. The pellet and the supernatant were boiled in SDS-sample buffer containing β-mercaptoethanol and analyzed by immunoblotting.

2.11 Detergent phase separation of membrane proteins

Method adapted from Bordier (1981).

2.11.1 Pre-condensation of Triton X-114

Materials

Triton X-114 (Sigma)

PBS

Method

10 ml of neat Triton X-114 were added to 500 ml of ice-cold PBS in a glass beaker and mixed until homogeneous. The beaker was then left at 37^{0}C for 16 hours. The detergent-depleted upper layer was discarded and the detergent-rich lower phase resuspended in 500 ml of ice-cold PBS. The mixture was left for another 16 hours at 37^{0}C. The detergent-depleted upper layer was again discarded and the whole process was repeated a further time. After the final pre-condensation the upper phase was discarded and the lower phase was placed into a glass bottle and stored at 4^{0}C until required.

2.11.2 Determination of the concentration of pre-condensed Triton X-114.

The absorbance of 1/100 dilution of pre-condensed Triton X-114 at 275 nm was determined. Since Triton X-114 was made up in PBS, the absorbance of PBS at 275 nm was also determined and subtracted from that of Triton X-114. By knowing the absorbance of 1% (w / v) Triton X-114 is approximately 28, the exact concentration of the pre-condensed Triton X-114 was calculated.

2.11.3 Separation of hydrophobic, hydrophilic and detergent-insoluble fractions of membrane proteins.

Materials

Adult worm membranes obtained by the method of freeze-thawing as

described in section 2.9.2.4.

Pre-condensed Triton X-114

PBS

Protease inhibitors : PMSF and TLCK (Sigma)

6% (w / v) Sucrose made up in PBS

Method

The membranes were washed with PBS and resuspended in 50 µl of 0.9% (w / v) cold, pre-condensed Triton X-114 to which 0.5 µl of PMSF and TLCK were added. The membranes were kept on ice for 1 hour with vortexing often. They were then centrifuged in a Beckman bench centrifuge on 'high' at 4^{0}C for 10 minutes. The pellet was washed with 0.9% Triton X-114 and resuspended in 20 µl reducing sample buffer for SDS-PAGE and boiled for 5 minutes. The supernatant was treated in the following way :

1. The supernatant was overlaid on 75 µl 6% sucrose in PBS and kept at 37^{0}C for 5 minutes and then centrifuged for 3 minutes in a Beckman Eppendorf bench centrifuge on 'low' at room temperature.
2. The top aqueous layer was taken off and 50 µl of 0.9% cold Triton X-114 were added to it. This was then overlaid on top of the previous sucrose cushion, incubated at 37^{0}C for 5 minutes and then centrifuged as above.
3. The top aqueous layer was taken off, 100 µl of 2% Triton X-114 were added and it was kept at 37^{0}C for 5 minutes. The detergent phase was retained.
4. After the incubation, the mixture was centrifuged as above and the top aqueous layer was freeze-dried to reduce its volume. The detergent phase was discarded.
5. 20 µl of reducing sample buffer were added to the aqueous and detergent phase (step 3) and they were boiled for 5 minutes.
6. The boiled samples were electrophoresed on a 10% (w / v) SDS-polyacrylamide gel, transferred to nitrocellulose and probed with anti-HSP70 antiserum.

2.12 Collection of proteins released by the adult worms

Materials

Econo-Pac 10 DG columns Bio-Rad

Protease inhibitors

PMSF and TLCK (both obtained from Sigma) made up as a 0.1M solutions in acetone (Analar grade ; May & Baker Ltd.).

Procedure

At the end of the culture period, the worms were transferred to ice, and the media containing proteins released by the worms during culture, were transferred to fresh 5 ml tubes. 1µl of each of the protease inhibitor solutions was added per ml of medium, and the medium was desalted on 10 DG columns.

Desalting by gel filtration on 10 DG columns

A 10 DG column (bed volume 10 ml) was washed with 20 ml distilled water. The culture supernatant, with added protease inhibitors, was added to the column. When the sample had run into the column,4 ml distilled water were used to elute the proteins into 13.5 ml centrifuge tubes. 1µl of each of the protease inhibitors was added per ml of eluant. After freezing in a bath of solid CO_2 and methanol, the eluants were freeze-dried overnight. The freeze-dried material was then analyzed by immunoblotting.

2.13 Immunoblotting

Immunoblotting with anti-HSP70 antiserum was performed using iodinated protein A, or, enhanced chemiluminescent method.

2.13.1 Immunoblotting with ^{125}I-protein A

Method adapted from Johnson & Thorpe (1987).

Materials

^{125}I-protein A : a kind gift from Dr. Lindsay, Biochemistry Department, University of Glasgow.

Samples and protein standards electrophoresed on polyacrylamide gels.

Electroblot tank, cassette, and Scotchbrite pads for blotting - obtained from Bio-Rad Labs Ltd.

Nitrocellulose sheets, 0.45 µm pre size - from Anderman & Co. Ltd.

3MM filter paper - Whatman.

Solutions

Transfer buffer : 25 mM Tris,
192 mM glycine,
20% (v / v) methanol.

Blocking buffer : Hb-PBS : PBS containing 3% (w / v) bovine haemoglobin (Sigma).

Amido black stain : 0.5% (w / v) amido black in 5% (v / v) acetic acid,
50% (v / v) methanol.

Amido black destain : 5% (v / v) acetic acid,
50% (v / v) methanol.

Method

Two pieces of filter paper and 1 piece of nitrocellulose were cut to the size of immunoblotting cassette. Scotchbrite pads, filter paper and nitrocellulose were all soaked thoroughly in transfer buffer. A Scotchbrite pad was placed on one half of the opened cassette, and a piece of filter paper laid on top. The polyacrylamide gel, removed from its glass plates immediately after electrophoresis, was placed on top of this first sheet of filter paper, and covered with a nitrocellulose sheet, expelling any air bubbles. The second piece of filter paper was laid on top of the nitrocellulose, and backed by a second Scotchbrite pad. The cassette was closed and secured firmly.

The immunoblotting tank was filled with cold transfer buffer, and the whole cassette slotted into place, with the gel facing the cathode, and the nitrocellulose facing the anode. Electrophoresis was carried for 4 hours at 400 mA constant current, or overnight at 40 mA.

The gel and the nitrocellulose sheet were then removed from the cassette. A strip of nitrocellulose to which protein standards should have transferred was cut off, immersed for 3 to 5 minutes in amido black stain, then destained. The rest of the nitrocellulose sheet was incubated on a gel shaker for 30 minutes at room temperature in blocking buffer. This buffer was then discarded, and the nitrocellulose cut into 0.5 cm wide strips. Each strip was placed in a slot in a multi-slotted plastic plate and covered with 2.5 ml Hb-PBS containing anti-HSP70 or pre-immune serum at a dilution of 1/250. The nitrocellulose strips were incubated in this solution for 2 hours at room temperature, with shaking, or, overnight at 4^0C. This was followed by 6 to 7 washes in Hb-PBS. The nitrocellulose strips were then incubated for 2 hours at room temperature, with shaking, in Hb-PBS containing 2×10^6 cpm of ^{125}I-protein A. Finally, the strips were washed 8 times in PBS and then allowed to dry at room temperature between 2 sheets of filter paper. Exposure to X-ray film was done as described in section 2.8.

2.13.2 Immunoblotting using enhanced chemiluminescent detection of proteins

Materials

Mini Trans-Blot Electrophoretic transfer cell, containing Electrode module, fibre pads, 2 gel holder cassettes, buffer chamber and lid, and cooling unit (Bio-Rad).

Horse radish peroxidase-conjugated donkey anti rabbit IgG (SAPU).

Samples and rainbow protein markers (Amersham), electrophoresed on SDS-polyacrylamide gels.

Nitrocellulose paper : Immun-Lite™ Blotting membrane.

3MM filter paper - Whatman.

Solutions

Transfer buffer : as before in section 2.13.1.

Blocking buffer : 5% marvel (Premier Brands UK Ltd.) in PBS Tween.

PBS tween : 7.5 mM disodium hydrogen orthophosphate (BDH),
3.25 mM sodium dihydrogen phosphate,
145 mM sodium chloride (Fisons),
0.05% (v/v) Tween 20 (Sigma).

0.9% Saline : 72 grams of NaCl in 8 litres of distilled water.

Substrate solution :

8 mg of 5-amino-2,3-dihydro-1,4-phthalazinedione(3-aminophthalhydrazide; Luminol; obtained from Sigma) were dissolved in 1ml 1M Tris base. To this 0.5 ml 1M Tris-HCl were added. The solution was made up to 20 ml with 150 mM NaCl. 200 µl of 10 mg / ml 4-iodophenol (Aldrich chemical Ltd.) made up in DMSO were added. Finally 6.5 µl of 100 volumes hydrogen peroxide (Fisons Analytical Reagents) were added.

Procedure

The tank set up was as in section 2.13.1 except that a cooling unit filled with frozen distilled water was installed in the buffer chamber, next to the electrode, a few minutes before starting the transfer.

Electrophoresis was carried out for 1 hour at 250 mA, 100 V, constant voltage.

The gel and the nitrocellulose paper were removed from the cassette. The nitrocellulose paper was blocked with blocking buffer for 30 minutes. Staining the markers with amido black stain was not necessary because the markers were coloured, and the colour showed on the paper.

After the 30 minute blocking period, the nitrocellulose paper was

probed for 1 hour with anti-HSP70 or pre-immune serum made up in blocking buffer at a dilution of 1/500. The paper was washed 5 times in PBS Tween, and then incubated for 1 hour with the horse radish peroxidase-conjugated antibody made up in the blocking buffer at a dilution of 1/250. The paper was then again washed for 10 times with PBS Tween and finally, it was rinsed briefly in 0.9% saline.

10 ml of substrate solution were mixed with the paper and it was put in a transparent plastic bag (eg. a money bag) and sealed, making sure there were no air bubbles present in the bag. The bag was then taped to an autoradiography cassette and exposed to an X-ray film. Exposure took between 1 second to 3 days depending on the amount of HSP70 present.

2.14 Resolving non-denatured protein complexes

2.14.1 Polyacrylamide gel electrophoresis under non-denaturing conditions

5, 6, 7, and 8% (w / v) acrylamide resolving gels were used. A 3% (w/v) stacking gel was used with each.

Non-denaturing PAGE was carried out as described for SDS-PAGE, except that SDS was omitted from all solutions.

2.14.1.1 Molecular weight markers

Protein marker	Molecular weight
a-Lactalbumin	14200
Carbonic Anhydrase	29000
Ovalbumin	45000
Albumin	66000 (monomer)
	132000 (dimer)
Urease	272000 (trimer)
	545000 (hexamer)

The protein markers were obtained from Sigma. Each marker was loaded individually in tracks of the different percentage gels.

2.14.1.2 Sample preparation

Sample buffer : 19.2 mM glycine,
2.5 mM Tris-HCl (pH 8.5),
1 mg / ml L-methionine

Sample preparation

Two batches of adult worms, 1 labelled overnight with ^{35}S-methionine, and 1 unlabelled were washed, and hand-homogenized in ice-cold sample buffer to give a final protein concentration of 1 mg / ml. 1µl of 0.1 M PMSF was added per ml of sample buffer.

20 µl aliquots of 20% (w / v) sucrose, and bromophenol blue, made up in sample buffer, were added to 20 µl aliquots of the homogenate to give a final sucrose concentration of 10%. Aliquots of the 2 samples were run on 5, 6, 7, and 8% gels alongside with molecular weight markers.

Electrophoresis was carried out as for SDS gels except that the electrophoresis buffer did not contain SDS and was diluted 5 times.

Each gel was cut into two; 1 part containing the labelled, and the other, the unlabelled samples. The labelled parts were stained, destained, and dried as in section 7.1.4, and the unlabelled parts were transferred to nitrocellulose papers and immunoblotted with anti-HSP70 and ^{125}I-protein A.

2.14.2 Resolving the non-denatured complexes on SDS gels

Equilibration buffer : 2% (w / v) SDS,
62.5 mM Tris-HCl pH 6.8,
0.5% (v / v) β-mercaptoethanol.

The autoradiographs obtained from blotting with anti-HSP70 were superimposed on the dried labelled gels and the bands which were

recognized by anti-HSP70 on the blots, were cut from the labelled gels. Each band was equilibrated for 15 minutes with 25 ml of the equilibration buffer, and against a fresh change of the same buffer for 30 minutes with gentle shaking.

The equilibrated gel slices were laid in slots of the stacker of a 10% polyacrylamide SDS slab gel. Molecular weight markers for SDS-PAGE were loaded alongside and the gel was electrophoresed, stained, destained, dried, and subjected to autoradiography as described in section 2.8.

2.15 Treatment with Praziquantel

Materials

Praziquantel (-) and Praziquantel (+) (from Dr. A. Harder, Bayer AG, Wuppertal, Federal republic of Germany.)

Stocks of praziquantel (-) were made up at 1mg, or 10 mg / ml in absolute ethanol and kept at -20^{0}C.

Stocks of praziquantel (+) were made up at 10 mg / ml in absolute ethanol and kept at -20^{0}C.

Adult worms were treated with varying concentrations of praziquantel ranging from 2 to 100 µg / ml of culture medium. In one experiment praziquantel (-) was present throughout the period with ^{35}S-methionine. In the rest of the experiments, the worms were treated with praziquantel for 15 minutes at 37^{0}C / 5% CO_2.

2.16 Heat shock procedure

The parasites were equilibrated for 30 minutes in methionine- free GMEM at 37^{0}C. The medium of the worms to be heat shocked was changed with : 1. GMEM preheated to 42^{0}C and the worms were incubated for 30 minutes or 2 hours at 42^{0}C / 5% CO_2. The medium was then changed with methionine-free medium preheated 42^{0}C and the worms were labelled with

^{35}S-methionine for one hour at 42^{0}C / 5% CO_2. Or, 2. Methionine -free medium preheated to 42^{0}C and labelled for 3 hours at 42^{0}C / 5% CO_2.

The medium of schistosomula to be heat shocked was changed with GMEM preheated to 42^{0}C and they were incubated for 3 hours before cytocentrifugation on poly L-lysine-coated slides at 650 rpm for 5 minutes.

2.17 Growth of HeLa cells

Materials

HeLa cells: A kind gift from Dr. R. Adams (Department of Biochemistry), University of Glasgow.

75 cm^2 tissue culture flasks

Trypsin solution (x1)

Versene

8-chamber tissue culture slides

Procedure

Cells were grown in approximately 31 ml GMEM containing 10% FCS 37^{0}C / 5%CO_2. The medium was changed every alternate day and the cells were kept till growth was satisfactory. 40 ml of Versene was mixed with 10 ml of Trypsin and 10 ml of this mixture was added to the cells. The cells were divided into 4 tissue culture flasks. These were then incubated at 37^{0}C / 5% CO_2. Medium was changed frequently till growth was satisfactory.
Cells from one flask were trypsinized as above and then counted using a haemocytometer. Cells were resuspended at 5x10^5 cell per ml of GMEM containing 10% FCS. 450 µl aliquots were placed in chambers of tissue culture slides overnight at 37^{0}C / 5% CO_2 to allow cells to stick to the slide.

2.18 Manufactures and suppliers

Amersham International Ltd., Amersham, Bucks., HP7 9LL.

Anderman and Co. Ltd., Kingston-upon-Thames, Surrey, KT26 6NH.

BDH Chemicals Ltd., Poole, Dorset, BH12 4NN.

Beckman Instruments Ltd., Glenrothes, Fife, Scotland.

Bethesda Research Labs., (Gibco/BRL), P.O.Box 35, Trident House, Renfrew road, Paisley.

Bio-Rad Labs. Ltd., Bio-Rad House, Maryland Avenue, Hemel Hempstead, Hertfordshire HP2 7TD.

Boehringer Mannheim Gmbh : Boehringer Corporation (London) Ltd., Bell Lane, Lewes, Sussex.

Eppendorf, Gerateban, Netheler and Heinz, Gmbh; U.K. Suppliers : Anderman and Co. (see above).

Fisons : F.S.A. Laboratory Suppliers, Bishop Meadow Road, Loughborough, England.

Flow Laboratories Ltd., P.O. Box 17, Irvine, Ayrshire, Scotland.

Farmachem (Research International) Ltd., 80 Kirk Street, Strathahaven, Scotland.

Gibco Ltd., Trident House, P.O. Box 35 Renfrew Road, Paisley.

Koch-Light Labs., Colnbrook, Berks.

Kodak Ltd., Station Road, Hemel Hempstead, Herts.

Leitz Instruments Ltd., 48 Park Street, Luton, Beds., LU1 3HP.

L.K.B. Instruments Ltd., 232 Addington Road, Croydon CR2 8YD.

May and Baker Ltd., Dagenham, England.

Millipore Corporation, Harrow, Middlesex, HA1 2YH.

Northumbria Biologicals Ltd., South Nelson Industrial Estate, Cramlington, Northumberland.

Pierce and Warriner (UK) Ltd., 44 Upper Northgate Street, Chester, CH1 4EF.

Pharmacia (Great Britain) Ltd., Prince Regent Road, Hounslow, Middlesex.

Scottish Antibody Production Unit (SAPU)-Law Hospital, Carluke, ML8 5ES, Lanarkshire, Scotland.

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, BH17 7NH.

Sky Plastics Ltd., Eastfield Side, Sutton in Ashfield, Notts.

Sterilin Ltd., Teddington, Middlesex, TW11 8AZ.

Technical Photo Systems : 55 Napier Road, Wardparic North, Cumbernauld, G68 OEK.

Whatman Chemical Separation Ltd., Springfield Mill, Maidstone, Kent.

Scottish Antibody Production Unit (SAPU)-Law Hospital, Carluke, ML8 5ES, Lanarkshire, Scotland.

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, BH17 7NH.

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Sterilin Ltd., Teddington, Middlesex, TW11 8AZ.

Technical Photo Systems : 55 Napier Road, Wardparic North, Cumbernauld, G68 OEK.

Whatman Chemical Separation Ltd., Springfield Mill, Maidstone, Kent.

Chapter Three

Detection of heat shock protein 70 (HSP70) in *Schistosoma mansoni*

3.1 Introduction

Heat shock protein 70 (HSP70) has been identified as one of the most abundant immunogenic molecules in many parasitic organisms. The *S. mansoni* HSP70 appears to elicit a strong and specific antibody response (Hedstrom *et al.*, 1987). This moiety has been shown to be one of the first proteins synthesized by schistosomula (Yuckenberg, Poupin and Mansour, 1987), production continuing through the adult stage (Hedstrom *et al.*, 1987).

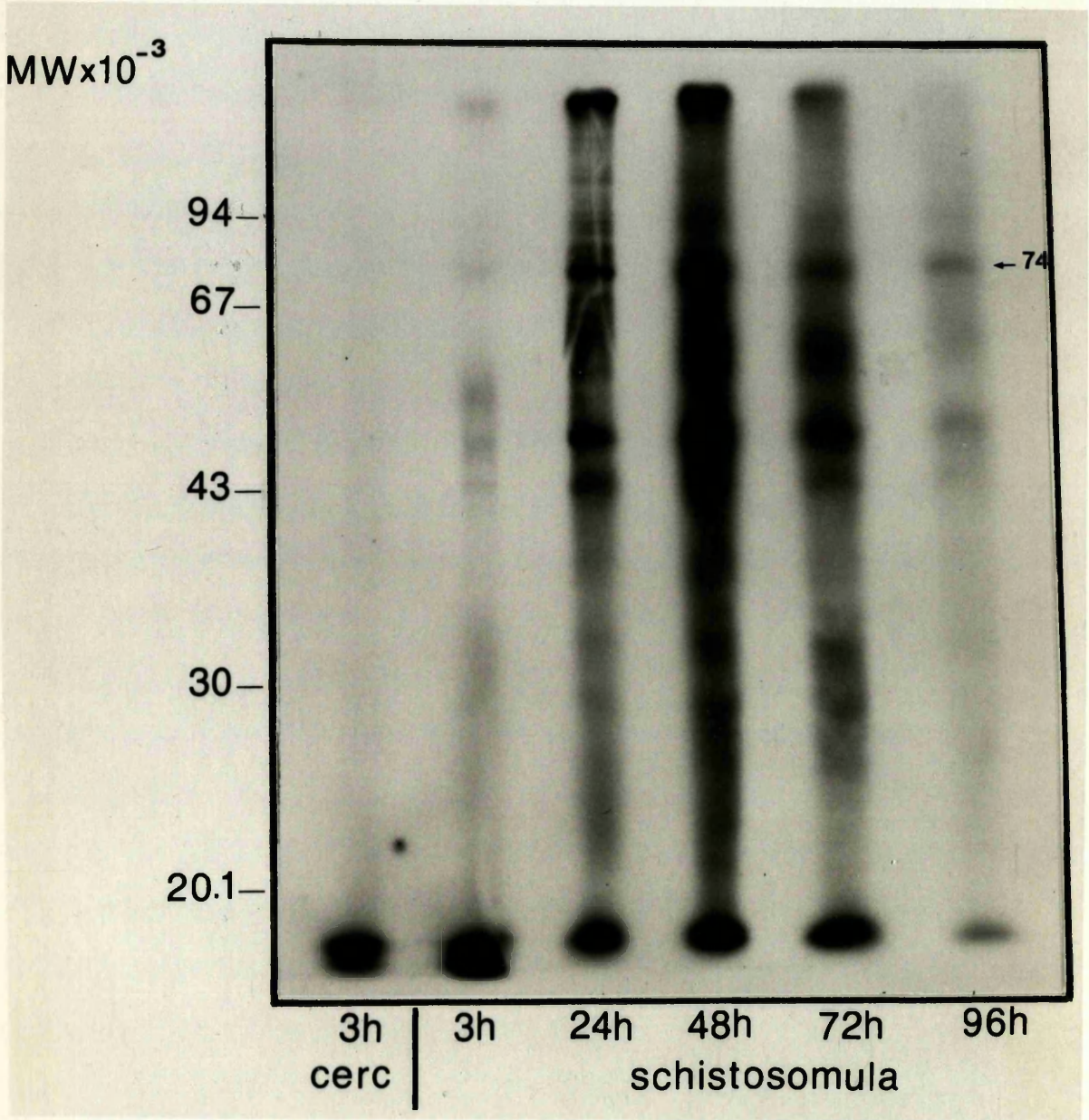
In this chapter, some experiments were performed to detect the induction or increase in the synthesis of HSP70 in different developmental stages of the parasite and then an antibody was selected to study HSP70.

3.2 Incorporation of ^{35}S-methionine into proteins of different developmental stages of *S. mansoni*

Mechanically transformed schistosomula were maintained *in vitro*. At different developmental stages, schistosomula were labelled with ^{35}S-methionine for 3 hours as described in Materials and Methods. Cercariae were also labelled in distilled water for 3 hours. Fractionating the labelled proteins on a 10% SDS-polyacrylamide gel (figure 3.1) showed that cercariae did not synthesize any proteins except for an intensely labelled material of M_r <14,000 Da (Blanton, Loula and Parker, 1987). 3 hour old schistosomula synthesized some minor bands, and a heavily labelled material of <14,000 Da. By the time the schistosomula reached 24 hour stage of development, they synthesized many proteins at an increased rate. The M_r 74 kDa protein was very prominent. At 48 hour stage of development the rate of protein synthesis increased. However, there were only few differences in the proteins synthesized by schistosomula at 48 hour and those at 24 hour stage of development. At 72 hour and 96 hour stages of development, protein synthesis decreased with the effect being very prominent at 96 hour stage of development. When the 72 and 96 hour old schistosomula were observed in culture, they appeared granular and their

Figure 3.1 ^{35}S-methionine-labelled proteins from different developmental stages of *S. mansoni* ; Fluorograph of 10% SDS-polyacrylamide gel.

Mechanically transformed schistosomula were cultured *in vitro* as described in Materials and Methods. At 3, 24, 48, 72 and 96 hours the schistosomula were taken and labelled with ^{35}S-methionine for 3 hours. Cercariae were also labelled for 3 hours in sterile distilled water to which antibiotics had been added. Labelling was with 25 μCi of ^{35}S-methionine per 1 ml of medium. Equal amounts of protein were analyzed by fractionating the proteins on a 10% (w / v) polyacrylamide gel and then fluorography.


MWx10-3
94
67
43
30
20.1
74
3h
cerc
3h
24h
48h
72h
96h
schistosomula

motility was highly reduced. Therefore, the decrease in protein synthesis was thought to have resulted from the decrease in ^{35}S-methionine uptake by the schistosomula due to culture conditions. Because of that, parasites were cultured *in vivo* by injecting the schistosomula into the abdominal cavity of mice as described in Materials and Methods. Mice were sacrificed at 3 days, 4 days, 8 days, 3 weeks and 8 weeks after injecting the cercariae to obtain the different developmental stages. Once obtained, they were labelled with ^{35}S-methionine for 3 hours in a methionine-free medium. Labelled proteins from these developmental stages were fractionated on a 10% SDS-polyacrylamide gel (figure 3.2). It is seen that protein synthesis in schistosomula cultured *in vivo* increased as the parasite developed except at the 8 week stage when it decreased, The M_r 74 kDa protein which was synthesized after transformation of cercariae to schistosomula could not be detected clearly because separation of protein bands was not very clear in this figure. To measure the rate of ^{35}S-methionine incorporation into the parasite proteins, the amount of TCA-insoluble radioactivity incorporated per 1 µg of protein from each developmental stage was plotted (figure 3.3). It is seen that the method of culture of schistosomula has different effects on the rate of protein synthesis. It seems that culturing schistosomula *in vitro* for long time periods causes decrease in protein synthesis which might result from the unfavorable conditions of culture which cause damage to schistosomula. Schistosomula cultured *in vivo*, however, show increase in protein synthesis as they develop except at 8 week stage of development when protein synthesis decreases.

3.3 Selection of an antibody to study HSP70

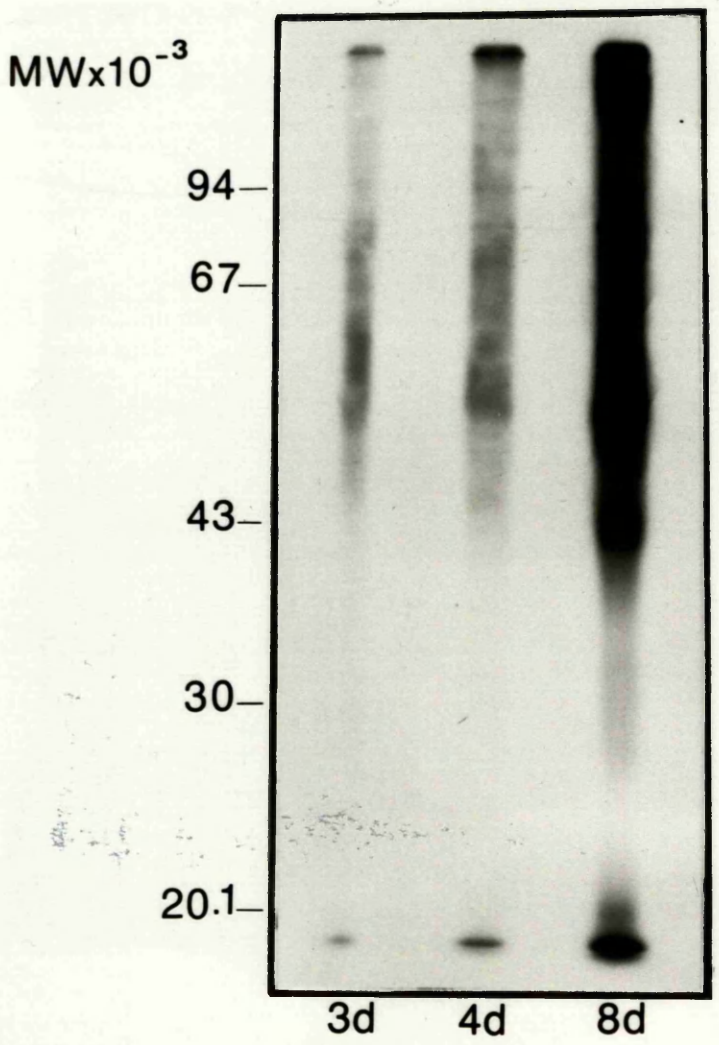
3.3.1 Monoclonal anti-72 kDa heat shock protein

The ability of a monoclonal antibody raised against a 72 kDa heat shock protein from HeLa cells to react with the HSP70 of the parasite was determined. Figure 3.4 shows an immunoprecipitation of metabolically labelled adult worm

Figure 3.2 ^{35}S-methionine-labelled proteins from *S. mansoni* cultured *in vivo* ; Fluorograph of 10% SDS-polyacrylamide gel.

Mice were injected with cercariae intraperitoneally as described in Materials and Methods. 3 days, 4 days and 8 days after injecting the cercariae, schistosomula were obtained from the mice as described in Materials and Methods. The schistosomula were washed and labelled with 100 μCi of ^{35}S-methionine / ml of methionine-free medium for 3 hours.

3 weeks and 8 weeks after injecting the cercariae, mice were perfused to obtain the worms. The worms were labelled with 50 μCi of ^{35}S-methionine / ml of methionine-free medium for 3 hours. Equal amounts of protein were analyzed on a 10% (w / v) polyacrylamide gel and then by fluorography.


MWx10^{-3}
94
67
43
30
20.1
3d
4d
8d


3W
8W

Figure 3.3 ^{35}S-methionine incorporation into proteins of different developmental stages of *S. mansoni*.

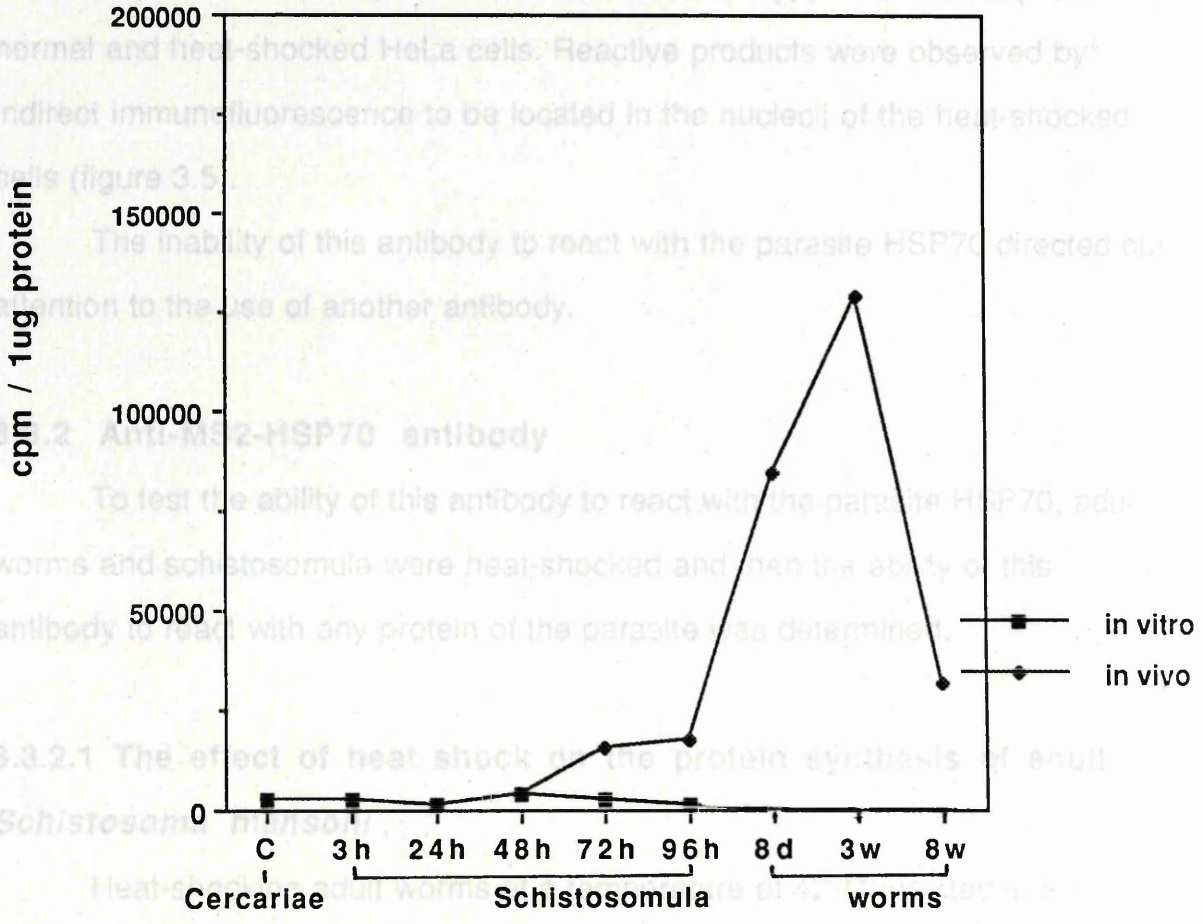
S. mansoni cultured *in vitro* and *in vivo* was labelled at different development stages with ^{35}S-methionine for 3 hours as described for figures 3.1 and 3.2. Samples were analyzed for TCA-insoluble radioactivity as described in Materials and Methods. The average values of cpm / 1 µg of protein were plotted.

C : cercariae

h : hour old schistosomula

d : day old schistosomula

w : week old worms


200000
150000
100000
50000
0
cpm / 1ug protein
in vitro
in vivo
C
3h
24h
48h
72h
96h
8d
3w
8w
Cercariae
Schistosomula
worms

proteins with this antibody. It seen that the antibody does not recognize any proteins different from those recognized by a normal mouse serum. The monoclonal antibody was also found not to recognize any proteins in the 3 day, 4 day, and 8 day old schistosomula, studied by indirect immunofluorescence.

To test the reactivity of the monoclonal antibody, it was used to probe normal and heat-shocked HeLa cells. Reactive products were observed by indirect immunofluorescence to be located in the nucleoli of the heat-shocked cells (figure 3.5).

The inability of this antibody to react with the parasite HSP70 directed our attention to the use of another antibody.

3.3.2 Anti-MS2-HSP70 antibody

To test the ability of this antibody to react with the parasite HSP70, adult worms and schistosomula were heat-shocked and then the ability of this antibody to react with any protein of the parasite was determined.

3.3.2.1 The effect of heat shock on the protein synthesis of adult *Schistosoma mansoni* .

Heat-shocking adult worms at a temperature of 42^{0}C resulted in an increase in the synthesis of two proteins of M_r 74 and 86 kDa (figure 3.6). This effect was apparent after a 30 minute heat shock for the 74 kDa protein (lane B) and after 2 hours for the 86 kDa one (lane C). The effect remained unchanged even after a 3 hour incubation at the elevated temperature (lane E). While the synthesis of these two proteins increased, that of other parasite proteins decreased (compare lanes D and E).

Figure 3.4 Immunoprecipitation of metabolically-labelled adult worm proteins with monoclonal anti-72 kDa heat shock protein from HeLa cells.

10 adult male worms were labelled with 50 μCi ^{35}S-methionine / ml in methionine-free medium for 3 hours. After that they were washed, homogenized and the proteins were immunoprecipitated with the antibody as described in Materials and Methods. The immunoprecipitates were analyzed by SDS-PAGE and then by fluorography.

NRS : normal rabbit serum

NMS : normal mouse serum

MCL : monoclonal anti-72 kDa heat shock protein from HeLa cells

HOM : worm homogenate

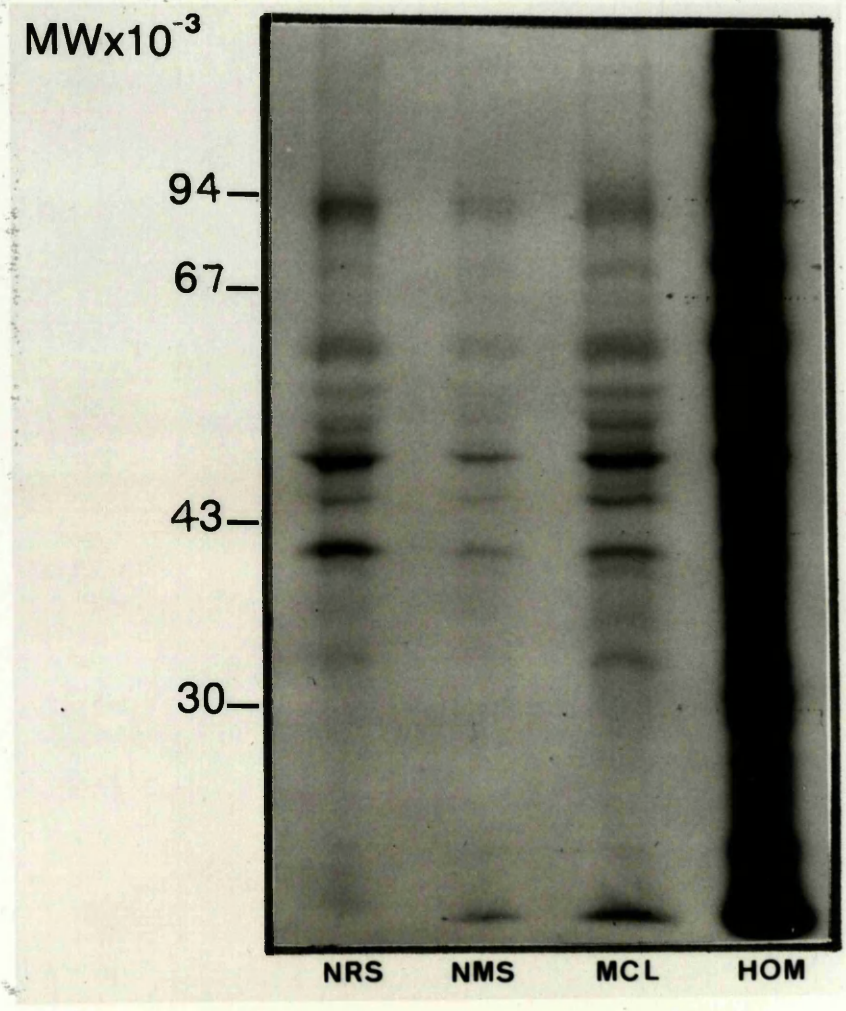

MWx10-3
94
67
43
30
NRS
NMS
MCL
HOM

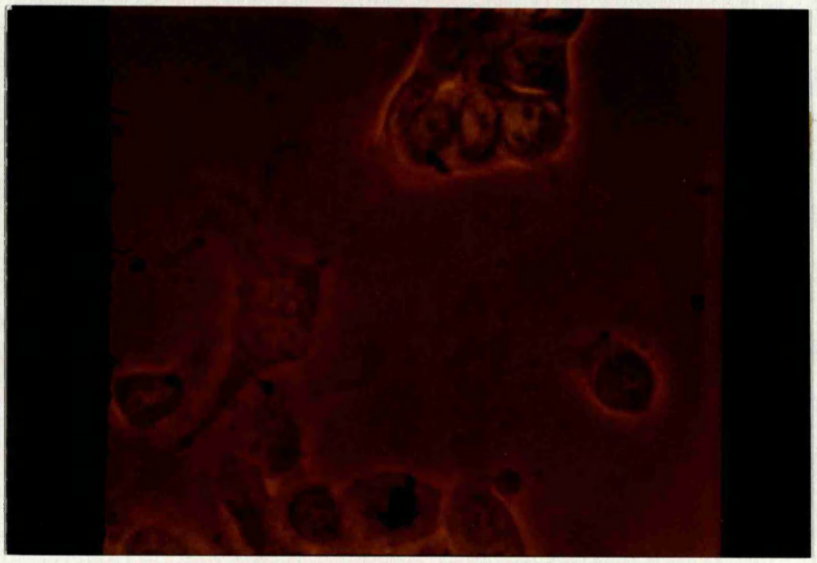
Figure 3.5 Distribution of HSP 70 in HeLa cells after heat shock, as determined by indirect immunofluorescence using monoclonal anti-72 kD heat shock protein.

HeLa cells growing on glass tissue culture slides were heat-shocked at 43^{0}C for 3 hours. After heat shock, cells were fixed and analyzed by indirect immunofluorescence as described in Materials and Methods.

a) Bright field + fluorescent micrograph of HeLa cells (x 1000) incubated with monoclonal anti- 72 kDa heat shock protein.

b) Fluorescent micrograph of HeLa cells (x 1000) incubated with monoclonal anti-72 kDa heat shock protein.

a)



b)

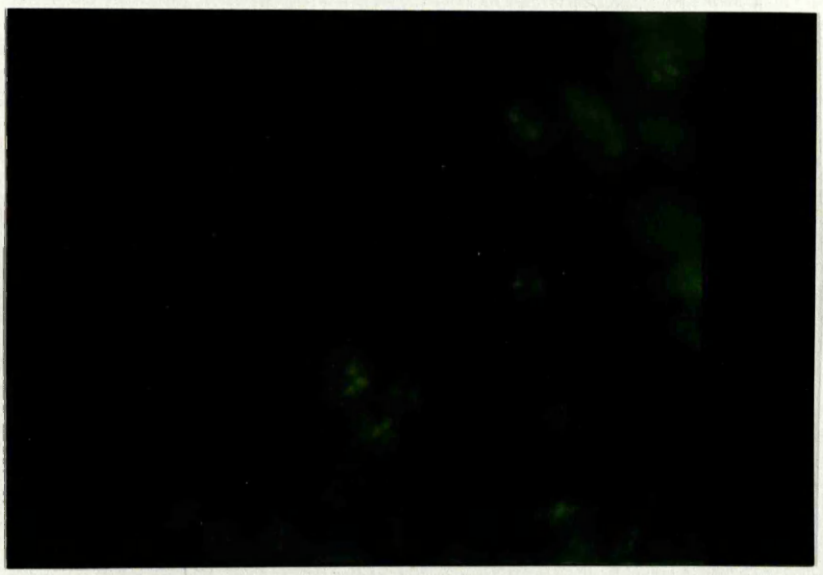


Figure 3.6 Fractionated ^{35}S-methionine-labelled proteins of *S. mansoni* adult worms heat-shocked from 37°C to 42°C ; Fluorograph of SDS-Polyacrylamide gel.

Worms were :

A : incubated at 37°C for 30 minutes and then labelled for 1 hour at 37°C / 5% CO_2.

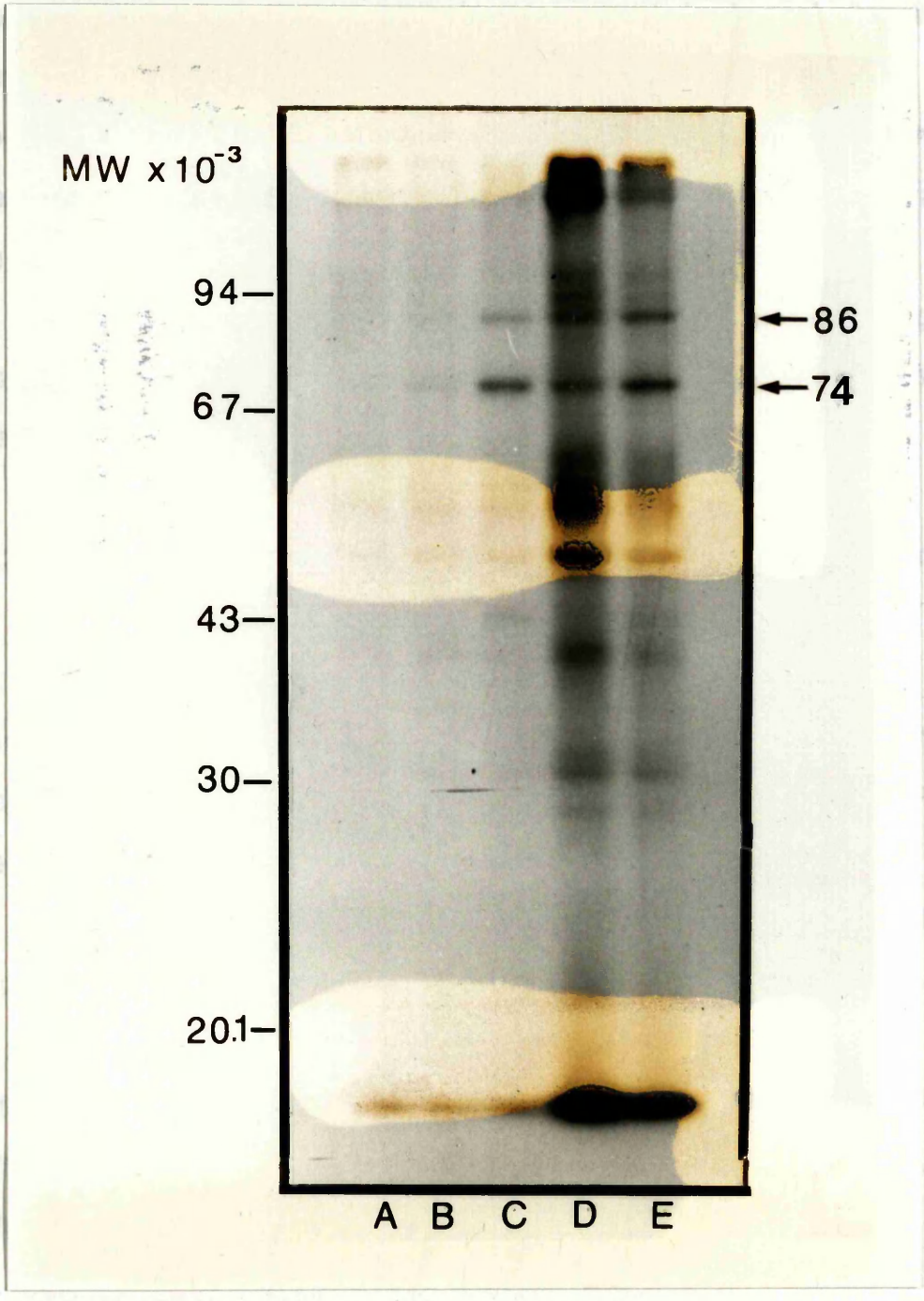
B : incubated at 42°C for 30 minutes and then labelled for 1 hour at 42°C / 5% CO_2.

C : incubated at 42°C for 2 hours and then labelled for 1 hour at 42°C / 5% CO_2.

D : labelled for 3 hours at 37°C / 5% CO_2.

E : labelled for 3 hours at 42°C / 5% CO_2.

Each group contained 18 worms (males and females) and was labelled with 66 µCi of ^{35}S-methionine in a total of 10 ml methionine-free medium. Equal amounts of protein were electrophoresed on a 10 % (w / v) SDS-polyacrylamide gel.


MW x10^{-3}
94
67
43
30
20.1
86
74
A B C D E

3.3.2.2 Immunological reactivity of anti-MS2-HSP70 to pre- and post-heat shock worms.

The result of an immunoprecipitation of metabolically labelled proteins from pre- and post-heat shock worms with this antibody is shown in figure 3.7. The antibody recognized a M_r 74 kD protein. It, also, precipitated more metabolically labelled HSP70 from the heat shocked worms than that from the normal ones. No immunoprecipitation was observed when pre-immune rabbit serum was used.

The antibody was also tested by the method of immunoblotting. Figure 3.8 shows that it recognized equal levels of HSP70 from pre- and post-heat shock worms.

3.3.2.3 Immunological reactivity of anti-MS2-HSP70 to pre- and post- heat shock schistosomula.

Figure 3.9 shows the result from an immunoblot of schistosomular proteins with anti-MS2-HSP70. It is seen that the antibody recognizes HSP70 from both normal and heat shocked schistosomula and that it reacts with more HSP70 from the heat shocked schistosomula.

Indirect immunofluorescence results from pre- and post-heat shock schistosomula, broken open by cytocentrifugation to allow access of the antibody to the inside of the parasite, confirm the above observation (figure 3.10). It is seen that in schistosomula which were heat-shocked the binding of anti-MS2-HSP70 antiserum was greater that that in schistosomula which were incubated at 37^{0}C.

3.4 Discussion

Biosynthetic activity, as assessed by labelled amino acid incorporation in *in vitro* culture, is very low in newly-prepared schistosomula. This "dormant" period was estimated at 6 hours by Nagai *et al.* (1977) and at 16 hours by

Figure 3.7 Immunoprecipitation of ^{35}S-methionine-labelled proteins from normal and heat shocked adult worms with anti-MS2-HSP70 antiserum.

15 adult male worms were labelled with 25 μCi / ml ^{35}S-methionine in a total of 10 ml methionine-free medium for 3 hours at 37^{0}C or 42^{0}C. The worms were washed with cold PBS and the proteins solubilized with Nonidet P40, immunoprecipitated, reduced and resolved on a 10 % (w / v) SDS-polyacrylamide gel.

hsp : immunoprecipitation with anti-MS2-HSP70 antiserum.
pre : immunoprecipitation with pre-immune serum.

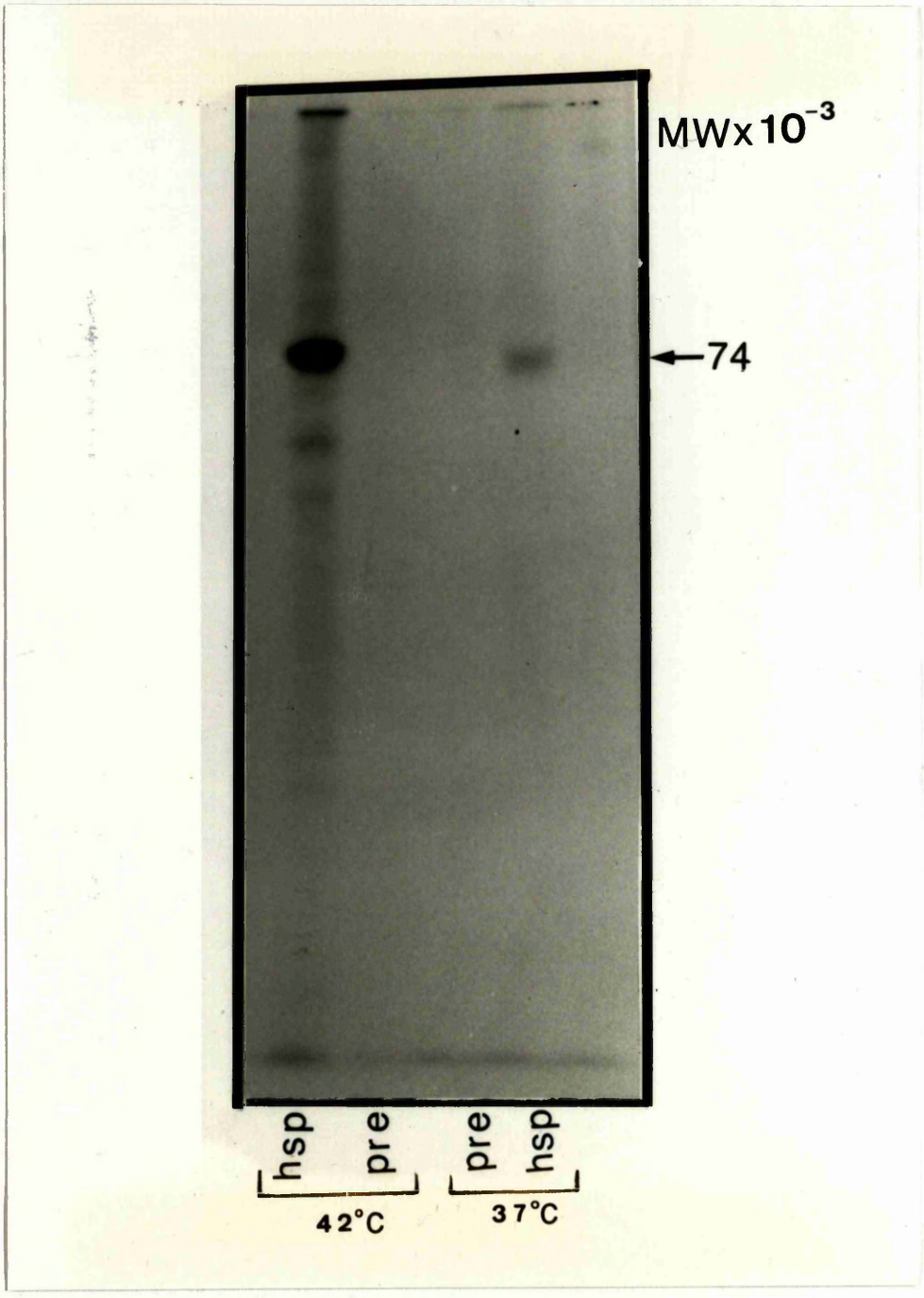

MWx10^{-3}
74
hsp
pre
pre
hsp
42°C
37°C

Figure 3.8 Immunoblot of proteins from normal and heat-shocked adult worms with anti-MS2-HSP70 antiserum.

10 adult male worms were maintained at 37^{0}C or incubated at 42^{0}C for 3 hours. The worms were washed, homogenized in 100 µl cold PBS, and boiled in reducing sample buffer. The proteins were electrophoresed on a 10 % (w/v) SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with anti-MS2-HSP70 antiserum followed by ^{125}I-protein A. The autoradiograph of the nitrocellulose filter is shown.

hsp : immunoblot with anti-MS2-HSP70 antiserum.

pre : immunoblot with pre-immune serum.

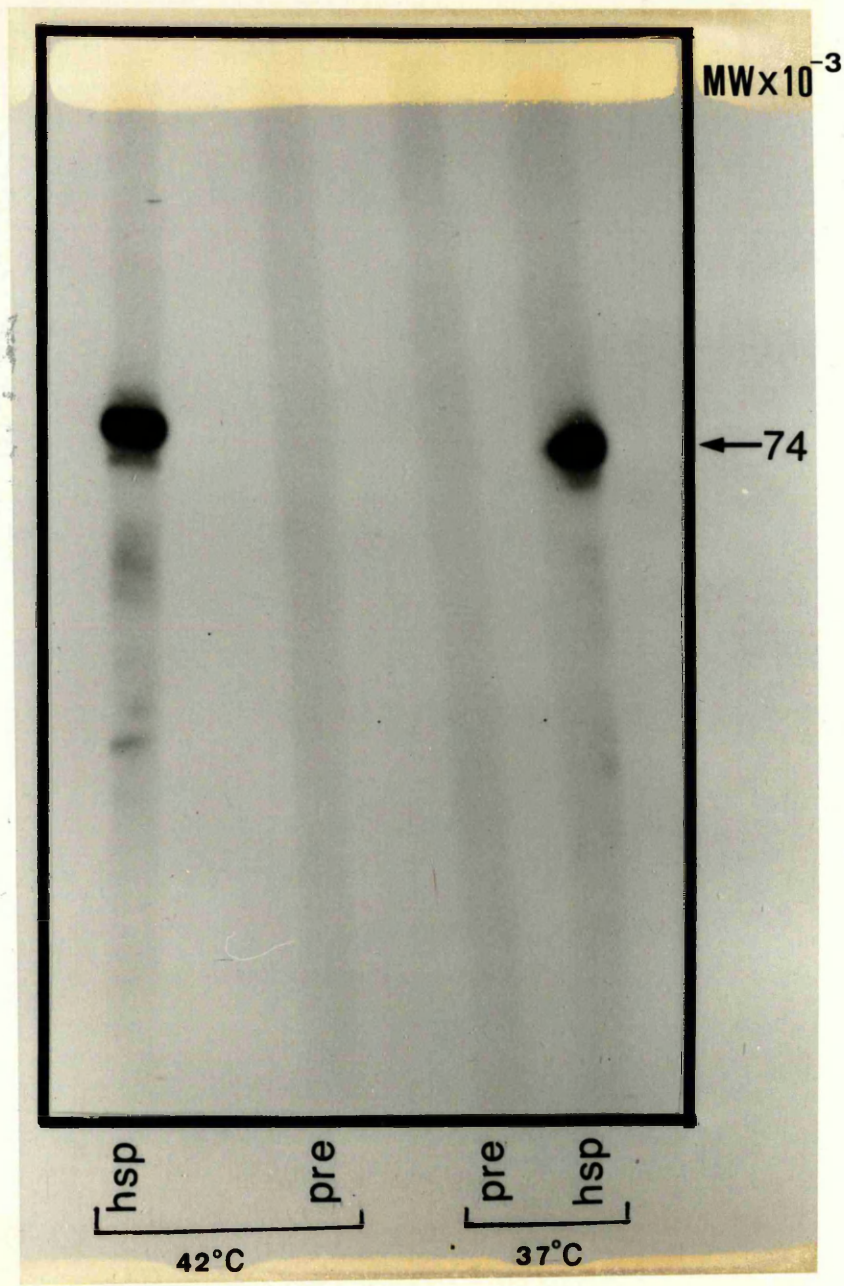

MW x 10^{-3}
74
hsp
pre
42°C
pre
hsp
37°C

Figure 3.9 Immunoblot of proteins from normal and heat-shocked schistosomula detected with anti-MS2-HSP70 antiserum.

Cercariae were transformed mechanically and the resulting schistosomula were incubated overnight at 37^{0}C / 5 % CO_2 in GMEM containing 10% FCS and 0.5 % LAH. After that the schistosomula were maintained at 37^{0}C or incubated at 42^{0}C for 3 hours. The schistosomula were then washed and prepared for immunoblotting as for the adult worms.

a : immunoblotting with anti-MS2-HSP70 antiserum

b: immunoblotting with pre-immune serum

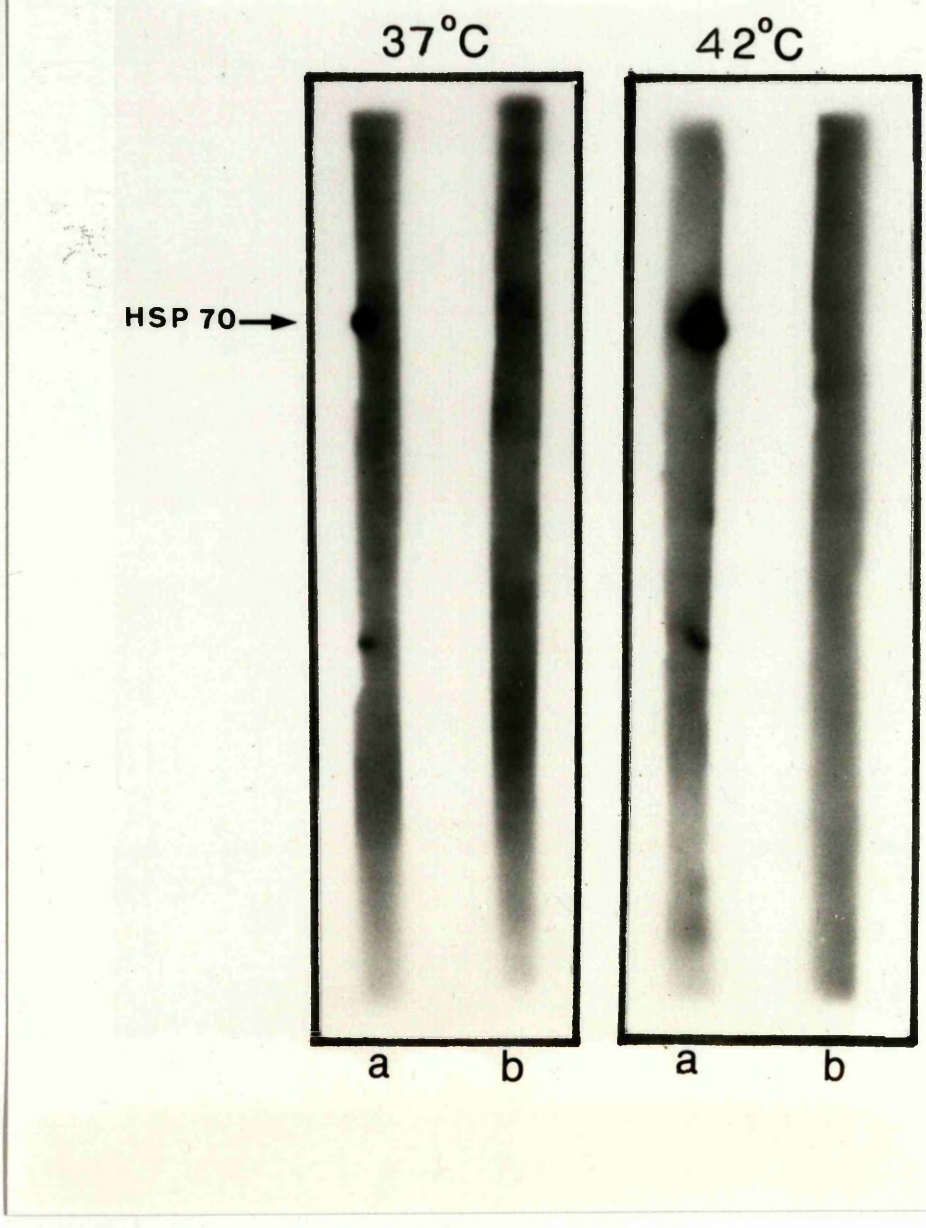

37°C
42°C
HSP 70
a
b
a
b

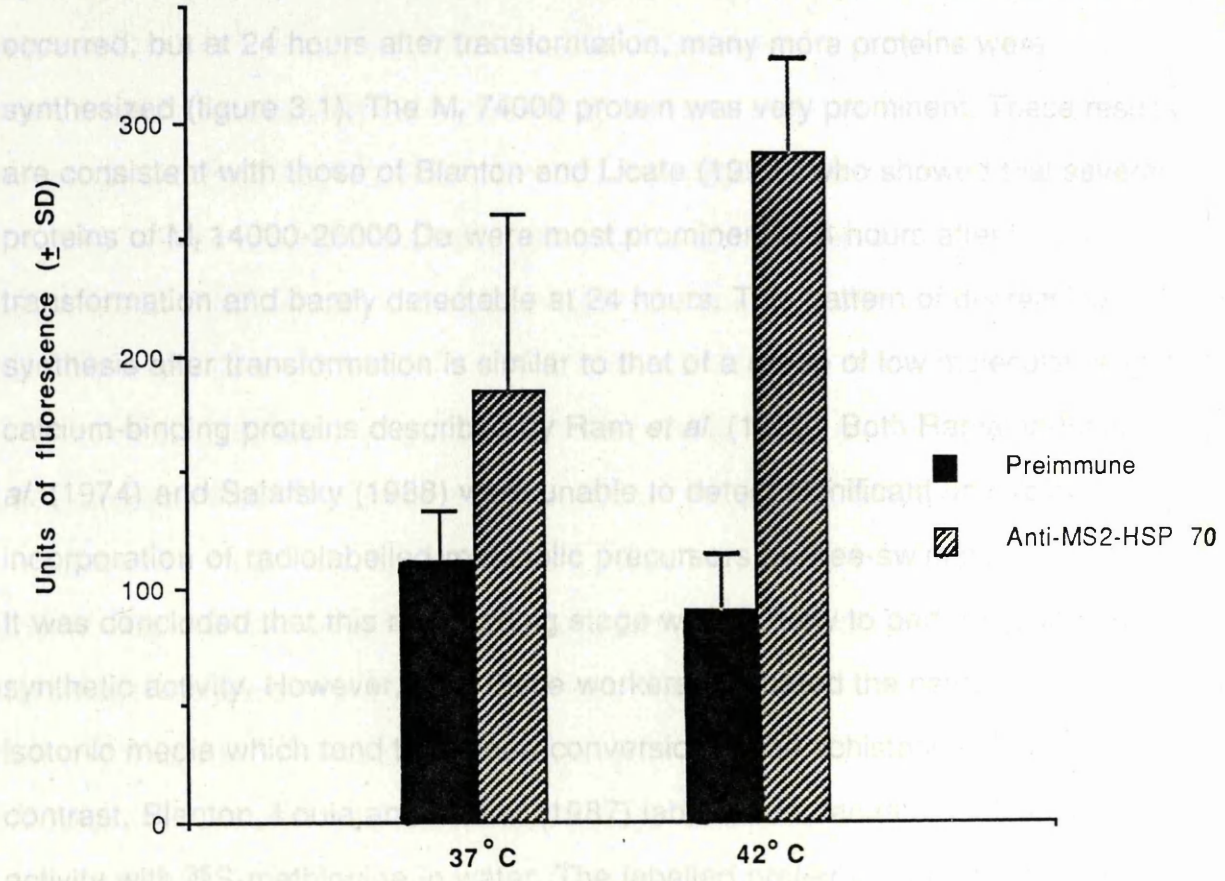
Figure 3.10 Binding of anti-MS2-HSP70 antiserum to normal and heat-shocked schistosomula detected by indirect immunofluorescence.

Newly transformed schistosomula were cultured overnight in GMEM containing 10 % FCS and 0.5 % LAH. Following that they were either maintained at 37^{0}C or heat-shocked at 42^{0}C for 3 hours as described in Materials and Methods. The schistosomula were then centrifuged, resuspended in 200 µl GMEM, and cytocentrifuged on poly L-lysine coated slides at 650 rpm for 5 minutes. The slides were fixed and prepared for immunofluorescence.

Statistical analysis

37^{0}C : pre v anti P = 0

42^{0}C : pre v anti P = 0


Units of fluorescence (± SD)
300
200
100
0
37° C
42° C
Preimmune
Anti-MS2-HSP 70

Yuckenberg, Poupin and Mansour (1987).Both groups observed a striking increase in protein synthesis after the initial quiescent stage. The present experiments also show that protein synthesis occurred only after several hours after transformation of cercariae to schistosomula. At 3 hours after transformation, synthesis of some minor bands and a heavily labelled material of <14000 Da occurred, but at 24 hours after transformation, many more proteins were synthesized (figure 3.1). The M_r 74000 protein was very prominent. These results are consistent with those of Blanton and Licate (1992), who showed that several proteins of M_r 14000-26000 Da were most prominent at 4 hours after transformation and barely detectable at 24 hours. This pattern of decreasing synthesis after transformation is similar to that of a group of low molecular weight calcium-binding proteins described by Ram *et al.* (1989). Both Ramalho-Pinto *et al.* (1974) and Salafsky (1988) were unable to detect significant uptake or incorporation of radiolabelled metabolic precursors by free-swimming cercariae. It was concluded that this non-feeding stage was unlikely to perform significant synthetic activity. However, both these workers incubated the cercariae in isotonic media which tend to support conversion to the schistosomulum stage. In contrast, Blanton, Loula and Parker (1987) labelled cercariae to high specific activity with ^{35}S-methionine in water. The labelled proteins were examined by SDS-PAGE. At 23^{0}C, methionine was incorporated into polypeptides of M_r 42, 38, 34, 25, 20 and 15 kDa. Material of M_r below 14000 was also very intensely labelled. Bands at M_r 84000 and 60000-58000 were faintly labelled.

HSP70 as well as other proteins continued to be synthesized and also their rate of synthesis increased as the parasite developed. The highest rate of protein synthesis was seen in the 3 week developmental stage (figure 3.3) which was consistent with that of Atkinson and Atkinson (1982) who showed that juvenile schistosomes exhibited a much higher rate of amino acid incorporation than adults. Synthesis of HSP70 after transformation has been observed by several workers. Yuckenberg, Poupin and Mansour (1987) showed that a protein

of M_r 69,000 Da was synthesized within a few hours after transfer of organisms from aquarium water to culture conditions equivalent to those in the mammalian host. This protein was immunoprecipitated by antiserum against HSP70 in a variety of eukaryotic cells. Blanton, Loula and Parker (1987) showed that 3 hour old mechanically transformed schistosomula synthesized a M_r 70,000 Da protein. This protein was not found in cercariae when their radiolabelled proteins were immunoprecipitated with anti-HSP70 serum. Hedstrom *et al.* (1987), however, detected HSP70 in cercariae by the method of immunoblotting. Together, these results show that while HSP70 is constitutively present in cercariae, it is not newly synthesized. Newly synthesized HSP70 is only detected after transformation.

Although several antisera to HSP70 from different organisms were used to immunoassay HSP70 from *S. mansoni* (Blanton, Loula and Parker, 1987; Yuckenberg, Poupin and Mansour,1987; Hedstrom *et al.*, 1987), a monoclonal anti-72 kDa heat shock protein from HeLa cells did not recognize HSP70 of the parasite in the experiments presented here. This could have been due to the fact that this monoclonal antibody reacts only with the induced 72 kDa protein (Amersham data sheet, 1990), rather than the constitutive form. However, reactivity with normal, unstressed cells was observed, but only in human cell lines (Amersham data sheet, 1990). It might also be that this antibody recognizes only the mammalian heat shock protein and therefore does not recognize the schistosome form. Consistent with this, the antibody has been shown to react with samples other than those of human, e.g., rat, mouse, hamster and monkey species. No reactivity has been observed with any avian samples (Amersham data sheet, 1990).

Examination of metabolically labelled proteins by polyacrylamide gel electrophoresis demonstrated that in adult worms subjected to a rise in temperature from 37°C to 42°C, heat shock proteins of M_r 74, and 86 kD were induced. These results were consistent with those of Johnsone *et al.* (1989) who

showed that adult *S. mansoni* respond to heat shock, showing increased synthesis of polypeptides of M_r 70 and 86 kDa. Other heat shock proteins were also induced in adult worms subjected to a rise in temperature from 37^{0}C to 42^{0}C, for example, proteins of M_r 100, 84, 43, and 34.5 kDa (Blanton, Loula, and Parker,1987). The M_r 70 kDa heat shock protein was very prominent in adult worm lysates both before and after a rise in temperature.

A rabbit polyclonal antiserum to a recombinant form of HSP70 gave a more intense immunoprecipitated protein band in post- than in pre-heat shock worms (figure 3.7) indicating that the protein is induced upon heat shock. However, the antibody reacted to equal amounts of HSP70 on protein blots from pre- and post-heat shock worms (figure 3.8). Since equal amounts of proteins were used for immunoprecipitation, this difference in the detection of HSP70 by the two methods indicates that the amount of HSP70 in pre- and post-heat shock worms does not change but its rate of synthesis increases upon heat shock. This increase in the rate of synthesis of new HSP70 is detected by immunoprecipitation of labelled parasite proteins.

In schistosomula no immunoprecipitations were performed due to the low levels of ^{35}S-methionine incorporated. However, indirect immunofluorescence and immunoblotting studies with anti-MS2-HSP70 antiserum showed an increase in HSP70 synthesis upon heat shock (figures 3.9 and 3.10).

Having obtained an antibody which recognized HSP70 in the parasite, it was used in all the subsequent experiments to study HSP70.

Chapter Four

Localization of heat shock protein 70 (HSP 70) in the adult schistosome

4.1 Introduction

Heat shock protein 70 (HSP70) is found to be a major immunogen detected in humans and animals infected chronically with *Schistosoma mansoni* (Hedstrom *et al.*,1987; Moser, Doumbo and Klinkert, 1990). This led us to think that HSP70 might be exposed at the surface of adults of *Schistosoma mansoni* , and hence be accessible to the host immune system. To investigate this and to try to localize HSP70, indirect immunofluorescence studies with anti-MS2-HSP70 were carried out on adult worms.

Proteins of the schistosome in direct contact with the exterior appear to decrease gradually as the parasite develops (Rumjanek, 1987). The process through which these proteins become unavailable for detection is thought to be due to the acquisition of host molecules by the parasites. This acquisition results in a general masking of the parasite's proteins. This is a striking and a well-documented feature of the schistosome surface (reviewed by Simpson and Smithers, 1985). This is thought to be one of the reasons for reduced antibody-binding to the schistosome surface. For this reason, adult worms were treated in a variety of ways to reveal molecules on their surface which could react with anti-MS2-HSP70 antiserum. Some of these treatments have been previously shown to displace host molecules (Harnett, Kusel and Barrowman, 1985).

4.2 Indirect immunofluorescence study on intact, formaldehyde-fixed, and Triton X-100- treated adult worms.

4.2.1 Intact, carbachol-treated adult worms.

Freshly perfused adult worms were probed with anti-MS2-HSP70 antiserum. Carbachol at a concentration of 1 mg / ml was used to immobilize the worms so that the fluorescence could be quantitated. No binding of the antibody was observed on the intact worms as shown in figure 4.1, lane A.

Figure 4.1 Binding of anti-MS2-HSP 70 antiserum to adult schistosomes.

Groups of 10 adult male worms were treated in different ways to reveal surface molecules as described in Materials and Methods. They were then labelled with anti-MS2-HSP 70 antiserum. The binding of the antiserum to the surface of the schistosome was detected by indirect immunofluorescence as described in Materials and Methods.

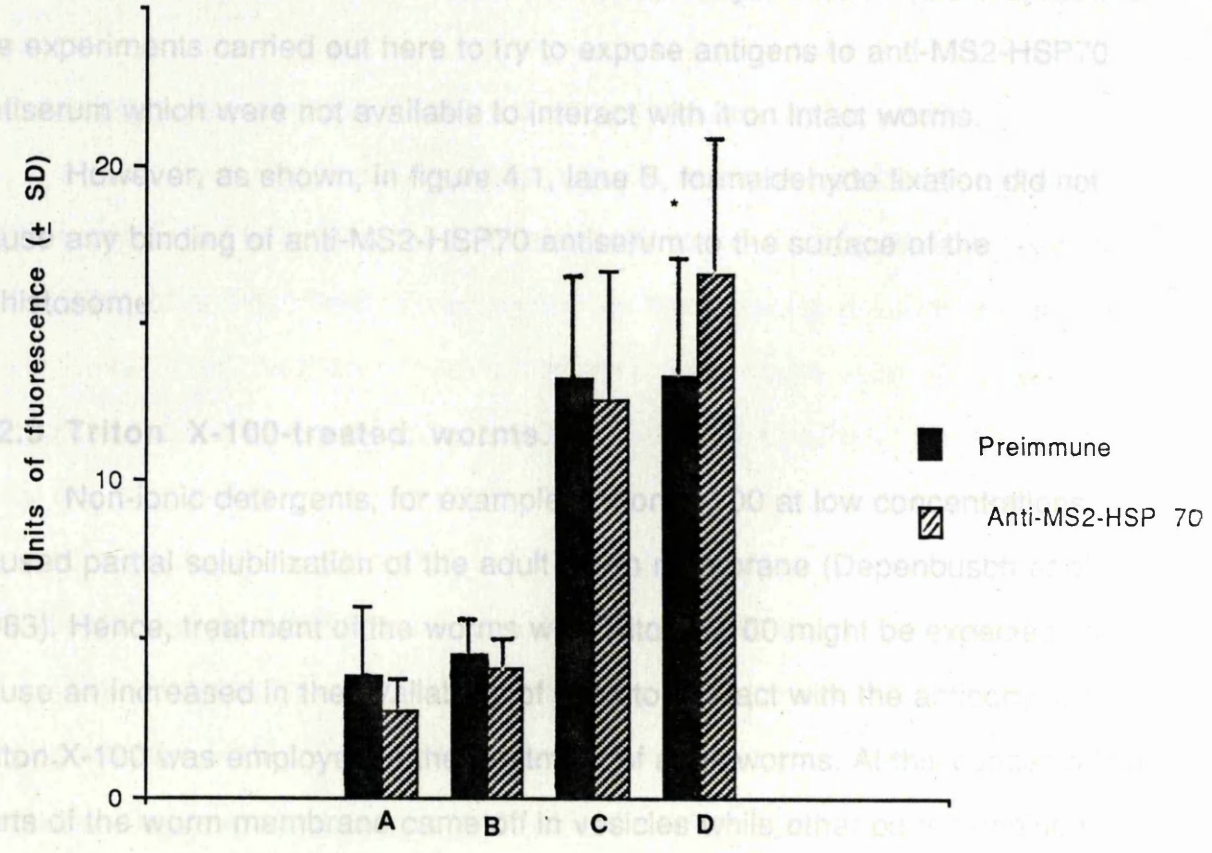
A : Intact, carbachol-treated.

B : Formaldehyde-fixed.

C : Triton X-100 treated.

D : Freeze-thawed.

* : P = 0


Units of fluorescence (± SD)
20
10
0
A
B
C
D
*
Preimmune
Anti-MS2-HSP 70

4.2.2 Formaldehyde-fixed worms.

Formaldehyde fixation may cause exposure of antigenic sites which are not normally able to interact with antibody (Harnett, Kusel and Barrowman,1985). A concentration of 0.1% formaldehyde appeared to induce the maximum exposure of hidden antigenic sites in this way (Harnett, Kusel and Barrowman,1985). This concentration of formaldehyde was therefore chosen for the experiments carried out here to try to expose antigens to anti-MS2-HSP70 antiserum which were not available to interact with it on intact worms.

However, as shown, in figure 4.1, lane B, formaldehyde fixation did not cause any binding of anti-MS2-HSP70 antiserum to the surface of the schistosome.

4.2.3 Triton X-100-treated worms.

Non-ionic detergents, for example, Triton X-100 at low concentrations caused partial solubilization of the adult worm membrane (Depenbusch *et al.*, 1983). Hence, treatment of the worms with Triton X-100 might be expected to cause an increased in the availability of sites to interact with the antibody. 0.1% Triton X-100 was employed in the treatment of adult worms. At this concentration parts of the worm membrane came off in vesicles while other parts remained unaffected. However, contrary to our expectations, figure 4.1, lane C, shows that there was no difference in labelling detergent-treated worms with pre-immune or anti-MS2-HSP70 sera.

4.3 Indirect immunofluorescence labelling of freeze-thawed adult worms.

As a consequence of the above results which show that HSP70 was not present on the surface of the worm, we thought it could be present underneath it. Since freezing and thawing strips the worms of their surfaces and leaves the

basement membrane of the bodies intact (Kusel 1972), this method was chosen to test the above idea.

Stripping the worms of their surfaces by freezing and thawing on solid CO_2 / methanol and then labelling the denuded worms with the antibody showed a significant difference between the binding of anti-MS2 HSP 70 and pre-immune sera (figure 4.1, lane D).

4.4 Localization of HSP 70 in frozen sections of the adult worm.

12 µm-thick frozen sections of adult worms were probed with anti-MS2-HSP70 antiserum in an attempt to localize it. There was a significant difference in the binding of anti-MS2-HSP70 antiserum as compared to pre-immune serum in the permeabilized, methanol-fixed, but not in the non-fixed sections (figure 4.2). Products reactive with anti-MS2-HSP70 were observed all over the section and were not localized at a specific place.

4.5 Detection of HSP 70 in isolated membranes.

Membranes of the worms were isolated and solubilized with Triton X-100 as described in Materials and Methods. Protein blots of detergent-soluble and insoluble fractions when reacted with anti-MS2-HSP70 antiserum showed that HSP70 was present in Triton X-100-soluble (figure 4.3,lane B) and not in the insoluble fraction (figure 4.3, lane A).

It was desirable to know how HSP70 participated in the structure of the membrane. Whether it was an integral or peripheral protein was investigated by phase separation in Triton X-114.

4.5.1 Phase separation of HSP70 in Triton X-114.

A solution of the non-ionic detergent Triton X-114 is homogeneous at 0^0C but separates into an aqueous phase and detergent phase above 20^0C. This detergent had been used to recover integral membrane proteins of *Schistosoma*

Figure 4.2 Binding of anti-MS2-HSP70 antiserum to frozen sections of the adult worm.

Frozen sections of adult worms were either fixed with 100 % methanol or left unfixed. They were then treated with pre-immune serum or anti-MS2-HSP 70 antiserum and prepared for immunofluorescence as described in Materials and Methods.

Statistical analysis

Unfixed : pre v anti	P = 0.041
Fixed : pre v anti	P = 0

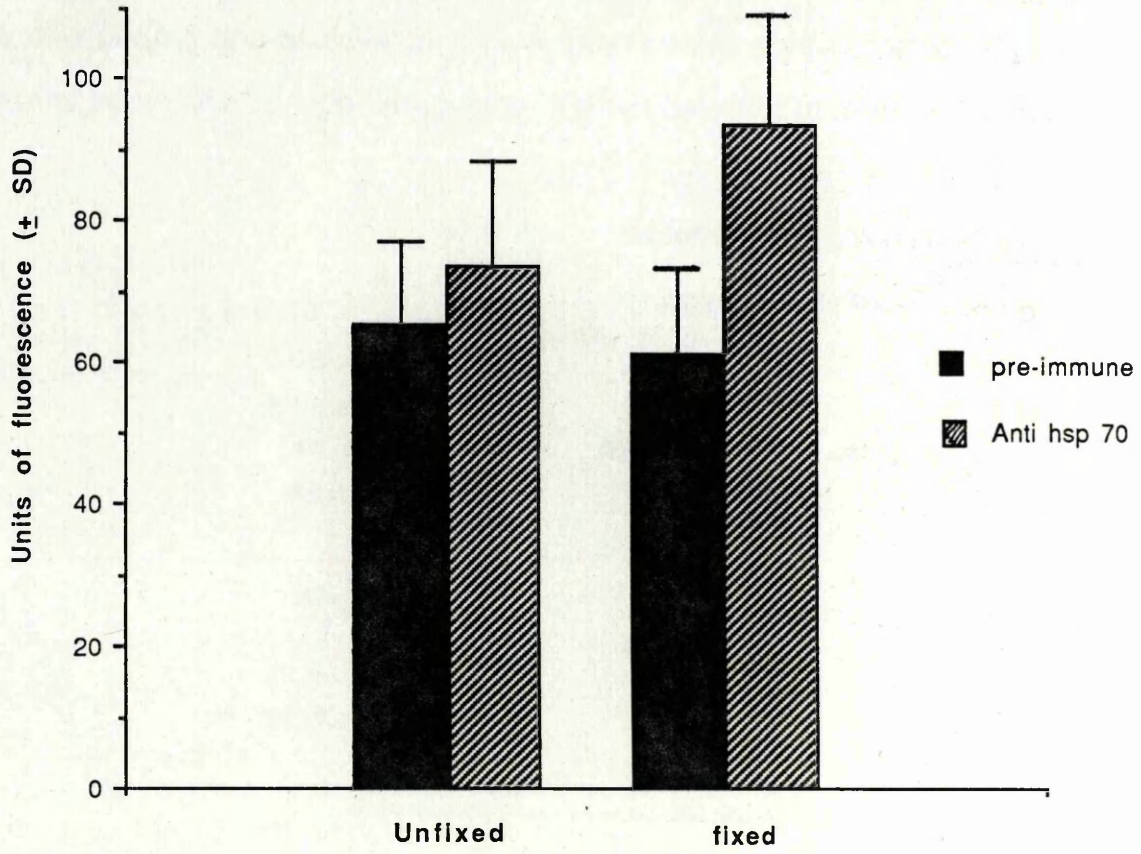
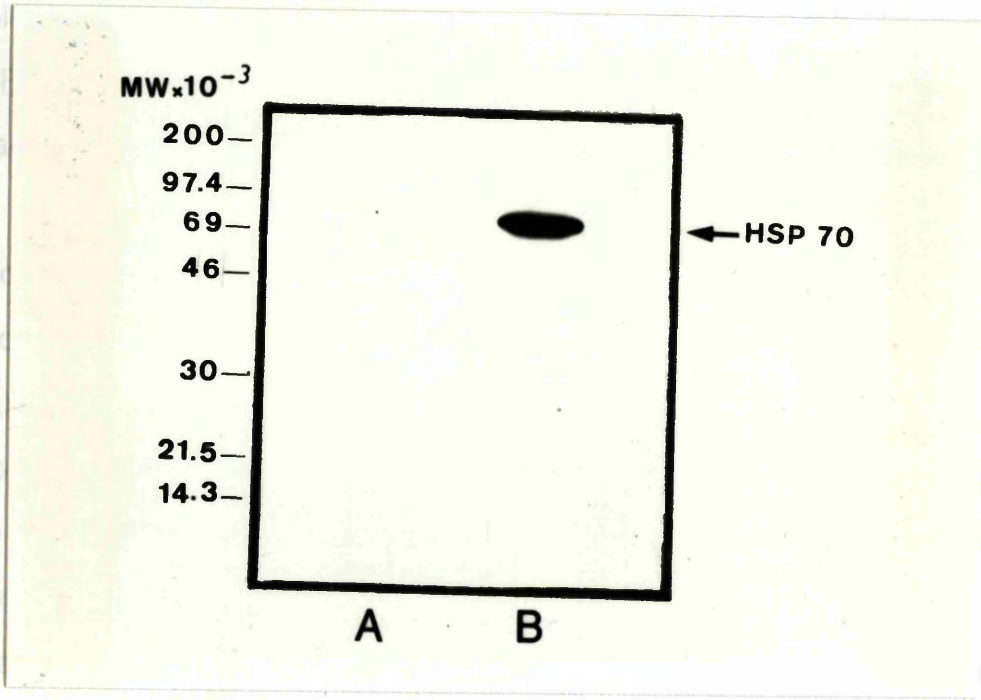

Units of fluorescence (± SD)
100
80
60
40
20
0
Unfixed
fixed
pre-immune
Anti hsp 70

Figure 4.3 Immunoblot of proteins from adult schistosome surface membranes with anti-MS2-HSP 70 antiserum.

Membranes from 50-60 adult worms were solubilized using Triton X-100. Triton- soluble and insoluble material was electrophoresed on a reducing 10 % (w/v) SDS-polyacrylamide gel, transferred to nitrocellulose and probed with anti-MS2-HSP70 antiserum followed by HRP-conjugated donkey anti rabbit serum.

A : Triton X-100-insoluble material.

B : Triton X-100-soluble material.


MW x 10⁻³
200
97.4
69
46
30
21.5
14.3
HSP 70
A
B

mansoni (Omer-Ali *et al.*, 1991) and *S. japonicum* adult worms (Rogers *et al.*, 1988), and to separate integral membrane proteins from hydrophilic ones (Bordier, 1981). Hydrophilic (aqueous) proteins remain in the aqueous phase, since they are unable to interact with Triton X-114, whereas integral membrane proteins with a hydrophobic domain remain closely associated with detergent (Bordier, 1981).

Here, adult worm membranes were isolated and solubilized with Triton X-114, and the soluble material was submitted to phase separation. The Triton X-114-insoluble, detergent, and aqueous fractions were immunoblotted with anti-MS2-HSP70 antiserum. Figure 4.4 shows that HSP 70 was present in all Triton X-114 fractions but the majority of it was present in the aqueous phase.

4.6 Discussion.

Indirect immunofluorescence studies using anti-MS2-HSP70 antiserum showed that HSP70 could not be detected on the surface of the adult schistosomes treated in different ways to reveal their surface molecules (figure 4.1). The increased binding of the antibody after stripping of the membranes of the worms (figure 4.1, lane D) might indicate the presence of HSP70 in the fibrous basal layer which becomes exposed after the membrane is stripped off. Increase in anti-MS2-HSP70 antiserum binding was also observed in frozen sections of the worms (figure 4.2). The ability of the antibody to detect HSP70 only when the cells in the frozen sections have been permeabilized by methanol treatment, indicates that the location of HSP70 is intracellular. This is not surprising, as HSP70 proteins studied in other organisms are found to be intracellular - they are either confined to certain compartments of the cell, or, are soluble cytoplasmic proteins (Craig, Kang and Boorstein, 1990). In some circumstances, however, heat shock proteins become expressed on the surface of some stressed cells (Kaufmann, 1990 a) and also on two important antigen presenting cells, namely B cells and macrophages (VanBuskirk *et al.*, 1990).

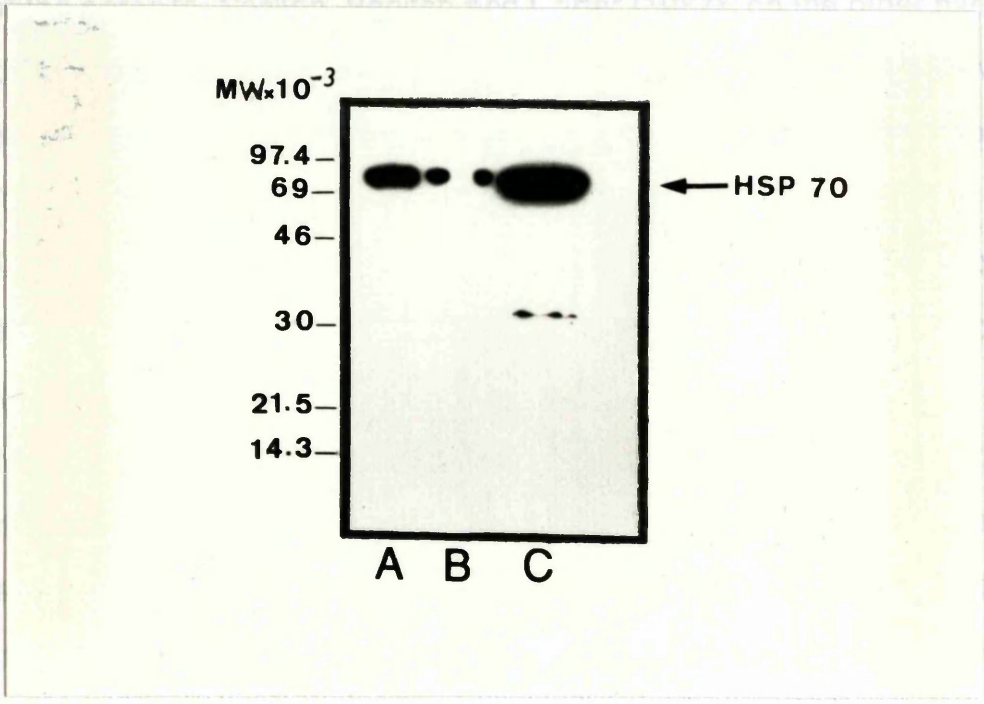
Figure 4.4 Immunoblot of phase-separated proteins from adult schistosome surface membrane with anti-MS2-HSP70 antiserum.

Membranes from 60 adult worms were solubilized and subjected to phase separation in Triton X-114. The insoluble, detergent-soluble, and aqueous fractions were electrophoresed on a reducing 10 % SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with anti-MS2-HSP70 antiserum followed by HRP-conjugated donkey anti-rabbit serum.

A : Triton X-114-insoluble fraction.

B : Triton X-114-soluble (hydrophobic) fraction.

C : Aqueous (hydrophilic) fraction.


MW x10^{-3}
97.4
69
46
30
21.5
14.3
HSP 70
A
B
C

HSP70 was distributed uniformly throughout the frozen sections and was not localized to a specific place. These results are consistent with those of Lanar, Pearce and Sher (1985) who showed that an antiserum against a fusion protein which immunoprecipitated a protein of M_r 70,000 in adults of *Schistosoma mansoni* localized this protein diffusely throughout the parenchyma and it did not appear on the tegument or dorsal surface of the worm near the tubercules. They also showed that the protein was highly concentrated near the surface of the male gynaecophoric canal. However, it is not known whether this protein is HSP70 of the parasite. Scallon, Bogitsh and Carter (1987), on the other hand, observed that HSP70 of *Schistosoma japonicum* was distributed in the nervous system of both males and females and in the dorsal and lateral teguments of males. Thus, a similar distribution of HSP70 in *S. mansoni* is likely. HSP70 of *S. mansoni* and *S. japonicum*, however, seem to be structurally dissimilar because antibodies directed towards HSP70 of each one of the two species are not cross-reactive (Hedstrom *et al.*, 1988).

Although HSP 70 cannot be found on the surface of the adult worm it can be detected in the membranes by immunoblotting (figure 4.3). This could have been due to HSP70 being present in amounts too low for it to be detected by indirect immunofluorescence, or, more likely, it could be present and distributed underneath the surface in a way that makes it inaccessible to the antibody even after formaldehyde fixation or detergent treatment of the worms.

An investigation of how HSP70 might participate in the membrane structure showed that the molecule was found in the membrane, partitioning in the hydrophilic, hydrophobic, as well as the detergent-insoluble fractions when the membranes were solubilized with Triton X-114 (figure 4.4). The presence of HSP70 in Triton X-114-insoluble fraction was surprising because, as shown previously, HSP70 did not occur in detergent-insoluble fraction when the membranes were solubilized with Triton X-100. This difference in the partitioning of HSP70 in the insoluble fractions of the two detergents could be due to the

incomplete solubilization of the membrane by Triton X-114. Indeed, extraction of mitochondrial proteins by various surfactants showed that Triton X-100 was the most effective surfactant as it extracted 70% of the total mitochondrial protein whereas Triton X-114 extracted about 60% (Egan 1976).

Although HSP70 is recovered in both hydrophilic and hydrophobic phases, the major fraction of HSP 70 in the adult membrane seems to be hydrophilic (aqueous), which indicates that it may be a peripheral membrane protein. The detection of a proportion of this molecule partitioning in the hydrophobic phase might be due to, (a) incomplete separation from integral membrane proteins during solubilization, (b) association with some integral membrane protein(s), or, (c) a small fraction of HSP70 itself being an integral membrane protein. The significance of this is explored further in the general discussion (eg. figure 7.1).

Chapter Five

Association of Heat shock protein 70 (HSP70) with different proteins of *Schistosoma mansoni*

HSP70 has also been shown to be involved in the process of antigen presentation (Pierce, DeNagel and VanBuskirk, 1991). Furthermore, the gene for human HSP70 was found located within the MHC (Guenther, 1991).

In this chapter, the association or interaction of HSP70 with proteins of *S. mansoni* was investigated. Usually co-immunoprecipitation is employed for the detection of HSP70 binding proteins. The disadvantages with this method are that antibodies against HSP70 may compete for the binding sites on the HSP70 molecule with the bound proteins, or the detergents used to solubilize the proteins can affect hydrophobic interactions between the proteins (Johnsone and Thorpe, 1987).Since some proteins bind to HSP70 by hydrophobic interactions (Schlesinger,1990), the interaction of HSP70 with parasite proteins were studied by the two following methods; a) by analyzing HSP70-protein complexes by non-denaturing gel electrophoresis, and b) by chemically cross-linking the parasite proteins and then immunoprecipitation.

5.2 Analyzing HSP70-protein complexes by non-denaturing gel electrophoresis

Denaturing polyacrylamide gel electrophoresis is a sensitive technique for the characterization of small amounts of protein. Urea and sodium dodecyl sulphate (SDS) cause the denaturation of proteins, typically dissociating oligomers to their subunits, and thus leading to losses of most biological properties. Non-denaturing polyacrylamide gel electrophoresis, on the other hand, permits the detection of non-covalently linked protein complexes because it causes no disruption or dissociation of these complexes.

5.2.1 Immunoblotting of non-denatured parasite proteins

Four different concentrations of non-denaturing gels were used because under non-denaturing conditions proteins differing in both size and charge were

found to migrate at the same rate at a given gel concentration (Hedrick and Smith, 1968). So, if HSP70 complex was migrating at a specific place in a certain gel concentration, other parasite proteins could be co-migrating with it. If this complex was then analyzed under denaturing conditions the possibility of other parasite proteins ,which are not part of the complex, being present cannot be eliminated. Therefore, proteins were run on several gel concentrations so that when the complexes from each gel concentration are analyzed on denaturing gels, only those proteins which appear in every track will be the ones involved in HSP70-protein complexes.

^{35}S-methionine-labelled and unlabeled worms were homogenized in a buffer for non-denaturing gels as described in Materials and Methods. The homogenate was then run on 5%, 6%, 7% and 8% non-denaturing polyacrylamide gels. The labelled proteins were run on one half of the gel while the unlabeled ones were run on the other half. After electrophoresis, the unlabeled half of the gel was blotted onto nitrocellulose and decorated with anti-MS2-HSP70 antiserum; the labelled half was stained with Coomassie blue, destained and dried.

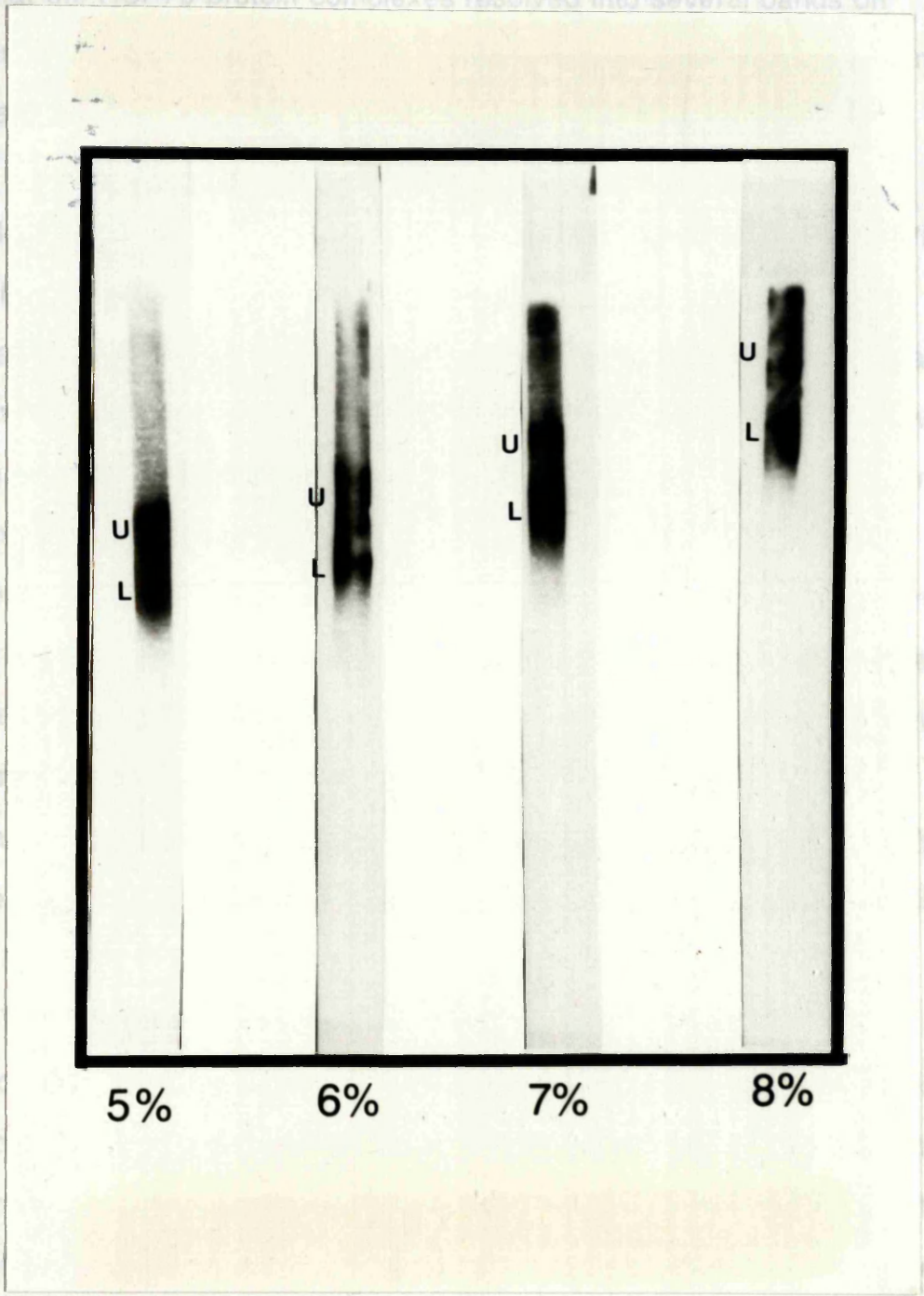
Figure 5.1 shows the result of immunoblotting non-denatured parasite proteins with anti-MS2-HSP70 antiserum. It can be seen that the antibody reacts with two major areas on the nitrocellulose; an upper and a lower area.

5.2.2 Resolving HSP70-protein complexes by denaturing polyacrylamide gel electrophoresis

In order to find out what these complexes consist of, this blot was superimposed on the labelled half of the gel and the areas corresponding to the upper and the lower areas were cut out from the labelled half of the gel. These cut out pieces of the gel were then equilibrated in a buffer containing SDS and β-mercaptoethanol to allow reduction and denaturation of the proteins. The equilibrated gel pieces were mounted in slots of a 10% SDS-polyacrylamide gel

Figure 5.1 Immunoblot of non-denatured adult schistosome proteins with anti-MS2-HSP70 antiserum.

Adult worms which were incubated overnight were homogenized on ice in 500 µl homogenate buffer for non-denaturing as described in Materials and Methods. The homogenate was loaded on 5%, 6%, 7% and 8% non-denaturing gels and electrophoresed. After electrophoresis, the gels were blotted onto nitrocellulose and decorated with anti-MS2-HSP70 antiserum, followed by ^{125}I-protein A. The autoradiograph of the nitrocellulose filter is shown. The areas to which the antibody reacted are shown as U = upper area, L = lower area.


U
L
U
L
U
L
5%
6%
7%
8%

and electrophoresed. After electrophoresis, the gel was stained with Coomassie blue, destained and dried and analyzed by fluorography.

The result of such an experiment is shown in figure 5.2 where it can be seen that the HSP70-protein complexes resolved into several bands on denaturing polyacrylamide gels. HSP70 (M_r 74 kDa on the figure) was found to associate with proteins of M_r >100, 65, 59, 49 and 30 kDa.

5.2.3 Identification of the proteins found associated with HSP70

In an attempt to identify some of these proteins or polypeptides associating with HSP70, antisera to some well known antigens of the parasite with similar molecular weight to the ones found associating with HSP70, were tried to react with these HSP70-protein complexes on blots of non-denatured proteins. However, these antisera did not react with the HSP70-protein complexes. Not only that, but the antisera did not react with any specific area on the nitrocellulose either; monoclonal anti-32-38 kDa antibody which is directed towards the 32-38 kDa surface molecule of schistosomula gave a very faint reaction which was concentrated on the top of the gel (figure 5.3, lane B), whereas the polyclonal anti-32 kDa antibody which is directed towards a 32 kDa surface glycoprotein from adult worms (Payares *et al.*, 1985) gave a faint smear (figure 5.3, lane A).

To see if these antisera actually reacted with their corresponding antigens, immunoprecipitations were performed using these antisera with ^{35}S-methionine-labelled proteins of the parasite. Figure 5.4 shows the monoclonal antibodies did not immunoprecipitate any band. There could be several explanations for this; a) The antibody has lost its reactivity and is no longer functioning, b) the proteins which are supposed to be immunoprecipitated by these antisera cannot be metabolically-labelled with ^{35}S-methionine, and hence no bands are seen on the fluorograph, and c) the reactive epitopes of the 32 kDa and 32-38 kDa antigens are masked by the other proteins associated with it and hence the antisera are

Figure 5.2 HSP70-protein complexes resolved by denaturing polyacrylamide gel electrophoresis ; Fluorograph of 10% SDS-gel.

20 adult worms were labelled with 500 µCi ^{35}S-methionine overnight in a total of 10 ml methionine-free medium containing 10% FCS. After that the worms were washed and homogenized in homogenate buffer as for figure 5.1. 40 µl aliquots of the homogenate were loaded on 5%, 6%, 7% and 8% non-denaturing gels. After electrophoresis, the gels were stained with Coomassie blue, destained and dried. The areas corresponding to the upper and lower areas on the autoradiograph in figure 5.1. were cut out from the labelled non-denaturing gels and were equilibrated in a buffer containing SDS and β-mercaptoethanol as described in Materials and Methods. The equilibrated pieces of the gels were loaded in slots of the stacking gel of a 10% SDS-polyacrylamide gel and electrophoresed. After electrophoresis, the gel was stained, destained, dried and subjected to fluorography.

The U and L letters on the figure correspond to the upper and lower areas to which HSP70 antibody reacts on the blots. The molecular weight shown with arrows corresponds to the proteins associating with HSP70.

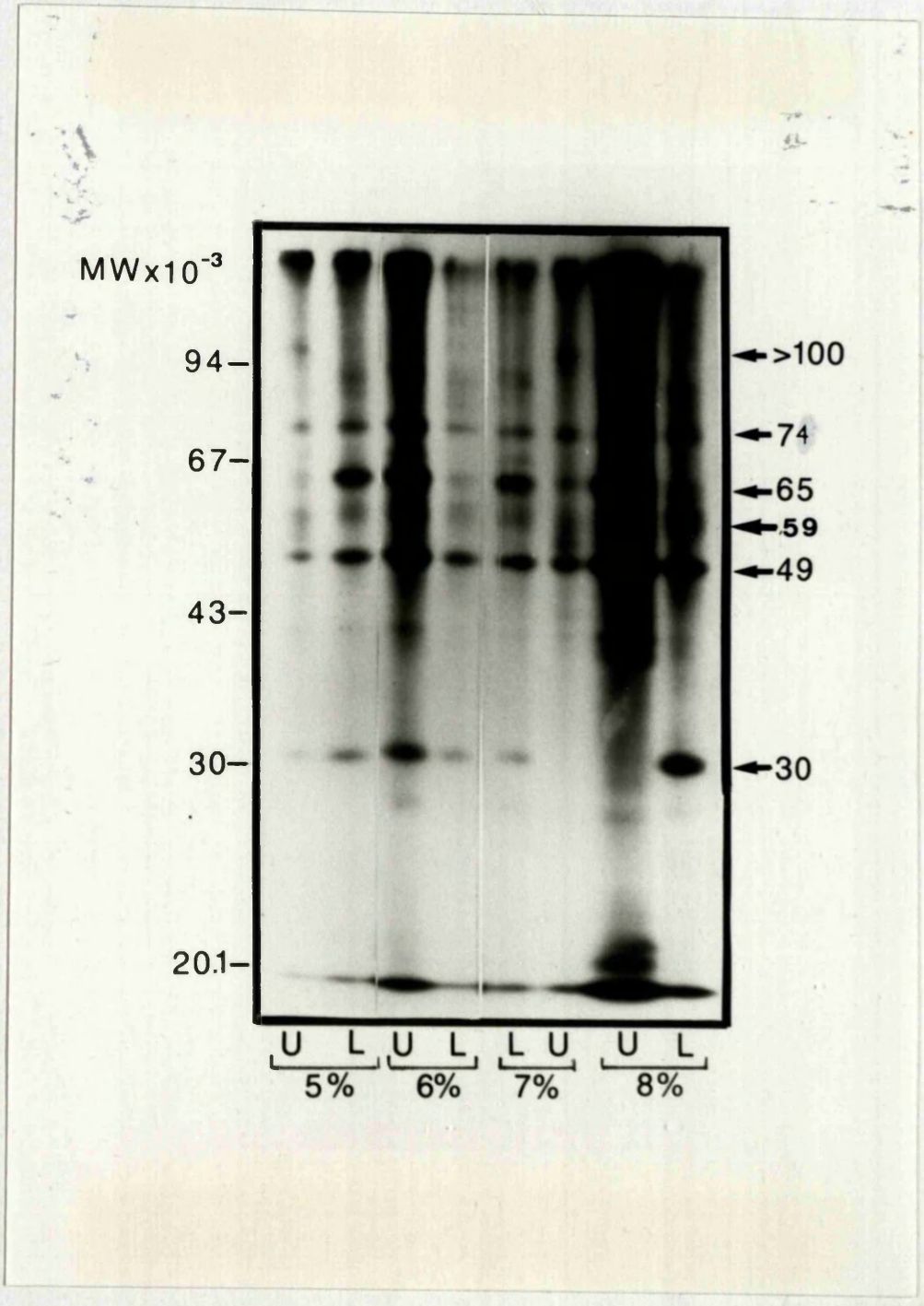

MWx10-3
94
67
43
30
20.1
>100
74
65
59
49
30
U L U L L U U L
5%
6%
7%
8%

Figure 5.3 Immunoblot of non-denatured adult schistosome proteins with monoclonal anti-30-38, and polyclonal anti-32, schistosome antigens.

Adult worms were homogenized and prepared for non-denaturing gel electrophoresis as described in figure 5.1. Only the 5% gel is shown.

Lanes A : monoclonal anti-30 kDa antigen

B : monoclonal anti-38 kDa antigen

C : anti-MS2-HSP70 antiserum.


A
B
C
D

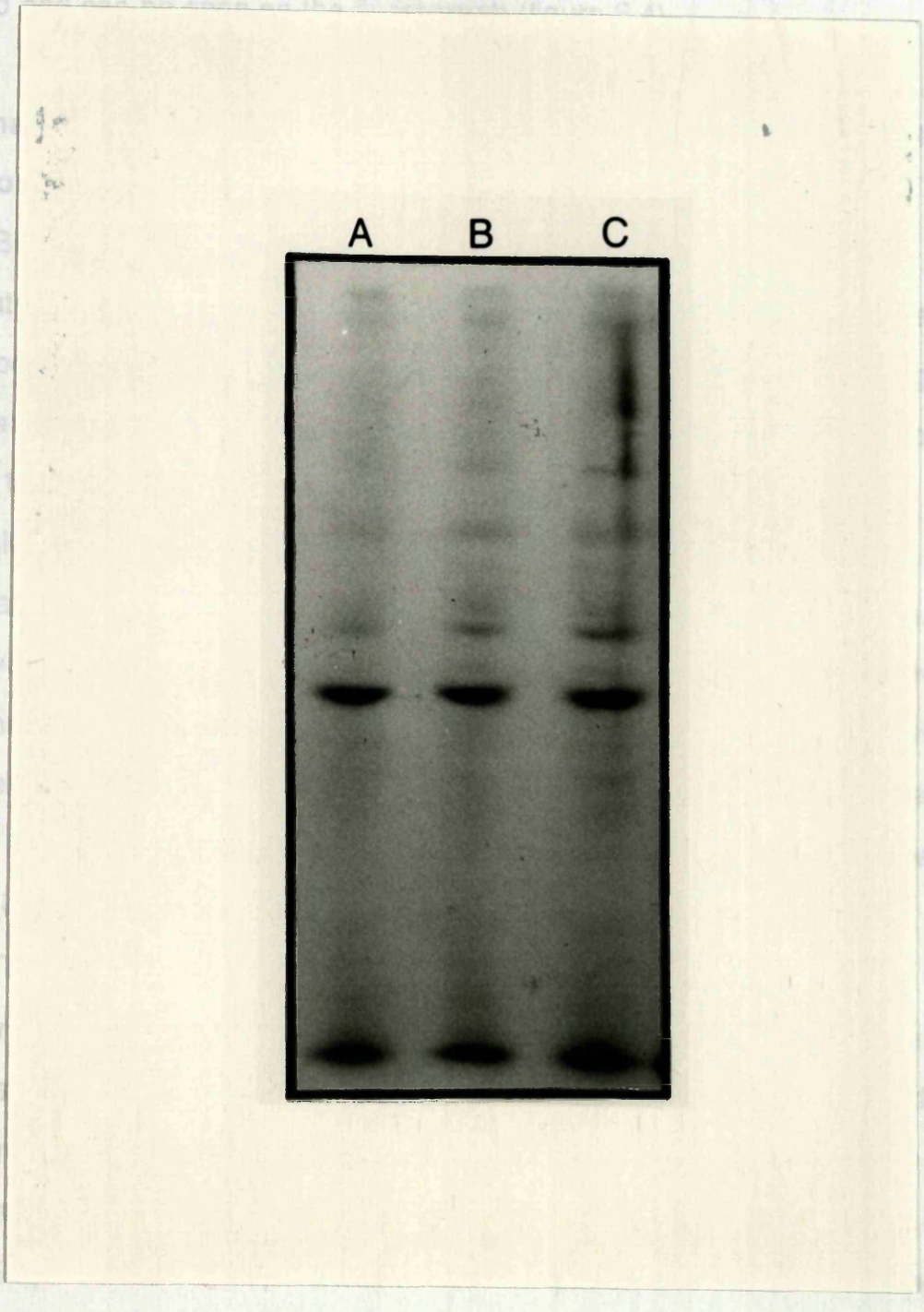
Figure 5.4 Immunoprecipitation of ^{35}S-methionine-labelled proteins of adult schistosomes with monoclonals anti-30-38, and polyclonal anti-32, schistosome antigens.

Adult worms were labelled with 20 µCi / ml ^{35}S-methionine. They were homogenized in 0.5% Nonidet P40 and the proteins were immunoprecipitated as described in Materials and Methods.

Lanes A : monoclonal anti-30 kDa antigen

B : monoclonal anti-38 kDa antigen

C : irrelevant monoclonal antibody.


A
B
C

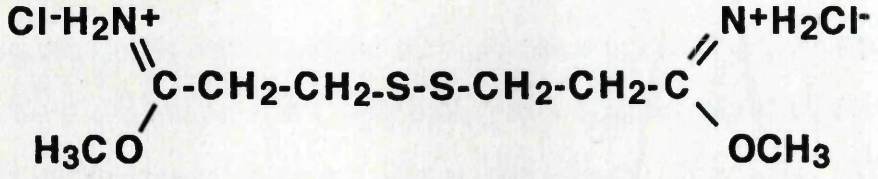
not reactive towards the antigens. If (b) was the case, then the 32 kDa M_r protein associating with HSP70 must be different from the one that should react with polyclonal anti-32 kDa antibody because the one that associates with HSP70 is labelled and can be seen on the fluorograph (figure 5.4).

5.3 Analyzing HSP70-complexes by chemically cross-linking adult schistosome proteins

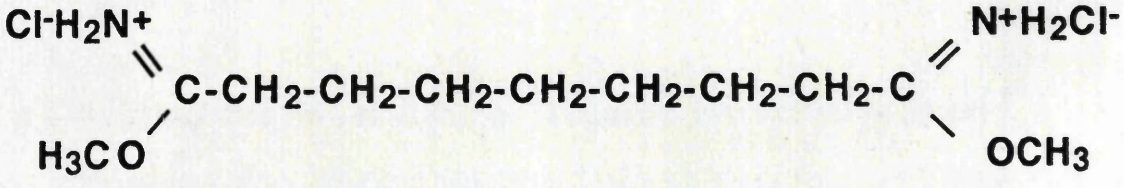
Bifunctional cross-linking agents which can chemically link closely associated amino groups have been used to investigate the spatial arrangement of components in multisubunit enzymes (Lumsden and Coggins, 1977; Coggins, Lumsden and Malcom, 1977), in complex structures such as ribosomes (Cover *et al.*, 1981), in membrane systems (Takemoto and Hansen, 1981), in viruses (Pepinsky *et al.*,1980), in actin-myosin interactions in rabbit muscle (Labbe' *et al.*, 1982), and in cross-linking HSP90 with v-erb A oncogene (Privalsky, 1991). The non-covalent associations which stabilize such structure are normally destroyed when these multicomponent aggregates are solubilized in a denaturing solvent. However, covalent cross-linking prevents the dissociation of opposed proteins and gel filtration or electrophoresis procedures can then be used to analyze for near neighbors in a complex.

The homobifunctional bis(imido esters), DMS and DTBP (figure 5.5) were used for the cross-linking experiments carried out here. DMS is non-cleavable whereas DTBP is its cleavable analogue. DTBP contains a disulphide bond that can be reduced by a mild reducing agent. DTBP and DMS react with lysine residues at pH's above 8 to form amidines. The mechanism of the reaction (figure 5.6) involves nucleophilic attack of the unprotonated imido ester. The rate of reaction is therefore critically dependent on pH.

Figure 5.5



Dimethyl 3,3'-dithiobispropionimidate (DTBP)



Dimethyl Suberimidate-2 HCl (DMS)

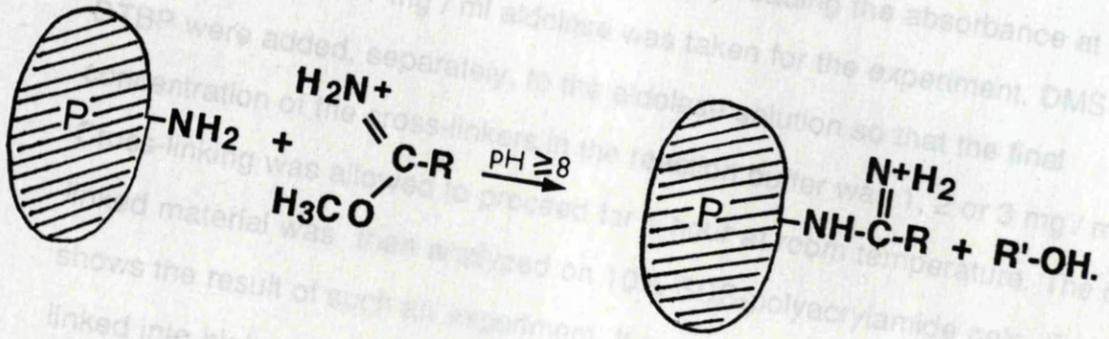


Figure 5.6 The reaction of protein amino groups with imido esters.

5.3.1 Testing the cross-linking reagents on a standard protein

To test if the cross-linking reagents were functional and that the cleavage of the cross-links formed by DTBP can be attained, a standard protein which has been shown to cross-link with bis(imido esters) was employed to perform the test. Aldolase can be cross-linked with DMS to give four species of molecular weights corresponding to the monomer, dimer, trimer and tetramer (Coggins, 1978).

1 mg of aldolase was dissolved in 1 ml of 50 mM TEA buffer. The exact concentration of aldolase was determined by reading the absorbance at 280 nm. A concentration of 1 mg / ml aldolase was taken for the experiment. DMS and DTBP were added, separately, to the aldolase solution so that the final concentration of the cross-linkers in the reaction buffer was 1, 2 or 3 mg / ml. Cross-linking was allowed to proceed for 1 hour at room temperature. The cross-linked material was then analyzed on 10% SDS-polyacrylamide gels. Figure 5.7 shows the result of such an experiment. It can be seen that aldolase was cross-linked into high molecular weight species.

5.3.2 Testing the cross-linking reagents on schistosomes

In cross-linking studies performed with intact human MH2 cells, cells were made permeable to the cross-linking reagents DTBP and DMS by incubating them in a hypotonic buffer to which the cross-linkers were added (Gillespie and Eisenman, 1989). A technical obstacle was thought to hinder the ability of cross-linking proteins of intact schistosomes and that is the membrane of the parasite. It is known that the parasite membrane is a double bilayer (McLaren and Hockley, 1977) and might not permeate the cross-linking reagents. Nevertheless, the protocol used by Gillespie and Eisenman (1989) was tried with the schistosomes and whether cross-linking occurred or not was looked at by reducing and non-reducing gel electrophoresis.

Adult worms were treated with 1 mg / ml DTBP or DMS for 1 hour at room temperature as described in Materials and Methods. Samples were washed to

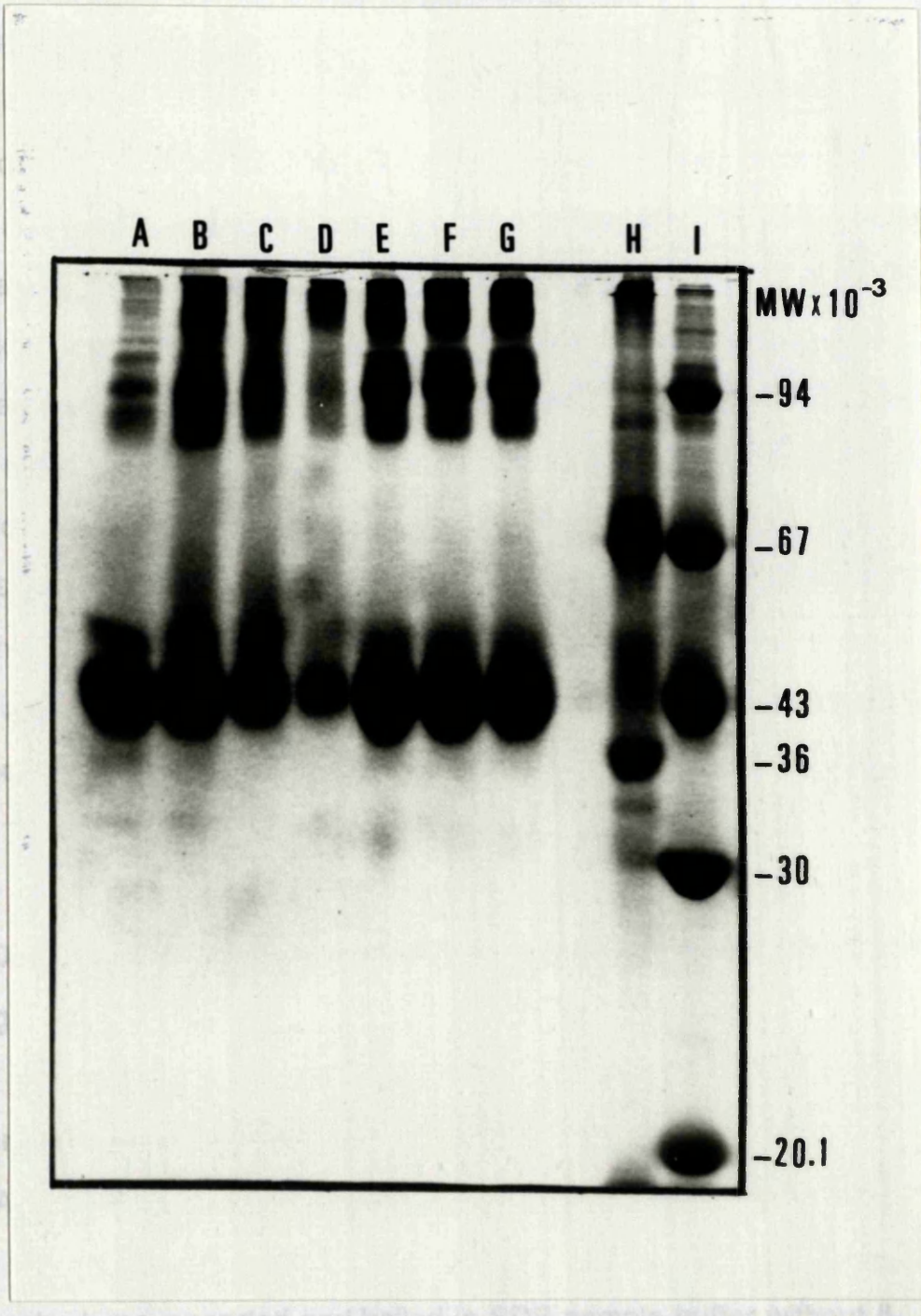
Figure 5.7 10% SDS-polyacrylamide gel of aldolase after treatment with the cross-linking reagents DTBP and DMS for 1 hour at room temperature in 50 mM TEA buffer pH 8.0.

Lanes A : Control (no cross-linker added).

B, C and D : 1, 2 and 3 mg / ml DTBP, respectively.

E, F and G : 1, 2 and 3 mg / ml DMS, respectively.

H, I : molecular weight markers of which the molecular weight is shown.


A
B
C
D
E
F
G
H
I
MW x 10^{-3}
-94
-67
-43
-36
-30
-20.1

remove excess cross-linker and the worms were solubilized in 0.5% Nonidet P40 in TEA buffer. One half of the Nonidet P40-soluble and insoluble material was boiled in SDS sample buffer containing β-mercaptoethanol (reduced samples), and the other half was boiled in SDS sample buffer without β-mercaptoethanol (non-reduced samples). The non-reduced samples were analyzed on a 7.5% (w / v) SDS-polyacrylamide gel and the reduced ones on a 10% (w / v) SDS-polyacrylamide gel. The result is shown in figure 5.8. It can be seen that the attempt to cross-link schistosome proteins was successful. Figure 5.8 a shows that after cross-linking with DTBP and DMS the pattern of fractionated proteins changes from discrete bands in the non-cross-linked sample (lane A) to an unresolved smear and a high molecular weight material which does not enter the resolving polyacrylamide gel, in the cross-linked samples (lanes B and C). After reduction with β-mercaptoethanol the material cross-linked with DTBP was resolved into separate bands in both Nonidet P40-soluble and insoluble fractions (figure 5.8 b). The material cross-linked with DMS, however, was not resolved because DMS is non-cleavable (lanes C and G).

5.3.3 Detection of HSP70-associated proteins by chemical cross-linking

5.3.3.1 Establishing the concentration of DTBP and DMS to be used with cross-linking studies on HSP70

Adult worms were treated with 1, 5, and 10 mg / ml DTBP or DMS for 1 hour at room temperature as described in Materials and Methods. After that they were washed, resuspended and boiled in SDS sample buffer without β-mercaptoethanol. The sample was run on a 7.5% (w / v) polyacrylamide gel. After electrophoresis the gel was blotted onto nitrocellulose and probed with anti-MS2-HSP70 antiserum. The result is shown in figure 5.9 in which it is seen that when DTBP and DMS were used at 1 mg / ml concentration HSP70 was cross-

Figure 5.8 SDS-polyacrylamide gels of schistosome proteins after treatment with the cross-linking reagents DTBP and DMS.

Groups of 6 adult worms were resuspended in 950 µl TEA buffer. 50 µl of 20 mg / ml of either DTBP or DMS stocks were added to give a final concentration of 1 mg / ml cross-linker. Cross-linking was allowed to proceed for 1 hour at room temperature after which the worms were washed and homogenized in 0.5% Nonidet P40 in TEA buffer. The Nonidet P40-soluble and insoluble fractions were either reduced (by boiling in SDS-sample buffer containing β-mercaptoethanol), or left unreduced (by boiling in SDS-sample buffer without β-mercaptoethanol). The non-reduced samples were analyzed on a 7.5% (w/v) SDS-polyacrylamide gel while the reduced ones were analyzed on a 10% (w/v) polyacrylamide gel.

Lanes M : molecular weight markers of which the molecular weight is shown.

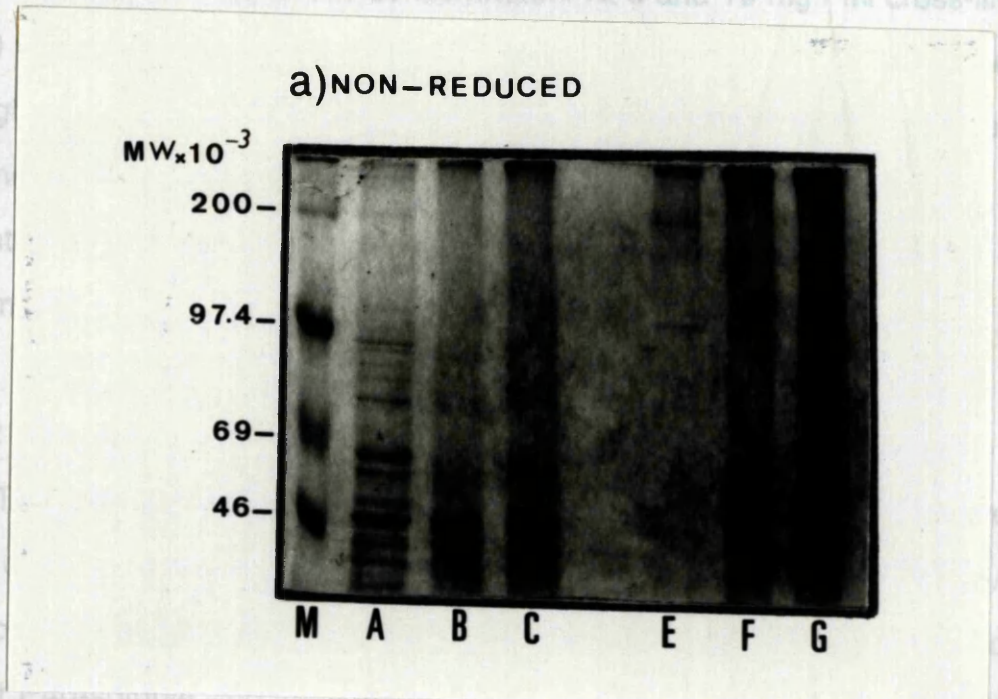
A and E : No cross-linker added.

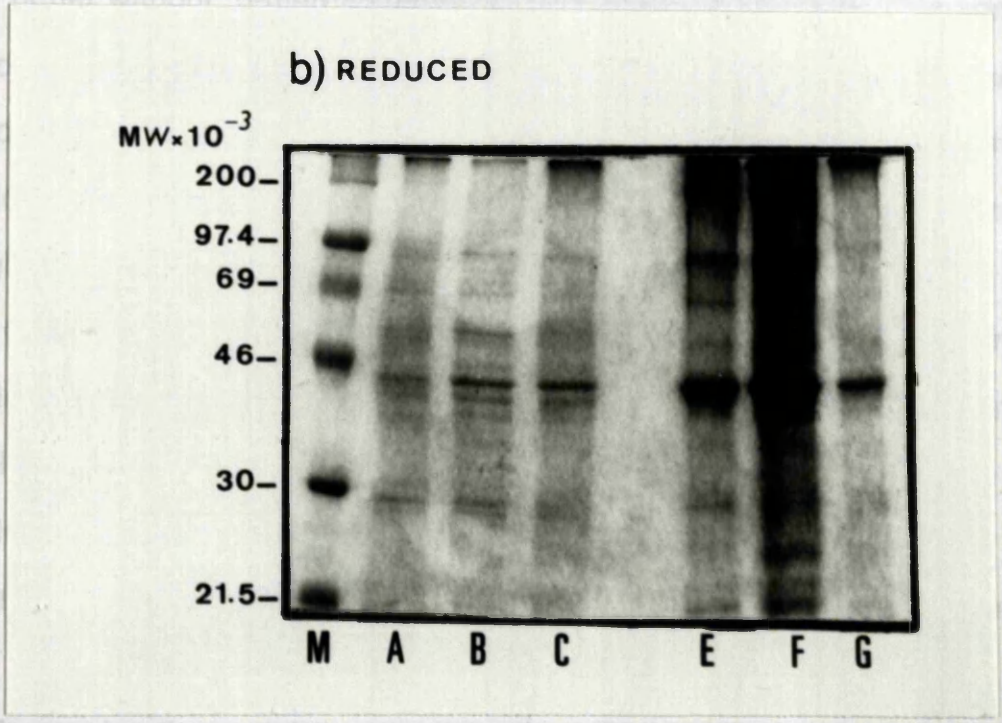
B and F : DTBP added.

C and G : DMS added.

A-C : Nonidet P40-soluble extract.

E-G : Nonidet P40-insoluble extract.


a) NON-REDUCED
MW x 10^-3
200
97.4
69
46
M A B C E F G


b) REDUCED
MW x 10^-3
200
97.4
69
46
30
21.5
M A B C E F G

linked to give higher molecular weight species. However, a large fraction of it remained uncross-linked at this concentration. At 5 and 10 mg / ml cross-linkers, HSP70 was so extremely cross-linked that little or no free HSP70 was observed. The high molecular weight material resulting from cross-linking did not even enter the stacking gel and thus it is not seen on the blot. Therefore, a concentration of 1 mg / ml cross-linkers were used in all the subsequent experiments.

5.3.3.2 Resolving the cross-linked HSP70-protein complexes

To confirm that the cross-links formed between HSP70 and other proteins can be cleaved (when DTBP is used), adult worms were treated with 1 mg / ml DTBP or DMS as above. The worms were then washed, homogenized, and the Nonidet P40-soluble and insoluble fractions were collected as described in Materials and Methods. One half of each of the Nonidet P40-soluble and insoluble fractions were boiled in SDS-sample buffer containing 5% (w / v) β-mercaptoethanol (reduced samples) and the other half was boiled in SDS-sample buffer without β-mercaptoethanol (non-reduced samples). The non-reduced samples were loaded on a 7.5% (w / v) polyacrylamide gel and the reduced ones on a 10% (w / v) polyacrylamide gel. After electrophoresis, the gels were blotted onto nitrocellulose and decorated with anti-MS2-HSP70 antiserum. The high molecular weight species formed with DTBP (figure 5.10 a, lanes B and E) was cleaved with β-mercaptoethanol in both the Nonidet P40-soluble and insoluble fractions (figure 5.10 b, lanes B and E) except a very small amount of it was still present in the NP40-soluble fraction. This might have been due to incomplete reduction of the cross-link. The material cross-linked with DMS was not cleaved, as expected, because DMS is non-cleavable (figure 5.10, lanes C and F).

To resolve the proteins cross-linked to HSP70, ^{35}S-methionine-labelled adult worms were treated with cross-linkers as above. The proteins were then

Figure 5.9 Immunoblot of adult schistosome proteins treated with different concentrations of the cross-linking reagents DTBP and DMS, with anti-MS2-HSP70 antiserum.

Groups of 10 adult worms were resuspended in 1 ml of 50 mM TEA buffer. DTBP and DMS were added from fresh stock solutions to give a final concentration of 1, 5 and 10 mg / ml. Cross-linking was allowed to proceed for 1 hour at room temperature after which the worms were washed , resuspended in SDS-sample buffer without β-mercaptoethanol, boiled and analyzed on a 7.5% (w/v) polyacrylamide gel.

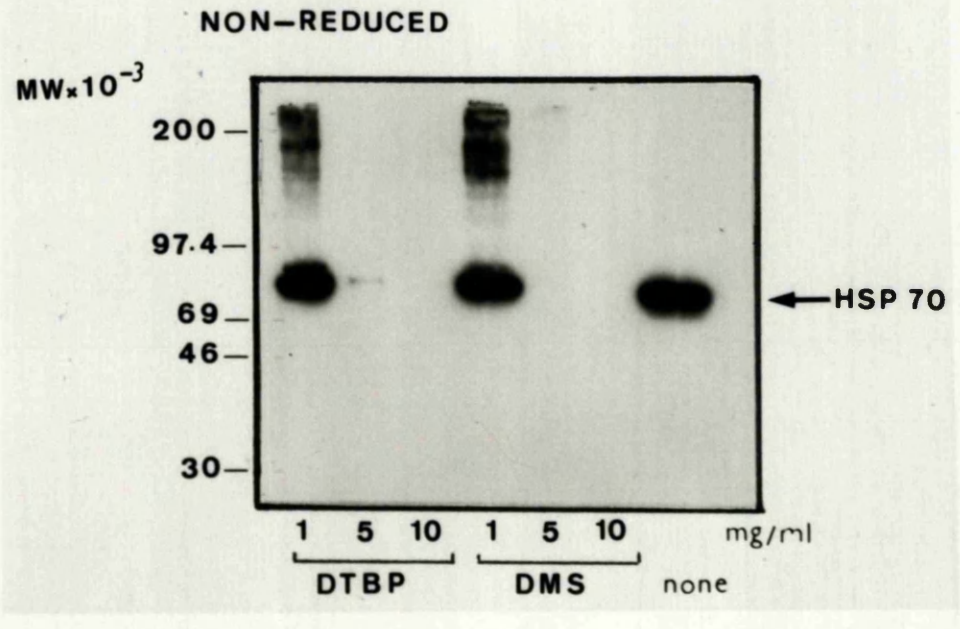

NON–REDUCED
MW×10⁻³
200
97.4
69
46
30
HSP 70
1 5 10 1 5 10
mg/ml
DTBP
DMS
none

Figure 5.10 Immunoblot of cross-linked schistosome proteins before and after reduction with β-mercaptoethanol, with anti-MS2-HSP70 antiserum.

Groups of 6 adult male worms were resuspended in 950 µl TEA buffer. 50 µl of 20 mg / ml of either DTBP or DMS stocks were added to give a final concentration of 1 mg/ ml cross-linker.Cross-linking was allowed to proceed for 1 hour at room temperature after which the worms were washed to remove excess cross-linker. The worms were then homogenized in 100 µl 0.5% Nonidet P40 in TEA buffer and the Nonidet P40-soluble and insoluble fractions were collected. One half of each of the fractions were boiled in SDS-sample buffer containing β-mercaptoethanol (reduced sample) and the other half was boiled in SDS-sample buffer without β-mercaptoethanol (non-reduced sample). The non-reduced samples were analyzed on a 7.5% (w/v) SDS-polyacrylamide gel while the reduced ones were analyzed on a 10% (w/v) polyacrylamide gel.

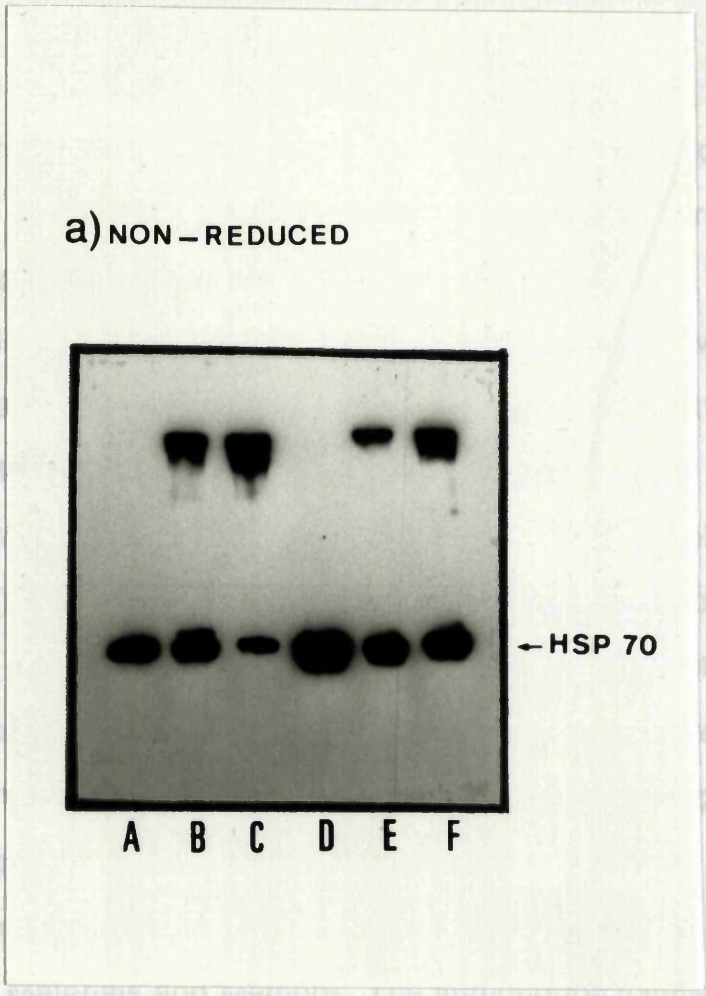
Lanes A and D : no cross-linker added (Control sample).

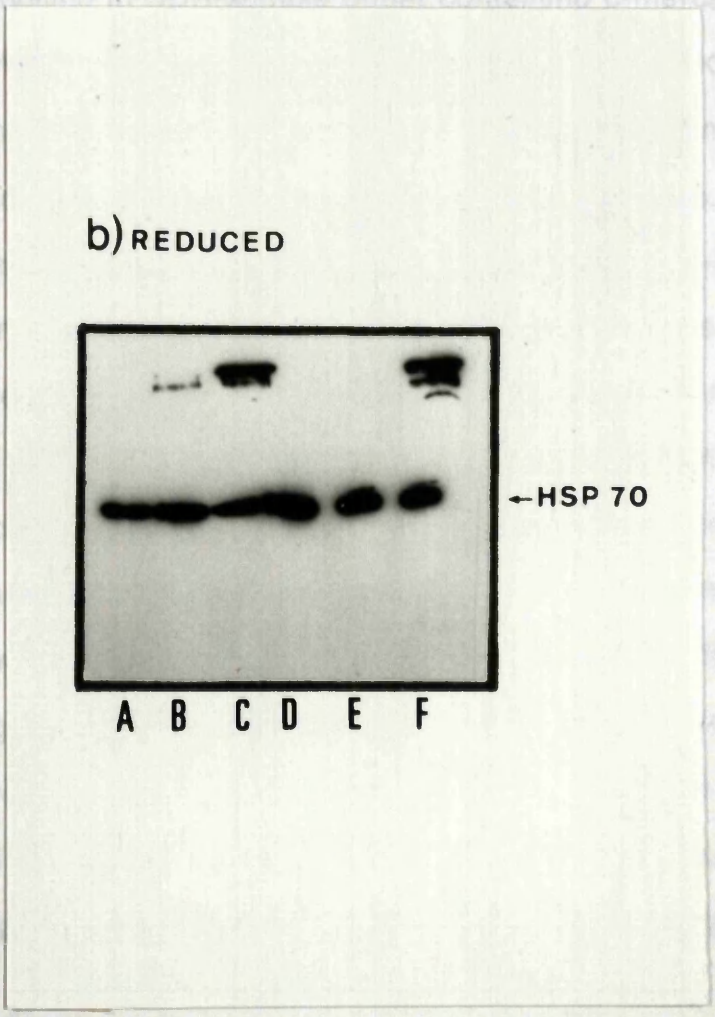
B and E : DTBP added.

C and F : DMS added.

A-C : Nonidet P40-insoluble fraction.

D-F : Nonidet P40-soluble fraction.


a) NON – REDUCED
←HSP 70
A B C D E F


b) REDUCED
←HSP 70
A B C D E F

solubilized, the amount of radioactivity incorporated in the Nonidet P40-soluble material was determined, and the proteins were prepared for immunoprecipitation as described in Materials and Methods. The amount of radioactivity incorporated into Nonidet P40-soluble material was shown to decrease after cross-linking (figure 5.11, lanes A-C). When the Nonidet P40-insoluble material was looked at (figure 5.11, lanes D-F), the amount of radioactivity was shown to increase in the cross-linked samples when DTBP was used (lane E). This might be due to either cross-linking of proteins to the cytoskeleton which then cannot be solubilized by Nonidet P40 and thus remain in the non-soluble fraction, or / and to cross-linking of soluble proteins in a way that they form very high molecular weight complexes which precipitate when the worm homogenate is clarified by centrifugation.

Immunoprecipitation with anti-MS2-HSP70 antiserum was carried out as described in Materials and Methods. The immunoprecipitated material was either reduced by boiling in SDS-sample buffer containing β-mercaptoethanol to cleave the cross-linker, or boiled in SDS-sample buffer without β-mercaptoethanol (non-reduced sample). The reduced and non-reduced samples were analyzed on 10% (w / v) polyacrylamide gels. Figure 5.12 shows the result of such an experiment. In the absence of cross-linking, anti-MS2-HSP70 antiserum immunoprecipitated newly synthesized HSP70 as well as a very high molecular weight complex which remained at the top of the resolving gel (figure 5.12 a, lane A). After cross-linking the high molecular weight complex was again immunoprecipitated but there was very little free HSP70 precipitated (lanes, B and C). When the high molecular weight cross-linked and non-cross-linked complexes were resolved on the reducing gel, it was found that in the absence of cross-linking the complex resolved into free HSP70 in addition to proteins of molecular weight >200, 62, 54, 52.5 kDa (figure 5,12 b, lane A). The complex cross-linked with DTBP was resolved into proteins of molecular weight 62, 58, 54, 52.5 kDa. However, there was still a high molecular weight compound

Figure 5.11 The amount of radioactivity incorporated in NP40-soluble and insoluble material from worms treated with the cross-linkers DTBP and DMS.

Worms were labelled overnight with 50 µCi / ml ^{35}S-methionine in a methionine-free medium. After that they were cross-linked and homogenized as described for figure 5.10. The amount of radioactivity incorporated in TCA-precipitable material from the Nonidet P40-soluble fraction was determined as described in Materials and Methods. The Nonidet P40-insoluble fraction was boiled in SDS-sample buffer and counted in a scintillation counter.

Lanes A and D : No cross-linker present.
B and E : DTBP present.
C and F : DMS present.
A-C : Nonidet P40-soluble fraction.
D-F : Nonidet P40-insoluble fraction.

The experiment was done in duplicate. The result shown is an average of the two.

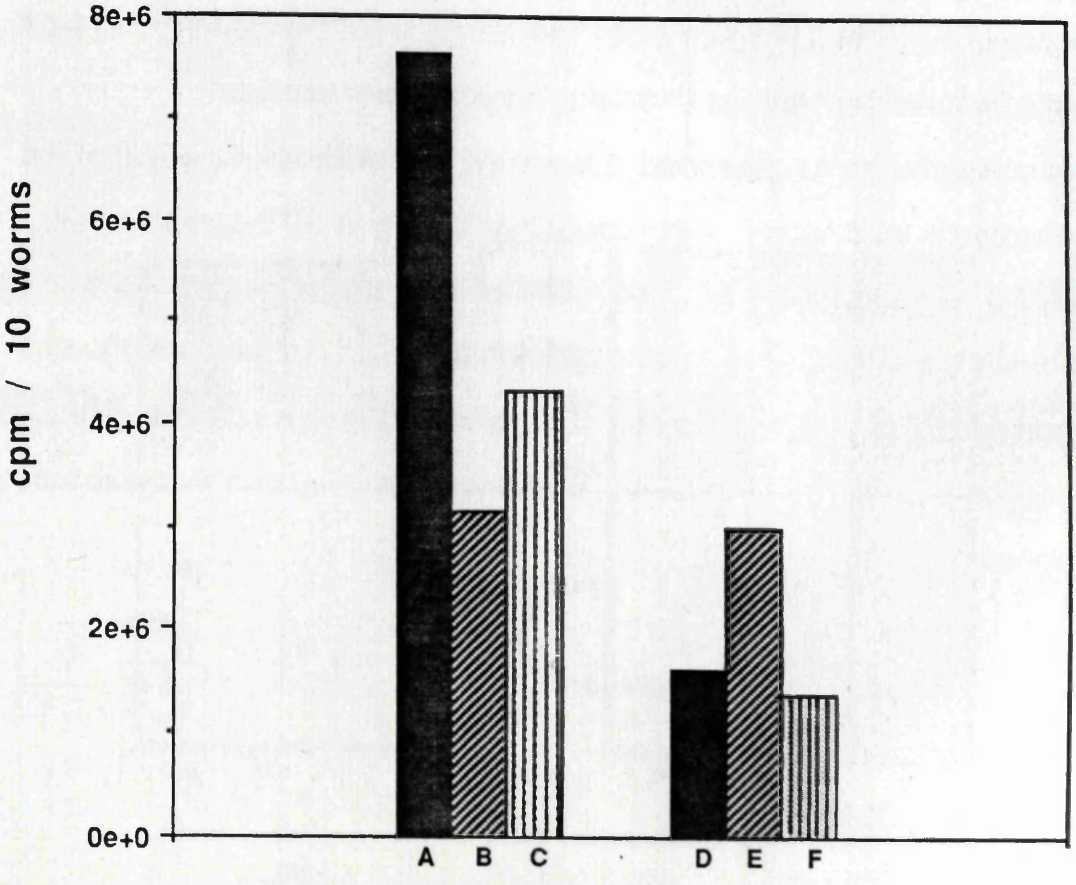
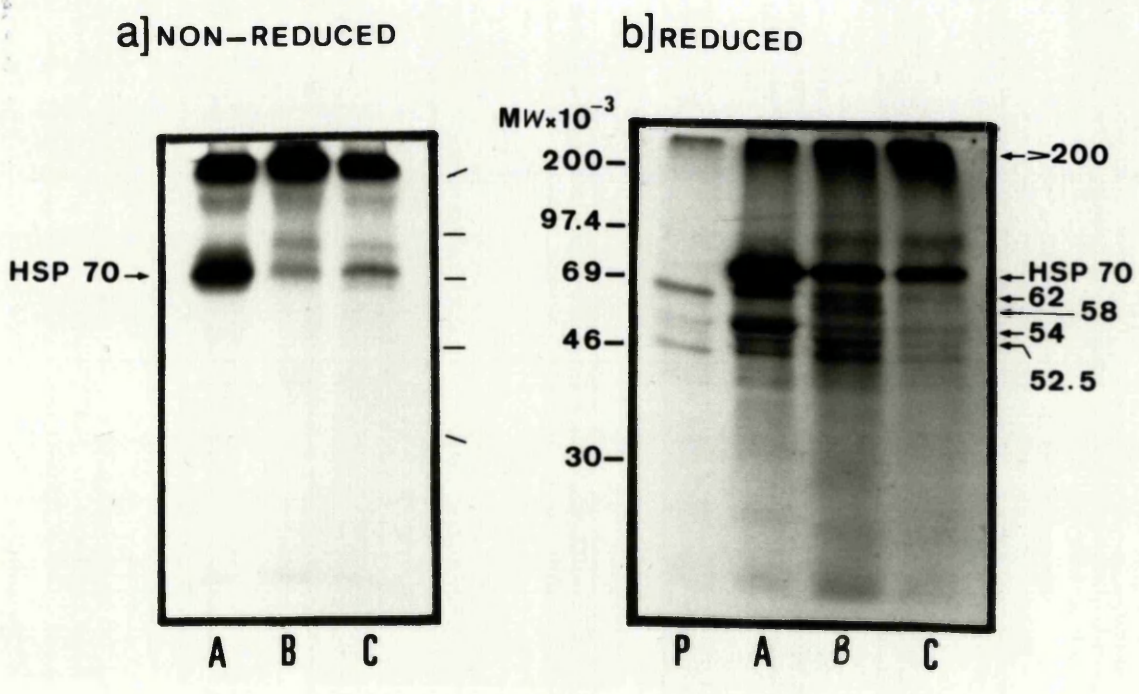

8e+6
6e+6
4e+6
2e+6
0e+0
cpm / 10 worms
A
B
C
D
E
F

Figure 5.12 Immunoprecipitation of cross-linked adult schistosome proteins before and after reduction with β-mercaptoethanol, with anti-MS2-HSP70 antiserum.

Worms were labelled overnight with 50 μCi / ml of ^{35}S-methionine in a methionine-free medium. After that they were treated with the cross-linking reagents as described in figure 5.10. The worms were homogenized in 0.5% Nonidet P40 and the Nonidet P40-soluble extract was used for immunoprecipitation as described in Materials and Methods. One half of the immunoprecipitated sample was reduced by boiling in SDS-sample buffer containing β-mercaptoethanol. The other half was left unreduced and was boiled in SDS-sample buffer without β-mercaptoethanol. The reduced and the non-reduced samples were analyzed on 10% (w/v) SDS-polyacrylamide gels. After electrophoresis, the gel was stained, destained and dried and then subjected to fluorography.

Lanes A : no cross-linker present
B : DTBP present
C : DMS present
P: immunoprecipitation with pre-immune serum
A-C : immunoprecipitation with anti-MS2-HSP70 antiserum.


a] NON-REDUCED
b] REDUCED
HSP 70
MW×10⁻³
200
97.4
69
46
30
>200
HSP 70
62
58
54
52.5
A B C
P A B C

present at the top of the resolving gel. The complex cross-linked with DMS was not resolved because DMS is non-cleavable. However, there was HSP70 as well as some faint bands corresponding to molecular weight of 62, 54, 52.2 kDa present.

5.4 Pulse-chase experiments with HSP70

In order to determine the turnover rate of HSP70 and other proteins associating with it pulse-chase experiments were performed.

5.4.1 *In vitro* pulse-chase experiment

Adult worms were pulsed with ^{35}S-methionine for 30 minutes and then chased for various periods of time in the presence of excess unlabeled methionine. HSP70 was then immunoprecipitated from equivalent amounts of TCA-precipitable counts, or from equivalent amounts of protein for each time point. Representative fluorographs are presented in figure 5.13. It is seen that anti-MS2-HSP70 antiserum immunoprecipitated HSP70 in addition to two other labelled bands, one of molecular weight >200 kDa and the other was a very high molecular weight compound (HMC) that did not enter the resolving gel. HSP70 and the high molecular weight compound continued to be precipitated throughout the chase period (figure 5.13 a). The M_r >200 kDa protein became coprecipitated only after a lag phase of 30 minutes and then continued to be precipitated throughout the chase period, although the amount of label incorporated by it decreased after 60 minutes chase period. To compare, proteins from the pulse-chased parasites were also analyzed on SDS-gels (figure 5.13 b). It is seen that the label incorporated into parasite proteins decreases by a small amount after 4 hour chase period. It is also seen that generally, all the parasite proteins behave in the same way.

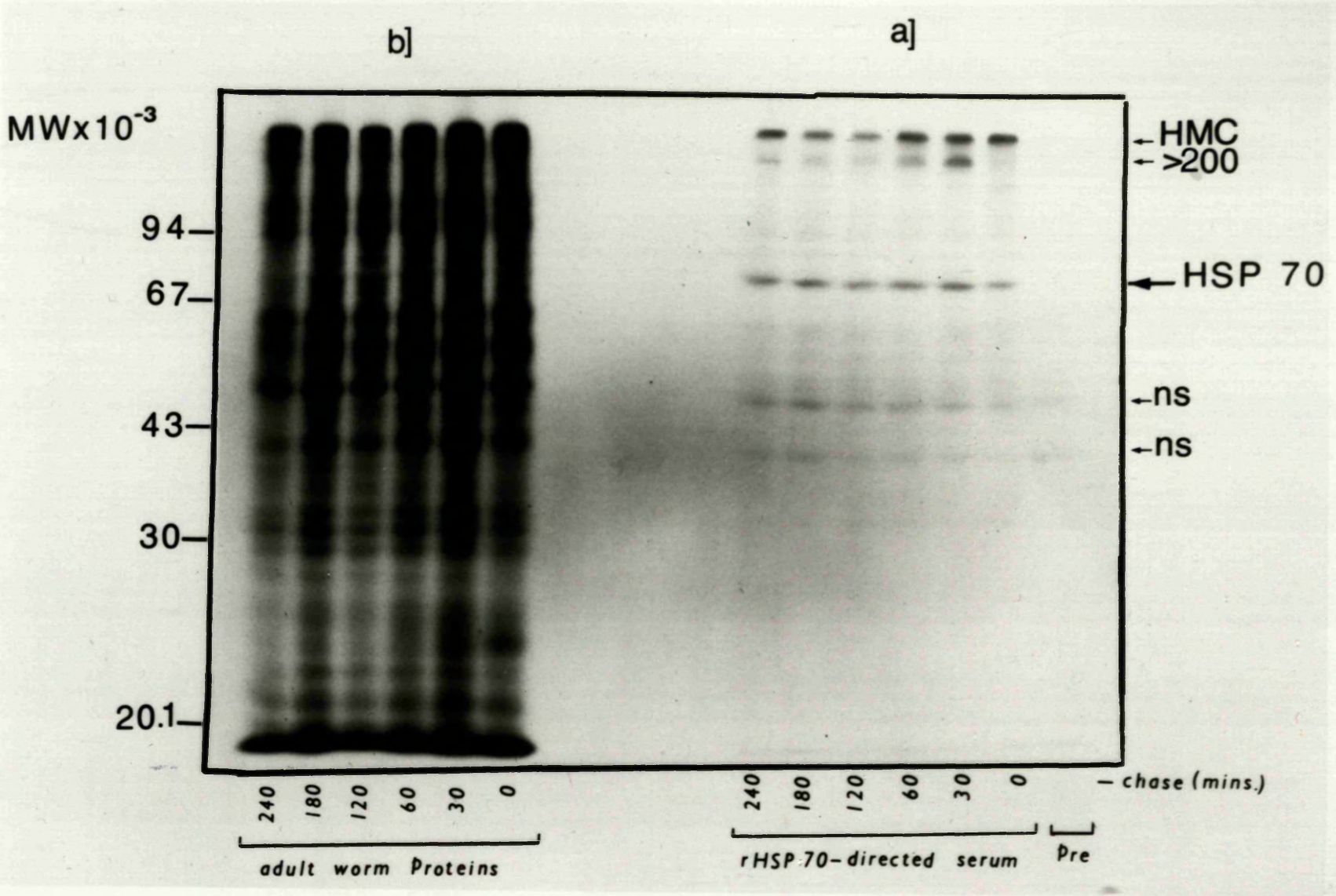
In another experiment in which the chase time was extended to 25 hours (figure 5.13 c) HSP70 and the high molecular weight compound (HMC)

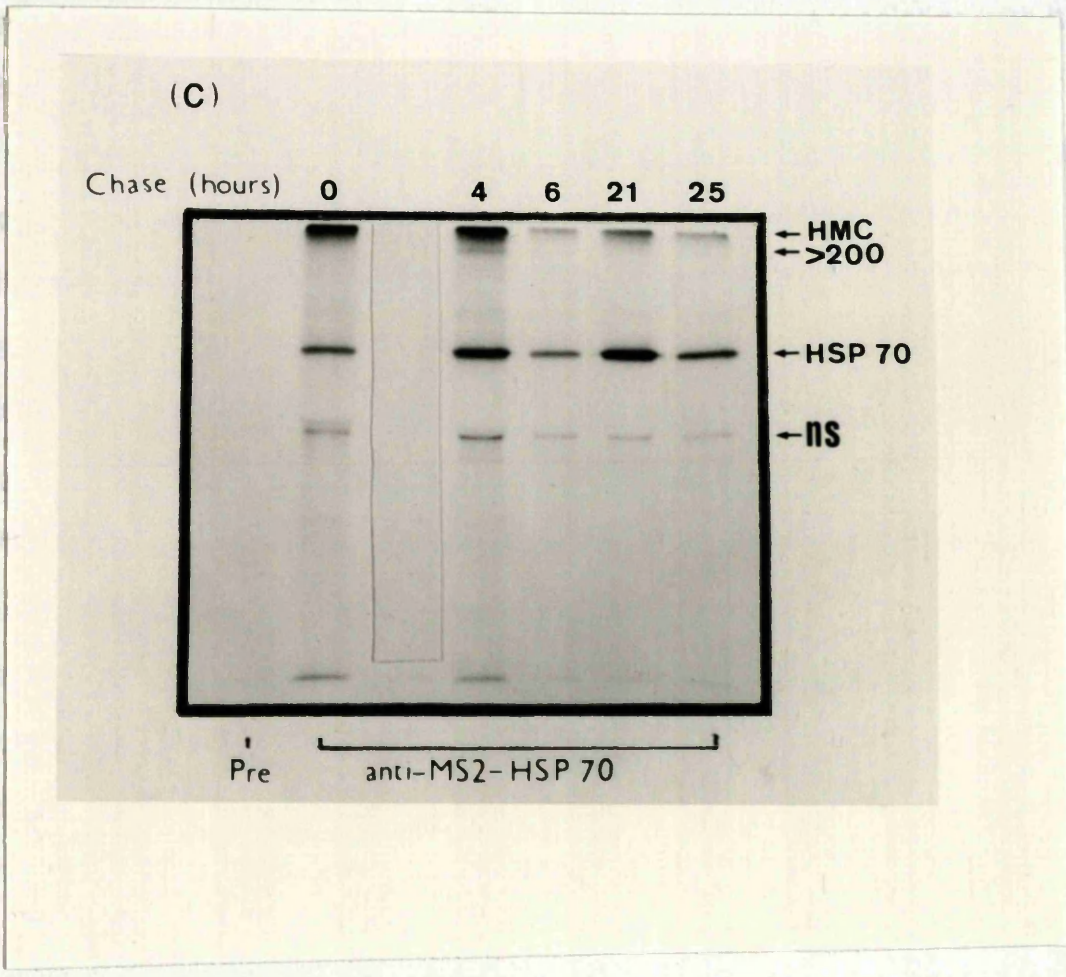
Figure 5.13 Pulse-chase analysis of HSP70 and proteins associating with it.

(a) Groups of 10 adults worms were pulsed for 30 minutes with 23 μCi / ml of ^{35}S-methionine and then chased as described in Materials and Methods. Chase times were for 0, 30, 60, 120, 180 and 240 minutes. Equivalent amounts of proteins from each time point were immunoprecipitated with anti-MS2-HSP70 antiserum. Proteins from the unchased (0) time point were also immunoprecipitated with pre-immune rabbit serum (pre). Molecular size markers are indicated (in kilodaltons).

(b) Adult worms were pulse-chased as above and then solubilized in 0.5% Nonidet P40. Equivalent amounts of proteins from the Nonidet P40-soluble extract from each time point were analyzed by SDS-PAGE. After electrophoresis, the gel was stained, destained and dried and then subjected to fluorography.

(c) Adult worms were pulse-labelled and chased for 0, 4,6,21 and 25 hours. Equivalent amounts of TCA-insoluble radioactivity (4.6×10^5) from each time point were immunoprecipitated with anti-MS2-HSP70 antiserum. Proteins from the unchased (0) time point were also immunoprecipitated with pre-immune rabbit serum (pre). ns, non-specific protein band. HMC, high molecular weight compound.


b]
a]
MWx10-3
94
67
43
30
20.1
HMC
>200
HSP 70
ns
ns
240
180
120
60
30
0
240
180
120
60
30
0
– chase (mins.)
adult worm Proteins
rHSP 70-directed serum
Pre


(C)
Chase (hours)
0
4
6
21
25
HMC
>200
HSP 70
ns
Pre
anti-MS2-HSP 70

associated with it did not seem to turnover over this chase period either. However, M_r >200 kDa protein was not detected after 6 hour chase period.

5.4.2 *In vivo* pulse-chase experiments

As shown above, HSP70 had a very low rate of turnover *in vitro* which was perhaps caused by the conditions of culture. Therefore, it was desirable to learn about the rate of turnover of HSP70 and other proteins associating with it *in vivo*. 3 experiments were performed in which mice were injected with 0.1, 0.5 and 1 mCi of ^{35}S-methionine. Mice were perfused at 2, 4, 6, 8 and 24 hours after injection of the label. The amount of label incorporated into equal amounts of parasite, serum and liver proteins was determined by TCA-precipitation. The results are shown in figure 5.14. It is seen that when mice were injected with 0.1 mCi of ^{35}S-methionine, parasite proteins did not seem to turnover (figure 5.14 a). The same effect was seen on liver proteins. When mice were injected with 0.5 mCi ^{35}S-methionine, parasite proteins seemed to turnover with a half life of approximately 2-3 hours. Liver and serum proteins also seemed to turnover at the same rate (figure 5.14 b). When mice were injected with 1 mCi ^{35}S-methionine, the label incorporated into parasite, surprisingly, seemed to increase with chase period. That of liver and serum proteins did not behave in a uniform pattern (figure 5.14 c). The pattern of fractionated, labelled proteins is shown in figure 5.15 (a and b). The amount of label incorporated into parasite proteins was, unfortunately, not enough to perform immunoprecipitation of HSP70.

Thus it is seen that from the 3 experiments performed,3 different results were obtained. A concentration of 0.5 mCi ^{35}S-methionine seemed to be the optimum concentration to be injected so that a chase of label could be detected. 0.1 mCi was too little to detect whereas 1 mCi was so high that it persisted in the mouse serum and was not chased.

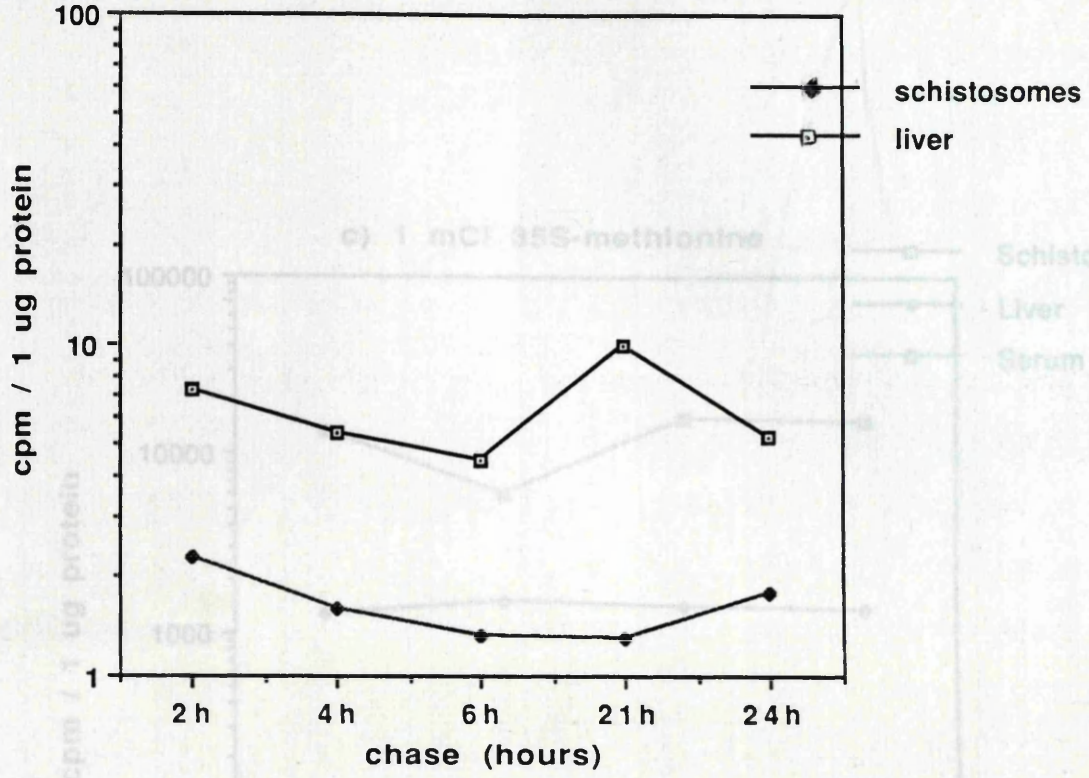
Figure 5.14 The amount of radioactivity incorporated into TCA-precipitable material from serum, liver and worms obtained from mice injected with different doses of ^{35}S-methionine.

(a) 10 mice were injected with 0.1 mCi ^{35}S-methionine. At 2, 4, 6, 21 and 24 hours after injection of the label mice were perfused and a sample of liver was taken for each time point. The amount of radioactivity incorporated into TCA-insoluble material was determined as described in Materials and Methods.

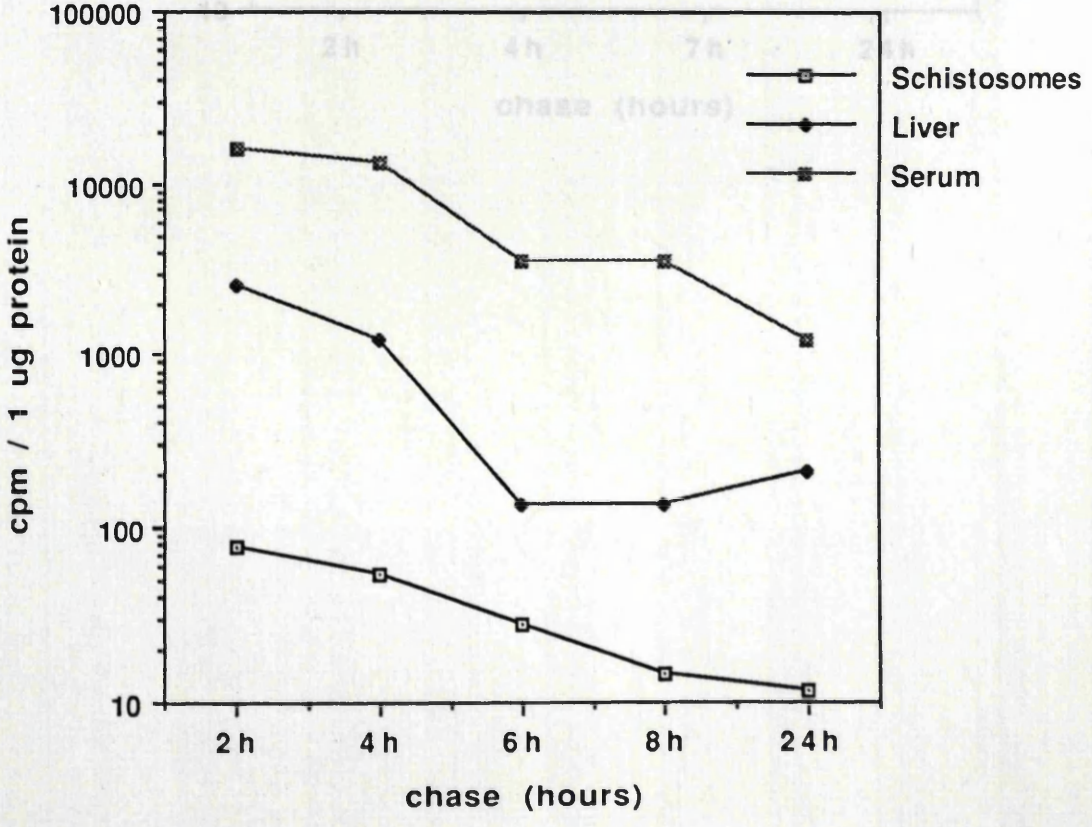
(b) Mice were injected with 0.5 mCi ^{35}S-methionine and then perfused at 2, 4, 6, 8 and 24 hours after injection of the label. Samples of liver and serum were taken for each time point and the amount of radioactivity was determined as before.

(c) Mice were injected with 1 mCi ^{35}S-methionine and then perfused at 2, 4, 7, and 24 hours after injection of the label. Samples of liver and serum were taken for each time point and the amount of radioactivity was determined as before.

a) 0.1 mCi 35S-methionine



b) 0.5 mCi 35S-methionine



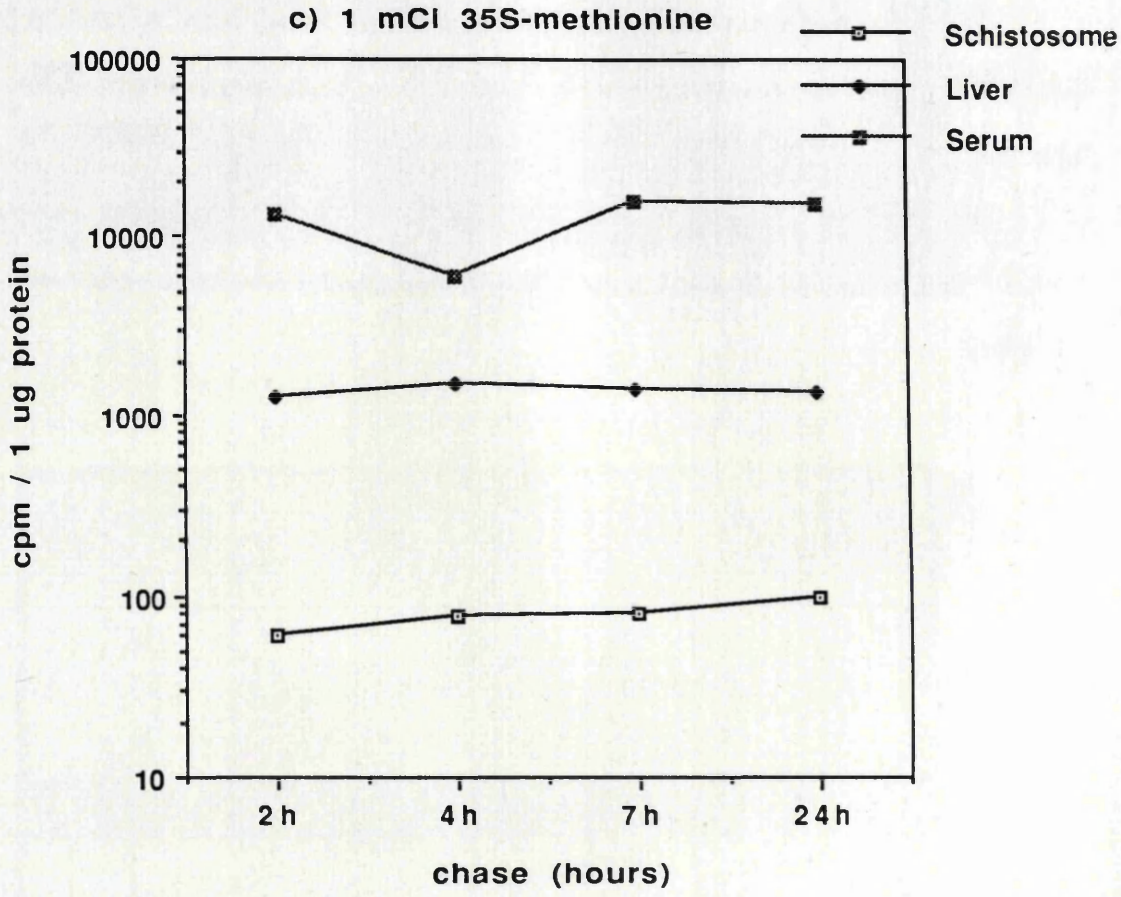

c) 1 mCi 35S-methionine
Schistosome
Liver
Serum
100000
10000
1000
100
10
cpm / 1 ug protein
2 h
4 h
7 h
24 h
chase (hours)

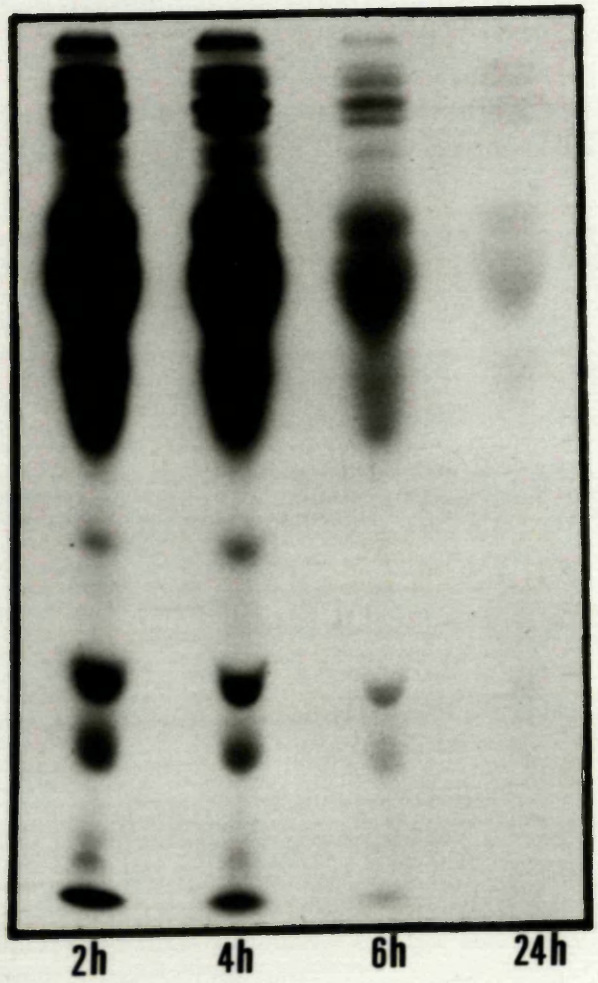
Figure 5.15 Fluorographs of *in vivo* pulse-chased, ^{35}S-methionine-labelled proteins from liver, serum and worms fractionated on 10% (w/v) SDS-polyacrylamide gels.

(a) Proteins from serum, liver and worms obtained from mice injected with 0.5 mCi ^{35}S-methionine and then perfused at 2, 4, 6, 8, and 24 hours after injection of the label.

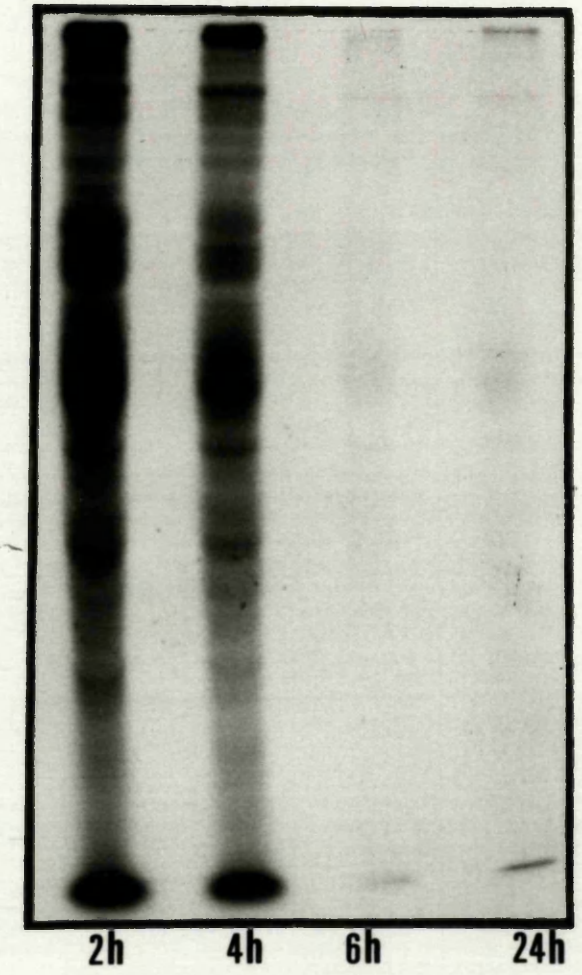
(b) Proteins from serum, liver and worms obtained from mice injected with 1 mCi ^{35}S-methionine and then perfused at 2, 4, 7, and 24 hours after injection of the label.

a]

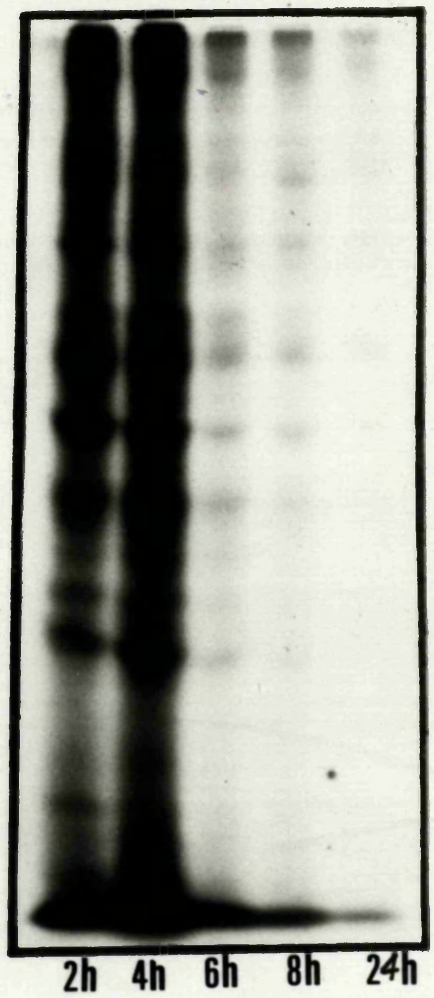
Serum



Liver

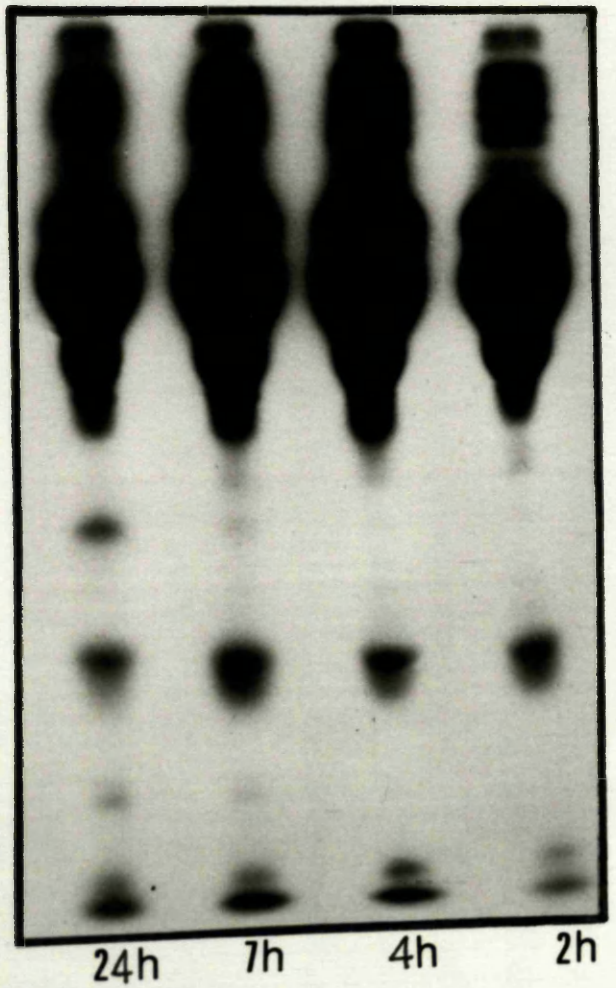


Schistosome

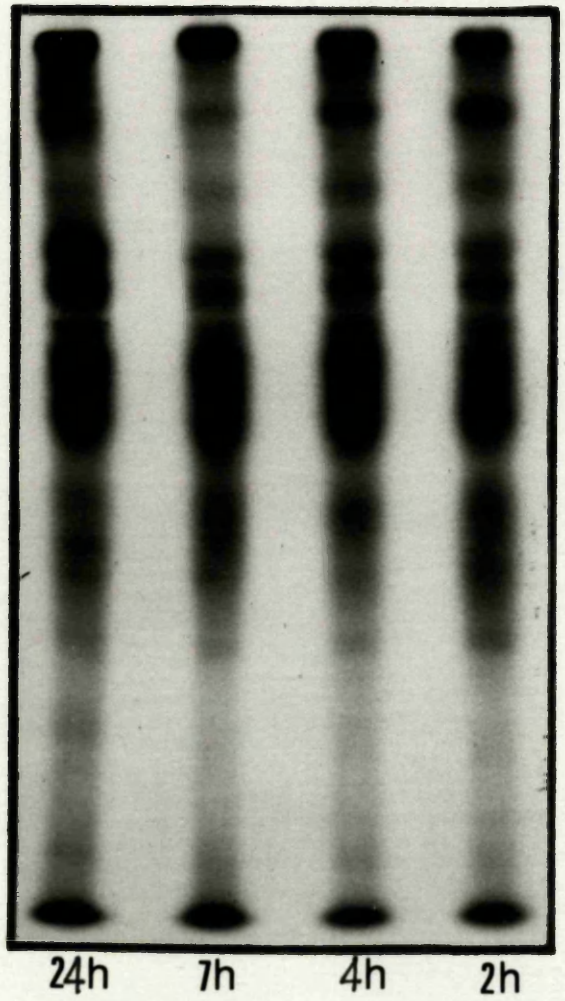


b]

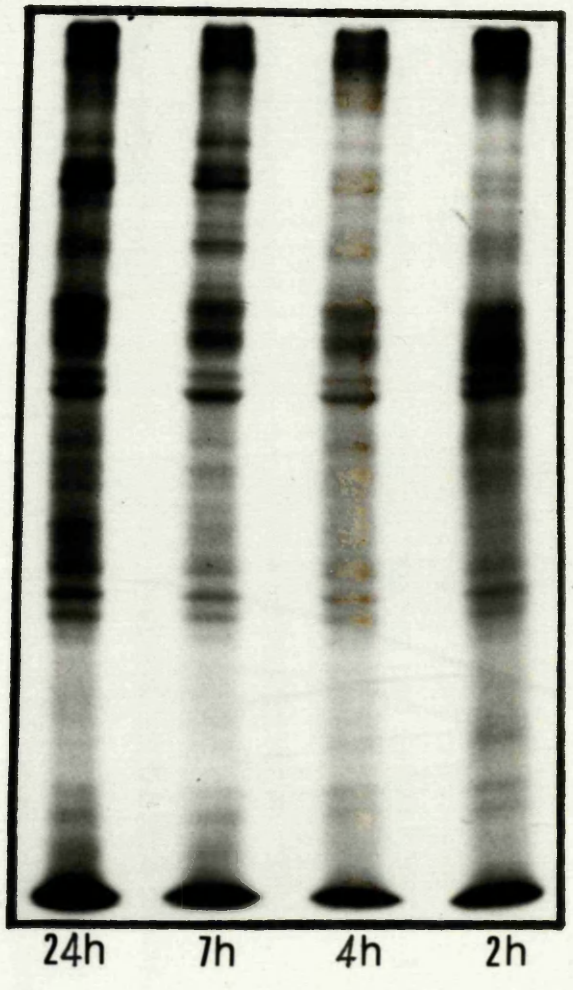
Serum



Liver



Schistosome



5.5 Discussion

Analysis of HSP70-complexes on non-denaturing and denaturing polyacrylamide gels revealed that HSP70 interacted with many newly synthesized polypeptides ranging in size from 30 to >100 kDa (figure 5.2). Proteins of defined size were associated along with a background smear of labelled material, perhaps representing unfinished nascent polypeptide chains. These results are consistent with those of Beckman, Mizzen and Welch (1990), who found that HSP70 of HeLa cells growing at 37^{0}C associated transiently with several newly synthesized proteins.

Interaction of HSP70 with other proteins was also examined by immunoprecipitation with anti-MS2-HSP70 antiserum of metabolically-labelled adult worm proteins which were chemically cross-linked . Non-reduced immunoprecipitates from control worms (ie worms which were not treated with cross-linkers) resolved into HSP70 and a high molecular weight complex on SDS-PAGE (figure 5.12 a, lane A). Those from the cross-linked worms resolved into a high molecular weight complex in addition to a very faint band representing HSP70 (figure 5.12 a, lanes B and C). Reducing the immunoprecipitates, however, resulted in resolving the high molecular weight complex from the control worms into proteins of M_r >200, 62, 54 and 52.5 kDa as well as HSP70 (figure 5.12 b, lane A) and from the cross-linked worms into proteins of M_r 62, 58, 54 and 52.5 kDa as well as HSP70 (figure 5.12 b, lane B). These results show that cross-linking did not significantly alter the pattern of co-precipitated proteins. Most of the co-precipitated proteins were present in both the control and the cross-linked samples, for example, the M_r 62, 54, 52.5 kDa as well as HSP70. The only difference between the two samples is the M_r 58 kDa protein which was precipitated from the DTBP-cross-linked sample. Thus, it seems that HSP70 exists in two forms in the parasite; one which is complexed with proteins of M_r >200, 62, 54 and 52.5 kDa, and the other which interacts with a M_r 58 kDa protein. This interaction, however, is not detected except after

chemical cross-linking of adult schistosome proteins.

The above results also show that HSP70 interacts with proteins in the high molecular weight complex (figure 5.12 a, lane A) by what seems to be disulphide bonds. This is somewhat unusual because HSP70 usually associates with other proteins by non-covelant, weak hydrophobic interactions (Flynn, Chappell and Rothman, 1989). However, it could be that HSP70 and the other molecules associating with it form non-specific disulphide bonds under non-reducing conditions. Indeed, if protein samples are not treated with β–mercaptoethanol before SDS-gel electrophoresis some cross-linked material is almost always observed, even if cross-linking reagents have not been used, owing to the non-specific formation of inter-chain disulphide bridges (Coggins, Hooper and Perham, 1976).

The HSP70-associated proteins detected by non-denaturing gel electrophoresis were slightly different from those detected by the method of immunoprecipitation. For example the M_r >200, 54 and 52.5 kDa proteins which were detected by immunoprecipitation, were not detected by non-denaturing gel electrophoresis. Since only the soluble proteins were analyzed on non-denaturing gels the proteins shown above might be membrane proteins which become solubilized only after detergent treatment of the worms and thus are not detected on non-denaturing gels. Other proteins, for example, the M_r >100, 49 and 30 kDa which were detected by non-denaturing gels, were not found by immunoprecipitation. These proteins could be associated with HSP70 by weak hydrophobic interactions which might have been disrupted when the detergent Nonidet P40 was used for solubilizing the parasite proteins or while using stringent washing procedures. The M_r 58 and 62 kDa proteins detected by immunoprecipitation could be the same as the M_r 59 and 65 kDa proteins detected by non-denaturing gel electrophoresis because of the similar molecular weight.

The identity of the proteins associating with HSP70 of the parasite is not known. The attempt to characterize one of them was unsuccessful (figures 5.3 and 5.4). Possible candidates for these proteins could be : the 200 kDa membrane protein of *S. mansoni* which was shown to be anchored to the adult worm membrane via a glycosylphosphatidylinositol (GPI) linkage (Sauma and Strand, 1990) ; glutathione S-transferase (GST) which is an intracellular soluble protein of M_r 28 kDa (Taylor *et al.*, 1988) ; Cathepsin B and Haemoglobinase of *S. mansoni* with apparent molecular weights of 31,000 and 32,000, respectively (Klinkert *et al.*, 1989, Ruppel *et al.*, 1985, Ruppel, Diesfeld and Rother, 1985) ; a M_r 188 kDa protein which is found in the soluble adult worm antigen preparation (James, Pearce and Sher, 1985) ; a M_r 53 kDa antigen from schistosomula (Kelly, 1987).

Fewer proteins were found to associate with HSP70 when parasites which were pulse-labelled were analyzed (figure 5.13) than those from parasites which were labelled overnight (figure 5.12). This shows that labelling the parasites for long time periods leads to more proteins being associated with HSP70. Consistent with these results Hughes and August (1982) showed that HSP72 coprecipitated with a surface glycoprotein (gp90) only from cells which had been biosynthetically labelled with ^{35}S-methionine for 12 hours and not from cells labelled only for 30 minutes. The changes in the immunoprecipitation pattern of HSP70-associated proteins indicates that different proteins may be in contact with HSP70 at different time points. Pulse-chase experiments showed that HSP70 and the high molecular weight compound associated with it had a very long half life. Also, the interaction of HSP70 with the high molecular weight compound was a stable one because both proteins were coprecipitated through the entire chase period (figures 5.13 a and c). The M_r >200 kDa protein, however, became associated with HSP70 after a lag phase of 30 minutes. The delay in association of this protein with HSP70 may represent the time necessary for the synthesis or transport of such a large molecule or alternatively, may reflect

some rate-limiting step in physical association. In contrast to the high molecular weight compound, this protein was not found associated with HSP70 after 6 hour chase period (figure 5.13 c) indicating that either the association of the M_r >200 kDa protein with HSP70 is transient or the protein itself has a half life of > 4 hours.

Many proteins having molecular weights similar to the ones found here, were found associated with HSP70 in other systems. For example, a polypeptide of M_r 55 kDa was co-eluted with HSP70 when its ability to bind peptides was studied (Flynn, Chappell and Rothman, 1989). The identity of this polypeptide is not known. Analysis of HSP70-associating proteins in HeLa cells by non-denaturing immunoprecipitation showed polypeptides of M_r 51, 43, 42 and 30 kDa interacting with HSP70 (Milarski,Welch and Morimoto, 1989). Also, studies by Margulis and Welsh (1991) showed several polypeptides of M_r 34, 40, 43, 51, 58, 93, 105 and 150 kDa associating with HSP70. Characterization of parasite proteins remains to be carried out.

It is conceivable that HSP70-binding proteins represent both nascent or denatured proteins that have been incorrectly folded, and thus bind to HSP70 in a nonspecific manner, but also proteins that bind through a specific and perhaps constitutive manner and which may participate in exerting the many functions of HSP70.During the last few years several HSP70-binding proteins were characterized such as mutant form of p53 tumor antigen, calmodulin, cell-cycle dependent proteins, 110 K surface glycoprotein and cytoskeletal proteins (reviewed by Schlesinger,1990). Also, several HSP70-binding proteins were isolated from bovine muscle recently. Two of them corresponding to M_r 43, 105 kDa were shown to be actin and α-actinin by immunoblotting (Margulis and Welsh, 1991). HSP70 homologue of *Escherichia coli*, DnaK was found to cooperate functionally with additional heat shock proteins DnaJ (HSP 43), GrpE and GroEl (HSP 60) (Langer *et.al.*,1992).

In conclusion, HSP70 in *S. mansoni* interacts with several newly synthesized proteins. Some of these interactions are transient while others are more stable. The identity of these polypeptides is yet to be determined.

Chapter Six

The effect of praziquantel on the synthesis and expression of heat shock protein 70 (HSP 70)

6.1 Introduction

Praziquantel appears to interact with lipid constituents of the tegumental membrane of the parasite and cause membrane destabilization (Andrews, 1985). Subsequently, the effects of praziquantel on schistosomes include contraction and paralysis (Pax, Bennett, and Fetterer, 1978), and tegumental damage (Becker *et al.*, 1980), possibly as a result of Ca^{2+} influx (Bricker *et al.*,1983, Xiao *et al.*,1984, 1985 b). These effects of praziquantel make the worms more vulnerable to host enzymes or antibody dependent immune effector mechanisms (Mehlhorn *et al.*,1981, Piper *et al.*,1991).

Praziquantel has been shown to increase parasite antigen exposure at the surface of adult *Schistosoma mansoni* when the worms are treated with the drug both *in vitro* (Harnett and Kusel, 1986), and *in vivo* (Brindley and Sher, 1987, Flisser and McLaren, 1989). A few of these exposed antigens have been characterized. A M_r 200 kDa glycoprotein (Brindley *et al* .,1989), and a M_r 27 kDa protein with esterase activity (Doenhoff *et al* .,1988), were both localized to the male tubercules and were shown to be directly involved in drug / antibody synergy. Other antigens, including an alkaline phosphatase were also revealed on the surface after praziquantel treatment.

In this chapter, the effect of praziquantel on overall protein synthesis and, in particular, on the synthesis and expression of HSP 70 was investigated.

6.2 The effect of praziquantel on HSP70 expression on the surface of the adult worm.

Harnett and Kusel (1986) employed praziquantel at a concentration of 10 µg / ml and found that it caused an increase in parasite antigen exposure. This concentration was therefore selected here to investigate HSP 70 expression.

The expression of HSP 70 was studied by indirect immunofluorescence with anti-MS2-HSP70 antiserum in exactly the same way as in chapter 4, sections 4.2. and 4.3., using an FITC-goat anti-rabbit antiserum and quantitative

fluorescence.

Freshly perfused worms were treated with praziquantel at a concentration of 10 µg / ml for 10-15 minutes at 37°C / 5% CO_2 and then the ability of anti-MS2-HSP 70 antiserum to bind to carbachol-treated, formaldehyde-treated, Triton X-100-treated, or to freeze-thawed worms was investigated. The result of such an experiment is shown in figure 6.1.

It is seen that treatment of the worms with praziquantel did not cause any binding of anti-MS2-HSP70 antiserum to the surface of worms treated with carbachol, formaldehyde, or Triton X-100. The binding was significant only after the surfaces of the worms had been removed by the method of freeze-thawing, a result which was obtained even when the worms were not treated with praziquantel (section 4.3.). These results seem to indicate that praziquantel does not cause any expression of HSP70 on the parasite surface.

6.3 Effect of praziquantel on the synthesis of HSP70

Treatment with the toxic drug praziquantel is undoubtedly traumatic for the parasite. It might be predicted that such stress would induce increased HSP70 synthesis. This possibility was therefore investigated.

Worms were treated with 10 µg / ml praziquantel for 15 minutes at 37°C as above. ^{35}S-methionine labelling was carried out for 1 hour and 3 hours after praziquantel treatment. Controls were performed with 10 µl / ml ethanol added to the culture medium, since praziquantel is dissolved in ethanol. Metabolically labelled proteins of the worms were then immunoprecipitated with anti-MS2-HSP 70 antiserum. The result of this immunoprecipitation is shown in figure 6.2. This gel shows that when the worms were labelled for 1 hour, no change in HSP70 synthesis was observed between the drug-treated and non-treated worms. However, when the labelling was continued for 3 hours, HSP70's synthesis increased in praziquantel-treated worms.

Figure 6.1 Binding of anti-MS2-HSP70 antiserum to praziquantel-treated adult schistosomes.

Groups of 10 adult male worms were exposed to 10 µg / ml praziquantel for 15 minutes at 37°C / 5% CO_2 and then subjected to treatments A-D, as described in Materials and Methods. Controls (worms which were not treated with praziquantel) were as seen in figure 4.1 (chapter 4). Worms were then labelled with anti-MS2-HSP70 and FITC-goat anti-rabbit antisera.

A : Intact, Carbachol-treated.

B : Formaldehyde-fixed.

C : Triton X-100-treated.

D : Freeze-thawed.

* : P = 0

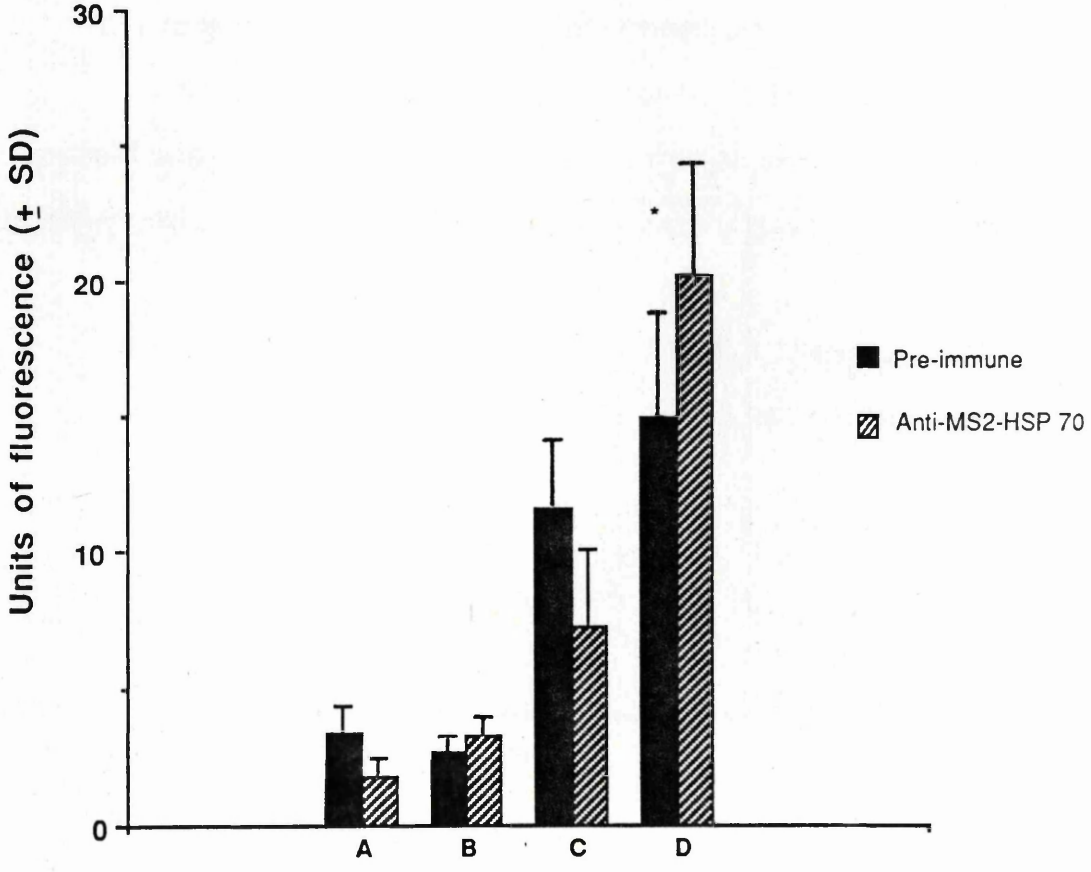
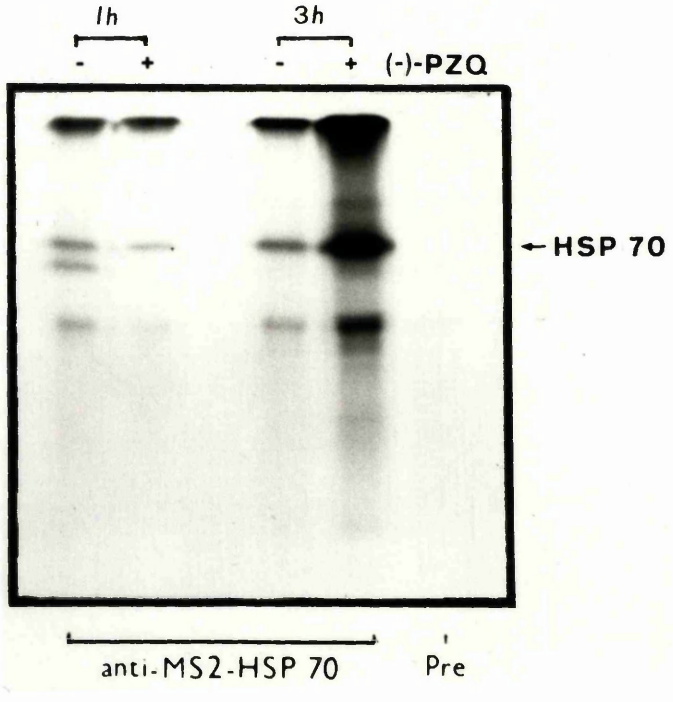

Units of fluorescence (± SD)
30
20
10
0
A
B
C
D
*
Pre-immune
Anti-MS2-HSP 70

Figure 6.2 Immunoprecipitation of ^{35}S-methionine-labelled proteins from praziquantel-treated and non-treated worms by anti-MS2-HSP70 antiserum.

2 groups of adult schistosomes were exposed to 10 µg / ml praziquantel for 15 minutes at 37^{0}C / 5% CO_2. Controls were performed with 10 µl / ml ethanol added to the culture medium. The worms were then washed and resuspended in methionine-free medium and labelled with 100 µCi ^{35}S-methionine / ml. 1 group was labelled for 1 hour and another for 3 hours. Equal amounts of TCA-precipitable counts were taken for each immunoprecipitate and the immunoprecipitation was carried out as described in Materials and Methods. The immunoprecipitates were analyzed on a 10% reducing SDS-polyacrylamide gel.

(-)-PZQ : Praziquantel

Pre : Pre-immune serum


1h
3h
- + - +
(-)-PZQ
HSP 70
anti-MS2-HSP 70
Pre

6.4 Effect of praziquantel on the secretion of HSP 70

Normal worms and worms treated with 10 µg / ml praziquantel for 15 minutes at 37^{0}C as above were cultured for different time periods in GMEM. The proteins secreted into the medium were collected, concentrated and immunoblotted with anti-MS2-HSP 70 antiserum as described in Materials and Methods.

From figure 6.3 a, it can be seen that untreated worms cultured for 4 hours showed some secretion of HSP70. However, in worms treated with praziquantel before culture, HSP70 secretion was greatly enhanced.

Adult worms vary with respect to a number of properties (Jones and Kusel, 1989).To ensure that this difference in secretion of HSP70 was not due to random variations in secretions in different worm batches , one batch of worms was cultured for 4 hours and then the medium was collected and analyzed for secreted HSP70 by immunoblotting. The same batch of worms was then treated with praziquantel for 15 minutes, washed, and cultured for a further 4 hours. The medium was then analyzed for secreted HSP 70 as before.

Figure 6.3 b shows the result of such an experiment. It can be seen that, again, there was a great increase in HSP70 secretion after praziquantel treatment.

Praziquantel is used as a racemate in therapy, and its effect is attributed to the R(-) isomer (Andrews *et al.*, 1985).To ensure that the secretion of HSP70 from the praziquantel-treated worms was specific to the R (-) rather than the S(+) enantiomer, worms were treated for 15 minutes with either the S (+) or R (-) enantiomers of praziquantel. Control worms were either left untreated or ethanol (10 µl / ml) was added to the culture medium. The worms were then incubated in drug-free medium for 4, 22, and 26 hours. HSP 70 secreted in the medium was detected by immunoblotting.

Figure 6.3 c shows that HSP70 secretion was very much enhanced only when the worms were treated with the R (-) but not S (+) enantiomer of

Figure 6.3 Immunoblots of proteins released in the culture medium of praziquantel-treated and non-treated worms with anti-MS2-HSP 70 antiserum.

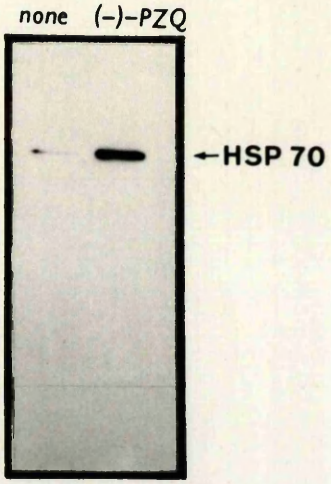
Equal numbers of adult worms were exposed to 10 µg / ml praziquantel for 15 minutes and then incubated for the required period of time in praziquantel-free medium. The proteins released in the medium were collected, concentrated and run on a reducing 10% (w / v) SDS-polyacrylamide gel. Immunoblotting was carried out as described in Materials and Methods.

a) Worms were either treated with praziquantel or left untreated and then incubated for 4 hours in praziquantel-free medium.

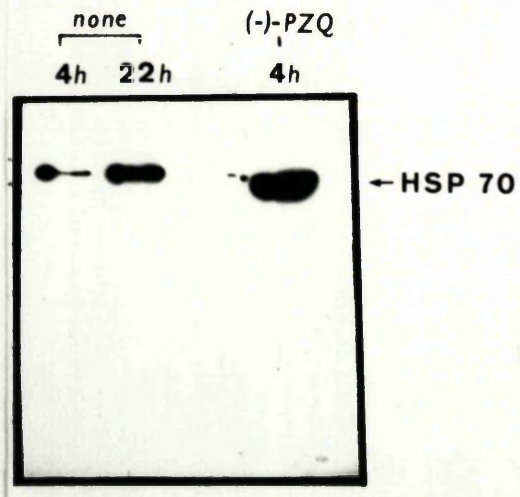
b) Normal worms were incubated in GMEM for 4 hours and 22 hours at 37^{0}C / 5% CO_2. After the released proteins had been collected, the worms from the 4 hour incubation were exposed to praziquantel for 15 minutes and then incubated in praziquantel-free medium for a further 4 hours.

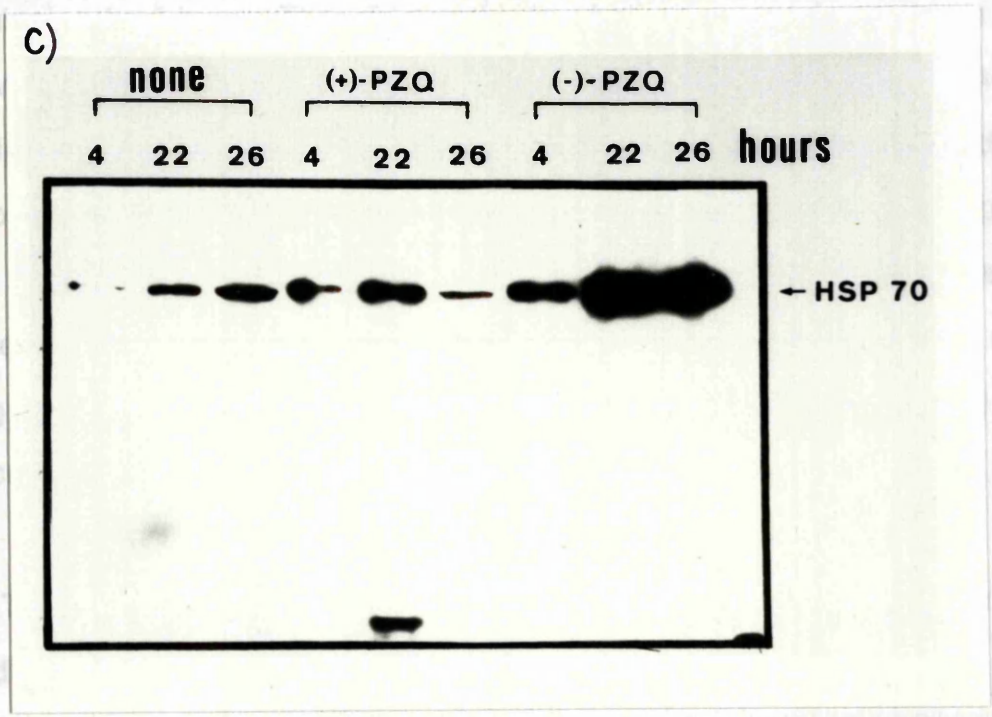
c) Worms were exposed to either 10 µg / ml (+)-praziquantel, (-)-praziquantel, or left untreated. Then they were incubated for 4, 22, and 26 hours in praziquantel-free medium.

a)



b)




C)
none
(+)-PZQ
(-)-PZQ
4 22 26 4 22 26 4 22 26 hours
HSP 70

praziquantel. Also, the secretion of HSP70 continues over the entire incubation period and does not stop after 4 hours of culture. Thus, there is more HSP70 in the 22 hour and 26 hour culture media than that in the 4 hour medium.

6.5 The effect on protein synthesis of incubating the parasites with increasing concentrations of praziquantel.

Adult worms were incubated with praziquantel at concentrations ranging from 0-5 µg / ml and their proteins were metabolically labelled with ^{35}S-methionine for 3 hours. The effect of this incubation on protein synthesis is shown in figure 6.4 a. which shows the radioactivity incorporated in TCA-precipitable material and in free methionine from the treated worms, and figure 6.4 b, which shows the corresponding labelling pattern of schistosome proteins. Both figures show that the amount of radioactivity incorporated into protein decreases with increasing praziquantel concentration in the incubation medium. In figure 6.4 b, the effect is seen when worms are incubated with PZQ at a concentration of 3 µg / ml and above.

6.6 The effect on protein synthesis of removing PZQ from the culture medium.

It was considered that the decrease in protein synthesis described above might be due to the fact that the worms were immobilized by praziquantel during the labelling period. In the following experiments, therefore, worms were treated with praziquantel at concentrations ranging from 0-100 µg / ml for 15 minutes, after which praziquantel was removed, and the worms labelled for either 3 hours or overnight.

6.6.1 Labelling for 3 hours

Figure 6.5 a shows that when worms were labelled for 3 hours after praziquantel removal, the amount of radioactivity incorporated in TCA-

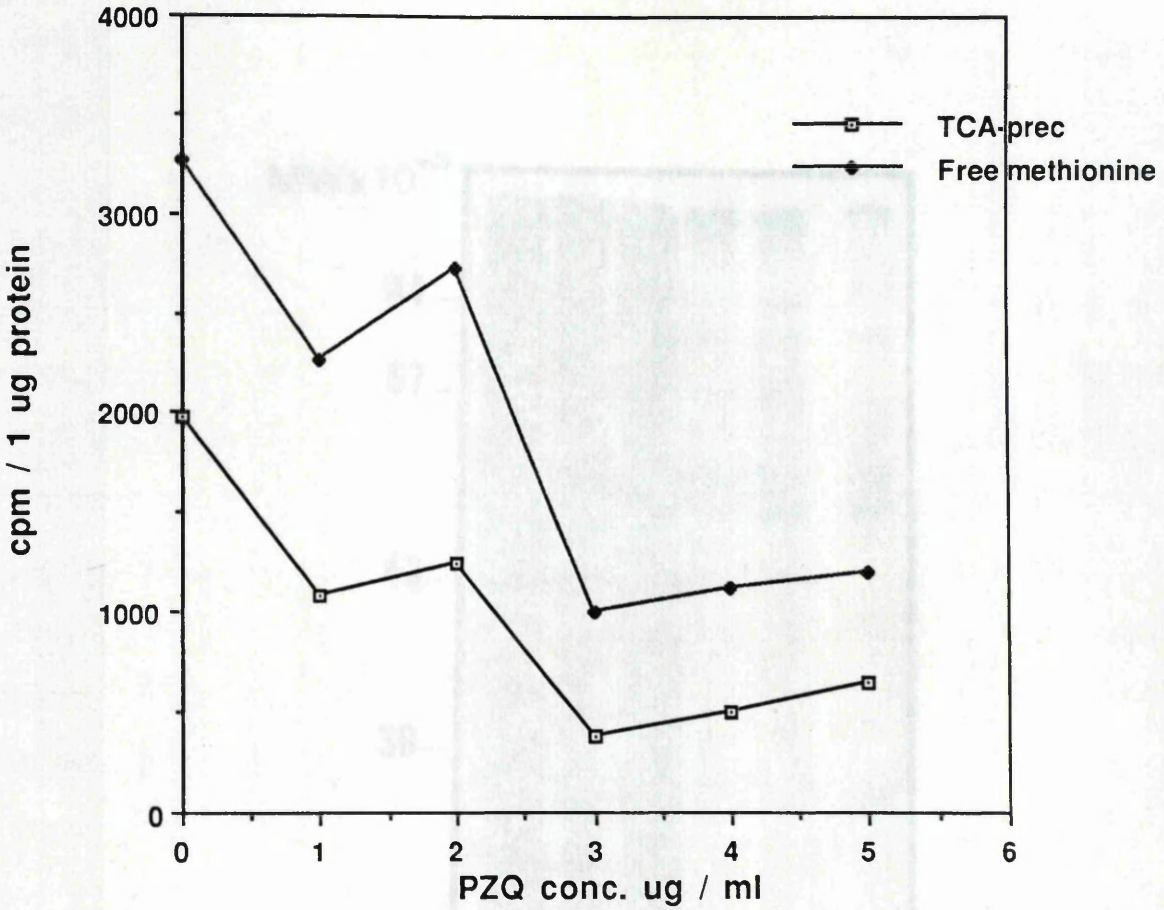
Figure 6.4 The effect on protein synthesis of incubating adult schistosomes with increasing concentrations of praziquantel.

a) The amount of radioactivity incorporated into TCA-precipitable material and in free methionine from worms treated with praziquantel.

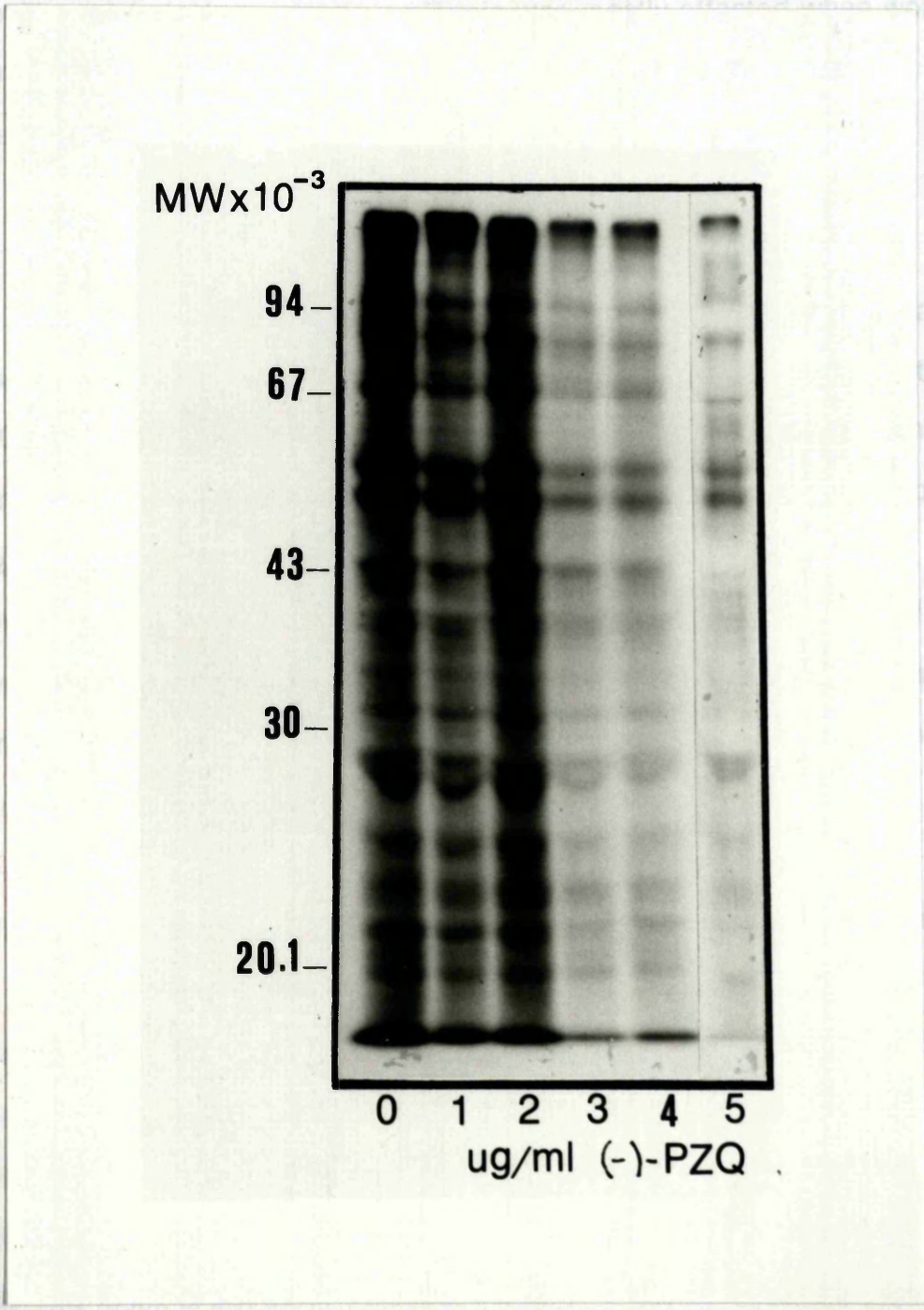
b) Fractionated, ^{35}S-methionine-labelled proteins of adult worms treated with praziquantel; Fluorograph of SDS-polyacrylamide gel.

6 groups of 15 worms / group were labelled with 25 µCi / ml ^{35}S-methionine for 1 hour. Praziquantel was then added at concentrations ranging from 0-5 µg / ml and then the labelling was continued for 90 minutes. Worms were washed, homogenized in 0.5% Nonidet P40, and the concentration of protein in each group was determined as described in Materials and Methods. Equal amounts of Nonidet P40-soluble proteins were counted and analyzed by 10% reducing SDS-PAGE.

a)


4000
3000
2000
1000
0
cpm / 1 ug protein
0
1
2
3
4
5
6
PZQ conc. ug / ml
TCA-prec
Free methionine

b)



precipitable material decreased with increasing praziquantel concentration. The pattern of labelled proteins was analyzed by SDS-PAGE, and is shown in figure 6.5 b. Again, it can be seen that the radioactive label incorporated into proteins decreased with increasing praziquantel concentration. However, the effect is less prominent here than in figure 6.4 b. where praziquantel was present throughout the labelling period. Here, protein synthesis was mostly affected when worms were treated with 100 µg / ml praziquantel. At this concentration very little protein synthesis was observed.

6.6.2 Labelling overnight

When the worms were labelled overnight after praziquantel removal, a slight increase in protein synthesis was seen when the worms had been treated with 10 µg / ml of praziquantel (figures 6.6 a and b). In another experiment, a similar increase was seen when worms were treated with 6 µg / ml praziquantel (figure 6.6 c). However, it seems that in worms which were treated with concentrations up to 10 µg / ml praziquantel, protein synthesis recovered after removal of the drug, and was restored to the same level as in untreated worms.. In worms which were treated with 30, 50, and 100 µg / ml praziquantel, however, protein synthesis remained low (figures 6.6 a and b).

6.7 The effect of praziquantel on synthesis of membrane proteins

Freshly perfused worms were treated with 0-100 µg / ml praziquantel for 15 minutes at 37^{0}C / 5% CO_2. Then they were labelled overnight with ^{35}S-methionine in drug-free medium. Membranes were isolated by freeze-thawing as described in Materials and Methods. The radioactivity incorporated into TCA-precipitable material was measured and the proteins were analyzed by SDS-PAGE. The result is shown in figures 6.7 a and b in which it is seen that, again, protein synthesis decreased with increasing praziquantel concentration, but did not recover after overnight labelling.

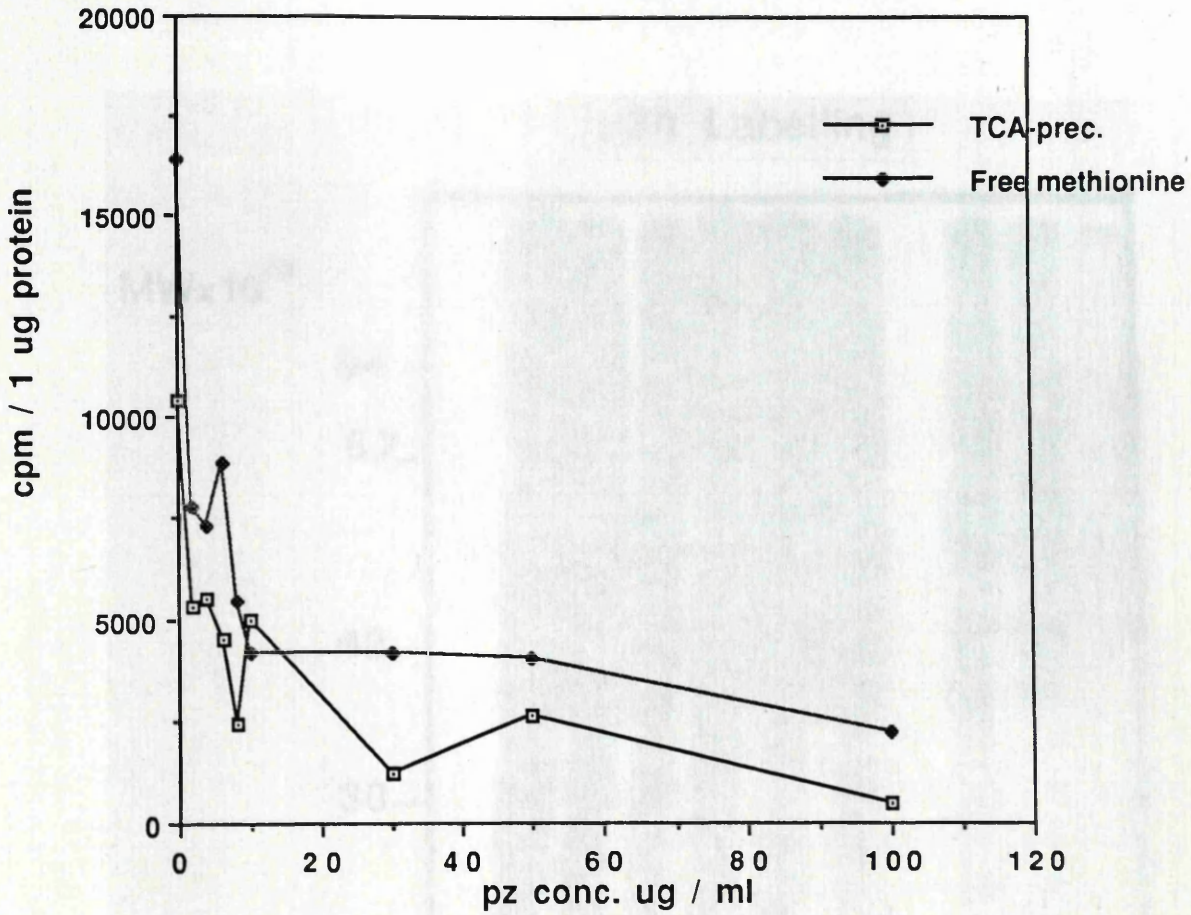
Figure 6.5 The effect on protein synthesis of removing praziquantel from the culture medium.

a) The amount of radioactivity incorporated into TCA-precipitable material and in free methionine from worms treated with praziquantel.

b) Fractionated, ^{35}S-methionine-labelled proteins of worms treated with praziquantel ; Fluorograph of 10% (w/v) SDS-polyacrylamide gel.

Groups of 5 worms were exposed to praziquantel at concentrations ranging from 0-100 µg / ml for 15 minutes at 37^{0}C / 5% CO_2. Worms were then washed and labelled with 50 µCi ^{35}S-methionine in praziquantel-free, methionine-free medium for 3 hours. After that the worms were washed, resuspended in sample buffer, and boiled for 10 minutes. The SDS-soluble material was collected and the protein concentration was determined in each group as described in Materials and Methods. Equal amounts of protein were counted and analyzed on a reducing 10% (w / v) SDS-polyacrylamide gel.

a)


20000
15000
10000
5000
0
cpm / 1 ug protein
0
20
40
60
80
100
120
pz conc. ug / ml
TCA-prec.
Free methionine

b)

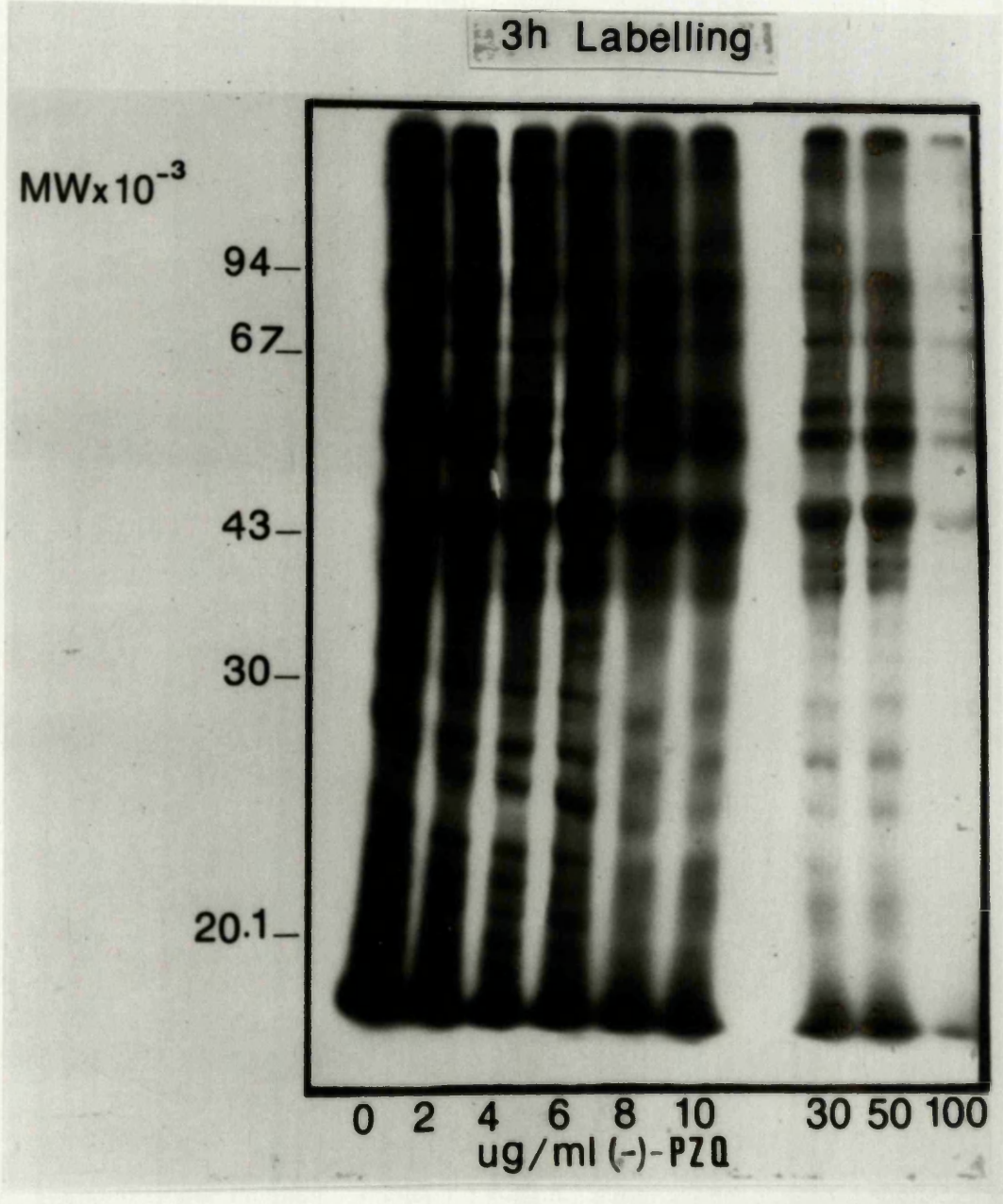


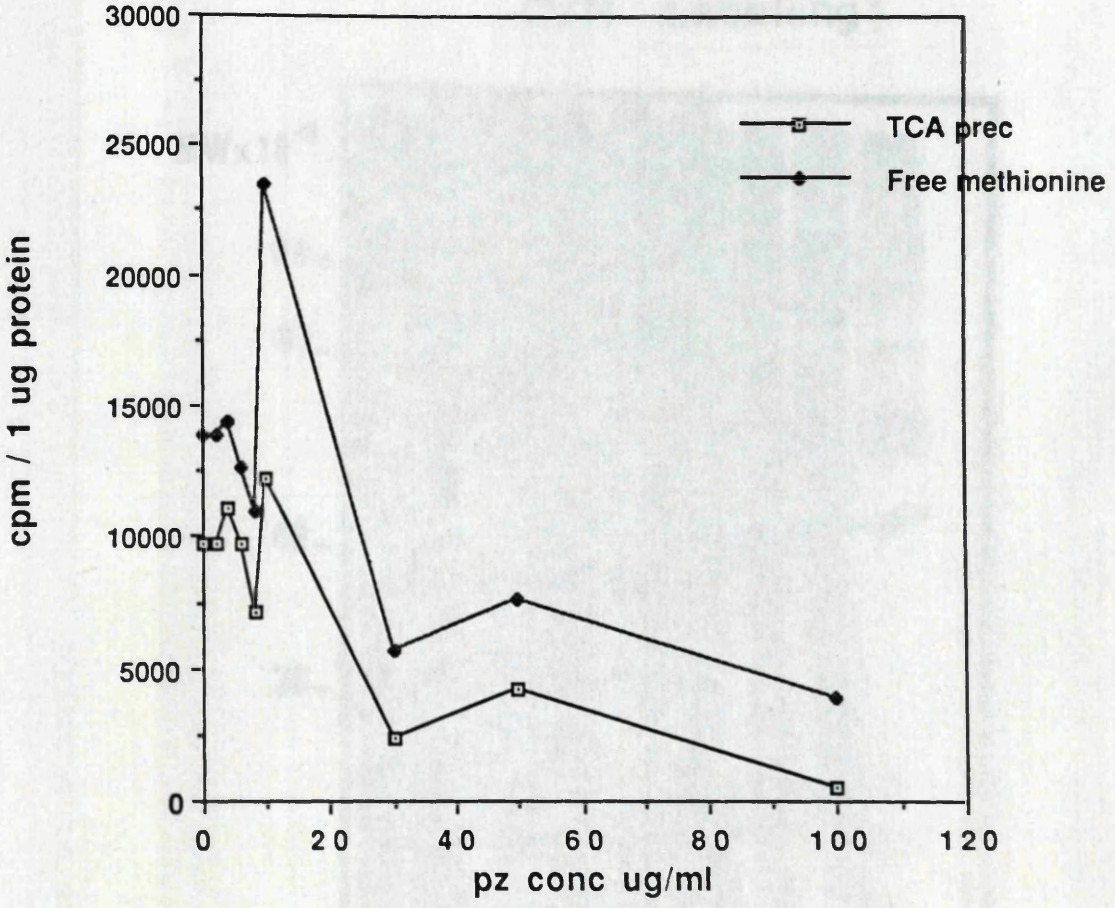
Figure 6.6 The effect on protein synthesis of removing praziquantel from the culture medium.

Legend as for figure 6.5, except the worms were labelled overnight.

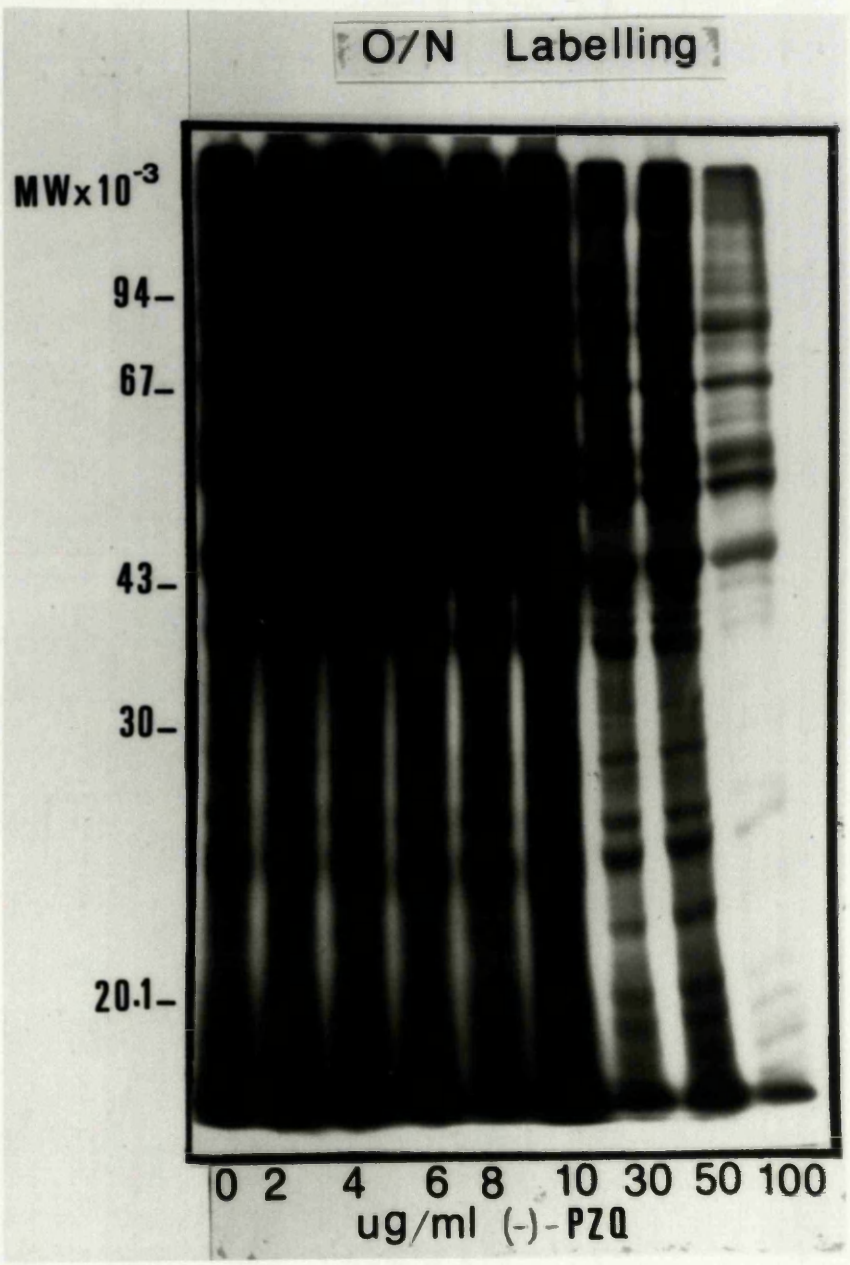
a) and c) The amount of radioactivity incorporated in TCA-precipitable material and in free methionine from worms treated with praziquantel.

b) Fractionated, ^{35}S-methionine-labelled proteins of worms treated with praziquantel ; Fluorograph of a reducing 10% (w/v) SDS-polyacrylamide gel.

a)


30000
25000
20000
15000
10000
5000
0
cpm / 1 ug protein
0
20
40
60
80
100
120
pz conc ug/ml
TCA prec
Free methionine

b)



c)

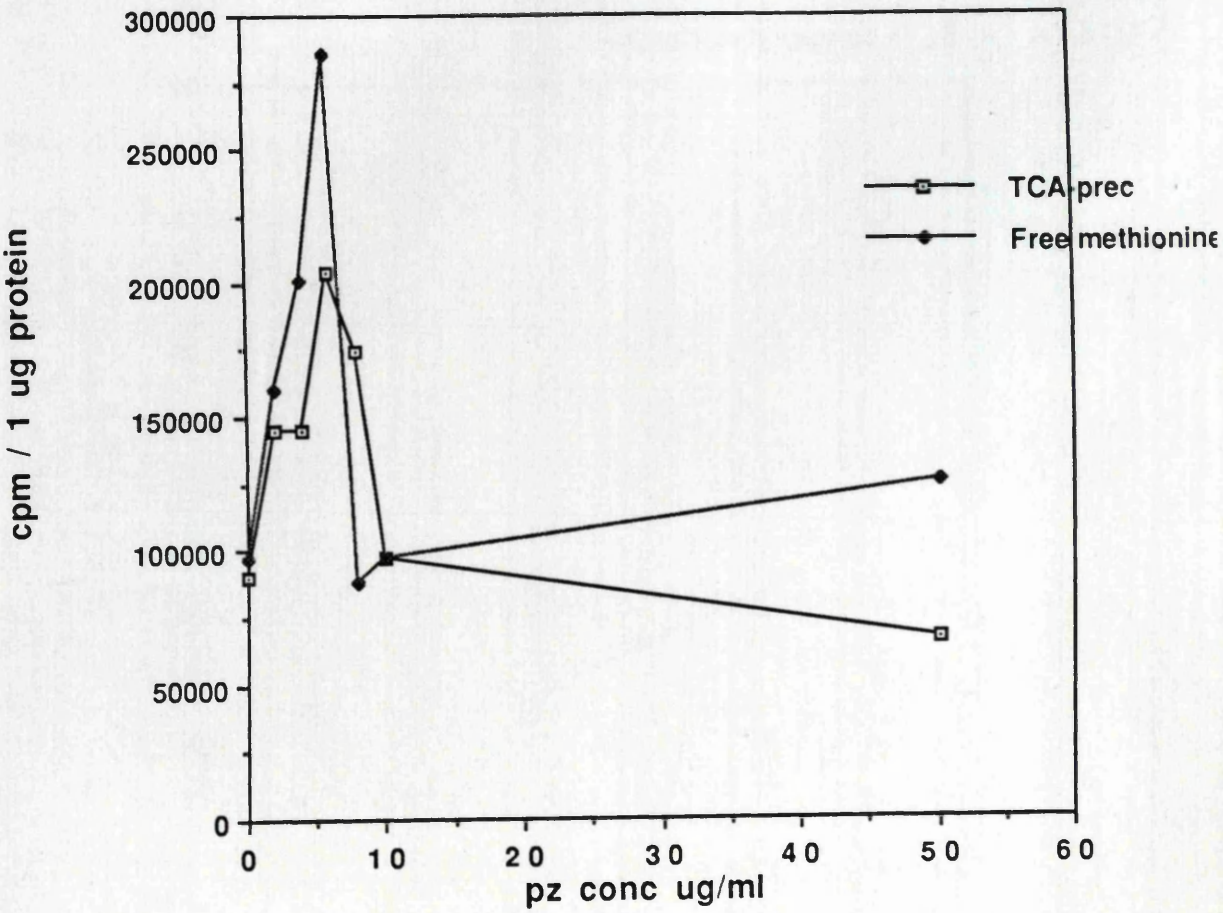
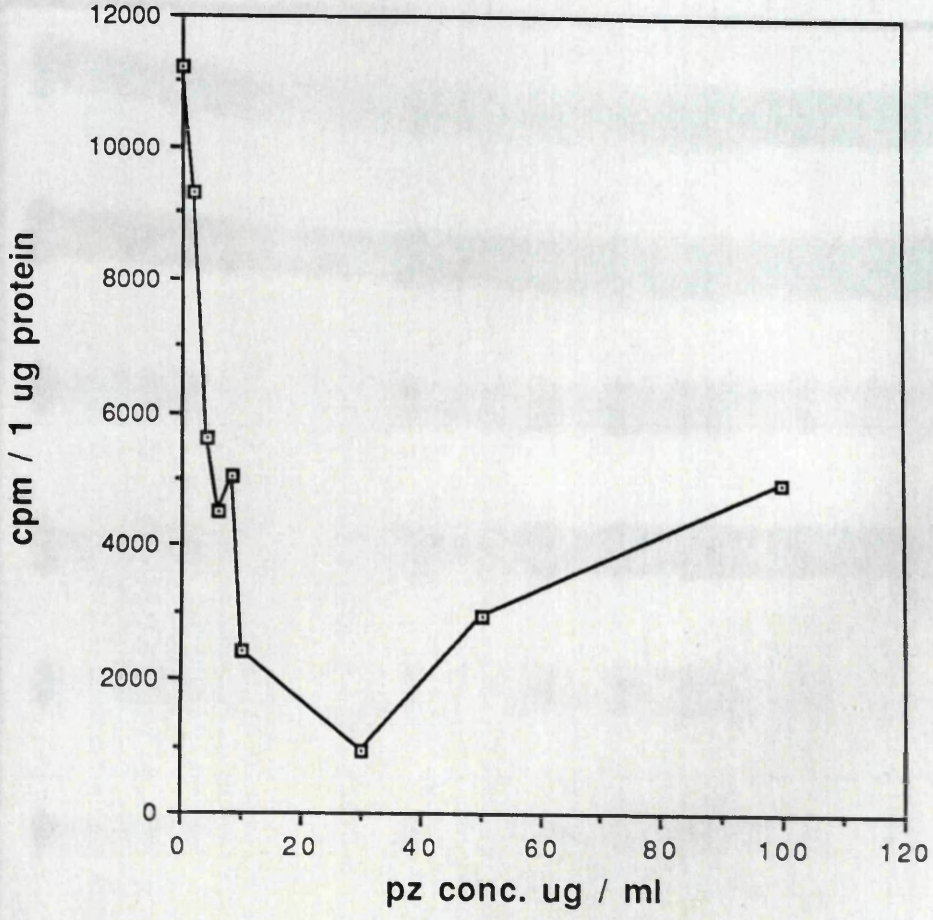

300000
250000
200000
150000
100000
50000
0
cpm / 1 ug protein
0
10
20
30
40
50
60
pz conc ug/ml
TCA prec
Free methionine

Figure 6.7 The effect of praziquantel on synthesis of membrane proteins.

Legend as for figure 6.6, except that after labelling, membranes of the worms were isolated as described in Materials and Methods and boiled in sample buffer for 10 minutes. The protein concentration was determined in each group and equal amounts of protein were counted and analyzed on a reducing 10% (w / v) SDS-polyacrylamide gel.

a)


12000
10000
8000
6000
4000
2000
0
cpm / 1 ug protein
0
20
40
60
80
100
120
pz conc. ug / ml

a)

6.8 Discussion

Treatment of parasites with praziquantel is associated with a diverse range of effects. These include muscle contraction or paralysis and tegumental damage - generally regarded as the two primary effects of praziquantel. Both effects arise from changes in the flux of divalent cations - particularly calcium - which follow drug-induced increases in membrane permeability.

The other effects of praziquantel (reviewed by Andrews, 1985), are often referred to as 'secondary' because they are considered to be related to, or a consequence of the primary effects. Such phenomena include changes in carbohydrate, protein and nucleotide metabolism, decrease in enzymatic activities, and changes in the properties of surface membranes.

Here the effect of praziquantel on schistosome protein synthesis was investigated. Praziquantel was found to inhibit protein synthesis when it was used at 3 µg / ml or higher concentrations, and when it remained in contact with the worms while they were being labelled (figures 6.4 a and b). This was thought to be caused by the continuous presence of praziquantel in the culture medium, causing paralysis and immobilization of the worms. Treating the worms with praziquantel for 15 minutes and then removing them to a praziquantel-free medium also caused a decrease in protein synthesis but the effect was not pronounced except at high concentrations of praziquantel , for example, 30 - 100 µg / ml (figure 6.5 a and b). When the worms were labelled overnight after drug treatment , however, protein synthesis seemed to recover. In worms which were treated with 2 - 10 µg / ml praziquantel , protein synthesis recovered to normal levels, but in those which were treated with 30 - 100 µg / ml of praziquantel protein synthesis remained low (figures 6.6 a and b).

It seems that the damage inflicted on protein synthesis by praziquantel at 2-10 µg / ml can be effectively repaired. However, praziquantel at higher concentrations affects protein synthesis in a way that it is not repaired, at least not after 24 hours in culture.

Xiao, Catto, and Webster (1985 b), found that when parasites exposed to praziquantel at 30 µg / ml for 15 minutes were observed 24 hours later, they were contracted, and either immobile or showed decreased movement. Whether protein synthesis is related to paralysis or immobilization of the worms is yet to be investigated using different agents which paralyze the worms, although it is known from literature that most inhibitors of known neurotransmitters of *S. mansoni* and various other pharmacologically active agents do not antagonize the action of praziquantel, for example, carbachol, spiroperidol, bromo-lysergic acid diethylamide, imipramine (at 0.1 mM), dopamine, noradrenaline, 5-hydroxytryptamine, arecoline, metrifonate, pentobarbital (at 1 mM), atropine, mecamylamine, and pempidine (at 10 mM) (Pax *et al.*, 1978, 1979 ; Coles, 1979 ; Xioa *et al.*, 1984).

Membrane protein synthesis decreased with increasing praziquantel concentrations (figures 6.7 a and b) and did not seem to recover after overnight incubation of the worms. Could this be related to the damage caused by praziquantel at the parasite membrane ?. The inhibitory effect of praziquantel on membrane protein synthesis seems to be so severe that recovery is slower than the total worm protein synthesis. In accordance with the observations described here, Staudt *et al.* (1992) showed that when pairs of schistosomes had been exposed to praziquantel at concentrations ranging from 0.01 to 100 µg / ml for 4 hours and then incubated in drug-free medium for another 20 hours, no repair of tegumental damage occurred during the period of incubation in drug-free medium.

Worms treated *in vivo* with subcurative doses of praziquantel survive, though often showing extensive structural damage, including damage to their tegument (Shaw and Erasmus, 1983 b), suggesting that the parasites are able to effectively repair limited tegumental damage. Studies on tegumental repair in *S. mansoni* (Popiel, Irvine, and Basch, 1985) have also indicated that, *in vitro* at least, the process of repair can occur within 3 days post-transection.

It is not surprising that the alterations which praziquantel causes so rapidly in the tegument and the musculature have repercussions for many other functions and traumatize the parasite. Praziquantel inserts readily in model lipid bilayers (Schepers *et al.*, 1988 ; Harder, Goosens, and Andrews, 1988), causing a rearrangement of the lipid molecules that lead to the formation of spaces and channels (Schepers *et al.*, 1988). Such destabilizing changes in parasite tegument could account for the influx of Ca^{2+} into the schistosome which follows exposure to praziquantel (Bricker *et al.*, 1983). If indeed praziquantel produces similar changes in the tegument of schistosomes, then it is very possible that the drug-induced rearrangement of the lipid constituents may alter the conformation of individual, integral membrane proteins.

Praziquantel also causes tegumental vacuolization and surface blebbing in *S. mansoni* (Becker *et al.*, 1980 ; Mehlhorn *et al.*,1983). Generalized tegumental damage has also been reported for *S. mansoni* (Bricker *et al.*, 1983). Such damaging effects of praziquantel on the parasite tegument must lead to denaturation of a proportion of parasite proteins. HSP 70 has a high affinity for denatured and aberrant proteins (Gaitanaris *et al.*, 1990)). Its function is to ensure that these individual polypeptides assume and maintain their native conformations, or, alternatively to target such molecules to lysosomes for proteolytic degradation if they are severely denatured and abnormal (Chiang *et al.*, 1989). Therefore, it is not surprising to find that the synthesis of HSP70 increases upon praziquantel treatment. This increase in HSP 70 synthesis in praziquantel-treated worms should allow them to renature or remove molecules which have lost their conformation and help the parasite to recover from the damaging effects of praziquantel.

There is very little known about the effects of praziquantel on protein synthesis as there are no previous reports describing it. It was shown in figure 6.2 that increased HSP 70 synthesis was not detected 1 hour after praziquantel treatment but was considerably increased 3 hours post-treatment. When total

protein synthesis was examined (figure 6.5) at a variety of concentrations of praziquantel, a decrease in synthesis was observed after 3 hours.

What is the mechanism by which praziquantel is decreasing protein synthesis, while at the same time increasing HSP 70 synthesis?

Experiments by Luckas *et al.* (1980) have shown that addition of praziquantel to an S. *mansoni*, cell-free, protein-synthesizing system had no effect on protein synthesis over the period of 90 minutes, hence an indirect mechanism must be sought. Praziquantel used at 2.7 mM (0.8 mg / ml) was sufficient to reduce 50 % of the RNA synthesis in isolated hamster liver nuclei but failed to inhibit completely the *in vitro* RNA synthesis (Wong *et al.*, 1990). Pre-incubation of the nuclei with praziquantel revealed that the inhibitory effect of the drug was reversible. Thus, it can be suggested that praziquantel is affecting parasite RNA synthesis over the period of 3 hours after treatment in which protein synthesis decreases, with the exception of HSP70, whose synthesis is stimulated. The reversibility of this inhibition of protein synthesis is shown in figure 6.6 , and the mechanism by which HSP70 synthesis increases is of great interest. (However, it should be noted that the inhibitory effect of praziquantel on protein synthesis observed here might not be related to the effect of praziquantel on *in vitro* RNA synthesis found by Wong *et al.* (1990) since the inhibition of RNA synthesis requires mM concentrations of praziquantel, whereas the inhibition of protein synthesis described above requires μ M concentrations.)

The effects of praziquantel appear to depend on changes in the permeability of membranes to cations, resulting in alterations in the movement of calcium and the regulation of its concentrations in cells and intracellular organelles. Calcium is a specific modulator of protein conformation and experiments by Mosser *et al.* (1990) have shown that addition of calcium to a cytoplasmic extract of HeLa cells activated heat shock factor (HSF) in a concentration- and time-dependent manner. Thus, it can be speculated that praziquantel causes increase in HSP70 synthesis by altering calcium

concentrations which then activate heat shock factor (HSF). The result is increased transcription of the heat shock genes, leading to an increase in the concentration of HSP70 in the cells.

Inhibition of protein synthesis after praziquantel treatment could be simply due to the low uptake of amino acids resulting from the paralysis and immobilization of the worms. Praziquantel used above 1 μM concentration inhibits glucose uptake (Harder, Andrews, and Thomas, 1987 b), and at 16 uM inhibits uptake of rubidium (Huang, 1984). The results presented in figures 6.4 a, 6.5 a and 6.6 a support this idea as they show that the pattern of radioactivity incorporated into TCA-precipitable material follows that of free methionine available from the worms. Once praziquantel is removed, the effect of paralysis is removed and the worms start synthesizing protein again as normal except when high concentrations of praziquantel are used. At these higher concentrations, praziquantel seems to cause long-term damage to either physiological or biochemical systems within the parasite which may inhibit or slow down protein synthesis.

Praziquantel did not expose HSP 70 on the surface of adult *S. mansoni* (figure 6.1). The results obtained here were similar to those obtained in chapter 4, which showed that HSP 70 was not detected on the adult worms except after stripping the surfaces away.

Because the damage inflicted by praziquantel is to the surface of the parasite, one might think that praziquantel treatment should expose HSP70 on the surface where the location might be important in renaturing those proteins which are denatured by praziquantel. However, it seems that the surface location of HSP70 is not a prerequisite for this.

Brindley *et al.* (1989) showed that praziquantel interaction with the surface of the parasite was restricted to exposing a small number of epitopes that were usually inaccessible to binding by host antibodies. These exposed epitopes

were found at or near the surface bilayer of the schistosome. HSP70 is not a surface located molecule as shown in chapter 4 and so it can be explained, in the view of the finding by Brindley *et al.*, that it can not be exposed by praziquantel.

A slight release of HSP70 was found in the culture medium of the parasite but after exposure to praziquantel the release of HSP 70 was very much enhanced (figure 6.3).

Release of surface components by both nematodes and trematodes is likely to be a consequence of their normal interaction with the environment. Philipp and Rumjanek (1984) concluded that in some cases qualitative and quantitative distortions of this phenomenon may be introduced by the conditions of culture of the parasites.

Adult worm tegument is unstable *in vitro* (Simpson *et al.*,1981; Smith, Reynolds, and Von-Lichtenburg, 1969). Radiolabelled tegumental antigens were found in culture fluids of radioiodinated (Kusel, Mackenzie, and McLaren, 1975; Shah and Ramasamy, 1982), or metabolically labelled (Kusel and Mackenzie, 1975) worms. Rather than being released individually, however, radiolabelled membrane proteins were found attached to membrane residues shed into the medium (Kusel, Mackenzie, and McLaren, 1975).

The enhanced release of HSP 70 after exposure to praziquantel might be due to the damage caused by praziquantel on the tegument of the parasite. Tegumental damage consisting of basal vacuolization of the syncytial tegument, blebbing of the surface membrane, and release of membrane bound vesicles appear to be normal reactions of schistosomes to several stresses encountered *in vivo* and *in vitro* (Philipp and Rumjanek, 1984). Furthermore, tegumental damage caused by praziquantel may become so severe that parts of the tegument are lost (Mehlhorn *et al.*, 1983).

It has been suggested by Coles (1979) that praziquantel may open pores in the membrane of the parasite. Also, praziquantel was found to cause a rearrangement of the lipid molecules in model lipid bilayers (Schepers *et al.*, 1988) which leads to the formation of spaces and channels. HSP70 could leak out from such pores or channels. However, exposure to 32 µM (10µg / ml) praziquantel did not cause tegumental leakage. ^{51}Chromium that had previously been incorporated by *S. mansoni* was not released by praziquantel (Harnett and Kusel, 1984).

The enhanced release of HSP70 into the culture medium is specific to the R (-) isomer of praziquantel. When worms were treated with the S (+) enantiomer HSP70's release was not enhanced (figure 6.3 c), although the effect of (+)-praziquantel in the contracture and immobilization of the worms was the same as (-)-praziquantel at the concentrations used in the experiment.

Praziquantel, as currently produced, is a racemic mixture. Preliminary studies, however, have shown that the primary therapeutic effect of praziquantel resides in its levorotatory (-) isomer (Andrews *et al.*,1983 ; Andrews, 1985). The efficacy of (-)-praziquantel is found to be superior to that of racemic praziquantel at equipotent doses, as the dextrorotatory (+) isomer apparently contributes little to the therapeutic efficacy of praziquantel (Andrews, 1985).

Comparative studies on the differences between enantiomorphs of praziquantel have been limited. Staudt *et al.* (1992) found that incubation of *S. mansoni* for 4 hours with 0.01 µg R (-)-praziquantel / ml caused surface blebs and lesions. In contrast to R (-)-praziquantel, even at a 105 times higher dose (100 µg / ml) S(+)-praziquantel caused no lesions in the surface of the worms.

Andrews *et al.* (1983) demonstrated marked quantitative differences between enantiomorphs both *in vivo* and *in vitro*. For example, they reported that in *S. mansoni*-infected mice a dose of 5 x 50 mg / kg of (-)-praziquantel resulted in complete reduction of worm burden compared with less

than 90 % worm reduction with a dose of 5 x 500 mg / kg of (+)-praziquantel. Studies by Bricker *et al.* (1983) suggest that the (-) isomer is more effective in inducing tegumental disruption, which is presumed to be responsible for the therapeutic effect of praziquantel (Xioa, Catto, and Webster, 1985).

In conclusion, praziquantel causes inhibition of parasite protein synthesis which is recoverable after praziquantel removal and incubation of the worms in praziquantel-free medium. The drug, however, causes an enhanced synthesis and release of HSP70. Bot these effects are attributable to the (-)-isomer of praziquantel.

Chapter seven
General Discussion

7.1 Introduction

The aim of this project was to try to understand the role that HSP70 plays in the metabolic processes of the parasite *Schistosoma mansoni*. For this, it was necessary to determine the distribution of HSP70 and then try to relate this to its function in interacting with different proteins or polypeptides of the parasite. This was important to understand as it was thought to indicate how HSP70 might participate in the synthesis and repair of the membrane of the parasite before and after treatment with agents that damage it, for example, the chemotherapeutic drug, praziquantel. Very recently, a possible involvement of HSP70 in the immunogenicity of irradiated parasite has been suggested (Wales, Kusel and Jones, 1992) which is consistent with its role in other systems.

7.2 The immune response to HSP70

The universal immune response to HSP70 in schistosome infections in a variety of hosts made it a good candidate for surface location in the parasite. Experiments designed to test this, by means of indirect immunofluorescence, however, clearly demonstrated that it was not available for labelling on the surface of adult schistosomes treated in a variety of ways (chapter 4, figure 4.1). Instead, HSP70 was found to be distributed inside the parasite. Analysis of secreted parasite proteins showed that HSP70 was secreted from the parasite, although in small amounts. (chapter 6). So, it seems that the universal immune response to HSP70 is directed towards the secreted molecule. Secreted parasite proteins, the source of which was the tegument, were indeed found to be very immunogenic (Lewis and Strand, 1991). The immune response to HSP70 (in the form of a detectable antibody titre), is observed only 5-6 weeks after infection with *S. mansoni* which is consistent with the finding that chronically infected patients recognize HSP70, whereas individuals with acute schistosomiasis do not (Moser, Doumbo and Klinkert, 1990). This delayed response suggests that the molecule only becomes accessible to the host immune system after being

secreted from the parasite, or following parasite death, due to its intracellular location. However, the presence of HSP70 in the hatching fluid of schistosome eggs (Scallon, Bogitsh and Carter, 1987) can also be the source for releasing HSP70 into the bloodstream as a circulating antigen (Fu and Carter, 1990).

7.3 HSP70 as a vaccine candidate

As a result of their strong immunogenicity, heat shock proteins have been suggested to represent potential vaccine candidates, perhaps even against multiple pathogens (Newport, 1991).

In order to qualify as vaccine candidates, heat shock proteins must elicit an immune response that can effectively deal with an intact, living parasite. Although HSP70 is generally immunogenic, it is unclear what is involved in the initial stages of its recognition by the host. It seems, as shown in chapter 6, that HSP70 is secreted by the parasite, either during the course of normal turnover or as a response to stress. Furthermore, a fraction of HSP70 actually represents a membrane component of the adult worm although it is not exposed on the surface (chapter 4). In any event, a surface location for the antigens is not a prerequisite for consideration as vaccine candidates, as effective non-specific delayed-type hypersensitivity reactions involving activated macrophages have been shown to be directed at cytoplasmic components of *Schistosoma mansoni* (Pearce *et al.*, 1988). An empirically identified schistosome vaccine target is glutathione S-transferase (GST) (Smith *et al.*, 1986 ; Taylor *et al.*, 1988), a descendant of the crystallin family of proteins which are represented during stress by the family of heat shock proteins. The enzyme appears to exemplify the evolutionary recruitment of a structural protein to perform enzymatic functions, and is peculiar in that, in addition to its typical cytoplasmic location, it is present on the parasite surface membrane ; like the parasite's HSP70, the antigen elicits a specific immune response that does not cross-react with the host (Taylor *et al.*, 1988).

7.4 Types of HSP70 in the parasite

It seems that two isoforms of HSP70 exist in the parasite. The occurrence of HSP70 in different fractions of the parasite (chapter 4), the detection of two complexes of HSP70 in non-denaturing gels and the detection of two forms of HSP70 when performing immunoprecipitation under non-reducing conditions on metabolically labelled proteins of the parasite (chapter 5) supports this notion. Future experiments analyzing HSP70 by two-dimensional gel electrophoresis should clarify the above. This is important to determine since the isoforms may have different functions in the parasite, or at different stages of development.

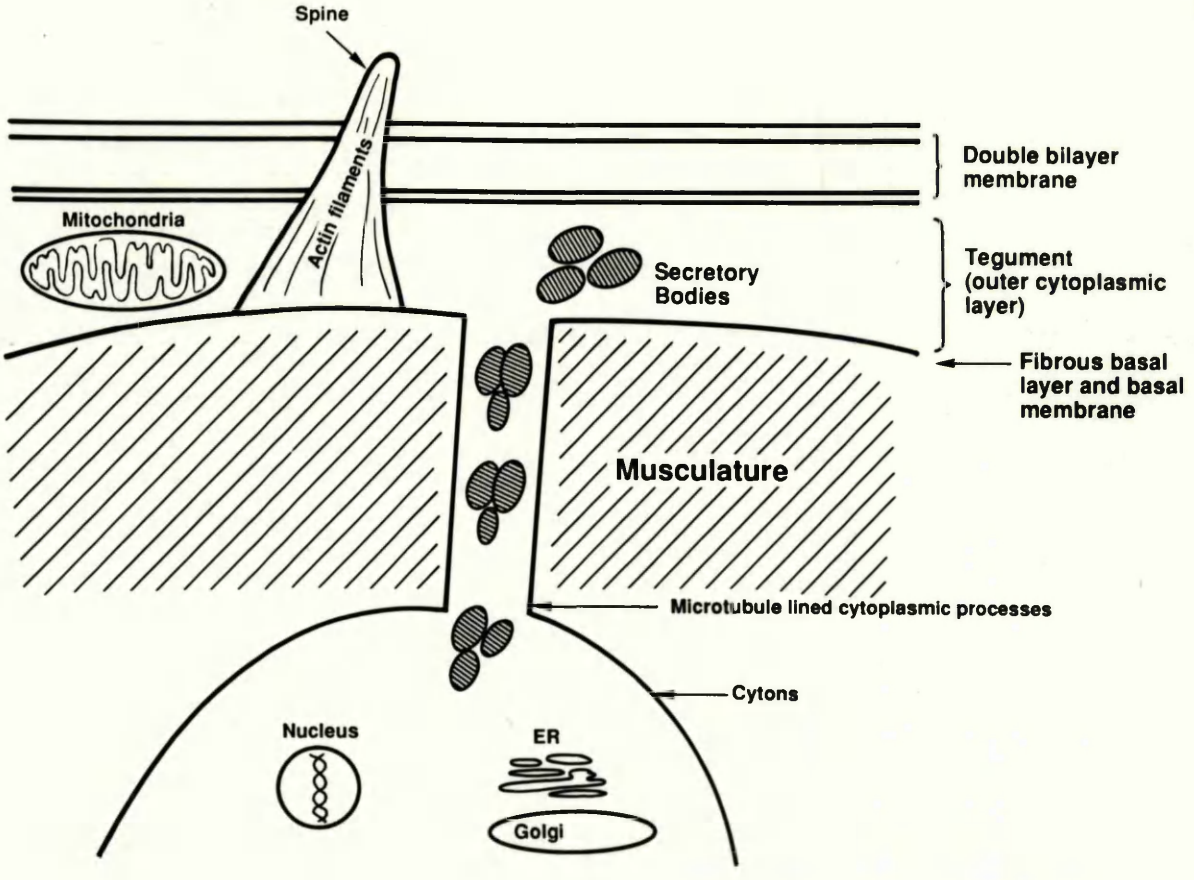
7.5 Distribution of HSP70 in the adult schistosome

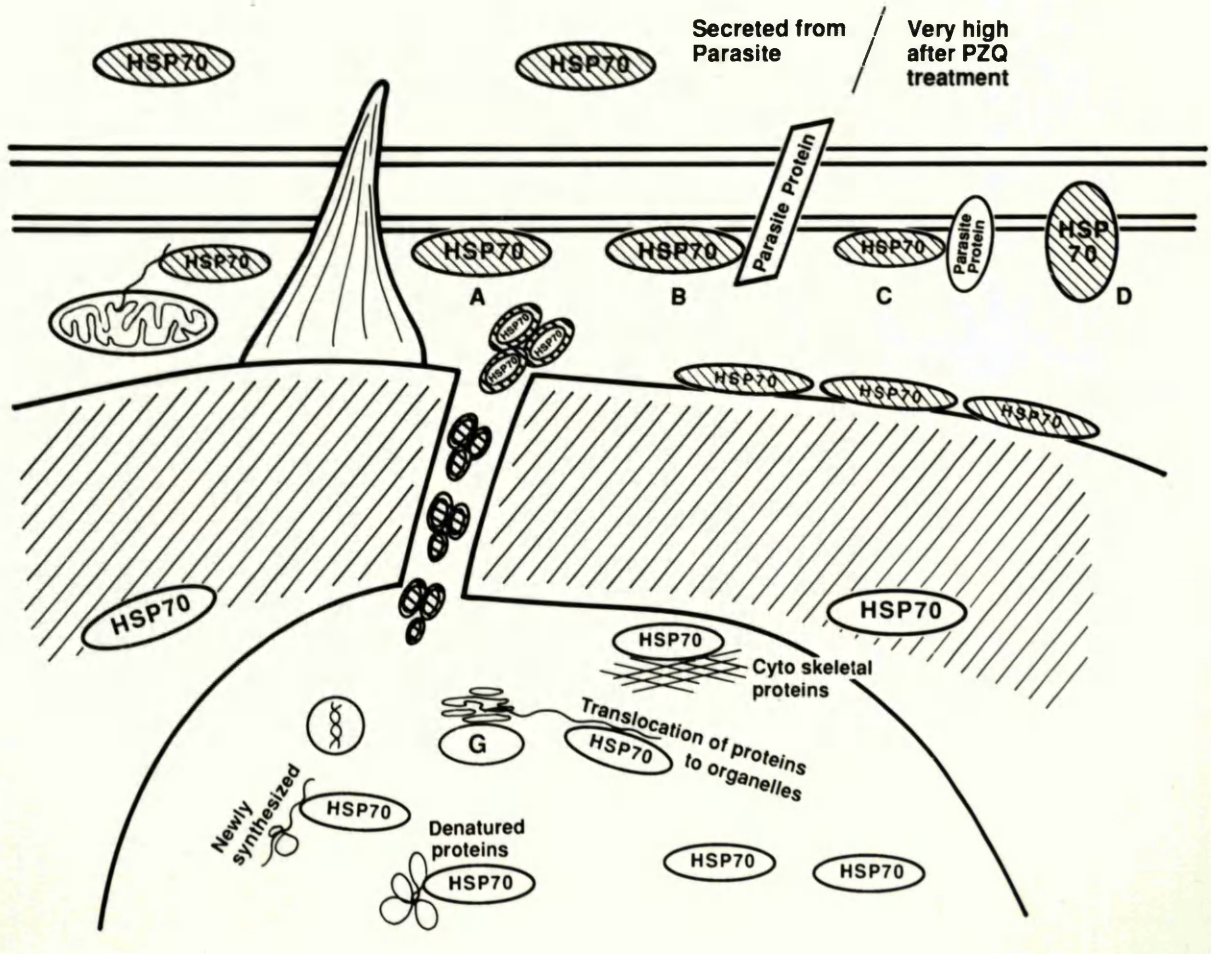
How HSP70 might be distributed inside the parasite and how it might function is illustrated in figure 7.1. The following discussion will be related to this figure.

7.5.1 Distribution of HSP70 in the membrane

A portion of HSP70 was shown to be a peripheral membrane protein (chapter 4) and this is shown in figure 7.1 as A, because a very large fraction of it was found in the aqueous (hydrophilic) phase after extraction with Triton X-114. The occurrence of a very small fraction of HSP70 in the detergent (hydrophobic) phase, however, indicates that the molecule might be an integral membrane protein shown in figure 7.1 as D. This implies that HSP70 could have a membrane anchoring domain, which, if so, would be unique among the known HSP70 proteins. Analysis of the amino acid sequence of the carboxy-terminus of malarial HSP70 showed that, at least, this part of the molecule did not contain any membrane spanning regions (Ardeshir *et al.*, 1987), yet, this molecule was detected on the surface of malarial parasite. Thus, the presence of a membrane anchoring domain in HSP70 of the schistosome is unlikely. Alternatively, HSP70 may partition in Triton X-114-soluble fraction by binding to, or, interacting with an

Figure 7.1 Proposed role for HSP70 in the parasite.


Spine
Actin filaments
Mitochondria
Double bilayer membrane
Tegument (outer cytoplasmic layer)
Secretory Bodies
Fibrous basal layer and basal membrane
Musculature
Microtubule lined cytoplasmic processes
Cytons
Nucleus
ER
Golgi


Secreted from Parasite
Very high after PZQ treatment
HSP70
Parasite Protein
HSP 70
A
B
C
D
Cyto skeletal proteins
Translocation of proteins to organelles
G
Newly synthesized
Denatured proteins

integral membrane protein of the parasite. Precedence for this comes from the observation that HSP70 associated with a protein of M_r >200 kDa (chapter 5), which might be the same protein shown by Sauma and Strand (1990) to be anchored to the adult worm membrane via a glysylphosphatidylinositol (GPI) linkage. Evidence for HSP70 being bound to membrane proteins in other systems comes from the observation that an HSP70 family member is bound to terminal transferrin receptor in membrane vesicles during reticulocyte maturation (Davis *et al.*, 1986).

It is not known to which side of the membrane bilayer HSP70 is located. Further investigations by immunoelectron microscopy should clarify this.

Comparing the distribution of HSP70 in *Schistosoma japonicum*, it would seem that HSP70 may be located on the inner side of the inner bilayer. In *S. japonicum*, HSP70 was found to be located in the tegument and the nervous system of the parasite (Scallon, Bogitsh and Carter, 1987). Whether the distribution of HSP70 in *S. japonicum* is similar to that in *S. mansoni* is not known. The two molecules seem to be structurally different as antibodies generated towards HSP70 of *S. japonicum* are not cross-reactive with that of *S. mansoni* (Hedstrom *et al.*, 1988).

The location of HSP70 in the tegument is consistent with its low rate of turnover (>24 hours) (chapter 5). The surface of the parasite has a low rate of turnover. This was studied by labelling the parasite internally with tritiated amino acids (Kusel *et al.*, 1975), binding of complement components and labelled antibodies to adult schistosomes (Ruppel and McLaren, 1986) and by loss of host antigens from the parasite surface (Saunders, Wilson and Coulson, 1987). However, loss of external label from the parasite membrane, studied by loss of radiolabelled molecules from adult worms (Kusel, Mackenzie and McLaren, 1975), loss of concanavalin A from schistosomula (Samuelson, Caufield and David, 1983) and of cationized ferritin from adult worms (Wilson and Barnes, 1977), showed that membrane turnover occurred rapidly with a half life of a

couple of hours. The rapid turnover observed in these cases may be due to repair of sub-lethal damage to the surface caused by some of the agents used to study it, for example, concanavalin A and cationized ferritin. This necessitates further studies on the rate of turnover of HSP70 after membrane damage which will be also valuable after praziquantel treatments of the worms.

7.5.2 Distribution of HSP70 inside the parasite

The results from chapter 4 showed increased anti-HSP70 antibody binding to denuded worms (ie worms of which the membranes were removed), and in frozen sections of the worm, which indicated the presence of HSP70 in the fibrous basal layer and inside the parasite, respectively.

Non-ionic detergent (Triton X-100, Nonidet P40)-insolubility is taken as a measure for cytoskeletal components. Thus,In the membrane, HSP70 did not seem to associate with the cytoskeleton because it did not partition in Triton X-100-insoluble fraction (chapter 4). However, in contrast, HSP70 in the total worm homogenate was shown to occur both in Nonidet P40-soluble and insoluble fractions, although a major fraction of it was detergent-soluble (chapter 5, figure 5.10). This indicates the association of HSP70 with some cytoskeletal components inside the parasite. The idea is supported by the finding that HSP70 forms stable interactions with some parasite proteins. These proteins might well be cytoskeletal proteins. Such a possibility is consistent with the findings in several laboratories of polypeptides of M_r about 70,000 associated with microtubules (Weatherbee, Luftig and Weihing, 1980; Whatley *et al.*, 1986), microfilaments (Bretscher and Weber, 1980), intermediate filaments (Wang, Asai and Lazarides, 1980; Clark and Brown, 1986) and a cell surface glycoprotein (Hughes and August, 1982). Association of HSP70 may suggest that this heat shock protein plays a role in the repair of reversible damage to the cytoskeleton. A cyto-architectural role for a heat shock protein similar to HSP70 has been suggested previously in studies examining its association with avian skeletal

myofibrils and intermediate filaments (Wang, Asai and Lazarides, 1980). Others have suggested that this heat shock protein may serve to mediate cytoskeletal and membrane interactions (Hughes and August, 1982).

7.6 Functions of HSP70 in the parasite

The general role of heat shock proteins in protein binding and translocation has recently been described by various workers (Chirico, Waters and Blobel, 1988; Deshaies *et al.*, 1988; Ellis, 1990; Nelson *et al.*, 1992). A similar function for it in the parasite is very likely as it was shown in chapter 5 that HSP70 in the adult worm associated with several newly synthesized proteins. Some of these associations were transient while others were more stable. Although the identity of these proteins is not known, they have similar molecular weight protein counterparts which bind HSP70 in other systems (see discussion for chapter 5). By analogy to the function of HSP70 in other organisms, one can speculate that some of these newly synthesized proteins are being translocated by HSP70 to different cellular compartments, held in conformations that will prevent incorrect interactions with other proteins (Ellis, 1990), or they are denatured parasite proteins that are targeted for lysosomal degradation.

The function of HSP70 in protein translocation may be very important in membrane synthesis and repair. The presence of HSP70 in the membrane and its occurrence in the Triton X-114-soluble (hydrophobic) fraction of the membrane might be important in inserting new peripheral and integral parasite membrane proteins and thus assist in the formation of a new membrane as the old one is shed off and replaced in response to immunological and chemical extracellular signals . This is supported by the observation that HSP70 may associate with integral membrane proteins of the parasite. These may be newly synthesized proteins that are targeted for insertion in the membrane.

HSP70 might also participate in membrane repair. Schistosomes are targets for an aggressive immune response which, not surprisingly, causes

membrane damage. The function of HSP70 in binding to damaged or denatured proteins might assist in the removal of these proteins and in the insertion of new ones. Membrane damaging agents, such as the chemotherapeutic drug, praziquantel, causes increase in the synthesis of HSP70. Whether this increase in synthesis is caused directly by tegumental damage or indirectly by some other process is discussed in chapter 6. The idea can be tested by employing some agents which cause membrane damage and disruption and see if that causes an enhanced synthesis of HSP70. However, one can speculate that the damage caused by praziquantel may lead to denaturation of proteins. The accumulation of these denatured proteins will be a signal that triggers the synthesis of HSP70. Indeed it is shown that production of excess substrates of HSP70 results in the induction of the heat shock response in both eukaryotes and prokaryotes. This is suggested by the fact that a number of inducers of the heat shock response are likely to function by doing just this. Denatured λ repressor, which is a substrate for DnaK *in vitro*, has been shown to induce the heat shock response in *Escherichia coli* (Parsell and Sauer, 1989 ; Gaitanaris *et al.*, 1990) ; injection of denatured proteins into *Xenopus* oocytes also induces the heat shock response (Ananthan, Goldberg and Voellmy, 1986). Protein synthesis inhibitors that cause an accumulation of missense proteins, amino acid analogues or protein fragments induce heat shock proteins (DiDominico, Bugaisky and Lindquist, 1982 ; Goff and Goldberg, 1985).The newly formed HSP70 would thus be necessary for removing these denatured proteins and in making way for new structures that are necessary for parasite survival.

Binding of HSP70 to denatured proteins of the parasite has been suggested recently (Wales, Kusel and Jones, 1992). Irradiated parasites confer a very high degree of immunity to challenge infections. The reason for this immunity is thought to be the presentation of denatured molecules which result from irradiation of the parasite, to the immune system. Irradiation causes an inhibition of parasite protein synthesis including HSP70 synthesis. The inhibition

of HSP70 synthesis may lead to the accumulation of denatured and aberrant proteins which are then presented to the immune system.

Heat shock proteins, in general, have the capacity to bind to different types of proteins. Therefore, it is possible that the denatured or damaged proteins of the parasite are continuously being removed with the help of these heat shock proteins. These denatured proteins may be generated as a result of the normal environmental stresses encountered upon transformation from cercariae to schistosomula. For example, the sudden change in temperature could be expected to stress the intruder, as it should dramatically alter rates of metabolic reactions and denaturation of proteins (Barret, 1986). Consistent with this, it is found that transforming schistosomula express very high levels of HSP70. The levels of HSP70 decline when the schistosomula is 24 hour after which the HSP70 is constitutively expressed in the following stages of the parasite life-cycle (Blanton and Licate, 1992 ; Newport, Culpepper and Agabian, 1988). Thus, it seems that HSP70 serves a unique and perhaps a remodelling function in transforming schistosomula by disaggregating unnecessary or outmoded macromolecular assemblies.

HSP70 might also function as a signal in differentiation or transformation during the life-cycle of the parasite. Messages for HSP70 are present before transformation of cercariae to schistosomula. However, translation of this message is not detected until 8 hours after transformation (Blanton and Licate, 1992). The induction of the synthesis of HSP70 at this time may be involved in adaptation of cercariae / schistosomula to the new environment.

7.7 Hypothesis of how secreted HSP70 after praziquantel treatment can affect host parasite relationship.

The chemotherapeutic action of praziquantel seems to be dependent upon the immune status of the host (Doenhoff *et al.*, 1987, 1988 ; Flisser, Delgado and McLaren, 1990). Treatment of adult schistosomes with the drug is

believed to erode the parasite surface and expose previously hidden antigens (Harnett and Kusel, 1986 ; Brindley and Sher, 1987 ; Flisser and McLaren, 1989), which are targets of immune effector mechanisms that mediate parasite death.

It was found in this work that praziquantel causes increase in the secretion of HSP70 (chapter 6, figure 6.3) and as illustrated in figure 7.1. What effect might this have on host / parasite interaction?. Our first thought was that HSP70 might be exposed on the surface of praziquantel-treated schistosomes. However, as shown in chapter 6, this was not the case. Instead, praziquantel caused a great increase in the secretion of HSP70. This enhanced level of HSP70 may boost the immune system and the antibodies generated might then show enhanced binding to HSP70 in the parasite. One way in which the antibodies could get into the adult schistosomes is through the lesions caused by praziquantel treatment. They could then attack HSP70 and cause parasite death in some way. However, praziquantel did not cause any enhanced binding of HSP70 antibody to the worms *in vitro* (chapter 6, figure 6.1). It is unclear why the antibody did not enter the lesions.

The enhanced secretion of HSP70 after treatment with praziquantel may also lead to the formation of antibody complexes and thus the precipitation of these near the parasite surface. This in turn, may destroy the parasite microenvironment and lead to its death (Kusel and Gordon, 1989).

Another thought that comes into mind is that the increase in the secretion of HSP70 after treatment with praziquantel might trigger an autoimmune response. A role for heat shock proteins in parasite-induced autoimmunity has been suggested (Abu-Shakra and Shoenfeld, 1991). However, although the antibodies generated to HSP70 during the normal course of infection are not cross-reactive with the host HSP70, antibodies which are generated after treatment with praziquantel might be directed to different epitopes of the molecule as praziquantel has been suggested to alter the conformation of certain

molecules that it exposes on the surface of the parasite (Brindley and Sher, 1989). However, no autoimmune disease or response correlating to praziquantel treatment has been reported.

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