BIOCHEMICAL EFFECTS OF IRRADIATION ON LARVAE OF *SCHISTOSOMA MANSONI*.

(2 VOLUMES)

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DOCTOR OF PHILOSOPHY

by

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ABBREVIATIONS.

Abbreviations are those recommended in the Instructions to Authors of the Biochemical Society (1986), with the following additions:

(1) Culture conditions: media and sera.

Elac: Glasgow's modification of Eagle's Minimal Essential Medium, supplemented with 0.5% (w/v) lactalbumin hydrolysate.

EMEM: Eagle's Minimal Essential Medium with Earle's salts.

EMEM w/o phosphate: EMEM without phosphate.

GMEM: Glasgow's modification of Eagle's Minimal Essential Medium.

GMEM w/o methionine: GMEM without methionine.

HEPES: N'-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid.

FCS: foetal calf serum

hiFCS: heat-inactivated foetal calf serum.

NHS: normal human serum.

hiNHS: heat-inactivated normal human serum.

(2) Analysis of protein synthesis.

cpm: counts (of radioactivity) per minute.

hsp: heat-shock protein.

hsp 70: heat-shock protein of molecular weight 70 000.

Mr: relative molecular weight.

R_f: relative mobility.

SDS: sodium dodecyl sulphate

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

TCA: trichloroacetic acid.

TEMED: N,N,N',N'-tetramethylenediamine.

PMSF: phenyl methyl sulphonyl fluoride. } Protease inhibitors

TPCK: L-1-tosylamide-2-phenylethylchloromethyl ketone

TLCK: N-α-p-tosyl-L-lysine chloromethyl ketone.

(3) Analysis of phospholipid synthesis.

P_i: inorganic phosphate.

PA: phosphatidic acid.

PC: phosphatidylcholine

PE: phosphatidylethanolamine

PG: phosphatidylglycerol

PI: phosphatidylinositol.

PS: phosphatidylserine.

TLC: thin-layer chromatography.

(4) Analysis of antigen expression.

4(i) Antisera

Anti-coat 1 } rabbit antisera raised against 2 fractions of the
Anti-coat 2 } cercarial glycocalyx. (Preparation described in 2.6.3.1.).

Anti-CMAG: rabbit antiserum raised against cercarial membrane antigens. (Preparation described in 2.6.3.1.)

IHS: Infected human serum.

NMS: normal mouse serum.

NRS: normal rabbit serum.

4(ii) Lectins

Con A: concanavalin A.

FBP: fucose binding protein (from *L. tetragonolobus*)

PNA: peanut agglutinin.

UEA: *Ulex europaeus* agglutinin.

WGA: wheatgerm agglutinin.

4(iii) Other abbreviations.

A_{492}: absorbance reading at 492 nm.

E.M.: electron microscopy

ELISA: enzyme-linked immunosorbent assay.

FITC: fluorescein isothiocyanate.

FITC-antibody: fluorescein isothiocyanate-conjugated antibody,

FITC-lectin, etc.: lectin, etc.

HRP: horseradish peroxidase.

HRP-antibody: horseradish peroxidase-conjugated antibody.

6-IAF: 6-iodoacetamidofluorescein.

OPD: *ortho* -phenylene diamine.

PI: sodium *meta* -periodate (context makes it clear when PI refers to phosphatidylinositol - see (3) above).

(5) Mouse protection experiments.

i.d.: intradermal
i.v.: intravenous
p.c.: percutaneous
s.c.: subcutaneous

} routes of administration of antigen or organisms.

(6) Immunological terms.

Ab : antibody

Ag : antigen.

ADCC: antibody-dependent cell-mediated cytotoxicity.

BCG: Bacille Calmette Guérin.

Fc: Portion of an antibody which binds to antibody receptors on cells, and to the C1q component of complement.

C3a, C5a: components of the complement system.

Ig: immunoglobulin

MHC: Major Histocompatibility Complex.

(7) Miscellaneous

5-AF: 5-aminofluorescein.

C1, C2, etc: clones number 1, 2, etc.

CYS: cysteine.

E.R: endoplasmic reticulum.

GSH: glutathione.

NEM: N-ethylmaleimide

PBS: phosphate-buffered saline.

$\bar{X} \pm$ S.E: mean, and standard error about the mean

Other abbreviations are occasionally used, and are accompanied by explanations in the text.

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Summary: Biochemical Effects of Irradiation on Larvae of *Schistosoma mansoni*.

Irradiation of *S. mansoni* cercariae causes parasite death well before maturity, yet the attenuated larvae stimulate potent protective immunity in experimental hosts. In contrast, resistance following a normal infection depends upon development of a mature, egg-laying worm burden. This study aimed to investigate how irradiation alters the biochemistry of schistosome larvae to enhance their immunogenicity so effectively.

The results presented in this thesis suggest that U.V.- or gamma-irradiated schistosomula may induce effective resistance because they express antigens in modified, non-native conformations. Such denatured antigens may be generated in a number of ways:

(1) by the normal environmental stresses brought to bear on the parasite during transformation from cercaria to schistosomulum.
(2) by the direct effects of irradiation on molecular structure.
(3) as a result of irradiation-induced inhibition of parasite metabolism, including synthesis of proteins and phospholipids and protein glycosylation.
(4) following synthesis on mRNA templates damaged or altered by irradiation.

These aberrant molecules are predicted to accumulate to especially high levels because irradiated schistosomula synthesize severely reduced amounts of heat-shock proteins. These enzymes assist non-native proteins to attain their mature conformations, or, alternatively, ensure their degradation and removal from the cell.

It is postulated that the altered conformation of antigens from irradiated larvae modifies the pattern of processing by host antigen-presenting cells, resulting in presentation of novel determinants to T-lymphocytes. In consequence, the T-cells stimulated by exposure to irradiated and normal cercariae are likely to differ in both antigen specificity and affinity. The characteristics of the immune response induced by irradiated parasites apparently ensure particularly effective elimination of a challenge infection.

Analysis of the changes in parasite protein synthesis and antigen expression during the four to five days following transformation made it possible to divide the abbreviated lifespan of irradiated schistosomula into several stages. It is proposed that, at each stage, different antigens may activate host immune responses in either the skin or the lungs. However, considerable variability is apparent, both in the potential of separate pools of irradiated cercariae to induce immunity at each of the postulated stages, and in the susceptibility of challenge larvae to the various immune responses.

The model presented in this thesis suggests that a successful vaccine might need to comprise antigens from several stages in larval development, stimulating multiple immune mechanisms which can eliminate challenge schistosomula at a number of points in their migration. The key to induction and expression of protective immunity at each stage appears to be effective presentation of the immunising antigens.

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1 Prevalence of schistosomiasis.

Human schistosomiasis is caused by helminths of the genus Schistosoma, which live within the blood vessels of the hepatic portal system around the gastrointestinal tract (S. mansoni, 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 freshwater 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 are actually infected (Iarotski and Davis, 1981). It is generally accepted that schistosomiasis is becoming more prevalent because of increased snail habitats created by water resource developments, of both large man-made lakes for irrigation schemes and generating hydroelectric power, and small dams to conserve water for use by humans and their livestock. Such developments attract increasing human populations that aggravate the spread and transmission of schistosomiasis (W.H.O., 1985). Current strategies for control of schistosomiasis are based on chemotherapy and snail control. Although these methods are undoubtedly useful, they suffer from some intrinsic disadvantages that render highly desirable the development of alternative measures; in particular, vaccination.

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. The most important snail genera and species, and their geographical distribution, are shown in table 1.1. Table 1.2 summarizes the broad geographic distribution, also the intermediate and definitive hosts of a number of schistosome species. As well as the three principal schistosomes of humans, details of some zoonotic schistosomes are listed. Species normally found in animals or birds may occasionally penetrate human skin and, in some cases, successfully reach maturity. Although these zoonotic infections are not of major clinical importance for humans, schistosomiasis in livestock is a significant economic problem in developing countries. Moreover, field trials with irradiated larvae of S. bovis and S. japonicum in cattle have demonstrated the potential of radiation-attenuated vaccines to stimulate protective immunity in the definitive host.

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.

Table 1.1. Principal intermediate hosts of human schistosomiasis.

	SNAIL SPECIES	GEOGRAPHICAL DISTRIBUTION
1.	HOSTS OF *S. mansoni.* (*Biomphalaria* spp.)	
1.a)	African *Biomphalaria* spp:	
	Alexandrina group:	
	Biom. alexandrina.	Nile Delta.
	Pfeifferi group:	Africa south of Sahara.
	Biom. pfeifferi	S.W. Asia.
	Sudanica group:	Equatorial Africa
	Biom. sudanica	
	Choanomphala group:	Lake Victoria.
	Biom. choanomphala.	
1.b)	American *Biomphalaria* spp:	
	Biom. glabrata.	Caribbean islands. Tropical S.America.
2.	HOSTS OF *S. japonicum.* (*Oncomelania hupensis* spp.)	
	O. h. hupensis	Mainland China.
	O. h. nosophora	Japan
	O. h. quadrasi	Philippines
	O. h. lindoensis	Sulawesi
3.	HOSTS OF *S. haematobium.* (*Bulinus* spp.)	
	Africanus group:	Ethiopia to S.Africa.
	Bul. africanus	
	Truncatus group:	North Africa.
	Bul. truncatus.	

Table 1.2. Schistosomes of man and some related parasites. Intermediate and definitive hosts, and geographical distribution.

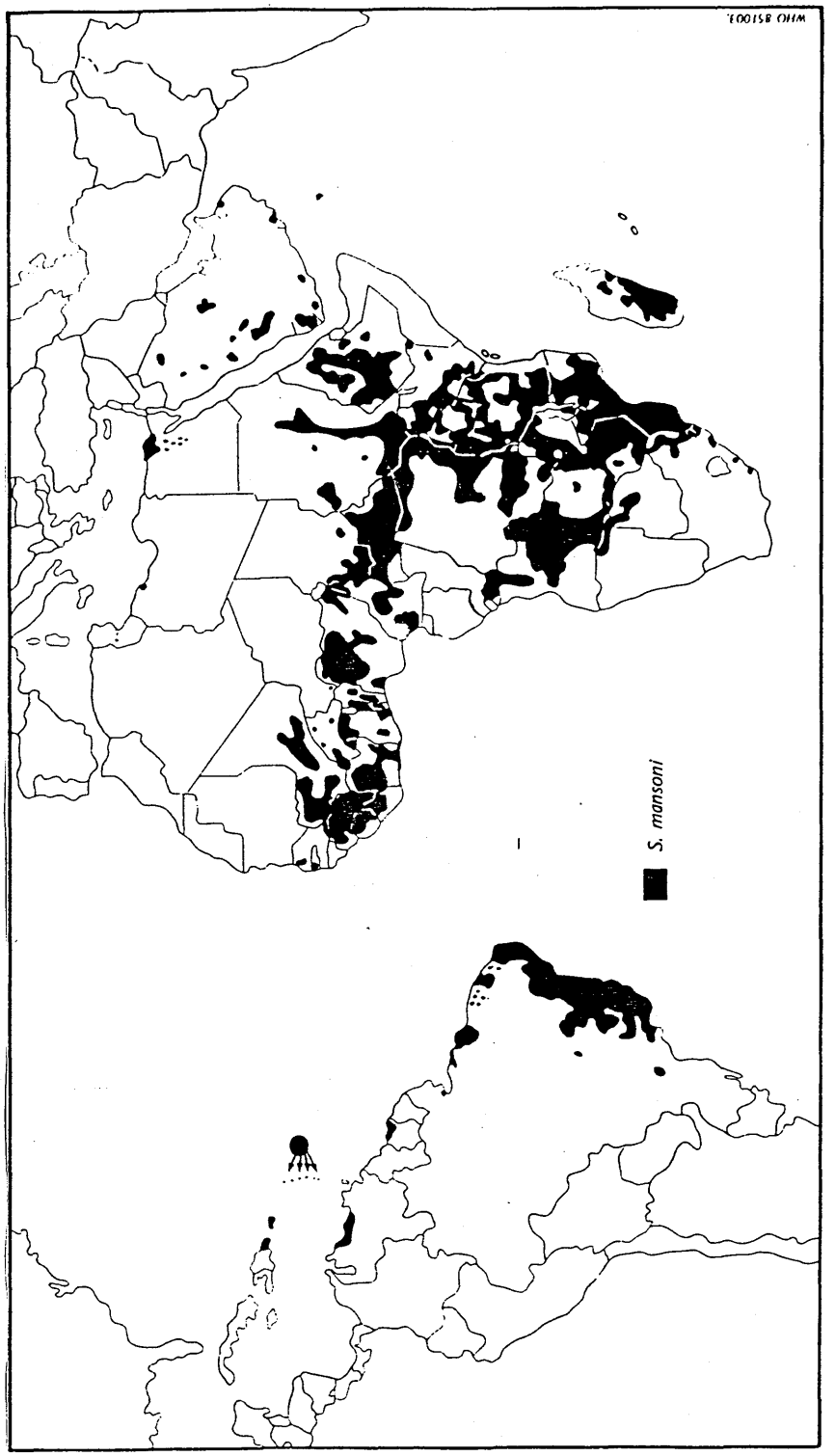
Table 1.2.

SPECIES	LOCATION IN MAN	SNAIL SPECIES	DEFINITIVE HOSTS	DISTRIBUTION.
1. Man is usual definitive host.				
S. mansoni	Lower bowel	*Biomphalaria*	Primates; rodents	Africa, S.W. Asia, S. America, Caribbean.
S. haematobium	U.G.S.	*Bulinus*	Primates	Africa, S.W. Asia.
S. japonicum	Upper bowel	*Oncomelania*	Primates; rodents; ruminants.	China, Japan, Philippines, Taiwan, Indonesia.
2. Zoonotic infections which can mature in man.				
S. mattheei	U.G.S.	*Bulinus*	Ruminants; primates.	S. Africa.
S. intercalatum	Bowel	*Bulinus*	Ruminants; primates.	W./Central Africa.
S. mekongi	Bowel	*Tricula*	Carnivores; primates.	S.E. Asia.
3. Zoonotic infections which do not mature in man.				
S. bovis	(skin)	*Bulinus*	Ruminants	North/Central Africa.
S. curassoni	(skin)	*Bulinus*	Ruminants	West Africa.
S. margrebowiei	(skin)	*Bulinus*	Ruminants	Central Africa.
"Avian" schistosomes	(skin)	*Bulinus*; *Lymnaea*	Birds	Worldwide.

U.G.S. = Urogenital system.
(skin) = Believed to die in human skin.

Figure 1.1: Global distribution of schistosomiasis due to *Schistosoma mansoni*.

(with modification, from WHO Technical Report Series No. 728 (1985)).


WHO 85100A
S. mansoni

1.3 The Parasite.

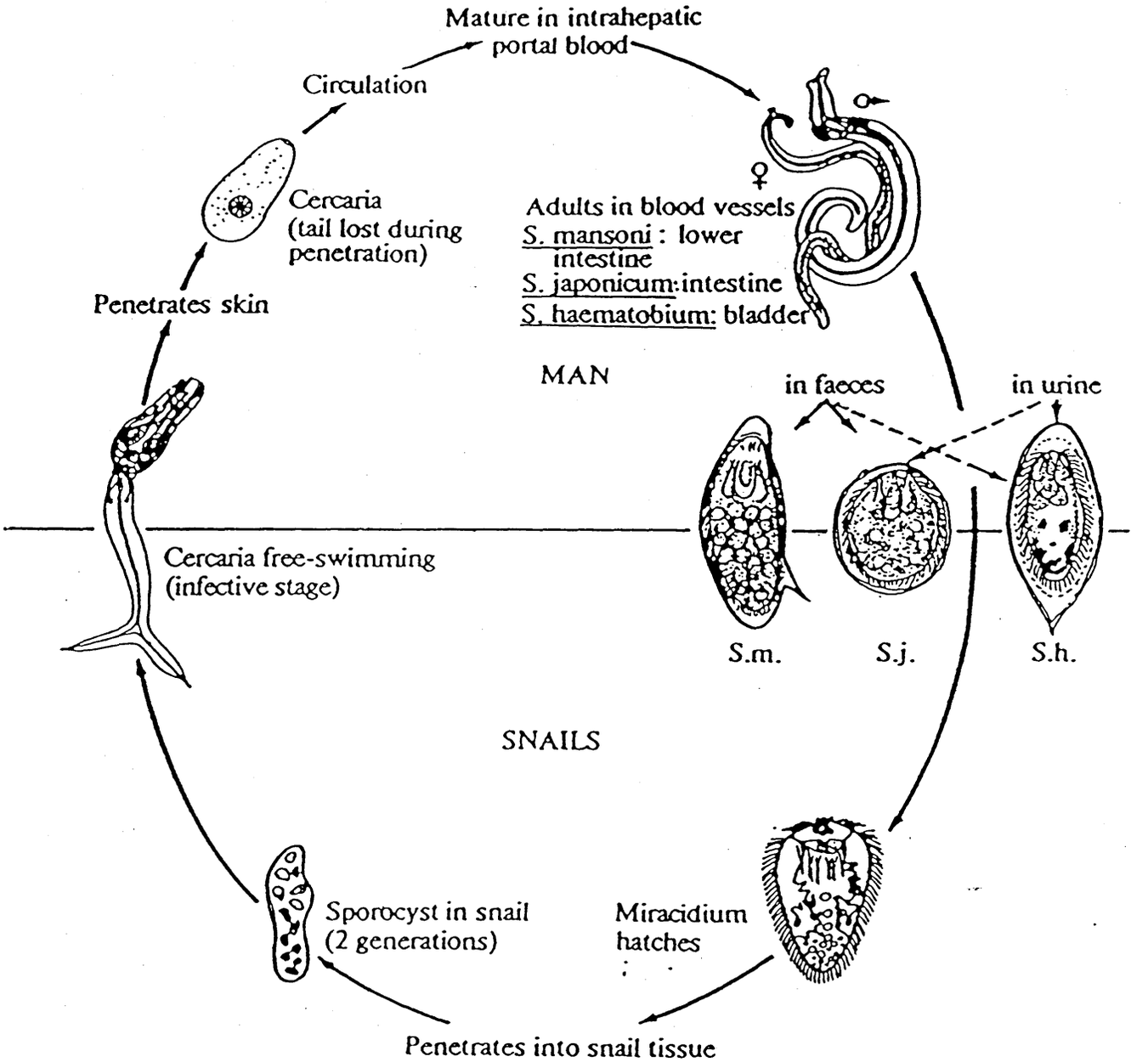
1.3.1. Life-cycle.

(see figure 1.2).

Schistosomes are digenetic trematodes of the family Schistosomatidae, transmitted by freshwater snails. Their name derives from the groove (schist) within which the adult male clasps the female. Adult worms mate in the mesenteric veins, and the female lays eggs which pass through the walls of the blood vessel and the intervening tissues to enter the lumen of the intestine or urinary system. The eggs are voided in the excreta, and must reach fresh water to continue the cycle by hatching to release a free-living larval form - the miracidium. This larva must then penetrate the exposed soft parts of a suitable snail, 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 the water, and penetrate human skin. The cercaria undergoes a substantial metamorphosis during and immediately after penetration to become a schistosomulum. The schistosomulum enters the blood system and passes through the right heart to the capillary beds of the lungs, then migrates to the liver, where it begins feeding and grows into an adult worm. Pairing takes place as the adults approach maturity, and the cycle is completed when the male, clasping the female, migrates against the venous blood flow to the veins around the bladder or intestines.

Thus, the schistosome life-cycle comprises two free-living stages which pass through hypotonic fresh water as they move between conditions of a higher osmotic pressure within the definitive host (human) and intermediate host (snail). During these two free-living

Figure 1.2 Life-cycle of human schistosomes: S. haematobium, S. mansoni and S. japonicum.



Life cycle of *Schistosome*.

transmission phases - vertebrate to snail, and snail to vertebrate - the parasites undergo significant reductions in number. These reductions are counterbalanced by the two parasitic, reproductive stages - asexual reproduction within the snail; sexual reproduction within humans. The success of these reproductive stages appears essential for restoring a population level adequate to maintain endemicity.

Estimated daily female egg production ranges from about 300 for S. mansoni to greater than 3 000 for S. japonicum. A high proportion of newly laid eggs fail to escape from humans. These eggs become trapped, either locally in the organ where they were laid, or in the liver or lungs, where they are carried by the venous blood flow. The host tissue reactions to these eggs give rise to pathological lesions responsible for the principal disease manifestations of schistosomiasis (Jordan and Webbe, 1982).

1.3.2. Morphology and Biology of S. mansoni

Figure 1.3 shows the principal features of adult worms, eggs, miracidia, cercariae and schistosomula of S. mansoni.

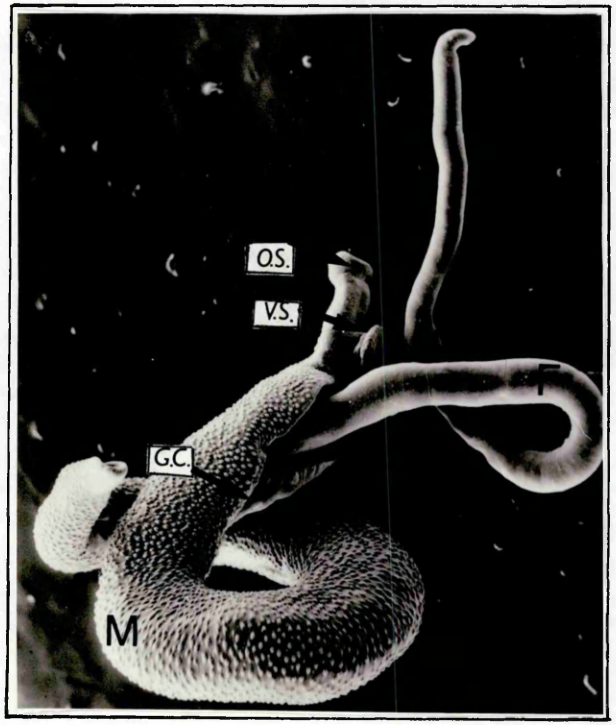
1.3.2.1. Adult worms.

The male and female worms of S. mansoni are slender, elongated parasites, 1-2 cm. in length. The female tends to be longer and 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.

Figure 1.3. Principal features of adult worms, eggs, miracidia, (1) to (5) cercariae and schistosomula of S. mansoni.

SCHISTOSOMA MANSONI: LIFE-CYCLE STAGES.

1. ADULT WORMS.



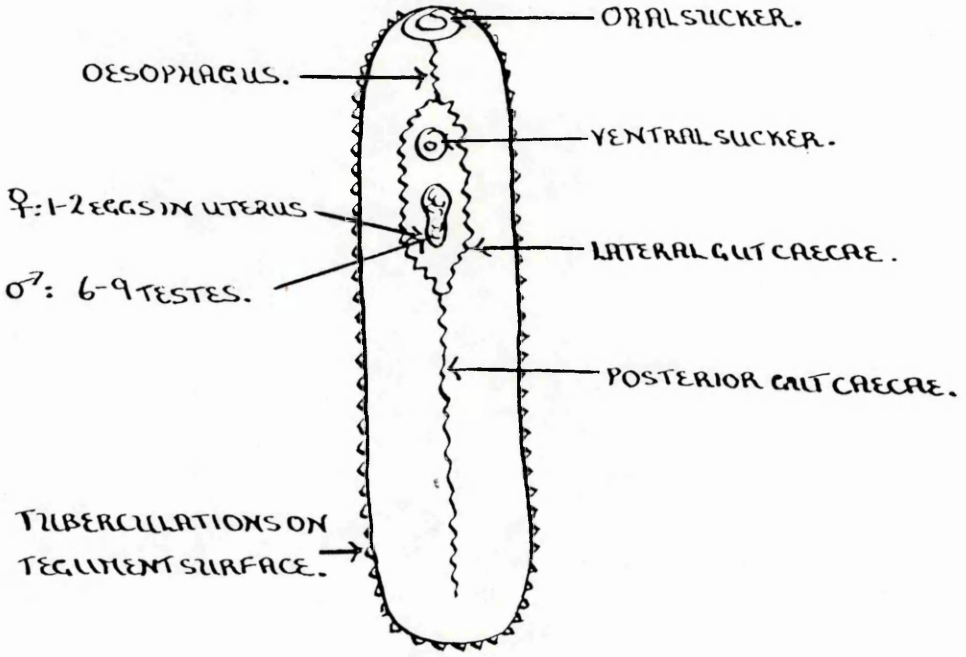
M: male **F**: female
G.C.: gynaecophoric canal
V.S.: ventral sucker
O.S.: oral sucker

Scanning E.M. courtesy of J.R. Kusel.

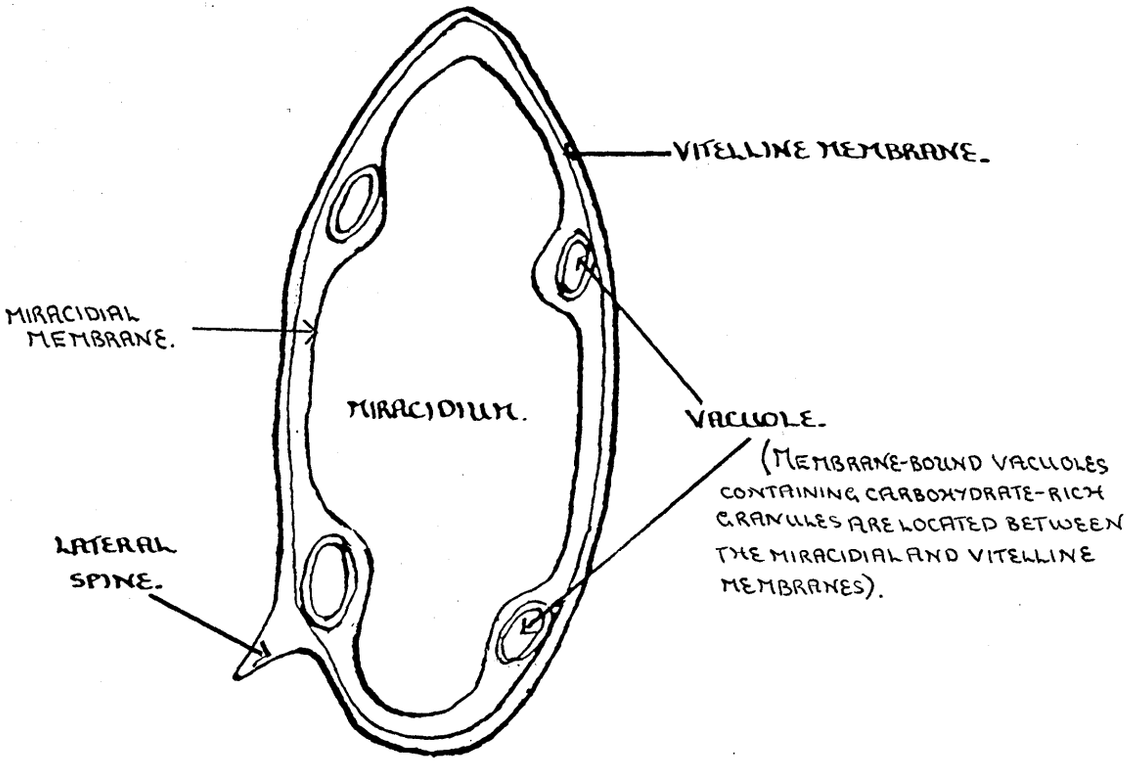
Dimensions: male: 1cm x 1mm

MALE: 5-11mm. LENGTH; 1mm. DIAMETER. (50-70 DAYS POST-INFECTION).

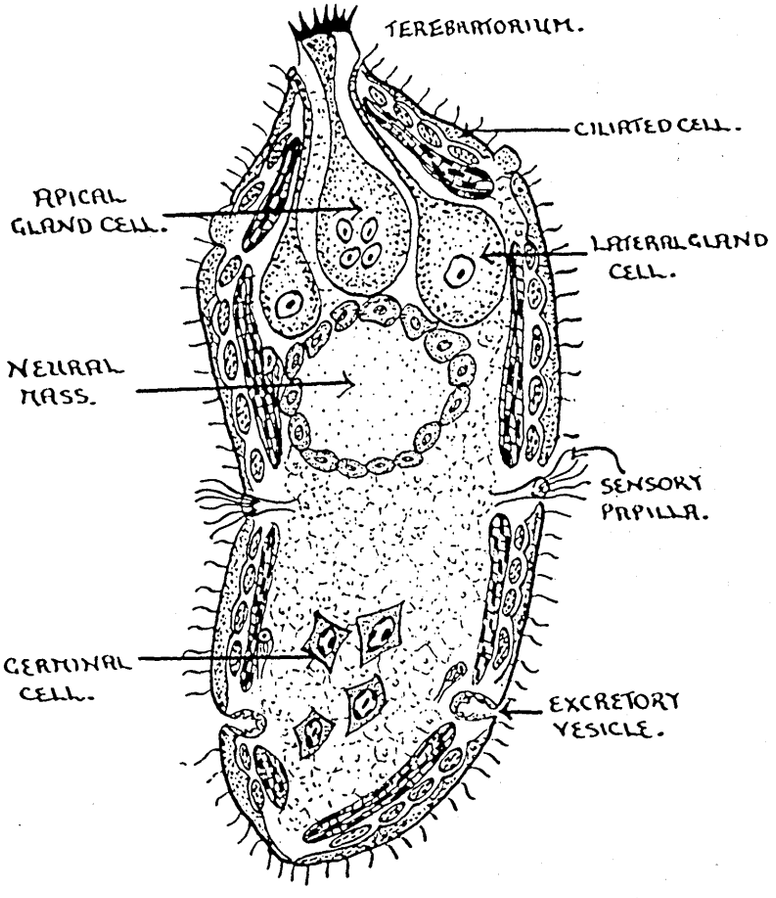
FEMALE: 10-20mm. LENGTH; 0·2mm. DIAMETER.



GENERAL STRUCTURE.
(after Stirrock, 1987).

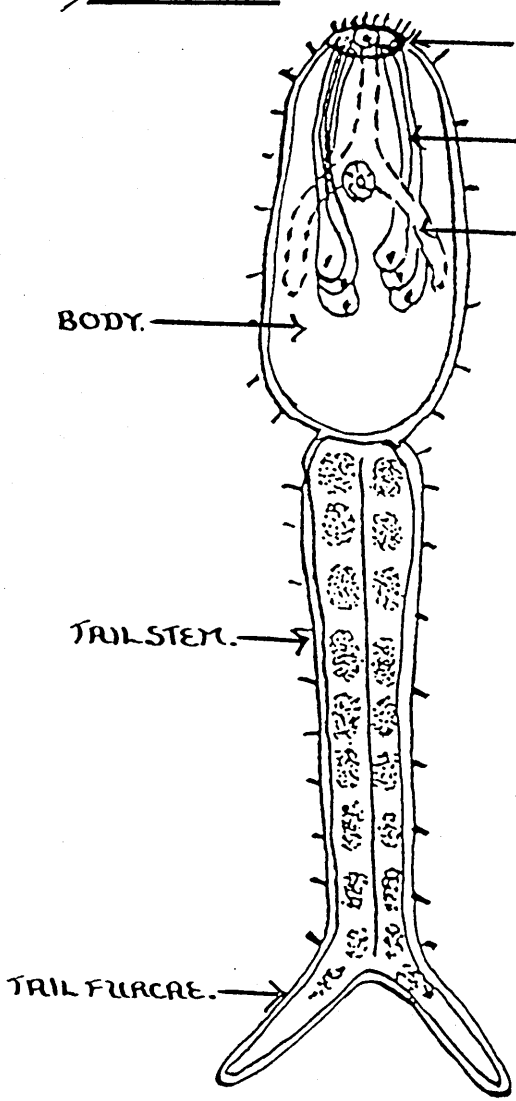


2/ EGG. 140 µm × 60µm. average.
(after Kusel, 1970a).



3/ MIRACIDIUM. 150-180 µm × 70-80µm.
(after Pan, 1980).

4. CERCARIA.



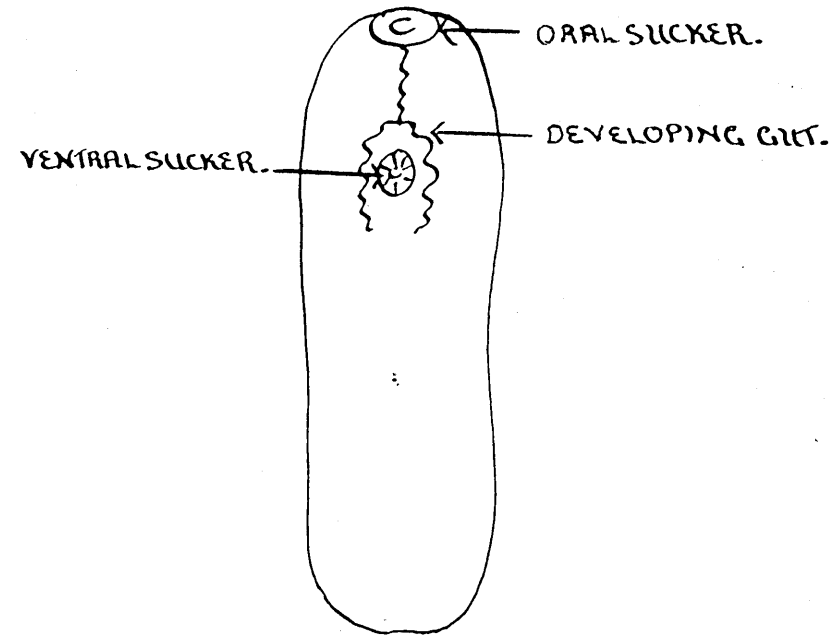
BODY: 214 x 94 µm.

TAIL STEM: 225 x 68 µm.

TAIL FURCAE: 105 µm.

(after Chappell, 1979).

5. SCHISTOSOMULUM (LESS THAN 4 DAYS POST-TRANSFORMATION).



DIMENSIONS: 214 x 94 µm.

a) The Surface

Scanning electron microscopy has revealed the surface topography of the adult worm. The dorsal surface of the male bears numerous large tubercles, each equipped with pointed spines. The entire surface of the female is pitted, with a few spines, but the tubercles 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.

Transmission electron microscopy indicates that the adult worm body is covered with an acellular, syncytial tegument. Using a uranyl acetate fixation method, Hockley and McLaren (1973) showed that the tegumental outer membrane is heptalaminate, apparently consisting of two closely apposed trilaminate membranes. These workers subsequently demonstrated that this double outer membrane is an adaptation characteristic of helminths which reside in intravascular habitats (McLaren and Hockley, 1977). Biochemical studies indicate that, as befits the interface between parasite and host environments, 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 turn over rapidly, replacement and repair being effected by membranous bodies within the tegument.

b) Role of the tegument in nutrition.

Glucose, amino acids, purines and pyrimidines from host blood can be absorbed transtegumentally across the exposed dorsal surface of male worms (Chappell, 1974; 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).

c) Role of the tegument in immune evasion.

- see section 1.5.7.

d) Internal structure.

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

The digestive system of adult worms 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. Host blood is ingested via the mouth; cells lining the gut secrete a globinase enzyme which digests serum proteins and haemoglobin to release tyrosine. Residue containing a black haematin-like pigment is regurgitated as a waste-product.

The reproductive organs are located between the two lateral gut caecae. S. mansoni males have 6-9 testes, located behind the ventral sucker. The testes lead to a seminal vesicle which the vas deferens connects to the gonopore. 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.

1.3.2.2. The Egg stage

S. mansoni eggs have average dimensions 142 µm. x 60 µm (Jourdane and Théron, 1987). The eggshell consists of sclerotin-tanned protein. Eggs of all species bear a spine, in either a lateral (S. mansoni and S. japonicum) or a terminal (S. haematobium) position. The inside of the eggshell is lined with a vitelline membrane which adheres to the shell by means of vacuoles pressing on the miracidium anteriorly and posteriorly (figure 1.3(2)).

A number of factors have been suggested to assist passage of schistosome eggs through the wall of blood vessels and the parenchyma of the intestine. Blood pressure, peristalsis, proteolytic enzymes secreted by the miracidia, and the spine of the egg, may all contribute (Bloch, 1980).

Certain environmental conditions are required to initiate hatching of schistosome eggs. Hypotonicity of the water appears to be the limiting factor (Kassim and Gilbertson, 1976), though light and temperature may also act as stimulants (Erasmus, 1972).

Hatching appears to occur by a purely physical mechanism. As water enters the vacuoles, increased turgor pressure causes the eggshell to split, and the miracidium escapes (Kusel, 1970 a).

1.3.2.3. The Miracidium.

The morphology of the miracidium has been investigated by both light and electron microscopy (Pan, 1965; 1980). A newly released miracidium varies between 150 and 180 µm. in length, and 70 to 80 µm, in width. It is covered by a series of tegumental plates bearing numerous cilia which, in concert with the longitudinal and circular muscles underlying the ciliated cells, propel the miracidium through the water. An anterior apical papilla or terebratorium bears various

sensory organelles. Ducts from the paired cephalic, or penetration, glands emerge at this point. These penetration glands secrete proteases involved in both passage of the egg through mammalian host tissues and, later, in penetration of the snail by the miracidium. The posterior region of the larva is occupied by some twenty germinal cells.

Miracidial behaviour is directed towards location and penetration of a suitable aquatic snail. S. mansoni miracidia display negative geotaxis and positive phototaxis (Chernin and Dunavan, 1962), thus tending to locate to the water surface, where Biomphalaria species are most often to be found. The proximity of snails causes an increase in velocity and rate of turning by the miracidia, which improves their chance of encountering a snail (Chernin, 1970). This behaviour seems to be a response to chemical stimuli from the snail host. Miracidia of S. mansoni may remain infective for 8 to 12 hours after hatching (Prah and James, 1977).

During penetration, the apical papilla becomes attached to the epithelium of the exposed soft parts of the snail, usually the foot, the other penetration points being either the tentacle or the edge of the mantle (Jourdane and Théron, 1987).

1.3.2.4. Intramolluscan stages.

As the miracidium penetrates the snail, the tegumental plates and cilia are shed. The ciliated epithelium is replaced by a syncytial tegument, and the body reorganises into a long sac - the mother sporocyst. The germinal cells of the miracidium grow and multiply, then individual daughter sporocysts comprising somatic and germinal cells develop. At the end of their differentiation in the mother sporocysts, the daughters look like vermiform larvae, 150-250 µm in length.

Between the tenth and seventeenth days after infection, the young sporocysts break through the tegumentary wall and migrate to the

digestive gland of the snail. Each daughter sporocyst contains between 50 and 100 germinal cells. Once settled in their permanent location in the digestive gland, daughter sporocysts increase greatly in size, and differentiation of cercarial embryos occurs.

Cheng and Bier (1972) described in detail the different stages in cercariogenesis for S. mansoni. Successive divisions of the germinal cells within daughter sporocysts lead to formation of the morula stage, comprising many somatic cells and a few germinal cells. This morula stage then develops into germ balls, covered by a primitive epithelium formed by fusion of the somatic cells. The embryonic mass divides into two unequal parts. The larger, anterior part develops into the cercarial body; the small posterior part into the tail. Complete development of S. mansoni cercariae takes about a week.

Jourdane et al (1980) demonstrated that the productivity of daughter sporocysts was not solely directed towards cercariae. They may also multiply to produce new generations of sporocysts. This aspect of intramolluscan development will be discussed in more detail in chapter 8.

Release of S. mansoni cercariae from the snail follows a circadian rhythm, occurring upon exposure to light during the photophase.

1.3.2.5. The Cercaria.

The cercarial tegument is a single continuous cytoplasmic structure covering the body and tail. The outer membrane is trilaminate, and invested with a 1-2 μm. thick, fibrillar glycocalyx. Beneath the tegument are, consecutively, a basal lamina, a thick layer of interstitial material, and circular and longitudinal muscle fibres.

A strong muscular terminal oral sucker and a weaker ventral sucker (acetabulum) are present on the head. Pre-acetabular and

post-acetabular gland cells open via ducts into apertures within the oral sucker. The pre-acetabular glands secrete enzymatic material; the post-acetabular ones, mainly mucus.

The principal means of movement for cercariae is by bursts of swimming - rapid lashing of the tail pulls the body along behind it. After a period of swimming, the cercariae sink slowly, body first. Another burst of activity then causes them to rise upwards through the water again. Cercariae change their activity pattern in response to shadows or turbulence, stimuli suggesting approach of a possible host through the water.

The lifespan and infectivity of cercariae is determined by their energy reserves, chiefly in the form of glycogen. Glycogen content declines in an exponential manner after shedding, diminishing rapidly after 6 hours (Olivier, 1966).

Having located their host, cercariae adhere to human skin by their suckers, aided by mucus secretions from the post-acetabular glands. Discharge of proteases from the pre-acetabular glands may occur when the oral sucker penetrates into the keratogenous zone of the skin. Free fatty acids in skin secretions, in particular, linoleic acid, seem to act as stimulants for penetration. Muscular contractions of the body carry the parasite into the dermis. The tail is shed as the free-living cercaria transforms into a parasitic schistosomulum adapted to life in the isotonic medium of the human bloodstream.

1.3.2.6. The Schistosomulum.

The schistosomulum is derived from the cercarial head, and develops over some four weeks into the adult worm. The invading cercarial head passes into the dermis, where it may remain for up to 120 hours (mouse model - Miller and Wilson, 1978). During this period, the organism adapts to parasitism in the vertebrate bloodstream. In

particular, the surface membrane changes from a single lipid bilayer into the typical adult double bilayer. The morphological and biochemical changes occurring during the transformation process will be described in detail in section 1.5.2.

Dynamic studies using isotope-labelled cercariae in experimental hosts show that schistosomula begin leaving the skin within a few hours of penetration, and that few remain after the fourth day. They are believed to migrate, mainly via the circulatory system, to the right heart, and on to the lungs, where they are delayed as they traverse the alveolar capillary beds. Peak numbers reach the lungs between 4 and 9 days after skin penetration. The first schistosomula reach the liver via the left heart, the systemic circulation and the hepatic portal system, about six days after penetration. Others, however, traverse different capillary beds, and return to the heart-lung circulation for a second circuit. The numbers of schistosomula in the liver build up over the 7 to 10 days after they first arrive. Schistosomula which do not reach the liver within three circuits probably die (Wilson and Coulson, 1986; Wilson *et al,* 1986).

Schistosomula start to elongate upon arrival in the lung. Metabolic studies of worms recovered from experimentally infected hosts suggest that parasite growth begins upon arrival in the liver (Lawson and Wilson, 1980a). However, it may be noted that, *in vitro,* signs of growth are detectable after as few as 4 days of culture (Clegg and Smithers, 1972). When growth begins, the gut becomes functional, extending to form two lateral caecae which reunite behind the ventral sucker. The parasites ingest red blood cells, and black haematin accumulates in the gut. By day 20 to 30, differentiation of the reproductive organs begins. Male and female worms mate, and start to lay eggs. The lifespan of worm pairs in humans has been variously estimated to average 3.5 to 12 years, with some worms surviving for 30 years or more (Vermund *et al,* 1983). Consequently, schistosomiasis is

a disease of long chronicity.

1.4 Pathology of human schistosomiasis.

The clinical manifestations of schistosomiasis in humans follow a time-course which corresponds, firstly, with parasite migration and development, then with establishment and persistence of an egg-laying infection. The pathology of chronic schistosomiasis is caused by schistosome eggs, rather than the worms themselves. Adult worms are impervious to host immune attack, giving rise to few focal lesions compared with the millions of eggs generated in the course of infection. Von Lichtenberg (1987) has reviewed the aetiology of schistosomiasis. Some of the immunopathology states are described below.

Cercarial dermatitis in human patients is characterized by a mild pruritis, followed 10 to 15 hours later by development of 3-5mm papules, erythema, oedema and severe pruritis (Cort, 1950). These symptoms appear to accompany cercarial reinvasion of the skin of already sensitized hosts. Using the mouse as an experimental model, it has been shown that primary exposure to cercariae elicits a mild inflammatory response, neutrophils being the predominant infiltrating cells. Secondary exposure, either to live cercariae or isolated cercarial antigens, stimulates an earlier, more intense and more durable cellular response, enriched in eosinophils (Von Lichtenberg *et al,* 1976).

As in the skin after cercarial penetration, schistosomula of a primary infection appear to cause few perturbations during their migration through the lungs of experimental hosts, whereas secondary and subsequent migrations through lung tissue elicit more intense cellular reactions (Von Lichtenberg, 1987). Reports of asthma-like episodes occurring in human patients within two weeks of exposure to

S. mansoni may correspond to this stage of immunopathology.

However, the onset of the symptoms characteristic of acute schistosomiasis - "Katayama fever" - coincides with the time of first appearance of eggs in the stools. Symptoms include fever, abdominal pain, nausea, diarrhoea, arthritic pains and swollen lymph nodes. Hepatosplenomegaly is often present. Blood counts show leucocytosis with pronounced eosinophilia. As the infection becomes chronic, these symptoms clear, while egg excretion rates remain unchanged.

No well-characterised experimental model for Katayama fever exists, and the pathogenesis of this state is unclear. Egg granulomas are very large at this time; lymphocyte blastogenic responses are high, and non-selective hypergammaglobulinaemia may be present, suggesting polyclonal B-cell activation. Circulating immune complexes may also occur. It has been suggested that this massive stimulation of the host immune response consequent upon egg-laying may induce the symptoms of acute schistosomiasis. Development of suppressive responses, and restoration of a degree of homeostasis as the disease progresses, may account for remission of these acute symptoms.

It may be noted, however, that comparatively few infected patients develop this acute febrile illness. Most infected children have only minor early symptoms. Their health may continue to appear satisfactory during the subsequent chronic phase of the disease, even while, in some individuals, lesions of the internal organs may be progressing as evidenced by objective clinical observations.

Chronic schistosomiasis mansoni may be either subclinical, or severe and symptomatic. Subclinical stages may present no definite disease manifestations, although hepatomegaly or hepatosplenomegaly may occur. Granulomatous lesions are frequently present in the liver and instestine, and generally contribute to pathogenesis.

Eventually, five or more years into infection, predisposed individuals with heavy parasite burdens begin to suffer advanced

fibrovascular lesions of the liver. In heavily infected populations, the prevalence of severe, symptomatic schistosomiasis may reach 5% or higher. The majority of infected persons, however, continue indefinitely in the subclinical state.

Portal fibrosis begins with diffuse inflammatory infiltration around portal radicles, proximal to presinusoidal vessels containing egg granulomas. Portal vein branches become blocked by granulomas, and disrupted by inflammation and fibrosis. Eggs and egg granulomas accumulate around these blockage sites, further contributing to portal enlargement. At the same time, the hepatic arteries enlarge, and send out neovascular branches. In this way, the presinusoidal portal hypertension resulting from fibrosis is compensated for by increased arterial flow. During this "compensated stage", liver cell function tests show few abnormalities, but splenomegaly and variceal haemorrhage occur as the result of portal hypertension.

Beyond this stage, hepatic decompensation and coma slowly develop.

In summary, the chronic nature of schistosomiasis means that the clinical manifestations of disease present a wide spectrum, ranging from the mild symptoms reported in surveys of endemic children to disablement or death in patients with advanced disease. This gradual evolution of schistosomiasis over a period of years, and the development of chronic pathology in a limited number of predisposed subjects, open the way for environmental control measures, chemotherapy, or immunomanipulation, to forestall development of the chronic state, and perhaps, ultimately, to abolish transmission.

1.5 Transformation of cercariae to schistosomula: structural and biochemical changes.

This project concentrates on a limited part of the schistosome life-cycle - the transition from free-living cercaria to lung stage

schistosomulum. The biochemical, physiological and morphological developments intrinsic to this transformation process are therefore described in some detail.

1.5.1. Definition of transformation

According to Stirewalt (1974), the strict definition of a schistosomulum is that stage of the schistosome into which a cercaria transforms after penetration of skin. However, recovery of schistosomula from skin is time-consuming and yields only limited numbers of organisms. Various methods have therefore been developed for producing schistosomula in quantity *in vitro* (reviewed by Stirewalt *et al*, 1974, 1983; Cousin *et al*, 1986; Salafsky *et al*, 1988). These techniques include penetration through excised skin membranes (dried or fresh rat, mouse, hamster or human skin); chemical stimulation using human or rat skin surface lipid, chloroform/methanol extracts of human skin lipid, or linoleic acid; mechanical stimulation by centrifugation and/or vortexing, and shearing off tails by syringe passage. Comparisons of the morphology and function of these artificially - derived schistosomula with *in vivo* forms have helped to define, firstly, the characteristics which distinguish a true schistosomulum, and secondly, the essential environmental triggers which stimulate transformation.

Stirewalt (1974) listed at least 20 characteristics which different investigators have used to define schistosomula. Changes in the structure of the surface tegument seem to be the major indicator of transformation. In a subsequent study (Stirewalt *et al*, 1983), *in vitro*- and *in vivo*-derived schistosomula were examined with respect to seven parameters selected as especially important. Loss of cercarial glycocalyx as indicated by absence of the cercarienhüllen reaction* and ultrastructural studies, development of a heptalaminate surface

membrane, migration of membranous vacuoles from subtegumental cells into the tegument, development of water intolerance and of stability to freezing, all indicated development of the surface structure characteristic of schistosomula. Another suggested criterion of transformation involved nuclear changes from heterochromasy (inert) to euchromasy (active). However, Salafsky et al (1988) claimed that euchromasy only occurred in damaged organisms containing necrotic cells. Stirewalt et al (1983) and Cousin et al (1986) concluded that the essential stimulus for cercaria to schistosomulum transformation, as defined by the above criteria, was incubation in isotonic medium at 37°C. Complex culture media were not necessary: PBS or even physiological saline were sufficient. Body-tail separation by mechanical stress, and gland exhaustion, were both irrelevant.

However, despite the apparent simplicity of the stimuli for transformation, reservations must be borne in mind when considering the in vivo relevance of chemically- or mechanically-transformed cercariae. Although, overall, the same surface changes seem to occur in artificially-derived schistosomula as in skin forms, the developments are significantly slower in the former. In vitro-prepared schistosomula may take three hours or longer to display the morphological characteristics evident within an hour of skin penetration. (Stirewalt et al, 1983; Cousin et al, 1983). The biochemical properties of artificially transformed schistosomula may also fail to represent the true in vivo situation. Salafsky et al (1988) showed that chemically- and mechanically-transformed schistosomula, identical by morphological criteria, differed with respect to eicosanoid production, RNA and protein synthesis. Although these workers did not perform comparative studies with skin forms, they emphasized that biochemical studies on in vitro-derived schistosomula should be interpreted with caution until the biochemical characteristics of genuine in vivo forms are better defined.

* Note: The cercarienhüllen reaction tests for the presence of the cercarial glycocalyx. Antibodies in infection serum react with glycocalyx antigens to form a complex visualized under the light microscope as an envelope sloughing from the parasite surface.

1.5.2. Changes in the tegumental outer membrane during transformation.

Transmission and scanning electron microscopy demonstrate that the structure of the parasite tegument is completely reorganized during transformation. The cercarial tegument is bounded by a single unit membrane and a thick glycocalyx which appears in the scanning electron microscope as a 1-2 μm thick mesh of 15-30 nm fibrils (Samuelson and Caulfield, 1985). The glycocalyx stains with ruthenium red, tannic acid, and by techniques that depend upon periodate oxidation. These reaction properties suggest that the glycocalyx is likely to be a complex carbohydrate structure - a proteoglycan or lipopolysaccharide (discussed by Samuelson and Caulfield, 1985). Biochemical studies (Caulfield et al, 1987) indicate that the glycocalyx is composed of approximately 80% carbohydrate and 20% protein.

Within 30 minutes of entering the skin, membranous vacuoles 100-150 nm in diameter appear in cell bodies located just below the cercarial tegument. These membranous vacuoles, usually limited by two closely apposed trilaminate membranes, pass into the tegument via cytoplasmic connections. They then fuse with the existing membrane, flowing and spreading over the surface to form the heptalaminate outer membrane characteristic of schistosomula. At the same time, the trilaminate plasma membrane of the cercarial tegument, with adherent glycocalyx material, is shed in the form of microvilli up to 4μm long. Formation and elimination of these microvilli occur, on average, between 20 and 90 minutes after entering the skin. By three hours after

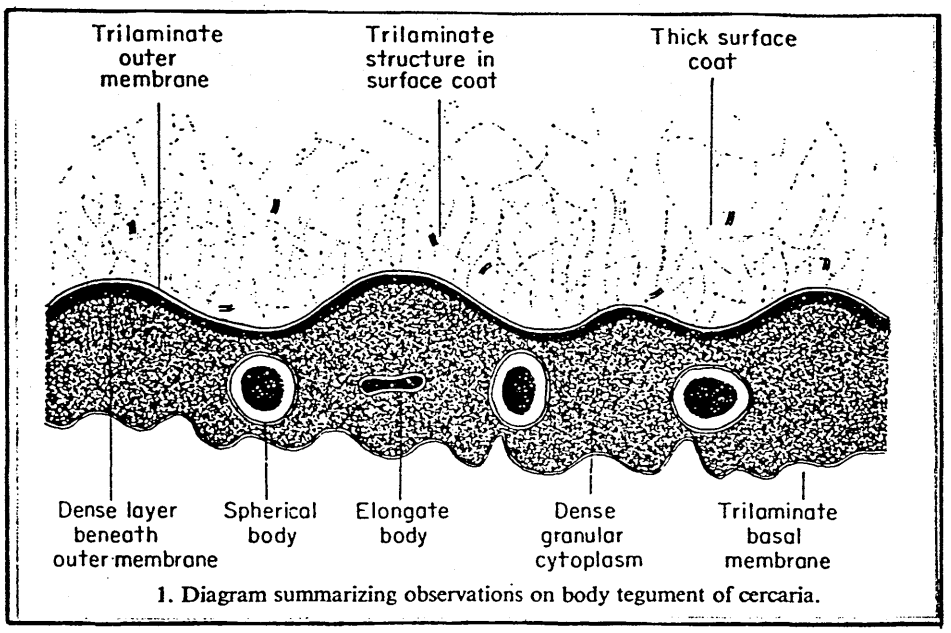
skin penetration, the new heptalaminate membrane is largely complete (Hockley and McLaren, 1973; McLaren, 1980). Figure 1.4 illustrates the development of the schistosomular membrane during transformation.

Samuelson and Caulfield (1985) reported from both ultrastructural studies and covalent labelling of the cercarial surface with ^{3}H or ^{125}I that some 40% of residual glycocalyx persists on the schistosomulum surface after the major period of microvilli formation is finished. The ultrastructural examinations and cercarienhüllen reaction tests of Stirewalt et al (1974, 1983) and Cousin et al (1986) also demonstrated the presence of glycocalyx material on schistosomula three hours after artificial transformation by various methods.

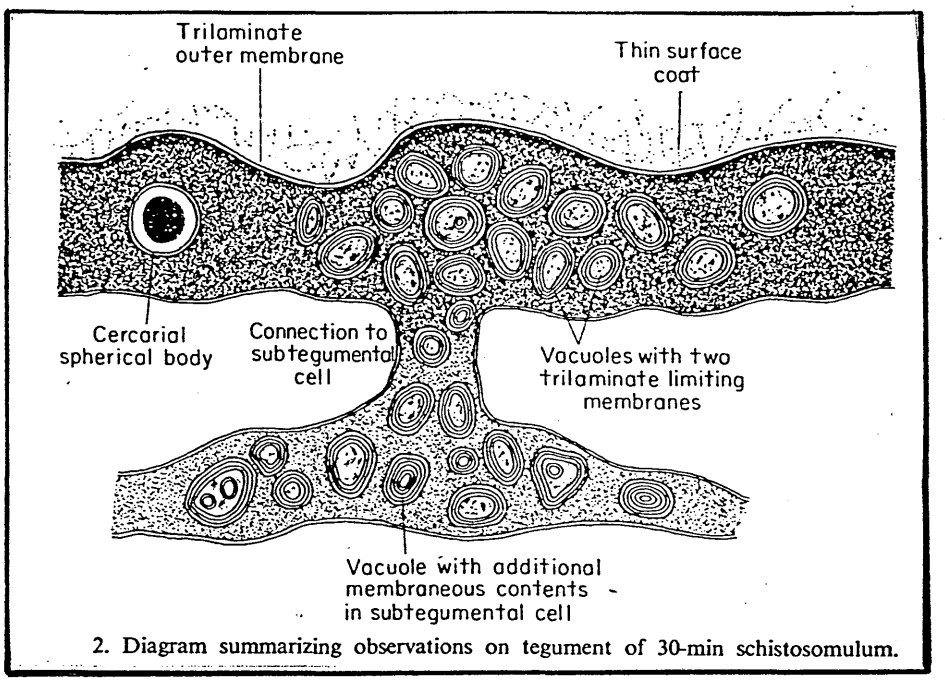
Changes in the chemical and biophysical properties of the post-transformation surface have also been noted. Kusel (1970b) reported that certain chemicals, such as urea, which cause rapid disintegration of the plasma membrane of cercariae, had no effect on the organisation of the schistosomular surface. It was suggested (Kusel, 1971) that this change in membrane stability might be due to a reorientation of chemical groups at the surface, possibly related to the incorporation of inorganic cations, such as Ca^{2+} from the surrounding medium. Foley et al (1988) investigated the biophysical changes accompanying transformation. Two separate experimental approaches - fluorescence recovery after photobleaching, and incorporation of the fluorescent lipophilic probe Mc540 - suggested that the cercarial outer membrane was predominantly in the gel (crystalline) state. Transformation was accompanied by a reorganisation such that, by 40 minutes, the outer membrane was mainly in the fluid (liquid-crystalline) phase.

Figure 1.4 (1) to (6) Transformation from cercaria to schistosomulum. Development of the schistosomular heptalaminate membrane. (Reproduced from Hockley and McLaren, 1973).

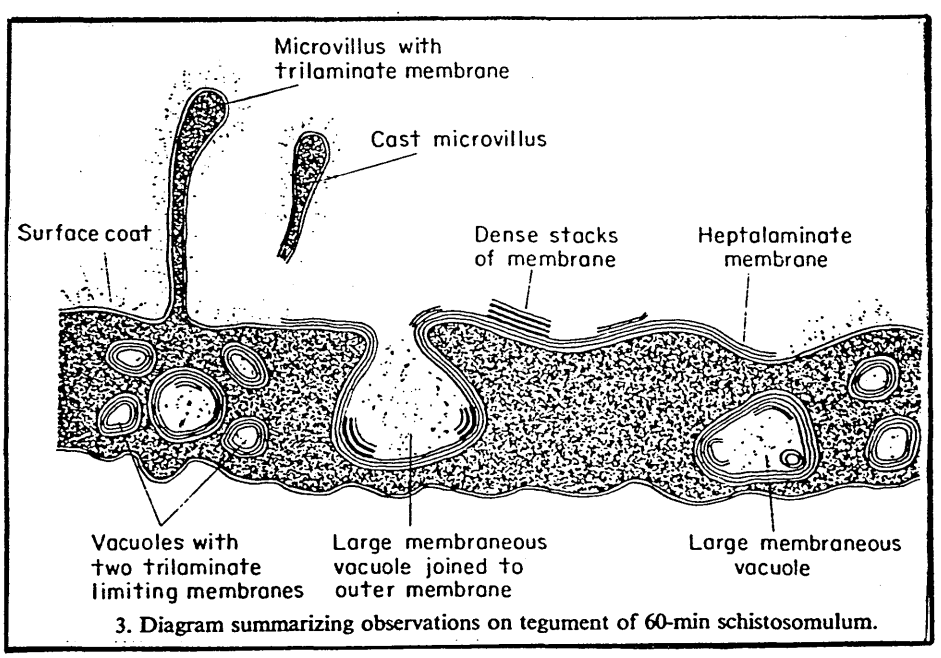
NOTE: The surface appears pentalaminate (4, 5, 6) when the central electron-lucent layer between the two bilayers is indistinguishable, and the two inner monolayers appear as a single thicker layer.



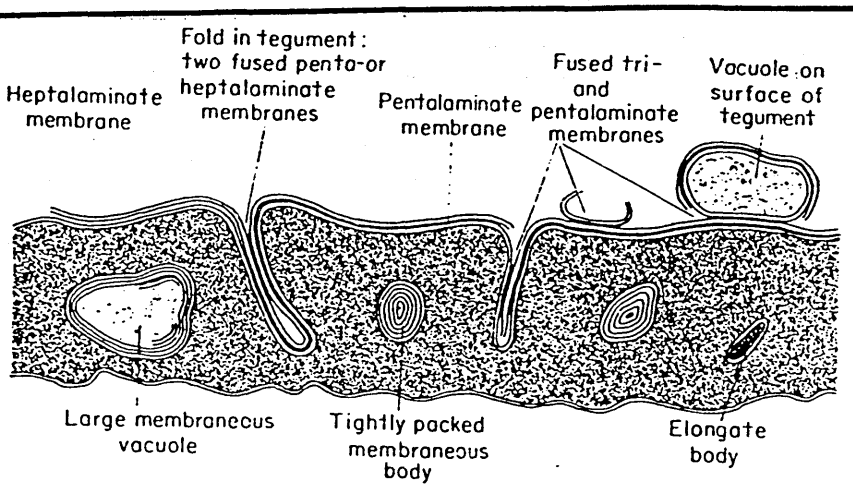
1. Diagram summarizing observations on body tegument of cercaria.



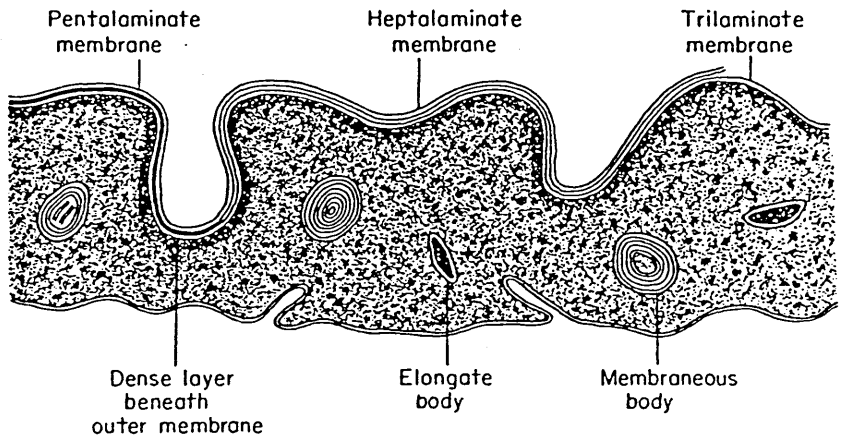
2. Diagram summarizing observations on tegument of 30-min schistosomulum.



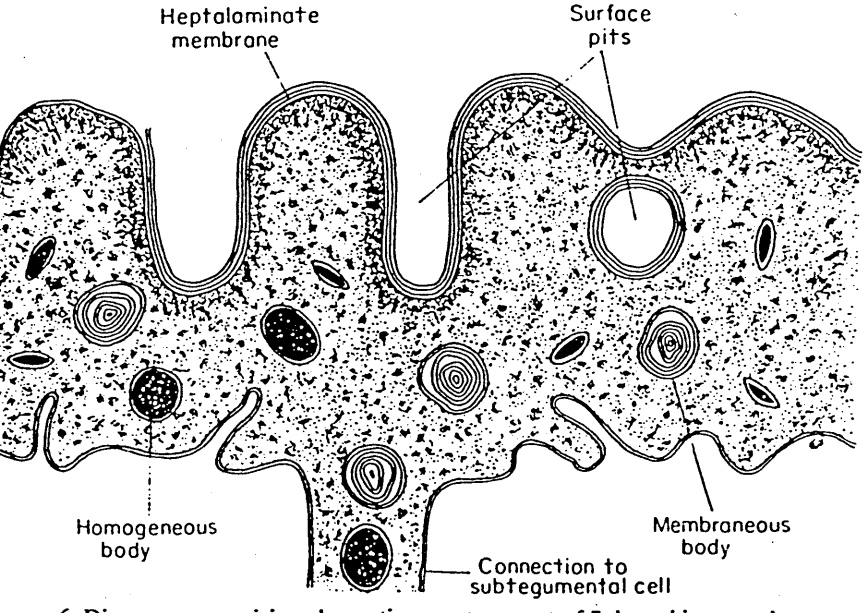
3. Diagram summarizing observations on tegument of 60-min schistosomulum.



4. Diagram summarizing observations on tegument of 3-h schistosomulum.



5. Diagram summarizing observations on tegument of 24-h schistosomulum.



6. Diagram summarizing observations on tegument of 7-day schistosomulum.

1.5.3. Structural changes in other parasite organs.

Aside from surface morphology, schistosomula show a number of other structural adaptations to their new parasitic way of life after skin penetration. One hour-old schistosomula show exhaustion of acetabular glands, alternate dilations and contractions of the digestive tract, suggesting imminent activity of the gut, and collapse of osmoregulatory tubules (Wilson, 1987). The same developments seem to occur in artificially-prepared schistosomula, though at a slower rate than *in vivo* (Cousin *et al*, 1981).

1.5.4. Changes in surface permeability and solute uptake.

Changes in membrane function would be predicted to accompany the restructuring of the surface membrane during transformation. For instance, the development of water intolerance and of stability to cryopreservation reflects a change in surface permeability (Stirewalt *et al*, 1983). Rumjanek (1987), Stirewalt (1974) and McLaren (1980) all concluded that surface permeability was a function of the glycocalyx. It was presumed that the glycocalyx excluded water from cercariae, but its removal during transformation allowed influx of water into schistosomula. This model was extended to include the assumption that free-living cercariae would be equally impermeable to small solutes such as amino acids and glucose. Transformation, however, should induce permeability to the nutrient molecules necessary to survival of the parasitic schistosomulum. In accordance with this scheme, Ramalho-Pinto *et al* (1974) reported that cercariae incorporated very low levels of radiolabelled amino acids. Salafsky *et al* (1988) reached the same conclusion for radiolabelled leucine, uridine and thymidine incorporation. The rigid state of the cercarial membrane implied by the work of Foley *et al* (1988) also predicts that it will be highly

impermeable.

However, various lines of experimental evidence point to discrepancies in this model, whereby loss of the glycocalyx converts the impermeable cercarial surface into a readily-permeable membrane in schistosomula. Firstly, Stirewalt et al (1983) observed that development of water intolerance and stability to freezing did not always coincide with glycocalyx degradation. They inferred that not only the glycocalyx, but also the surface membrane and underlying syncytial tegument might determine cercarial permeability characteristics.

Secondly, neither the impermeability to solutes of cercariae nor the increased permeability of schistosomula are firmly established. Neither Ramalho-Pinto et al (1974) nor Salafsky et al (1988) used the natural cercarial environment - aquarium water - for their labelling experiments which suggested low uptake of solutes by cercariae. Both groups used an isotonic medium - Hanks' saline or Elac - which favours the development of schistosomula rather than cercariae (Stirewalt et al, 1983; Cousin et al, 1986). Some earlier experiments suggest that cercariae in water may in fact be capable of internalizing solutes. Bruce et al (1969) showed that cercariae in aquarium water buffered with 0.01M Hepes took up and metabolized high amounts of ^{14}C - pyruvate. Moreover, after depletion of glycogen reserves by 18 hours after shedding, they readily internalized ^{14}C - glucose. Finally, incorporation of essential fatty acids, in particular, linoleic acid, by free-swimming cercariae in water is a well-established phenomenon (Haas and Schmitt, 1978) which questions the concept of the completely rigid gel state of the cercarial membrane envisaged by Foley et al (1988). Thus, the transport characteristics of the cercarial surface are, as yet, ill-defined.

As regards newly-transformed schistosomula, they do not display the high solute uptake predicted by the model in which the glycocalyx

is the chief determinant of surface permeability. On the contrary, a number of different workers agree that uptake of radioactive precursors - both amino acids and nucleotides - is remarkably low for the first six hours following either skin or mechanical transformation (Yuckenberg et al, 1987; Nagai et al, 1977; Chappell, 1974).

Transport mechanisms in schistosomula have been studied by Chappell (1974), who investigated the kinetics of methyl-^{14}C-methionine uptake by three-hour skin forms. There appeared to be two kinetically distinguishable systems, one saturable, and the second, simple diffusion. As regards other solutes, Levy and Read (1975) demonstrated that cytosine, thymine and uracil entered adult worms entirely by diffusion. Adenine, guanine, hypoxanthine, and the nucleosides adenosine and uridine were absorbed in part by mediated systems. We might speculate that these, or similar transport systems, could also operate in earlier schistosome stages. Podesta (1983) drew attention to the fact that transport into helminth parasites such as schistosomes is a multi-stage process. Firstly, solutes must be transported across the two unit membranes forming the double outer bilayer. After traversing the syncytial epithelium, another set of transport mechanisms must operate in the trilaminate basal membrane to transfer the solutes to the parasite interior.

In summary, while it is generally accepted that the structural changes accompanying transformation from cercariae to schistosomula entail altered permeability characteristics, the transport mechanisms specific for the two larval stages are not yet defined.

1.5.5. Protein synthesis by cercariae and newly-transformed schistosomula.

1.5.5.1. Protein synthesis by cercariae within the snail.

The synthetic activity of developing cercariae within the snail host may be investigated by supplementing the snail water with radioactive metabolites. In this way, different workers have traced the uptake and retention by cercariae of radiolabelled amino acids, nucleosides, acetate, glucose and other citric acid cycle intermediates (Lewert and Para, 1966; Bruce *et al,* 1969; Para *et al,* 1970; Reid *et al,* 1977; Knight *et al,* 1968). Atkinson and Atkinson (1981) labelled cercariae *in vivo* with ^{14}C - leucine by this technique, then analysed the proteins synthesized at different stages in cercarial embryogenesis by 1- and 2-dimensional SDS-PAGE and fluorography. Three polypeptides of high molecular weight were synthesized by cercariae three days before reaching maturity and release from the snail. The most prominent cercarial polypeptides at this stage were two which comigrated on electrophoresis with actin (Mr 43 000) and the heavy chain of myosin (Mr 200 000). The rate of actin synthesis decreased at 48 and 24 hours before emergence, and only minimal synthesis was detectable in the four hours immediately preceding emergence. The authors suggested that the synthesis of myosin and actin observed at 72 hours before release might be required for growth of the tail musculature, since Cheng and Bier (1972) observed that the cercarial tail was actively growing at approximately this stage of development. In the four hour period immediately preceding escape, the most intensely labelled cercarial polypeptides had molecular weights 35 000, 53 000, 58 000. These workers were also able to precipitate labelled proteins from the water into which the cercariae emerged. As assessed by migration on 2-D gel electrophoresis, these polypeptides were

identical to several found in both shed, washed cercariae and the infected snail hepatopancreas. It was inferred that cercariae released a significant proportion of their radiolabelled proteins, both during their development and migration in the snail, and, after emergence, while swimming freely in water.

1.5.5.2. Protein synthesis by free-living cercariae.

Both Ramalho-Pinto et al (1974) and Salafsky et al (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. Thus, Salafsky et al (1988) stated that "cercariae showed no significant synthesis of DNA, RNA or protein". However, as pointed out above, both these workers incubated the cercariae in isotonic media which tend to support conversion to the schistosomulum stage. In contrast, Blanton et al (1987) labelled cercariae to high specific activity with ^{35}S-methionine in water. The labelled proteins were examined by SDS-PAGE. At 23°C, methionine was incorporated into polypeptides at molecular weights 42 000, 38 000, 34 000, 25 000, 20 000, 15 000. Material of molecular weight below 14 000 was also very intensely labelled. Bands at molecular weights 84 000 and 60 000-58 000 were faintly labelled. These authors also investigated how protein synthesis by cercariae in water responded to heat-shock. At 37°C and 42°C, there was pronounced induction of synthesis of the 60-58 000 Mr doublet. Other less prominent induced proteins included ones at molecular weights 84 000, 70 000 and 43 000. Synthesis of other proteins, including those below molecular weight 14 000, declined. The 60-58 000 molecular weight proteins were assigned the role of the major heat-shock proteins of free-living cercariae. Interestingly, a developmentally regulated 58000 molecular weight protein was also

detected in cercariae prior to their release from the snail host by Atkinson and Atkinson (1981).

Thus, earlier conclusions notwithstanding, protein synthesis by free-living cercariae is both significant and highly responsive to environmental signals.

1.5.5.3. Protein synthesis by newly-transformed schistosomula.

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 six hours by Nagai *et al* (1977) and at sixteen hours by Yuckenberg *et al* (1987). Both groups observed a striking increase in protein synthesis after the initial quiescent stage. Since the cercarial trilaminate membrane is entirely replaced by the schistosomular heptalaminate membrane during the period of low protein synthesis, it seems that the proteins inserted into the new schistosomular membrane must have been synthesized and stored by developing cercariae.

More information on the mechanisms of protein synthesis operating during this early transformation period was derived from the use of protein synthesis inhibitors by Nagai *et al* (1977). Puromycin, which prevents translation of proteins on mRNA transcripts, inhibited protein synthesis by more than 70% during the five hours immediately following transformation. In contrast, Actinomycin D had no effect on protein synthesis during this early stage, but an eighteen hour incubation with Actinomycin D induced 50-60% inhibition. Actinomycin D has no effect on previously synthesized RNA, but prevents production of new RNA messages, by intercalating into DNA.

Drawing together the results of Nagai *et al* (1977) and Yuckenberg *et al* (1987), we might suggest that, during the inital 5 to 6 hours following transformation, schistosomular development relies

chiefly on pre-synthesized proteins and, to a small extent, on translation of previously synthesized mRNA. With time, however, as the pre-synthesized proteins and RNA transcripts are utilized, a progressive requirement for *de novo* protein synthesis and DNA-dependent RNA synthesis becomes evident.

According to both Yuckenberg *et al* (1987) and Blanton *et al* (1987), the major protein synthesized by newly-transformed schistosomula has Mr 70 000, and cross-reacts with antibodies raised against bovine heat shock proteins (hsp's) and a *Drosophilla* heat shock protein of Mr 70 000 (hsp 70). When 18 hour old schistosomula were raised from 37°C to 42°C, synthesis of the molecular weight 70 000 protein was further induced to 3 to 4 times its level at 37°C, again implicating this protein in the heat-shock response. Confirmation of the identity of the Mr 70 000 protein as a hsp comes from the genetic work of Hedstrom *et al* (1987). Adult schistosome cDNA clones expressing fusion peptides that specifically absorbed antibodies capable of immunoprecipitating the 70 000 molecular weight protein were isolated from λgt11 expression libraries. Sequencing of the corresponding genomic DNA identified this antigen as a homologue of human and *Drosophila* hsp 70's. It seems that this hsp helps the schistosomulum to adapt from the cercarial environment - fresh water at approximately 23°C - to the isotonic mammalian internal milieu at 37°C.

As regards the general nature of the proteins synthesized by newly-transformed schistosomula, Nagai *et al* (1977) found that synthetic activity during the six to twenty-four hours following transformation was predominantly devoted to surface proteins. Schistosomular teguments incorporated two to three times as much radio-labelled amino acid precursor as the bodies. These authors suggested that the relatively small amount of radioactivity in the body proteins might represent chiefly the presence in subtegumental cells of membranous bodies destined to contribute later to the schistosomular

surface.

1.5.6. Surface antigens expressed by cercariae and schistosomula.

As the tegumental surface changes during transformation from cercaria to schistosomulum, two distinct generations of surface antigens are presented to the host over a period of just a few hours.

Samuelson and Caulfield (1985) labelled the cercarial surface by periodate oxidation in combination with NaB^3H_4. The labelled material was acidic, molecular weight greater than 1 000 000, and reacted with antibodies in mouse infection serum. On SDS - PAGE, 70-80% of the labelled material was retained in the stacking gel, while the remainder ran with the dye-front. No proteins could be detected within the molecular weight limits of the resolving gel. It was concluded that this high molecular weight, acidic material corresponded to the cercarial glycocalyx, and was the major cercarial surface antigen.

However, using surface iodination and immunoprecipitation techniques, other workers have identified polypeptides of lower molecular weight on the cercarial surface. Apart from less consistently recognised proteins at molecular weights $>$ 140 000, 24 000-30 000 and 15 000-20 000 (Payares et al, 1985), the major antigens immunoprecipitated by various antisera form a complex of glycoproteins ranging from Mr 32 000 to 38 000 (Shah and Ramasamy, 1982; Payares et al, 1985; Dissous et al, 1982). Dissous et al (1982) demonstrated that a single monoclonal antibody identified both a 38 000 and a 150 000 molecular weight antigen on the cercarial surface. The target epitope of this monoclonal was shown to be an oligosaccharide also present in haemolymph from the snail host B. glabrata (Dissous et al, 1986). It was suggested that the carbohydrate structure concerned might be involved in osmotic adaptation by cercariae in fresh water. Some continuity between the surfaces of cercariae and schistosomula is

suggested by the close correlation between the immunoprecipitation pattern of a series of glycoproteins at Mr 38 000-32 000 in cercariae and three hour old schistosomula (Dissous et al, 1981; Simpson et al, 1983 b; Payares et al, 1985). As the schistosomula mature, a Mr 32 000 antigen within this complex becomes progressively more dominant at the surface and the higher molecular weight members gradually cease to be detectable by surface labelling techniques (Payares et al, 1985; Simpson et al, 1984).

Fluorescent antibody binding techniques and in vitro cytotoxicity assays demonstrate that schistosomula immediately after transformation express antigens, and are susceptible to a variety of killing mechanisms, whereas older schistosomula are less antigenic and less susceptible to immune killing (McLaren, 1980; Smithers and Doenhoff, 1982). The greater part of research effort has therefore been directed towards identification of antigens exposed at the surface of freshly-transformed schistosomula.

Rumjanek et al (1983) identified a doublet at molecular weight 45 000 on the newly-transformed schistosomulum surface which only became accessible to labelling reagents after incubation in medium containing human serum. It was proposed that this protein acted as a lipid receptor, transferring lipid from host serum to the schistosomulum.

Various workers have detected a protein of Mr about 66 000 on the surface of 3-hour schistosomula (Samuelson and Caulfield, 1982; Taylor et al, 1981; Simpson et al, 1983b). Wilson (1987) suggested that this molecule might be the enzyme alkaline phosphatase, which has molecular weight approximately 66 000. Its presence in the schistosomulum was confirmed by Taylor and Wells (1984) who used alkaline phosphatase activity as an aid to isolation of 3-hour schistosomulum surface membranes.

By using glucose oxidase or periodate in combination with

NaB^3H_4, Samuelson and Caulfield (1982) ensured the identification of only glycosylated antigens on the schistosomulum surface. The labelled glycoproteins were then separated by gel electrophoresis, and their molecular weights identified as 105 000, 67 000, 54 000, 39 000, 34 000, 27 000, 22 000, 19 000, 17 000. Surface iodination techniques have also identified surface proteins, of unknown function, at Mr 92 to 94 000 and 15 000 (Simpson et al, 1983 b). The contributions of these different proteins in stimulating a protective immune response will be discussed in section 1.8.

1.5.7. Reduction in surface antigenicity with time.

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. Thus, schistosomula recovered from the skin or lungs on day 4 after infection no longer activate complement via the alternative pathway, and will not bind specific anti-schistosome antibodies (Ramalho-Pinto et al, 1978; McLaren, 1980). Antibodies which kill 3-hour schistosomula in vitro via complement-mediated mechanisms are ineffective against 4-day lung worms, or against schistosomula cultured in vitro for four days in the presence of serum or serum and erythrocytes. Dean (1977) reported that schistosomula maintained in serum-free, chemically-defined media also became refractory to the effects of lethal antibody. However, a subsequent study by Tavares et al (1978b) did not demonstrate acquisition of significant protection in serum-free medium. In contrast, these workers found that culture in medium supplemented with serum did confer significant protection. Eosinophils adhere in quantity to 3-hour schistosomula in vitro via either antibody or complement receptors, and inflict lethal damage, but 4-day lung worms

are not susceptible to eosinophil killing. McLaren (1980) reported a significant reduction in eosinophil-mediated killing of schistosomula cultured for only 24 hours in serum-supplemented medium.

Some of this reduction in surface reactivity seems to be attributable to turnover and shedding of surface components. During culture at 37°C, anti-schistosome antibody and C3 bound to the surface of newly-transformed schistosomula are lost exponentially with a halftime of 5 hours (Samuelson et al, 1980). Schistosomula cultured for 18 hours at 37°C in defined medium, or in medium supplemented with serum, then labelled, also failed to bind significant antibody or C3 to their surfaces. Using a different approach, Samuelson and Caulfield (1982) calculated a halftime of 10 to 15 hours for turnover of covalently labelled glycoproteins and glycolipids from newly-transformed schistosomula in vitro. Presumably, this surface turnover involves both discarding of residual glycocalyx material and secretion of the newly-formed heptalaminate membrane.

Reduction in surface antigenicity seems to depend, at least in part, on schistosomular metabolic activity. Thus, there seems to be a relationship between serum-induced stimulation of protein synthesis and protein turnover, and development of resistance to the effects of complement-dependent lethal antibody binding (Tavares et al, 1978b; Cordeiro et al, 1984). Moreover, the protein synthesis inhibitor puromycin blocks the induction of protection by serum. (Tavares et al, 1978b).

Another mechanism which appears to contribute to the loss of antigenicity of the parasite surface involves acquisition of host antigens which adhere to, or integrate into, the parasite outer membrane (Smithers et al, 1969). According to this theory, host antigens mask parasite epitopes from immune recognition and attack.

The antigen masking hypothesis was initially advanced to help explain the phenomenon of concomitant immunity - continued survival of

adult worms in face of an immune response which destroys invading cercariae of a challenge infection (section 1.7). Smithers *et al* (1969) demonstrated that when adult worms grown in mice were transplanted into monkeys which had been immunised previously with mouse red blood cells, virtually all the worms were destroyed. In the control system, where mouse worms were transferred to unimmunised monkeys, the parasites developed normally. The conclusion was drawn that worms grown in mice possessed mouse antigens on their surface which rendered them recognisable to anti-mouse monkeys.

In subsequent experiments, juvenile schistosomula recovered from the skin and lungs of infected animals, or cultured *in vitro* in the presence of red blood cells, were shown to have acquired host antigens (Goldring *et al*, 1976, 1977; Sher *et al*, 1978; Smith and Kusel, 1979). The majority of acquired antigens appeared to be in the form of glycolipids or megalolipids. A, B, H and Lewis blood group substances were all taken up as lipids rather than glycoproteins (Goldring *et al*, 1976, 1977). In contrast, the M, N, A, rhesus and Duffy antigens, which are thought to be glycoprotein constituents of the erythrocyte membrane, are not acquired by the developing parasite. As Clegg (1972) pointed out, the hydrophobic ceramide end of a glycolipid molecule will facilitate its insertion into the parasite membrane.

However, Sher *et al* (1978) demonstrated by immunofluorescence that schistosomula recovered from mouse skin and lungs expressed gene products of the K and Ia regions of the MHC at their surface. These alloantigens are glycoproteins. In contrast, other protein antigens, such as Thy 1, Ly 1 and C3, could not be detected. Smith and Kusel (1979) showed that schistosomula which had penetrated mouse skin, either *in vitro* or *in vivo*, acquired antigens specific for the glycocalyx of squamous cells of the epidermis. These "intercellular substance" (ICS) antigens are also thought to be glycoproteins. Increasing quantities of these antigens were acquired over the initial

24 hour period spent in the skin. However, ICS antigens were lost during migration of schistosomula to the lungs. Schistosomula prepared by mechanical transformation in the absence of skin penetration did not express skin antigens.

As regards the stage of development with which this project is chiefly concerned - cercariae to lung forms - the nature and quantity of host antigens acquired will depend upon the methods of preparation and culture. Schistosomula recovered from mouse skin *in vivo*, or passed through a skin preparation *in vitro*, will express the skin-specific antigens detected by Smith and Kusel (1979). As for blood-group antigens, McLaren *et al* (1975) demonstrated that schistosomula recovered from mouse skin at as early as 3 hours stained positively for the presence of mouse erythrocyte antigens. In contrast, 3 hour schistosomula obtained by penetration of excised skin *in vitro*, or transformed mechanically and cultured in the presence of red blood cells, did not express blood group antigens. After 24 hours of culture in the presence of red blood cells, however, acquired blood group antigens were just detectable, and increased with further time in culture. 4-day lung worms gave a very strong positive reaction for the presence of host antigens. (McLaren, 1984).

Concomitantly with increased expression of host antigens as the schistosomula develop, binding of anti-schistosome antibody to the surface decreases. Nevertheless, all schistosome stages recovered from the vertebrate host, up to and including 6-week old, fully mature adults, continue to bind infection serum weakly. One apparent exception are lung-stage parasites. Most workers have reported that lung worms are refractory to antibody binding (reviewed by McLaren, 1980). However, the P388D monocyte-like cell, which bears F_c receptors, has been used to demonstrate the binding of antibodies from vaccinated mice and rats to 6-day lung worms (Bickle and Ford, 1982).

While circumstantial evidence thus strongly implies a role for

host molecules in shielding the parasite against immune attack, conclusive proof of this protective function is lacking. The continued binding of anti-schistosome antibody to schistosomes in the presence of host antigens suggests that, if protection is indeed afforded by the acquired host antigens, it may be less than complete.

1.5.8. Changes in energy metabolism during transformation.

Cercariae are aerobic organisms, relying on glycogen as energy substrate (Oliver *et al,* 1953; Bruce *et al,* 1969). Use of inhibitors indicates the presence of a functional citric acid cycle, and cytochromes a/a_3, b and c have all been demonstrated in cercariae (Coles, 1972). Glycogen reserves are sufficient for penetration, but, according to Lawson and Wilson (1980b), will support less than 24 hours *in vivo.* Thus, the parasite must begin to rely on host metabolism from shortly after penetration.

In post-infective stages, metabolism is predominantly anaerobic, and schistosomula excrete lactic acid. Thompson *et al* (1984) measured the time-course of transition from cyanide-sensitive to cyanide-insensitive metabolism. At 3 hours, the energy metabolism of schistosomula was similar to that of cercariae, but by 24 hours, lactate production predominated. Since this change was not accompanied by increased levels of lactate dehydrogenase, and was not puromycin-sensitive, it must be effected by proteins already synthesized by cercariae. Indeed, cercariae are capable of homolactic fermentation under anaerobic conditions (Oliver *et al,* 1953).

A number of suggestions have been made as to the environmental trigger which switches the fully aerobic metabolism of cercariae to the excretion of lactic acid by schistosomula. Temperature is unlikely to be the main stimulus, since O_2 uptake by cercariae is unaffected by raising the temperature above 30°C (Coles, 1972, 1973). Interestingly,

increase in osmotic pressure causes a temporary production of lactate by cercariae (Coles, 1972). Rumjanek (1987) also suggested that the increased concentration of solutes and electrolytes upon entering the mammalian environment might initiate activation of enzymes of the glycolytic pathway.

Adenylate cyclase could well act as a second messenger in initiating changes in enzyme activity during schistosomular development. Adenylate cyclase activity in cercariae is unresponsive to serotonin, but within four days of *in vitro* culture of schistosomula, serotonin activation of adenylate cyclase is evident (Kasschau and Mansour, 1982). Rumjanek (1987) noted that cyclic AMP can activate a number of key enzymes in the glycolytic pathway, including phosphofructokinase. Thus, adenylate cyclase might indeed be involved in signalling the transition from free-living stage to parasitism in the definitive host.

1.6 Potential for vaccines against human schistosomiasis.

1.6.1. The need for a vaccine.

Current strategies for control of schistosomiasis are based primarily on chemotherapy. However, as Butterworth (1987b) pointed out, in the long term, development of alternative measures is highly desirable, for the following reasons:

(1) Reinfection may occur rapidly following treatment, especially in areas of high transmission, since it is reported that intensities of infection in some individuals may rise to almost half pre-treatment levels within a year after chemotherapy. Chemotherapy control would therefore require constant surveillance and retreatment in areas of high transmission. In contrast, it seems that, once immunity develops in humans under conditions of natural exposure, it is long-lasting (see

below). It would therefore be predicted that a single immunisation should suffice.

(2) It is anticipated that, under conditions of extensive drug usage, drug-resistant schistosome strains will emerge. Development of resistance in some parasites to the commonly used drug oxamniquine has already been demonstrated under field conditions. A vaccine would obviate such problems of drug resistance.

Considerable research effort has therefore concentrated on the immunology of schistosomiasis. Studies have been directed, firstly, at determining whether protective immunity can be demonstrated, in humans or experimental animals; secondly, at establishing the effector mechanisms and target antigens of such immunity.

1.6.2. Immunity in human schistosomiasis.

Early epidemiological studies in endemic areas showed that both prevalence and intensity of S. mansoni and S. haematobium infections declined in older age groups (Fisher, 1934; Clarke, 1966). The inference was drawn that older individuals had developed immunity to reinfection. Warren (1973) questioned this conclusion, arguing that the decline in infection might equally well be due to natural death of adult worms from primary infections, in combination with a reduced level of exposure to new infection, due to decreased water contact. Levels of water contact are indeed closely related to age (Dalton and Pole, 1978). However, this explanation was, to some extent, contradicted by the study of Kloetzel and da Silva (1967) on infection of adult immigrants to an endemic area. The time-course of infection prevalence and intensity followed the same pattern in the immigrants as in individuals who had resided in an endemic area since childhood.

Thus, development of resistance seemed to depend on duration of exposure, rather than age-related water contact.

The most convincing evidence for acquired immunity in man has come, however, from recent reinfection studies. This approach involves initial clearing of the worm burdens from infected individuals by chemotherapy. Intensities of reinfection are then measured, and, at the same time, levels of exposure of individuals to contaminated water are monitored. Although treatment itself may alter the subjects' original immune state, this procedure does allow accurate determination of reinfection levels. This approach has been applied to S. haematobium infections in The Gambia and S. mansoni infections in Kenya. Both studies have demonstrated an age-dependent, acquired resistance, and have indicated possible immunological mechanisms.

1.6.2.1. Reinfection studies on schistosomiasis haematobium.

In The Gambia, two treatment/reinfection studies have been performed. Levels of cercarial exposure were assessed on the basis of duration of water contact, the number of cercariae at water contact sites, and the extent and nature of contact with water (Hagan et al, 1985a, 1987; Wilkins et al, 1987).

The first study (Hagan et al, 1985a) involved a group of 50 children, aged 8 to 13 years, whose original infections were effectively cleared by chemotherapy. Twenty-six subjects were classed as reinfected, with >1 egg per 10 ml of urine, and twenty-four were described as not reinfected, having <1 egg per 10 ml after the transmission period. Levels of exposure and age alone were not sufficient to account for the reduced incidence of reinfection in the "non-reinfected" children. Other factors were also implicated, suggesting the development of protective immunity in these subjects.

In the second study, individuals were selected from a wider age

range. Intensities of reinfection were tenfold lower in the 10 to 14 year age group than among 5 to 9 year old children,and were 100-fold lower in females over 15. In contrast, levels of exposure were only slightly less in the 10 to 14 year age group than in the 5 to 9 year group, and were only 5-fold less for females over 15. When individuals were stratified according to levels of exposure, a marked age-dependence of reinfection was evident, heavily exposed 2 to 9 year old children showing 100-fold greater intensity of reinfection than individuals of over 15 years experiencing comparable levels of exposure.

Thus, some age-dependent factor other than level of exposure seems to be responsible for resistance to reinfection. It is proposed that this factor is the development of protective immunity.

1.6.2.2. Reinfection studies on schistosomiasis mansoni.

Extensive studies based on the same principle have been performed for schistosomiasis mansoni in Kenya. A pilot study by Sturrock *et al* (1983) demonstrated that a proportion of *S. mansoni* - infected children treated with hycanthone failed to become reinfected. Age, pretreatment intensities of infection, and sex had no influence on reinfection rates. Although the distance of the child's home from contaminated water contact sites did have some influence, this was insufficient to account completely for the reinfection pattern observed.

These initial findings were subsequently extended by Butterworth *et al* (1984, 1985). A group of 129 infected children, aged 9 to 15, received chemotherapy. Observations were made of the duration, nature and extent of contact of each child with water bodies known to contain infected snails. This allowed identification of two extreme subgroups among the children. Twenty-two individuals were classified as

susceptible to reinfection, with over 100 eggs per gram of faeces by 6 months after treatment. By contrast, in another 70 children, reinfection was either undetectable or occurred at only low levels, less than 30 eggs per gram of faeces, throughout the study period. Half of these lightly reinfected children showed high levels of water contact, thus their relatively low rate of reinfection could not be attributed to a lack of exposure. It was therefore concluded that this group must display resistance to reinfection. These two susceptible and resistant groups did not differ in mean pretreatment intensities of infection, but did differ in age, the resistant children being on average two years older. Thus, the observed resistance was an acquired and age-dependent event, presumably reflecting development of immunity.

Subsequently, a broader age-group was examined, treatment being offered to all infected individuals in the community. The incidence and intensity of reinfection, together with levels of water contact, were observed over the following 9 months. (Sturrock *et al*, 1987; reviewed by Butterworth, 1987a). Among children of 0 to 9 years, incidence of reinfection was higher among individuals who had been infected before treatment than among those who had not. This pattern was correlated with higher levels of water contact among the previously infected children. In subjects older than 9 years, however, the pattern was reversed. Previously uninfected individuals went on to display an even incidence of new infections, independent of age, throughout their adult life. In contrast, those who had previously been infected showed a steady decline in incidence of reinfection with age, even though they showed consistently higher levels of water contact than previously uninfected individuals. Thus, it seemed that only recent experience of infection induced resistance to reinfection. Resistance was therefore not attributable simply to some physiological change associated with aging.

In summary, the results of these reinfection studies with *S.*

haematobium and S. mansoni supply strong evidence for the existence of immunity to schistosome infections. It seems that development of immunity plays an important role in determining the prevalence and intensity of infection in subjects in endemic areas.

1.6.3. Mechanisms of immunity in human schistosomiasis.

1.6.3.1. Cellular cytotoxicity mechanisms

Human eosinophils and neutrophils have both been shown to act in concert with antibody to kill schistosomula in vitro (Butterworth et al, 1975, 1980; Moser and Sher, 1981). Activated macrophages from infected humans are also capable of killing schistosomula in vitro, independently of antibody (Mahmoud et al, 1979).

Reinfection studies have allowed some insight into immunological correlates of human resistance in vivo. The earliest indications of immune mechanisms in human schistosomiasis came from the studies on S. mansoni performed by Sturrock et al (1983). In this case, children with high eosinophil counts, and antibody levels which, in concert with eosinophils, were sufficient to mediate significant damage to schistosomula in vitro, showed lower levels of reinfection than children with only high eosinophil counts or antibody levels, who, in turn, had lower levels of reinfection than those with neither.

Similar observations were made on S. haematobium infections in The Gambia. In the studies on resistance among 8 to 13 year old children, low levels of reinfection were associated with high circulating eosinophil counts. (Hagan et al, 1985a). Other studies on both S. haematobium (Hagan et al, 1985b) and S. mansoni (David et al, 1980) demonstrated enhanced antibody-dependent schistosomulicidal activity of eosinophils from subjects with high counts. However, antibodies mediating eosinophil-dependent killing of schistosomula were

not present at especially high levels in the subset of children with high eosinophil levels in the study of Hagan et al (1985a).

Thus, while the indications are that expression of resistance to reinfection following treatment may be associated with antibody-dependent cellular cytotoxicity reactions against the young schistosomulum, the precise mechanisms operating in vivo are yet to be determined.

1.6.3.2. Blocking and protective antibodies.

In contrast to the earlier reports by Sturrock et al (1983), later studies on S. mansoni in Kenya (Butterworth et al, 1985), showed no significant differences between resistant and susceptible groups, either in circulating eosinophil counts or in levels of antibodies mediating eosinophil-dependent killing of schistosomula. However, more detailed analysis of the serum responses of children in this study revealed some differences which might help explain the slow development of protective immunity (Butterworth et al, 1987a).

The younger children, susceptible to reinfection, showed high levels of anti-egg antibodies, including antibodies specific for a major egg polysaccharide antigen, K3 (Dunne et al, 1987). These antibodies cross-react with carbohydrate epitopes on schistosomulum surface antigens. Previous studies in the rat model (Grzych et al, 1984) had shown that an IgG2c monoclonal antibody specific for an oligosaccharide moiety on a Mr 38 000 surface antigen of schistosomula blocked the capacity for an IgG2a monoclonal, specific for the same antigen, to mediate eosinophil-dependent killing of schistosomula in vitro, and to confer protection in vivo. These observations suggested that similar blocking antibodies, induced in response to egg carbohydrates and cross-reacting with the schistosomulum surface, might also block expression of immunity in young children.

In support of this hypothesis, Khalife et al (1986) demonstrated directly the presence, in sera from the subjects of this study, of IgM antibodies which acted in vitro to block the eosinophil-dependent schistosomulicidal action of IgG antibodies from the same sera. Such IgM antibodies were present in significantly higher levels in sera from young, susceptible children than in older, resistant individuals.

These observations were drawn together into the following hypothesis to explain the development of protective immunity in human schistosomiasis. During early infections of young children, it is proposed that the major immunogenic stimuli are antigens released from eggs. These egg antigens, largely polysaccharide in nature, elicit high and persistent levels of IgM antibodies. These antibodies cross-react with schistosomulum surface glycoproteins, inhibiting the binding of effector IgG or IgE antibodies that may simultaneously be formed in response to glycoproteins released from schistosomula or adult worms. The young child therefore remains susceptible to reinfection. As the child ages, it is proposed that the IgM and other blocking antibodies spontaneously decline, while protective IgG responses are maintained or increased. The child is then free to express his capacity to resist reinfection. As egg loads decline in consequence of this resistance, blocking antibody levels fall still further. Thus, the child now develops effective and prolonged immunity. This hypothesis has the advantage of explaining the very slow development of immunity, over a period of several years, in subjects in endemic areas. Observations on S. mansoni in the mouse model further support and extend this hypothesis (section 1.7.1.9).

According to the studies of Hagan et al (1987); see also Hagan (1987), such anti-carbohydrate blocking antibodies are unlikely to play a major role in resistance to S. haematobium infection. These workers tend to favour the hypothesis that the determining factor in resistance

is the development of high levels of protective IgG antibodies. They contend that development of high antibody levels alone after many years of exposure might be sufficient to explain the slow development of immunity, without postulating a role for blocking antibodies. Wilkins and Capron (1977) found that high anti-parasite antibody titres were associated with a subsequent reduction in egg count in S. haematobium-infected patients. Also consistent with this theory is the observation by Dunne et al (1988) that only older, resistant subjects express significant levels of IgG4 antibodies. These authors also pointed out that, in healthy European children, IgG4 antibody production only becomes significant in the second decade of life. Thus, an age-dependent increase in antibody levels, perhaps of particular isotypes, might also contribute to development of protective immunity.

1.6.3.3. Lymphocyte responses.

Immunological studies by Colley et al (1986), though preliminary, have suggested a role for lymphocyte responses in limiting reinfection after treatment. The incidence of reinfection following treatment in 18 patients was examined in relation to responses to egg, adult worm and cercarial antigen preparations. 7 of the 18 patients became detectably reinfected over a 2-year follow up period. These patients did not differ from those who failed to become reinfected with respect to pretreatment intensity of infection, or distance of their homes from water contact sites. However, they did differ in lymphocyte proliferative responses. Patients who failed to become reinfected maintained high responses to all three antigen preparations throughout the study period. In contrast, at the time of the last blastogenesis assay before they became reinfected, these 7 patients showed significantly lower responses to cercarial and egg antigens, and somewhat lower responses to adult worm preparations, than those who

failed to become reinfected.

1.6.4. Conclusions

In conclusion, field studies indicate that acquired immunity to schistosome infection does occur in man. Induction of immunity by vaccination may therefore be possible. Although several correlations between immunological parameters and resistance to reinfection have been reported, the actual mechanisms of protection in man have still to be defined.

However, investigations in animal models have made considerable progress in identifying potential immune mechanisms. Such studies should serve as a basis in directing the future development of a vaccine for humans.

1.7 Immunity to schistosomula in animal models.

1.7.1. Immunity following a natural infection - concomitant immunity

1.7.1.1. Introduction

In mice, guinea-pigs and primates, development of a patent, egg-laying infection induces resistance to a second cercarial challenge. Resistance develops at the time egg-laying commences, reaching a peak some 4 to 6 weeks later (Smithers and Miller, 1980; Dean, 1983). Exposure to migrating larval stages is not essential to development of protective immunity, since surgical transplantation of adult worms alone into the portal vessels of naive monkeys or mice induces protection against challenge (Smithers and Terry, 1967; Peresan and Cioli, 1980). Resistance mechanisms are induced which attack and eliminate invading challenge schistosomula, while adult worms of the

primary infection persist, unaffected by the immunity which they themselves stimulate. This situation, where a patent infection survives in the face of immunity to secondary challenge, is described as "concomitant immunity" (Smithers and Terry, 1969).

In the mouse model, concomitant immunity is clearly correlated with the presence of eggs in the tissues, or with physiological changes accompanying egg-associated pathology, for instance, portal hypertension (Harrison et al, 1982; Dean, 1983). Concomitant immunity is as effective against heterologous challenge, with a different schistosome species from the immunizing one, as against homologous challenge. The involvement of liver damage in concomitant immunity, and its non-specific nature, have led to the suggestion that this model of resistance may be based, at least in part, on non-immunological factors. Thus, it seems that vascular changes in the liver, consequent upon egg-laying, impede the migration of challenge schistosomula in mice (Wilson et al, 1983). However, various workers have demonstrated that at least a proportion of the resistance developed in chronically infected mice is immunologically-mediated. James and Cheever (1985) demonstrated that defective immune responses were responsible for the failure of certain mouse strains to develop high levels of concomitant immunity. Incani and McLaren (1984) and McLaren et al (1987) showed the importance of immune effector mechanisms operating in the skin in destroying a proportion of challenge larvae in chronically infected mice.

1.7.1.2. Sites and targets of attrition in the concomitant immunity model.

Experimental approaches to determine the site of immune elimination in infected animals include recovery of parasites from different host tissues, injection of challenge parasites via routes

which bypass the skin and/or lungs, autoradiographic tracking of the migration of radiolabelled challenge parasites, and administration of protective or inhibitory serum at various times post-challenge. The following summary applies mainly to the mouse model.

a) The skin

Since the newly-transformed schistosomulum is the parasite stage most susceptible to immune effector mechanisms *in vitro*, the skin seems a likely site to look for challenge attrition. However, studies on cutaneous attrition in infected mice have given ambiguous results.

Counting of schistosomula recovered from minced, incubated skin indicated that mice with long-standing primary infections eliminated some challenge worms in the skin (Smithers and Gammage, 1980). Mice infected for 4, 8 or 12 weeks prior to challenge gave recoveries approximately equal to those of normal mice on the second day after challenge. In mice with 15-17 week infections, however, the yields were reduced by 20-30%, while liver perfusion counts of adult worms were reduced by more than 50%. These data suggested that mice infected for less than three months eliminated very few challenge worms as they migrated through the skin, while mice with older infections eliminated a significant fraction of the challenge at this site.

Incani and McLaren (1984) demonstrated the occurrence of cell-mediated immune effector mechanisms in the skin of 16-week chronically infected mice. Following up this observation, McLaren *et al* (1987) used a monoclonal antibody that depletes cutaneous effector cells to demonstrate that 20-40% of challenge infection is normally killed in the skin of chronically infected mice.

In contrast, autoradiographic tracking experiments with ^{75}Se-labelled cercariae indicate that nearly all challenge cercariae that penetrate the skin of chronically infected mice eventually leave the

skin and arrive in the lungs (Dean and Mangold, 1983; Georgi et al, 1983).

The discrepancies between these results may reflect the use of different mouse strains - CBA in the first two cases; C57/BL in the second two - or else the use of different sensitization or challenge sites. Incani and McLaren (1984) and McLaren et al (1987) used abdominal skin for challenge infection, as opposed to tail skin by Dean and Mangold (1983) and Georgi et al (1983). It may be relevant that rodent tail skin is deficient in Langerhans cells, which play a central role in skin-associated immunity (Bergstresser et al, 1980).

b) The Lung.

Using a mincing and incubation technique, a number of workers have found that the lung schistosomulum counts from challenged, infected mice are reduced compared to controls (Olivier and Schneidermann, 1953; Blum and Cioli, 1981; Smithers and Gammage, 1980; Dean and Mangold, 1983; reviewed by Dean, 1983). In an overview of these experiments, Dean (1983) observed that significant lung count reductions were not detected until at least seven weeks after primary infection, and maximal reductions were obtained at 12 weeks, at the earliest.

However, a number of lines of evidence suggest that, at least in some cases, the lung schistosomulum recovery technique does not accurately reflect resistance to reinfection. Autoradiographic tracking experiments indicate that, even under conditions in which lung schistosomulum recoveries are greatly reduced, most of the worms which cannot be recovered are viable (Dean and Mangold, 1983). Mice with 16- and 25-week previous infections yielded considerably reduced numbers of schistosomula by the lung recovery technique, while autoradiographic data for the same groups of mice indicated that nearly all schistosomula migrated through the lungs to arrive in the liver.

Moreover, in almost all experiments where worm recoveries have been measured at both lung and adult stages, lung schistosomulum recoveries have accounted for considerably less than the total resistance. Thus, it seems that a major phase of challenge elimination must occur after the lung stage.

c) The liver

Autoradiography of liver preparations 3 weeks after challenge with radiolabelled cercariae indicates that most challenge worm elimination in previously infected mice occurs in the liver (Dean and Mangold, 1983). Although numbers of worms detected by autoradiography in the livers of previously infected mice at 3 weeks after challenge infection were only moderately reduced in comparison with controls, very few of the worms present in the livers of previously infected mice could be recovered by portal perfusion, whereas almost all worms present in naive controls could be recovered. Identical groups of mice did not yield greater recoveries when perfused at later times, indicating that these worms did not mature, or migrate from the liver to mesenteric veins.

Perfusion of adult worms from the hepatic portal system and mesenteric veins shows a significant reduction in the number of worms derived from challenge when administered as early as 3 weeks after initial infection, recovery declining to a minimum at 6 to 8 weeks after primary infection (figures listed by Dean, 1983). Levels of protection of 60-100% are then maintained for more than a year.

In summary, it seems that the major phase of challenge attrition in the concomitant immunity model operates against challenge parasites arriving in the liver. The onset and development of this stage of resistance coincide with the kinetics of egg laying by adults of the primary infection. The liver damage associated with egg granulomas

seems to be involved in blocking maturation and migration of challenge worms. However, it does seem that immunologically mediated resistance also develops after primary infection. Some weeks after egg-laying has peaked, involvement of a second, specific stage of immune attrition can be detected in elimination of challenge larvae in the skin.

1.7.1.3. Effector mechanisms in concomitant immunity.

a) *In vitro* studies.

In vitro studies have revealed potential effector mechanisms which may operate in immunity to schistosomiasis, although the relation of these studies to *in vivo* responses must be interpreted with caution.

In the "lethal antibody" system of Clegg and Smithers (1972), schistosomula opsonized with antibody from infection serum were efficiently killed in the presence of complement. Ultrastructural examination showed that damage was directed against the surface and tegument of the parasite. However, Sher *et al* (1974) subsequently found that, although high levels of lethal antibody could be induced in rats by immunisation with adult worm membrane preparations, resistance to cercarial challenge was not evident. Thus, the presence of lethal antibody did not correlate with immunity *in vivo.*

Cellular effector mechanisms against young schistosomula have chiefly been examined in antibody- or complement-dependent cellular cytotoxicity systems *in vitro.* In these assays, antibodies from infection (or vaccine) sera bind to target antigens on the schistosomular surface. If complement is present in the system, it too will be activated by the classical pathway. Various effector cells adhere, via Fc or complement receptors. The cells then release toxic mediators which damage and kill the target schistosomula (figure 1.5a).

According to McLaren (1980), the eosinophil is the most effective cytotoxic cell acting against skin-stage schistosomula in such systems. Human and rodent eosinophils have been shown to adhere to the surface of young schistosomula via complement or Fc receptors, then to secrete cationic proteins which are lethal to the parasites (Butterworth et al, 1975; McLaren, 1982). Purified eosinophil cationic proteins are also highly toxic when schistosomula are exposed to them directly (Butterworth et al, 1979; McLaren et al, 1981).

Neutrophils also kill schistosomula in vitro by release of neutrophil cationic proteins. This cell type requires complement for its activation, and is ineffective in the presence of antibody alone (Incani and McLaren, 1984).

Mast cells have been shown to adhere to the surface of young schistosomula in the presence of complement from infected rat serum (Sher, 1976). Cellular degranulation and parasite damage did not occur directly in consequence of mast cell binding in vitro. However, this cell type did appear to enhance eosinophil-mediated schistosomular damage in the presence of antibody or complement (Capron et al, 1978; McLaren, 1980).

Macrophages have also been shown to damage schistosomula in antibody-dependent systems in vitro. Capron et al (1980) used rat sera and cells to demonstrate that IgG2a and IgE antibodies can cause macrophage adherence and damage to the parasite.

As described in section 1.5.7, newly transformed schistosomula rapidly cease to bind antibody and become refractory to antibody-dependent killing mechanisms. Thus, it seems unlikely that ADCC mechanisms can fully account for the in vivo pattern of challenge attrition in the concomitant immunity model, whereby the majority of challenge schistosomula are killed when they are older than 24 hours, in the skin, lungs or liver. As yet, only one immune mechanism has been

Figure 1.5: Four types of cellular reactions which may mediate immune attrition against schistosomula.

(a) Type II hypersensitivity: Antibody-dependent cell-mediated cytotoxicity (ADCC). This type of reaction requires direct contact between effector cell and target schistosomulum.

(b) to (d) Inflammatory reactions - do not require cell/parasite contact.

(b) Type I hypersensitivity.

(c) Type III hypersensitivity.

(b) and (c) both involve antibody and Fc-bearing effector cells - e.g. eosinophils, neutrophils, mast cells, macrophages.

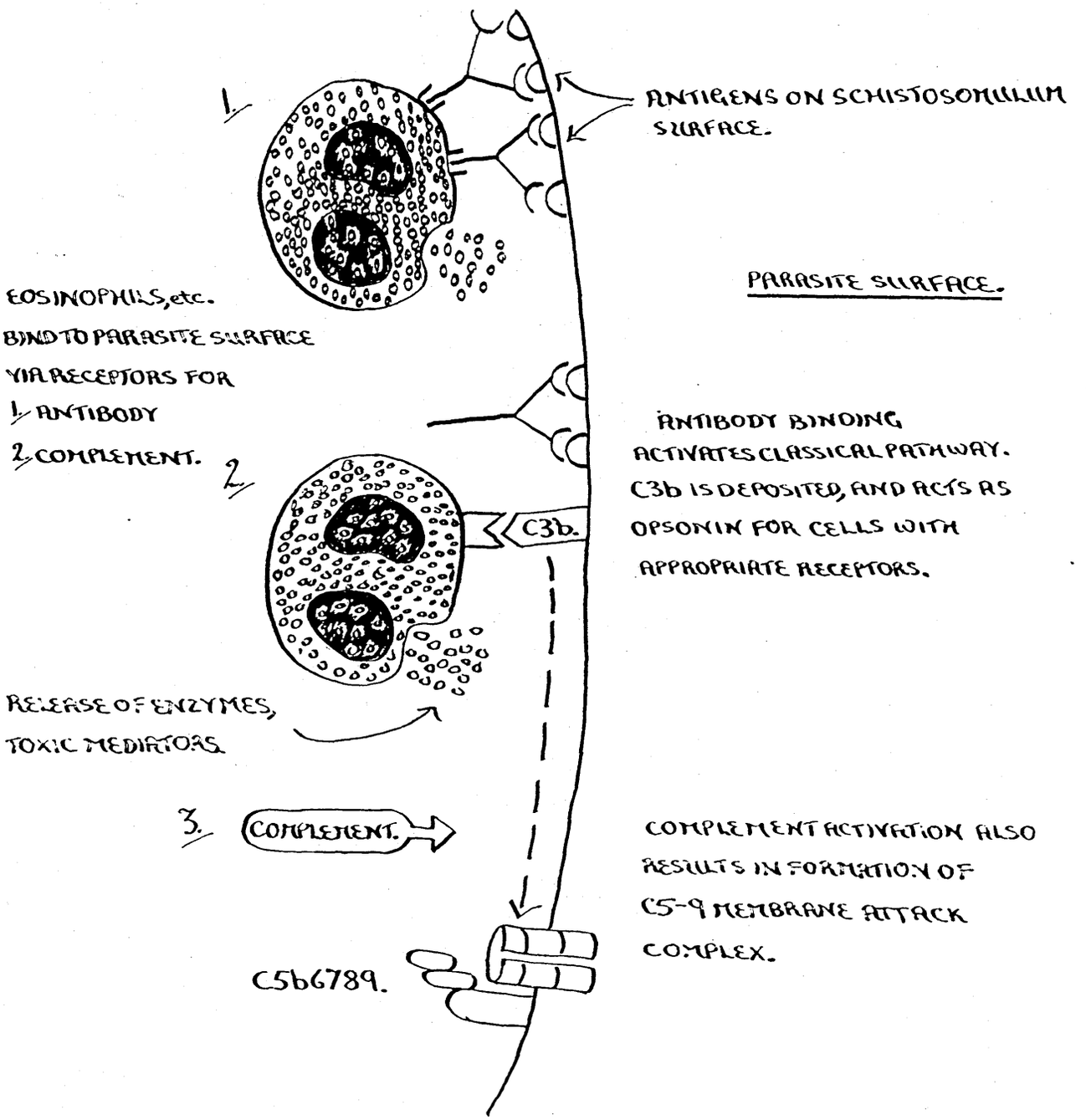
(d) Type IV hypersensitivity.

This mechanism is wholly cell-mediated, with lymphokine - activated macrophages as effector cells.

(a) Type II hypersensitivity: Antibody-dependent cell-mediated cytotoxicity (ADCC)

ADCC reactions involve direct cell contact with the parasite target, via antibody and/or complement receptors. The effector cells - eosinophils, neutrophils, macrophages, platelets - release mediators which damage the schistosomulum.

Activation of complement at the parasite surface, via the classical pathway, may also cause membrane damage by formation of the C5-9 membrane attack complex.


1.
ANTIGENS ON SCHISTOSOMULUM SURFACE.
PARASITE SURFACE.
EOSINOPHILS, etc.
BIND TO PARASITE SURFACE
VIA RECEPTORS FOR
1. ANTIBODY
2. COMPLEMENT.
2.
ANTIBODY BINDING
ACTIVATES CLASSICAL PATHWAY.
C3b IS DEPOSITED, AND ACTS AS
OPSONIN FOR CELLS WITH
APPROPRIATE RECEPTORS.
C3b.
RELEASE OF ENZYMES,
TOXIC MEDIATORS.
3.
COMPLEMENT.
COMPLEMENT ACTIVATION ALSO
RESULTS IN FORMATION OF
C5-9 MEMBRANE ATTACK
COMPLEX.
C5b6789.

(b) Type I hypersensitivity (IgE-mediated hypersensitivity).

This inflammatory response requires participation by antibody. Direct cell/parasite contact is not necessary.

Cells bearing Fc^E receptors - eosinophils, basophils, mast cells, some macrophages, platelets - bind IgE. On secondary encounter of antigens released in schistosomular secretions, or derived from dead and disintegrating schistosomula, with IgE antibodies become cross-linked, inducing cellular degranulation with release of enzymes and mediators.

These mediators

(1) damage the parasite directly;

(2) increase vascular permeability;

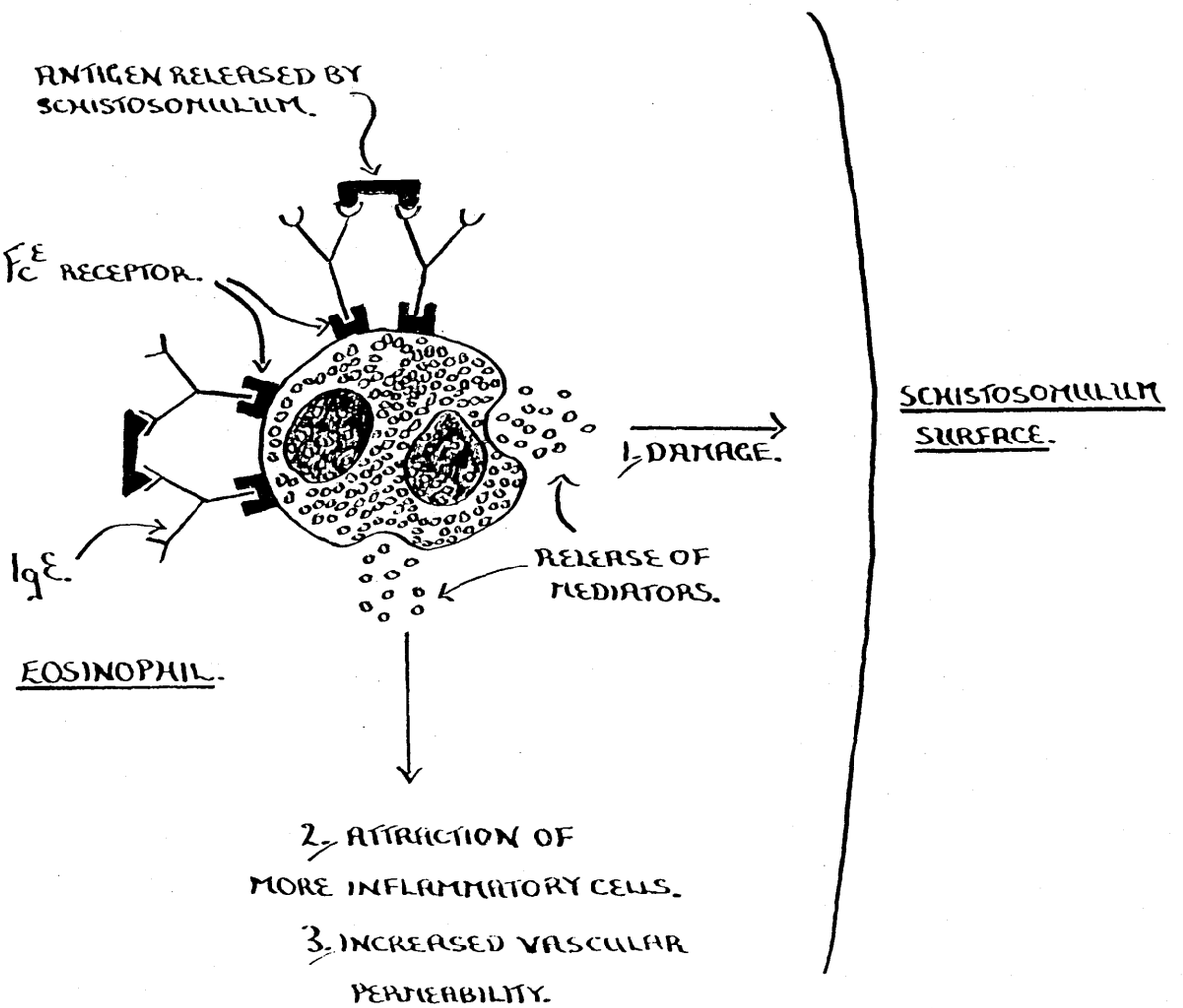
(3) attract more inflammatory cells to the area.

Anaphylatoxins - C3a, C5a - can also stimulate degranulation of these cells.

DIMENSIONS:

EOSINOPHIL: 10-15 μm. DIAMETER.

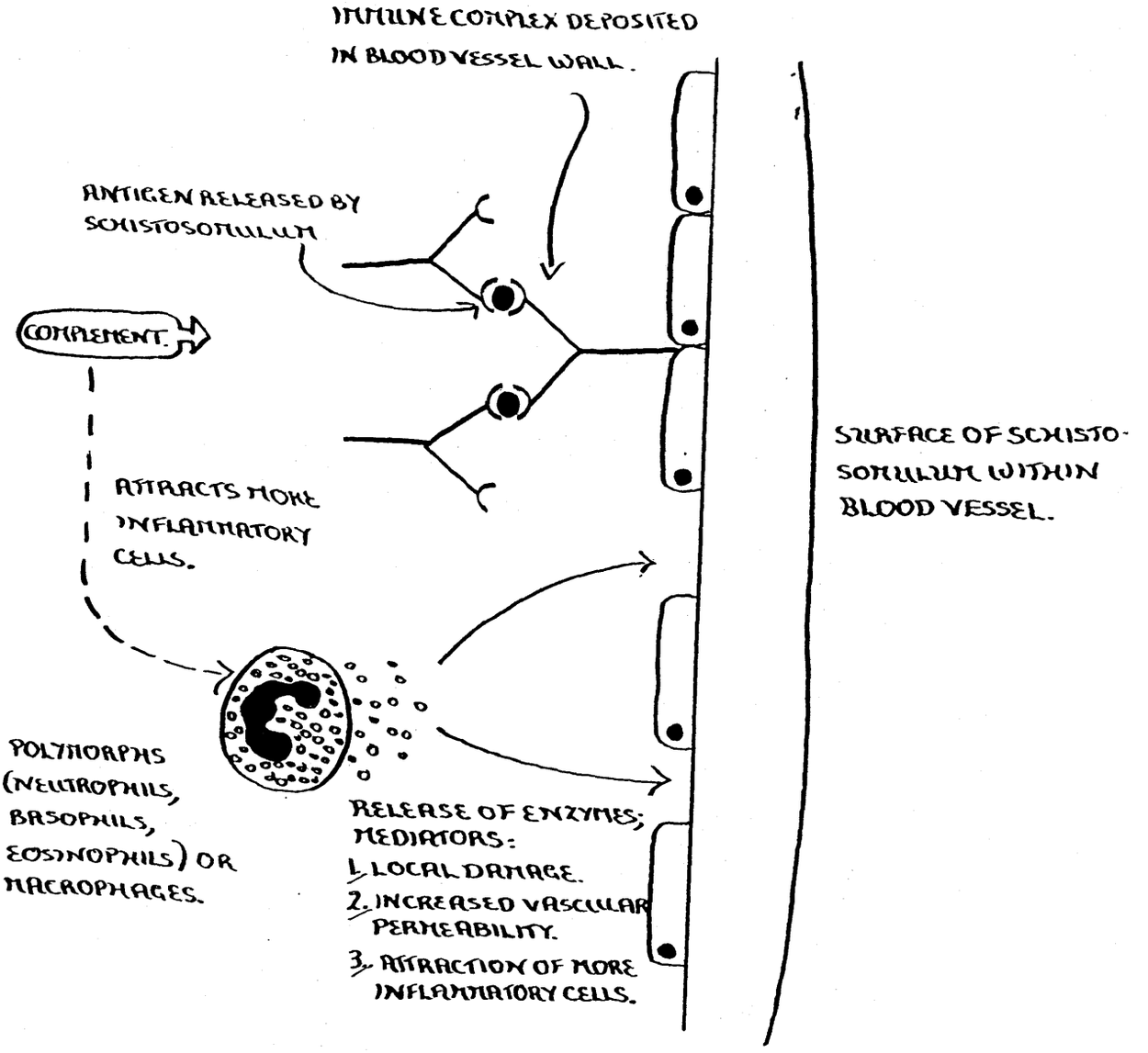
SCHISTOSOMULUM: 214 x 94 μm.



(c) Type III hypersensitivity (Immune complex hypersensitivity).

This inflammatory response requires participation by antibody and/or complement. Direct cell/parasite contact is not necessary.

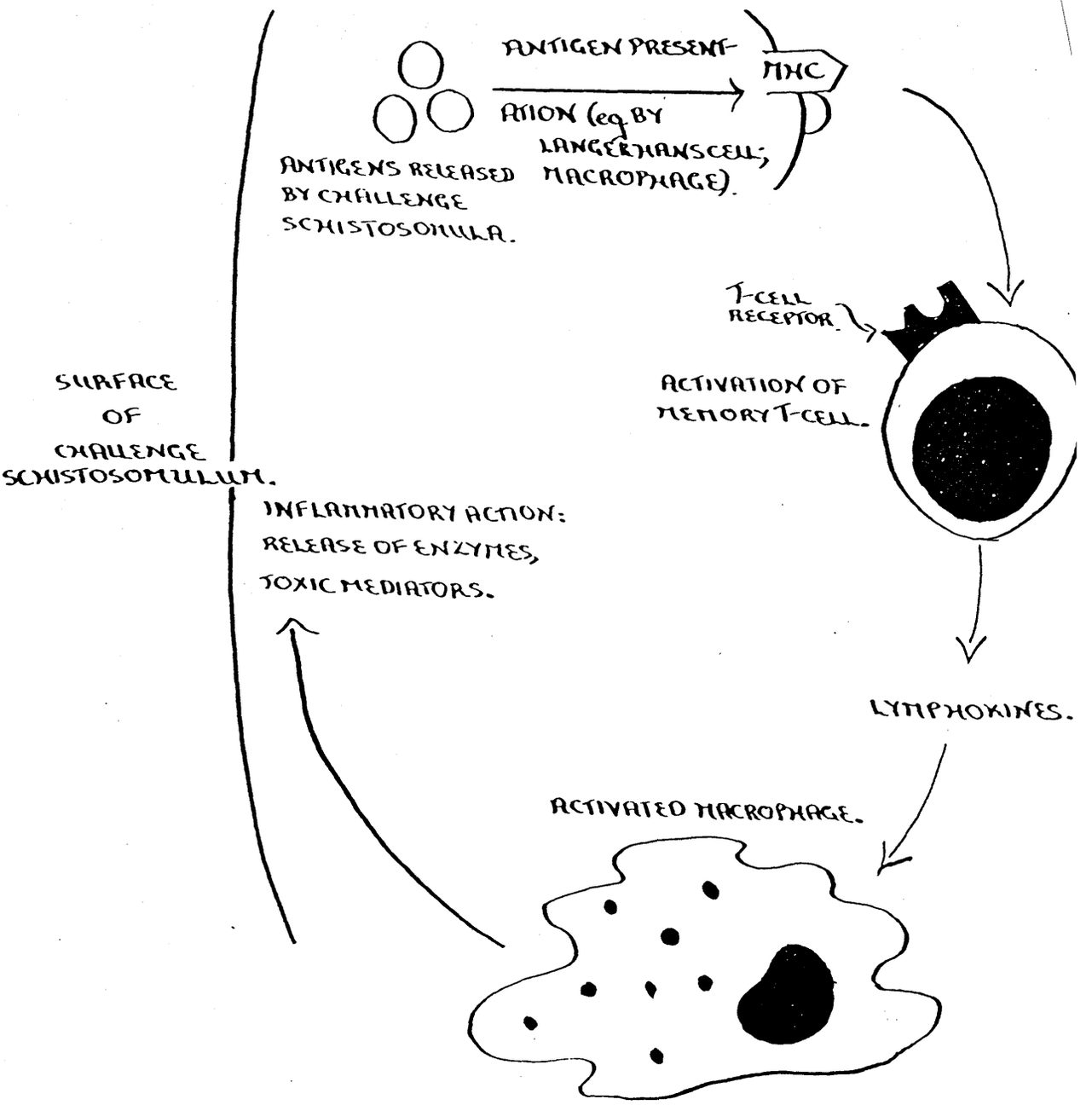
Antigens released by challenge schistosomula may result in deposition of immune complexes (cross-linked antibody/antigen) in a variety of tissues, for instance, skin, blood vessel walls, lung alveoli. These immune complexes may interact with the complement system, leading to generation of C3a and C5a, which have anaphylatoxic and chemotactic properties. They may also activate cells bearing Fc or C3 receptors - eosinophils, basophils, neutrophils, macrophages, platelets - causing release of lytic enzymes and toxic mediators, as well as vasoactive amines which increase vascular permeability.


IMMUNE COMPLEX DEPOSITED IN BLOOD VESSEL WALL.
ANTIGEN RELEASED BY SCHISTOSOMULUM
COMPLEMENT
ATTRACTS MORE INFLAMMATORY CELLS.
SURFACE OF SCHISTOSOMULUM WITHIN BLOOD VESSEL.
POLYMORPHS (NEUTROPHILS, BASOPHILS, EOSINOPHILS) OR MACROPHAGES.
RELEASE OF ENZYMES; MEDIATORS:
1. LOCAL DAMAGE.
2. INCREASED VASCULAR PERMEABILITY.
3. ATTRACTION OF MORE INFLAMMATORY CELLS.

(d) Type IV hypersensitivity.

This type of hypersensitivity is wholly T-cell mediated, and does not involve participation by antibody. Activated macrophages act as the predominant effector cells. Direct contact of macrophages with the parasite surface is not necessary.

Antigen-sensitized T-cells release lymphokines following secondary contact with antigens released by challenge schistosomula. Lymphokines induce inflammatory reactions, and activate and attract macrophages which release lytic enzymes and toxic mediators.


ANTIGEN PRESENTATION (eg BY LANGERHANSCELL; MACROPHAGE).
MHC
ANTIGENS RELEASED BY CHALLENGE SCHISTOSOMULA.
T-CELL RECEPTOR.
ACTIVATION OF MEMORY T-CELL.
SURFACE OF CHALLENGE SCHISTOSOMULUM.
INFLAMMATORY ACTION: RELEASE OF ENZYMES, TOXIC MEDIATORS.
LYMPHOKINES.
ACTIVATED MACROPHAGE.

shown to be effective in killing late-stage schistosomula *in vitro*. Macrophages activated by lymphokine exposure *in vitro*, or *in vivo* by previous infection, can kill both 3-hour schistosomula (Bout *et al*, 1981; James *et al*, 1982; Pearce and James, 1986) and 2 week old, juvenile worms (Pearce and James, 1986) in *in vitro* assays.

b) *In vivo* studies.

A number of studies have aimed to relate immune effector mechanisms *in vivo* to the systems defined *in vitro*.

Attempts to transfer resistance by intravenous injection of antibody have succeeded in some experiments, but failed in others. Sher *et al* (1975) partially protected naive mice of several strains by transfer, just before infection, of serum from mice with 12- to 15-week old infections. Statistically significant protection was demonstrated by reduced worm counts at both lung worm and adult stages. Serum injected from 3 to 11 days after challenge was not protective, indicating that the transferred serum was only effective against the skin schistosomulum stage. Successful transfer of protection with homologous serum was also obtained by Hillyer *et al* (1975), Rombert and Trinca (1979) and Mahmoud *et al* (1975). In the rat model, protection can be consistently transferred to naive recipients with the anaphylactic antibodies IgG2a and IgE from infection serum (Capron *et al*, 1980). Partial protection has also been obtained by injection of various monoclonal antibodies derived from infected rodents (section 1.8).

In other studies, however, serum transferred from infected to normal mice by several routes and schedules failed to reduce significantly the worm counts of the recipients (Maddison and Kagan, 1978, 1979; Doenhoff and Long, 1979). Exchange of blood between mice by

parabiosis also failed to confer resistance to reinfection in the experiment of Dean et al (1981b). Moreover, mice treated with anti-μ chain antiserum to abolish antibody-producing cells developed the same degree of resistance as untreated controls (Maddison et al, 1980).

The role of complement in concomitant immunity is also not clearly defined. Increased numbers of challenge worms compared with controls were obtained in mice deficient in the fifth component of complement (Sher et al, 1975), or by treatment of mice with cobra venom factor (CVF) (Tavares et al, 1978a). On the other hand, Doenhoff and Long (1979) reported that CVF treatment did not significantly affect the number of worms recovered by portal perfusion 10 days after challenge. Contradictory results were also obtained by Sher et al (1982).

As regards cellular involvement in concomitant immunity, a role for eosinophils in elimination of secondary S. mansoni infections was indicated by experiments in which the injection into resistant mice of rabbit antiserum raised against mouse eosinophils led to an increase of approximately 75% in challenge worm counts (Mahmoud et al, 1975).

Mast cells may also play a role in resistance to reinfection by S. mansoni. Repeated injection of a mast cell disrupting agent, compound 48/80, into chronically infected mice before and after challenge restored adult worm counts almost to control levels (Dean et al, 1976).

Histological studies have demonstrated typical antibody-dependent cellular cytotoxicity reactions involving neutrophils and eosinophils around challenge schistosomula in the epidermis of previously-infected mice (Incani and McLaren, 1984). It was suggested that larvae which become trapped in these foci may be sloughed from the skin. However, such ADCC reactions, involving parasite-cell contact, are seen only rarely in dermal, subcutaneous and pulmonary tissues. Infiltration of eosinophils, neutrophils and macrophages is, however, clearly demonstrable at these 3 sites in previously-infected hosts (von Lichtenberg et al, 1976; Savage and Colley, 1980; Incani and McLaren,

1984).

It is proposed that attrition at these later stages in schistosomular development is based on cellular inflammatory reactions in response to antigens released by migrating or dying schistosomula of the challenge infection. Local release of toxins by the cells in the inflammatory foci may cause parasite damage and death without necessitating direct contact between effector cells and schistosomula (Smithers et al, 1987; McLaren and Smithers, 1987) (see figure 1.5). Such inflammatory reactions could be induced by type I or type III hypersensitivity mechanisms, both of which involve antibody and effector cells bearing Fc receptors - eosinophils, neutrophils, mast cells, macrophages. Evidence for the importance of the cutaneous inflammatory response in concomitant immunity in mice was obtained by administering a monoclonal antibody directed against mouse neutrophils at the time of challenge (McLaren et al, 1987). This treatment depleted the cutaneous reaction around the challenge parasites, reducing the level of concomitant resistance by 45% compared with infected but untreated controls.

Type IV hypersensitivity, wholly cell-mediated, with lymphokine-activated macrophages as effector cells, also seems to contribute to concomitant immunity, for James and Cheever (1985) found that mouse strains with genetic defects in cell-mediated immunity and macrophage activation failed to develop resistance to challenge after a primary infection.

In vitro studies have shown that mouse IgM antibodies which react with carbohydrate epitopes on both the schistosomular surface and the egg block ADCC mechanisms against young schistosomula (Omer-ali et al, 1986, 1988; Yi et al, 1986a,b). It has also been proposed that these blocking antibodies may modulate the other cellular inflammatory responses hypersensitivity: types I, III, IV - see figure 1.5). which appear to trap and damage migrating schistosomula (Smithers et al,

1987).

These inflammatory responses which form the specific immune component of concomitant resistance, and their modulation by anti-carbohydrate antibodies, will be discussed in more detail in the section on irradiated vaccine immunity (section 1.9), for it seems that common immune effector mechanisms may operate in both models (Smithers et al, 1987; McLaren and Smithers, 1987; James et al, 1987).

In summary, while antibody- and cell-mediated responses are implicated in concomitant immunity, the precise roles and relative importance of each are not clearly defined. Attempts to induce resistance in animal models with defined antigens have helped identify mechanisms and targets of protective immunity which might be essential considerations in a future vaccine.

1.8 Role of defined antigens in protective immunity.

1.8.1. Protective antigens identified by passive transfer of monoclonal antibodies.

A number of potentially protective parasite antigens have been identified by monoclonal antibodies which are able to transfer passive resistance to challenge with S. mansoni cercariae. Such protective monoclonal antibodies have been produced from both rat and mouse lymphocytes. The level of protection in passive transfer experiments ranges from 27 to 70% in mice and rats. All the antigens recognised by these protective monoclonal antibodies appear on the surface of early schistosomula.

Several of these monoclonals recognise schistosomular surface antigens of molecular weight 200 000 and 38 000. Grzych et al (1982) produced a rat IgG2a monoclonal which immunoprecipitated both these antigens. Passive transfer of this antibody reduced challenge worm

survival in rats by 53-63%. Zodda and Phillips (1982) produced a mouse IgG1 monoclonal which, when transferred in mice, produced a 48% reduction in worm burden. This antibody recognized several molecules in addition to the schistosomulum surface antigens at molecular weight 200 000 and 38 000. It also reacted with 17 000 and 20 000 Mr species on the schistosomular surface and with 160 000 and 130 000 Mr antigens within a cercarial extract (Kelly et al, 1986). Tarrab-Hazdai et al (1985) induced over 40% immunity in mice with an IgG1 monclonal which identified the surface antigen of molecular weight >200 000. Harn et al (1984) produced a panel of monoclonal antibodies from mice injected with egg antigens. The resulting monoclonals reacted with both egg, and schistosomular surface, antigens. One antibody was selected, which transferred 41% immunity to mice. On Western blotting of whole schistosomular extracts, this antibody identified 160 000 and 130 000 molecular weight antigens. However, after surface iodination of schistosomula and immunoprecipitation, antigens at Mr 200 000 and 38 000 were again recognised (Harn et al, 1987). The protective monoclonal antibody produced by Smith et al (1982) also recognised the antigens of molecular weight > 200 000 and 38 000.

These two antigens appear to be highly glycosylated, expressing carbohydrate epitopes also common to egg antigens (Smithers et al, 1987; Dissous and Capron, 1983). Since a number of the monoclonals recognize other antigens in addition to the molecular weight > 200 000 and 38 000 ones, it cannot be excluded that other molecules might express the same carbohydrate epitopes, though multispecificity of antibody binding means that a single monoclonal may recognize a number of quite different epitopes (Ghosh and Campbell, 1986). Although these carbohydrate epitopes shared between eggs and schistosomula are targets of protective antibodies, it seems that they may simultaneously induce antibodies of blocking isotypes which modulate the immune response (see sections 1.7, 1.9; chapter 7).

Protective antibodies recognising schistosomular surface antigens of other molecular weights have also been described. A monoclonal recognising antigens of Mr 45 000 and 30 000 on the schistosomular surface transferred protection to mice in the study of Tarrab-Hazdai et al (1985). An antibody which precipitates 22 000 - 26 000 molecular weight antigens released by schistosomula in culture is reported to protect against challenge in the rat model (reported by Simpson and Cioli, 1987). Bickle et al (1986) characterised IgG3 and IgG2a monoclonals recognising schistosomulum surface antigens of molecular weight 16 000 and 32 000, respectively, which transferred 28-70% and 27-58% immunity to mice.

Levels of protection induced by transfer of monoclonal antibodies range on average from 30 to 60% The failure to achieve higher levels of immunity might suggest that other, presumably cellular, components of the immune response are required for fully effective kiling of challenge parasites. Possible in vivo mechanisms of parasite attrition induced by antibody transfer include antibody-dependent cellular cytotoxicity reactions and other inflammatory responses in the skin and/or lungs (Smithers et al, 1987; see figure 1.5). Transfer of immune serum has been shown to induce both resistance and lung inflammation in mice (Olveda et al, 1981). Similar inflammatory responses are likely to be induced by the transfer of protective monoclonal antibodies. Whatever the mechanisms, passive transfer of protection with these monoclonal antibodies does indicate that schistosomular surface antigens can mediate immunity. Active immunization experiments have revealed other protective antigens.

1.8.2. Identification of protective antigens by direct immunisation.

Immunoaffinity chromatography with protective monoclonal antibodies has been used in a number of instances to isolate antigens

for vaccination experiments in experimental hosts. The carbohydrate epitope of the > 200 000 molecular weight species which appears to be such a dominant antigen on the schistosomular surface has been involved in two active immunisation experiments. Smith and Clegg (1985) performed the first such immunisation study with purified antigen. These workers used their protective monoclonal antibody which recognised the schistosomular surface antigens of molecular weight > 200 000 and 38 000 (above) to purify a cross-reacting antigen of molecular weight 155 000 from adult worm extracts. Immunization of mice with graded amounts of antigen (2-50 μg/mouse) in alhydrogel adjuvant resulted in a low but overall statistically significant level of protection (25%, $p < 0.01$). A higher reduction in worm burden (42%, $p < 0.01$) was achieved by vaccinating 3 cynomolgous monkeys.

Tarrab-Hazdai et al (1985) used one of their protective monoclonals to purify the schistosomular surface antigen of molecular weight > 200 000 by immunoaffinity chromatography. The isolated antigen was then used to immunise mice, with Freund's Complete Adjuvant for a primary immunization, followed by a boost without adjuvant. A maximum of 34% immunity was obtained.

Auriault et al (1985) induced extremely high levels of protection, up to 89%, in rats by direct vaccination with crude released products from cultured schistosomula, or with the complex of molecular weight 22 000-26 000 isolated from this material (Capron and Capron, 1986). These antigens elicit strong IgE responses in vivo and their efficacy in vaccinatoin may be due to stimulation of eosinophil-mediated killing.

Harn (1987) reported preliminary experiments in which mice immunized with immunoaffinity-purified schistosomulum surface antigens of molecular weight 22 000, 28 000 and 38 000 showed 15-27%, 38% and 35% resistance, respectively.

While a number of potentially protective antigens have thus been

identified, a major problem in large-scale production of vaccines against eucaryotic parasites such as schistosomes is that the shortage of parasite material, and complexity of production by passage through both definitive and intermediate hosts, precludes the preparation of antigens directly from the parasite itself. The most promising approach for the immediate future appears to be molecular cloning of genes encoding protective peptide antigens, and expression of such peptides in appropriate procaryotic or eucaryotic vectors. In contrast, expression of cloned genes for glycosylated proteins, for instance by introduction of parasite DNA into immortal mammalian tissue culture cell lines, is as yet in its initial stages (Beardsell and Howell, 1987), although some other possible means of circumventing the problem of large-scale production of carbohydrate antigens have been suggested (see below).

Four protective antigens have been selected and extensively characterized with a view to large-scale vaccine production. The mechanisms by which these antigens induce immunity have also been investigated in some detail.

1.8.3. Defined protective antigens of schistosomula.

(1) The 38 000 molecular weight surface antigen of schistosomula.

The carbohydrate nature of the Mr 38 000 schistosomular surface antigen, and its capacity to induce blocking as well as protective antibodies, both limit its potential as a vaccine candidate (see sections 1.7, 1.9). Grzych *et al* (1985) attempted to overcome both these problems by immunizing with anti-idiotype antibodies instead of the original antigen. A monoclonal anti-idiotype antibody was produced which appeared to mimic the epitope recognized by the protective rat IgG2a monoclonal described above, and which apparently corresponded to

a carbohydrate epitope present on the surface antigens of molecular weight 38 000 and >200 000. Active immunization with the anti-idiotype antibody produced 50-76% protection in rats.

Although encouraging, these results are as yet of limited applicability for vaccination against human disease. An alternative method for large-scale production of this antigen was suggested by cross-reactivity studies. Dissous et al (1986) showed that the protective epitope on the Mr 38 000 antigen was also expressed in tissues of Biomphalaria glabrata, the intermediate snail host for S. mansoni, where it was associated with a snail component of molecular weight 90 000, as well as in 3 other species of freshwater snail, two of which were also schistosome hosts. These observations led the authors to suggest that this carbohydrate structure might have some role to play in adaptation to changes in osmolarity during the free-living aquatic stages of the schistosome life-cycle. The epitope was also present on keyhole limpet hemocyanin (KLH) (Grzych et al, 1987). Indeed, immunization with KLH protected rats against S. mansoni infection, and induced production of anti-38 000 antibodies which conferred immunity by passive transfer to rats. Deglycosylation of KLH abolished its immunological and biological properties, and confirmed the involvement of its carbohydrate moieties in the cross-reactivity. Not only does the availability of commercially-purified KLH greatly facilitate determination and chemical synthesis of the cross-reactive carbohydrate structure, but it has even been suggested that farming of snails to produce a schistosome vaccine might be possible.

While protective antibodies specific for the Mr 38 000 antigen from rats, mice and humans have been shown to mediate eosinophil-dependent killing of schistosomula in vitro, this activity is inhibited, either sterically or directly, by blocking antibodies recognising the same epitope (Grzych et al, 1984; Butterworth et al, 1985; Smithers et al, 1987). Clearly, a vaccine based on this

antigen would require to be presented to the host in such a way as to stimulate a protective rather than a blocking response.

(2) Glutathione-S-transferases (molecular weight 26 000, 28 000).

Mitchell et al (1984, 1985) found that a proportion of JAX 129/J mice showed a high level of resistance to S. japonicum and S. mansoni infections when bred and maintained under conventional conditions. When the antibody responses to adult worm antigens in resistant and susceptible mice were compared, only the resistant ones were found to be high responders to an S. japonicum antigen of molecular weight 26 000 (Sj26). Clones corresponding to this antigen were identified in a cDNA library in λ-gt11, and the full amino acid sequence of the antigen determined (Smith et al, 1988). On the basis of its amino acid sequence and enzyme activity, Sj26 was identified as the enzyme glutathione-S-transferase. Vaccination of mice with the β-galactosidase - Sj26 fusion protein (30-10 μg of protein/mouse in 3 equal doses, with Freund's Complete Adjuvant for the primary immunisation) produced 30% immunity in responder mouse strains. As indicated by its amino acid sequence, Triton X-114 phase partitioning and studies on antibody-binding to intact parasites, Sj26 is a cytosolic enzyme rather than an integral membrane protein (Mitchell, 1989).

Balloul et al (1985) followed a different line of research to identify an S. mansoni glutathione-S-transferase. These workers isolated a 28 000 molecular weight antigen (Sm28) by fractionation of adult S. mansoni proteins on SDS-PAGE, and electrophoretic elution of the protein from polyacrylamide gels. Antisera produced in rats against the 28 000 molecular weight protein recognized in vitro translation products from adult worm mRNA showing the same electrophoretic mobilities as the extracted protein on SDS-PAGE and

isoelectric focussing. This result suggested that the 28 000 molecular weight protein was not subject to post-translational processing, hence was a suitable candidate for large-scale production by molecular cloning. Immunofluorescence experiments indicated that the molecular weight 28 000 antigen was mainly located in the parenchyma of schistosomula and adult worms. However, immunoprecipitation of an Mr 28 000 labelled molecule from surface-iodinated schistosomula by anti-28 000 antibodies indicated that the antigen could be briefly exposed at the schistosomular surface. It was suggested, therefore, that the 28 000 molecular weight antigen might be a secreted molecule (Dissous et al, 1987). Sm28, either purified from whole worm extracts, or as a cloned product from a λ-gt11 expression vector, induced partial (35-65%) protection against challenge in rats, mice, hamsters and monkeys (Balloul et al, 1987 a, b, c). Taylor et al (1988) identified Sm28 as a glutathione-S-transferase.

By passing extracts of S. japonicum and S. mansoni adult worms over glutathione-conjugated agarose columns, it was demonstrated that each species possesses both 26 000 and 28 000 molecular weight glutathione-S-transferases (Davern et al, 1987; Mitchell, 1989). The 28 000 molecule predominates over the 26 000 one in both species. Cross-reactivity has been shown between the dominant Sm28 and Sj28 antigens, but Sm26 and Sj26 apparently show less homology (Davern et al, 1987).

Mitchell (1989) described a possible mechanism by which vaccination with glutathione-S-transferase as antigen might induce protective immunity against challenge. It seems that antibodies against glutathione-S-transferase protect only in combination with other, aggressive immune responses to the schistosomes. It was proposed that the parasite's glutathione-S-transferases normally are transported to the surface to protect the worm against damage by detoxifying foreign substances - for instance, products released from immune effector

cells, or oxidised lipids and proteins generated at the worm surface in consequence of immune attack. High levels of antibodies specific for glutathione-S-transferase could bind to the enzymes exposed at the surface and prevent such detoxification, leading to exacerbation of immune damage, and parasite death.

This hypothesis predicts that a vaccine based on anti-glutathione-S-transferase responses would require, at the least, to be bipartite. One or more antigens capable of eliciting an initial aggressive immune response, and a glutathione-S-transferase to induce antibody responses which neutralize the worms' detoxification functions would both be essential components.

(3) Paramyosin and induction of cell-mediated immunity

Most of the immunogens described above were selected for study because of their potential to induce a protective humoral response. On encountering a challenge infection, surface antigens of the young schistosomulum appear to be the target of antibody-dependent cellular cytotoxicity, with eosinophils and macrophages acting as the chief effector cells.

However, James et al (1987) consider that induction of T-cell mediated anti-schistosome immunity could be equally important in protection. A vaccination protocol based on cell-mediated immunity was developed, which consistently stimulated strong resistance to challenge S. mansoni infection in mice.

A crude antigen preparation composed of the saline soluble components of whole adult worms or schistosomula, administered intradermally (i.d.) with a bacterial adjuvant, induces consistent protection (30-50%) against S. mansoni infection in C57BL mice (James, 1985). Both the route of inoculation and the adjuvant were critical to the success of vaccination, suggesting that induction of

effective immunity by this procedure is highly dependent on antigen presentation. Administration of the same antigens intravenously or intramuscularly was ineffective. Only low and inconsistent levels of protection were achieved by intradermal injection of antigen in the absence of adjuvant. Adjuvants which were effective in i.d. vaccination included viable *M. bovis*, BCG, Complete Freund's Adjuvant, whole *B. pertussis* vaccine and saponin, while *C. parvum*, tetanus or diphtheria toxoids, alum, lentinan and yeast glucan all failed to induce resistance. Resistance seemed to be directed against pre-lung stage parasites in this model, since i.d.-vaccinated mice were protected against percutaneous cercarial challenge, but not against intravenous injection of lung-stage larvae.

Protection by this vaccine was shown to be highly correlated with cell-mediated immunity. Indeed, studies on hapten-specific immunity indicate that the i.d. route of antigen presentation favours development of cell-mediated immunity (Greene and Benacerraf, 1980). Mice protectively vaccinated by James' crude intradermal vaccine were shown to have developed strong antigen specific cell-mediated immune responses, including delayed hypersensitivity, lymphokine production and lymphocyte proliferation (James, 1986). These mice also displayed minimal antibody responses to larval or adult schistosome antigens, only recognising a single internal parasite protein of molecular weight 97 000 (James *et al*, 1985; Pearce *et al*, 1986). The absence of antibody reactivity with the schistosomular surface antigens, which appear to be those involved in antibody-dependent cell-mediated cytotoxicity mechanisms, suggests that such humoral responses do not contribute to resistance in this model. The i.d.-immunized, protected mice responded to antigen challenge by recruitment and activation of macrophages that could kill schistosomula *in vitro* in an antibody-independent fashion (James, 1986) - a possible effector mechanism in resistance based on cell-mediated immunity.

A series of other experiments implicated cell-mediated, rather than humoral, responses in immunity in this vaccine model. Intravenously immunized mice failed to develop the cell-mediated responses described above, but produced high levels of anti-larval antibodies. These animals were not protected against challenge. Congenitally athymic (T-cell-deficient) mice were not protected by i.d. vaccination, while μ-suppressed mice were fully responsive. Mice with inherited defects in immediate hypersensitivity response (IgE production, mast cells) became resistant to challenge after i.d. immunisation with this vaccine, as did strains with defects in endotoxin responsiveness, complement and NK (Normal Killer) cell function (James and Deblois, 1986). Moreover, P and BALB/c strain mice, which did not develop antigen-specific cell mediated immunity following i.d. vaccination, but did produce antibodies, were not protected.

When the soluble adult worm antigenic preparation was fractionated by gel filtration, the immunogenic fraction was found to contain the 97 000 molecular weight species which represents the only antigen recognized by antibodies from i.d.-vaccinated mice. Indirect immuno-fluorescence localized this 97 000 molecular weight antigen (Sm97) to the muscle layer just below the tegument in adult parasites (Pearce et al, 1986). cDNA clones expressing products reactive with antibodies against native Sm-97 were identified. The deduced amino acid sequence identified this molecule as paramyosin, an invertebrate muscle protein. Purified schistosome paramyosin was capable of stimulating lymphocyte proliferation and gamma-interferon production by cells from mice immunized with crude antigen. Conversely, i.d. immunisation with affinity-purified Sm97 plus BCG sensitized mice to respond to either crude antigen or Sm97 in these assays (Sher et al, 1986). Thus, the isolated Mr 97 000 molecule was both antigenic and immunogenic for cell-mediated immunity under these conditions, although direct protection experiments with this antigen have yet to be reported.

It is proposed that intracellular antigens such as paramyosin could be presented to the host following the spontaneous parasite damage and attrition seen even in naive hosts, or possibly as a consequence of normal surface membrane turnover (Kelly, 1987). The activated macrophages characteristic of cell-mediated immunity inflict damage on the muscle layers, rather than the tegument, of young schistosomula (McLaren and James, 1985). This action of the macrophages may impede migration of challenge larvae, trapping them in the skin (Smithers et al, 1987; McLaren and Smithers, 1987).

Table 1.3 lists the schistosome antigens defined as protective.

Overall, these attempts to define targets and mechanisms of protective immunity in schistosomiasis suggest that, for optimal effectiveness, a vaccine must incorporate a number of different antigens - internal, surface-associated and secreted - which act in concert to induce both humoral and cellular immune effector mechanisms. Clearly, the difficulties of generating such complex molecular interactions in defined systems will be considerable. At present, the subtleties of antigen presentation essential for stimulating both arms of the immune response are best displayed by live but attenuated larval vaccines. Attenuation may be effected by various types of irradiation - gamma, X-, U.V. - or by drug-treatment.

It is worth comparing the attempts to induce resistance using drug-cured schistosome infections with the well-established immunising effects of irradiated cercariae. In only one instance have drug-attenuated larvae constituted an effective vaccine, suggesting that premature abbreviation of an initial infection does not induce protective immunity unless the attenuation process allows antigens be presented in such a way as to render them specially immunogenic.

Table 1.3 Some schistosome antigens defined as protective.

References:

1. Grzych et al (1982)
2. Zodda and Phillips (1982)
3. Harn et al (1984)
4. Kelly et al (1986)
5. Dissous et al (1982)
6. Smith and Clegg (1985)
7. James et al (1985)
8. Tarrab-Hazdai et al (1985)
9. Bickle et al (1986)
10. Balloul et al (1987a)
11. Mitchell (1989)
12. Sher and Pearce (1987)
13. Harn (1987)

Table 1.3.

MOLECULAR WEIGHT OF ANTIGEN	PROTECTIVE BY PASSIVE TRANSFER OF MONOCLONAL ANTIBODY?	PROTECTIVE BY DIRECT IMMUNISATION?	GENE CLONED	COMMENTS; REFERENCES
>200 000	Yes (rats and mice)	Yes (mice) Anti-idiotype protects rats.	No	Carbohydrate epitope is protective; common to other lifecycle stages and to molluscs. May also induce blocking antibodies (1-5).
155 000	Yes	Yes (monkeys)	No	Ref. (6)
97 000	No monoclonal produced	Yes (mice)	Yes	Identified as paramyosin. Induces cell-mediated immunity in association with BCG, by i.d. inoculation. (7)
45 000	Yes (mice)	Yes (mice)	No	Ref (8).
38 000	Yes (rats and mice)	Not reported	No	Same carbohydrate epitope as >200 kDa antigen.

Table 1.3. Continued

MOLECULAR WEIGHT OF ANTIGEN	PROTECTIVE BY PASSIVE TRANSFER OF MONOCLONAL ANTIBODY?	PROTECTIVE BY DIRECT IMMUNISATION?	GENE CLONED	COMMENTS; REFERENCES
32 000	Yes (mice)	Not reported	No	Ref (9).
30 000	Yes (mice)	Yes (mice)	No	Ref (8).
28 000, 26 000	Yes (mice & rats)	Yes (mice, rats, monkeys)	Yes	Glutathione-S-transferases. Refs (10), (11).
22 000-26 000 complex	Yes (rats)	Yes (rats)	No	Released by schistosomula in culture. Induce high IgE response Ref. (12).
22 000	Yes (mice)	Yes (mice)	No	Ref. (13).
16 000	Yes (mice)	Not reported	No	Ref. (9).

1.9 Attenuated larval vaccines.

1.9.1. Drug-attenuated primary infections.

In the majority of studies. S. mansoni infections cured prior to parasite maturation have failed to induce resistance. In the early study by Campbell (1963), mice exposed to heavy single or multiple infections one to twenty-eight days prior to curative treatment with a 2-phenyl quinolone drug were afterwards given a heavy challenge infection, then assessed for prolonged mouse survival and reduced severity of the granulomatous response. It was concluded that the number of granulomas, and therefore, apparently, surviving challenge worms, was not significantly reduced, although in some cases mouse survival was slightly prolonged.

Pellegrino and Katz (1974) obtained no resistance in mice cleared of their immunizing infections (500 cercariae percutaneously) by treatment with the drug UK 3883, administered 2 or 4 hours before, or 2, 4 or 8 hours after exposure to 500 cercariae, and challenged 15 days later with 200 cercariae. However, mice treated during the hour of infection did show a 63% reduction in mean challenge worm burden, although the authors did not discuss the significance of this observation. Bickle and Andrews (1985) later confirmed that treatment with oxamniquine (a derivative of UK 3883) did not render prematurely-killed larvae immunogenic. Mice were infected with 500 cercariae percutaneously, treated with oxamniquine 24 hours later, then challenged with 200 cercariae 5 weeks post-infection. No resistance to challenge infection was observed.

Tribouley et al (1977) attempted to protect mice by eliminating immunizing infections with high ambient temperature. Mice were maintained at 35°C, a temperature shown to kill migrating schistosomula during the first 2 weeks of development. Four subcutaneous injections

of 2000 cercariae were administered over a period of 15 days. 20 days after the last exposure, mice were returned to room temperature, and challenged subcutaneously with 500 cercariae. Lung worm recoveries over the next 17 days were not significantly different from those of control mice exposed to the same temperatures or to 22°C throughout the course of the experiment, indicating that these prematurely-terminated infections also had not induced resistance.

In contrast to these results, Bickle and Andrews (1985) demonstrated significant resistance following attenuation of early S. mansoni infections in mice with the benzodiazepine derivative Roll-3128. Drug treatment at 3 hours after primary exposure failed to produce significant resistance, but there was a marked rise in the resistance induced when treatment was delayed until 24 hours. Delay in treatment until 2-3 days post-infection produced the maximum level of resistance (60-75%), which was maintained following treatment at up to 5 days post-infection. Significantly lower levels of resistance occurred with later (6-15 day) treatments. Autoradiographic tracking (Mastin et al, 1985a) demonstrated that Roll-3128 treatment arrests parasites of the primary infection in the skin. The authors suggested that the peak resistance induced by drug-treatment during the 2-5 day period might reflect optimum expression of protective antigens at this time. It was proposed that these immunogens are not normally exposed by the parasite, but that Roll-3128 treatment renders them accessible to the host immune system.

1.9.2. Resistance induced by irradiated cercariae or schistosomula.

1.9.2.1. Introduction.

Resistance to reinfection can be induced in experimental animals

by exposure to radiation-attenuated larvae which die before reaching maturity. Clearly, this type of immunity is not associated with the adult stage of the parasite, nor with egg-associated pathology, which contribute to concomitant resistance, but is induced by the radiation-damaged schistosomula during their curtailed migration. Vaccine-induced immunity develops very rapidly compared to the concomitant immunity model. Resistance is detectable as early as 2 weeks after immunisation, plateaus at about week 5 (Dean, 1983), and has been shown to persist essentially undiminished for a year and a half (Hsü et al, 1981).

As in concomitant immunity, the targets of irradiated vaccine-induced resistance are the immature, migrating schistosomula of the challenge infection. Irradiated vaccine immunity, unlike concomitant immunity, is species specific (Bickle et al, 1985; Moloney and Webbe, 1987). The specific nature of vaccine immunity, and the absence of complicating factors such as egg-associated pathology, allow us to infer that protection in this model is based wholly on lymphocyte-associated immunity. This conclusion is confirmed by the demonstration by Sher et al (1982) that irradiated vaccine immunity in mice is T- and B-cell dependent.

1.9.2.2. Irradiated vaccines in domestic animals and primates.

Irradiation is an established method of attenuation in a wide range of parasite systems. In every case, the attenuated parasite induces a high level of immunity during its abbreviated lifespan, and dies before reaching the mature stage associated with pathogenicity. Successful vaccination of laboratory animals has been achieved with irradiated protozoans - Anaplasma, Babesia, Theileria, (IAEA, 1967), Plasmodia (Wellde and Sadun, 1967), Trypanosoma (Duxbury and Sadun, 1970), Leishmania (Liew et al, 1984), as well as a number of

helminths - Nippostrongylus brasiliensis (Jennings et al, 1963), Ancyclostoma caninum (Miller, 1964; Miller et al, 1970); Trichinella spiralis (Gould et al, 1957), Echinococcus granulosus (IAEA 1967), Trichostrongylus colubriformis (Jarrett et al, 1960 b), Taenia saginata (Urquhart,1961), Dictyocaulus filaria (Tavari et al, 1971), Dictyocaulus viviparus (Jarrett et al, 1958, 1959, 1960a). We might speculate that some unifying factor could account for this striking capacity of irradiation to generate safe and effective vaccines from so many different parasite species.

The paradigm for a successful radiation-attenuated larval vaccine is that introduced for control of parasitic bronchitis in cattle (caused by Dictyocaulus viviparus). It is therefore worth briefly considering the development of this vaccine (reviewed by Poynter, 1968). The pathogenic stage in the life-cycle of this parasite is the adult worm. Adult worms live in the bronchioles of the lungs and induce severe inflammatory responses. Animals which survive an initial Dictyocaulus infection become highly resistant to reinfection. Study in an experimental host, the guinea-pig, indicated that one particular larval stage - L_4 - was responsible for inducing immunity. Thus, it became clear that it might be possible to induce immunity artificially, but avoid the pathology associated with the adult worms, by restricting the development of the worm to the larval stages by irradiation. The guinea-pig model was used to develop a suitable immunization protocol using infective third-stage larvae irradiated at 40 krad. This level of irradiation allowed larvae to develop only to the L_4 stage before dying. It was possible to induce greater than 90% protection against challenge in guinea pigs by administering 2 oral doses, each of 100 irradiated larvae, at an interval of 3 to 4 weeks (Poynter, 1968). This immunisation protocol was found to be equally successful in cattle, thus providing a basis for development of the vaccine on a commercial

scale. The vaccine has been in use in cattle since its first introduction in 1958. A similar vaccine has been employed to control parasitic bronchitis in sheep and goats (D. filaria species) in India since 1971 (Sharma et al, 1988).

Human schistosomiasis shows sufficient similarity to Dictyocaulus infection in cattle to suggest that an irradiated vaccine could be equally effective against this disease. Firstly, as with Dictyocaulus, immature parasite stages cause little or no pathology; maturation and egg-laying cause the disease symptoms. Secondly, natural infection also induces resistance, at least partly immunologically-based, to larval challenge. Finally, mice, rats and guinea-pigs all serve as convenient laboratory hosts for study of immune mechanisms in schistosomiasis, and have clearly demonstrated the efficacy of the irradiated vaccine in protecting rodents against infection. Indeed, irradiated schistosomiasis vaccines have already been employed with considerable success in cattle.

Animal schistosomiasis is a problem of serious economic significance in some parts of Africa and Asia. Several species of schistosomes are involved, including S. bovis in the Sudan, S. mattheei in southern Africa, Ornithobilharzia turkestanicum and S. spindale in the Middle East, S. nasalis and S. indicum in India, and S. japonicum in the Far East.

Under laboratory conditions, sheep vaccinated with cercariae of S. mattheei subjected to gamma irradiation at 6 krad showed a 74% reduction in survival of challenge worms. 6 krad-irradiated, skin-transformed schistosomula, administered intravenously or intramuscularly, and 6 krad-irradiated, syringe-passaged schistosomula injected intramuscularly, all induced highly significant levels of immunity to challenge (56-78%). Vaccination against S. bovis in cattle has also been extensively investigated. Prior vaccination of calves with 10 000 3 krad gamma-irradiated cercariae or schistosomula of S. bovis induced 43-50% resistance to laboratory challenge with

10 000 normal cercariae (Bushara et al, 1978). Immunising schistosomula were given intramuscularly; cercariae percutaneously. These protection studies all used relatively low doses of irradiation which do allow a small proportion of the immunising parasites to mature (Dean, 1983).

Subsequently, higher irradiation doses (20-50 krad) which consistently cause parasite death before adulthood were shown to be effective in producing attenuated vaccines against both S. bovis and S. japonicum (Taylor, 1980; Hsü et al, 1984). As with the lower irradiation doses, artificially-transformed schistosomula appeared to be as effective in inducing resistance as the original irradiated cercariae from which they were derived. Since schistosomula, unlike cercariae, are suitable for injection, this observation partially overcame the difficulties of administering a cercarial vaccine. A further technical advance was the demonstration that these irradiated schistosomula could be stored in cryopreserved form in liquid nitrogen (James, 1981). When re-warmed, the attenuated schistosomula were viable for long enough to stimulate resistance.

A vaccine consisting of irradiated, cryopreserved S. bovis schistosomula was tested in a 10-month field trial in the Sudan (McCauley et al, 1984). Groups of vaccinated and non-vaccinated calves were exposed to S. bovis infection under natural conditions. Thus, the animals were exposed to parasites of a much greater genetic diversity than laboratory-maintained strains. The results clearly indicated that the vaccine was protective. Although all the calves did eventually become infected, mean adult worm counts were reduced by 69%, and egg counts by 80%, compared with non-vaccinated controls.

A parallel experiment aimed to vaccinate cattle against S. japonicum using irradiation-attenuated schistosomula (not cryopreserved). 71.6% protection was demonstrated against laboratory challenge, and a 65.1%-75.7% reduction in worm counts in the

corresponding field trial (Hsü et al, 1984).

These successes in vaccinating domestic animals encouraged attempts to induce immunity in primates using similar irradiated larval vaccines. Initial attempts to immunise baboons with S. mansoni cercariae or schistosomula irradiated at low doses (2-6 krad) were unsuccessful (Taylor et al, 1976). However, high doses of irradiation were effective in rendering the larvae immunogenic in primates. Stek et al (1981b) immunised baboons with 2 doses of approximately 5000 20 krad gamma-irradiated cercariae, percutaneously, at 21-day intervals. The animals were challenged with approximately 250 cercariae 21 days later. A 70% reduction in worm burden and a 82% decrease in egg excretion rates compared to unimmunized controls were observed.

Schistosomula could also be protective: Murrell et al (1979b) immunized cynomolgous monkeys by i.m. injection of 10^4 schistosomula derived from 50 krad gamma-irradiated cercariae of S. mansoni. Challenge worm survival was reduced by 52%. Webbe et al (1982) extended investigation of irradiated schistosome vaccines to S. haematobium. Groups of 5 baboons were vaccinated 3 times at 6-weekly intervals at a rate of 1000 20 krad gamma-irradiated organisms per kg of body weight. Animals were challenged percutaneously with 7500 cercariae 3 months after challenge. 64-89% reductions in challenge worm burden were observed when immunisation was with percutaneously applied cercariae or i.m.-injected artificially-transformed schistosomula. Cercariae tended to be more effective. 20 krad irradiated, cryo-preserved schistosomula and 3 krad irradiated larvae did not protect in this study. High levels of resistance have also been obtained in rhesus monkeys with S. japonicum cercariae irradiated with both low and high doses of X-irradiation. (Hsü et al, 1965, 1969).

Although these results do suggest that a live, attenuated vaccine of this type could well induce partial protection in man, the difficulties of obtaining, storing and administering live parasite

material, and the paramount importance of vaccine safety considerations mean that molecularly-defined vaccines would be greatly preferred. The major role of irradiated larval vaccines has been to elucidate, chiefly in rodent models, potential mechanisms and molecular targets of protective immunity.

1.9.2.3. Laboratory models of irradiated vaccine immunity.

1.9.2.3.1. Dose and type of irradiation.

S. mansoni larvae attenuated with gamma-, U.V.- and X- irradiation have all induced resistance to reinfection in mice. Gamma-irradiated vaccines have received the most attention. A wide range of doses of gamma-irradiation - 2 to 100 krad - has been effective (Dean, 1983). Levels of protection induced by larvae exposed to low doses of gamma-irradiation - 2 to 7.5 krad - vary from 91.2% to minus 33.5% in individual studies (Villella et al, 1961; Erickson and Caldwell, 1965). Most authors observed a small percentage of immunizing, irradiated worms surviving to autopsy at these low irradiation doses (Villella et al, 1961; Radke and Sadun, 1963; Erickson and Caldwell, 1965; Minard et al, 1978a, Bickle et al, 1979; Dean et al, 1981c). The survival of these breakthrough worms makes it difficult to assess whether the same non-specific mechanisms which contribute to resistance after normal infections (section 1.7) are also involved in the protection induced by cercariae at these low doses.

Perlowagora-Szumlewicz and Olivier (1963), Minard et al (1978a) and Bickle et al (1979) all found that maximum resistance was obtained with higher doses of gamma-irradiation, which prevented parasite maturation. Different research groups have achieved optimum protection with larvae attenuated at different radiation doses. Bickle et al (1979) obtained maximal resistance with parasites irradiated at

20 krad, while a second research group found that 50-56 krad was the optimal attenuating dose (Minard *et al*, 1978a; Murrell *et al*, 1979a). Several parameters differed in the separate studies, including dose rate, interval between vaccination and challenge, mouse strain and parasite strain. Winches Farm Field Station (WFFS) parasite strain was used in the work of Bickle and colleagues, as opposed to the Naval Medical Research Institute (NMRI) strain in the other two studies. James and Dobinson (1985) later compared directly the immunizing effect of the two parasite strains, WFFS and NMRI, both irradiated at 20 krad and 56 krad, under identical conditions at the same dose rate, then used to immunize the same mouse strains. The first experiment seemed to confirm previous results, in that optimal protection against challenge was generated with WFFS strain *S. mansoni* using the 20 krad dose, and with NMRI strain *S. mansoni* irradiated at 56 krad. In three subsequent experiments, however, a higher degree of protective resistance was consistently achieved with 20 krad - irradiated larvae of both the WFFS and NMRI strains. Thus, it would seem that the irradiation dose which generates optimal immunogenicity is not a fixed parameter for a given parasite strain, but may vary from experiment to experiment.

As regards radiation dose rate, both Minard *et al* (1978a) and Murrell *et al* (1979c) observed that varying the rate of gamma exposure between 0.65 and 8 krad/minute did not detectably influence the level of resistance.

Overall levels of protection induced by highly gamma-irradiated cercariae in responsive mouse strains range from approximately 30 to 90% in individual experiments (reviewed by Dean, 1983).

A number of authors have also studied X-irradiation as a means of attenuating *S. mansoni* cercariae (Perlowagora-Szumlewicz and Olivier, 1963; Perlowagora-Szumlewicz, 1964; Hsü *et al*, 1981, 1982). Perlowagora-Szumlewicz (1964) found that, as with gamma-irradiation, a

low dose (2.5 krad) allowed a proportion of the immunizing parasites to mature. However, the other studies did not report any survival of immunizing parasites at low X-irradiation doses. Levels of protection induced by cercariae X-irradiated at low doses were highly variable, ranging in different experiments from minus 106.2% (Perlowagora-Szumlewicz, 1964; 1 immunization with 300 2.5 krad X-irradiated cercariae) to plus 85.2% (Hsü et al, 1981; 5 immunisations with 500 3 krad X-irradiated cercariae). Levels of protection of 70-90% have been consistently produced by 3 to 5 immunisations with cercariae irradiated at 24 to 48 krad (Hsü et al, 1979, 1981, 1982). In the 1981 experiment, the dose-protection curve showed a broad plateau, from 20 to 100 krad. 5 immunizations with cercariae exposed to X-irradiation within this range induced 80 to 90% resistance to challenge. At 160 krad, however, the immunizing potential of the cercariae declined.

U. V.-irradiation has received the least attention as a means of attenuation. Murrell et al (1975) induced 66% resistance to cercarial challenge by immunisation with 500 cercariae exposed to U. V.-irradiation for 3 minutes at 100 ergs/sec. (= total dose of 3 μW mins). It was shown that approximately 5% (ie. 25) of these immunising cercariae survived, and could be recovered by portal perfusion at the end of the experiment. Ghandour and Majid (1978) obtained a 28.2% reduction in challenge worm survival in mice immunized with 500 cercariae subjected to U. V.-irradiation. In this study, the dose of radiation was not stated, and no attempt to assess survival of immunizing, irradiated parasites to adulthood was reported. Thus, neither of these studies exclude some contribution to resistance by egg-laying adults from the primary infection. Dean et al (1983) subsequently performed a more extensive study on immunizing mice with U. V.-irradiated cercariae of S. mansoni. At a dose of 220 μW min cm^{-2}, a few immunizing worms survived to adulthood. At doses of 330 μW min cm^{-2} or higher, attenuation was complete. In 2 successive experi-

ments, the irradiation doses inducing optimal resistance were 330 μW min cm^{-2} and 440 μW min cm^{-2}, yielding 68.8% and 52.2% protection against challenge, respectively. An attenuating dose of 880 μW min cm^{-2} rendered cercariae considerably less immunogenic - reductions in challenge worm counts were 14.9% and 26.6% in the 2 experiments.

U. V.-irradiated cercariae of S. japonicum have also been used in mouse immunisation studies. Moloney et al (1985) found that a much higher irradiation dose than for S. mansoni - 40 seconds at an intensity of 42.2+1 mW cm^{-2} = 28.1 mW min cm^{-2} - was necessary to prevent parasite maturation and induce optimal resistance. Exposure to 300-800 cercariae, irradiated at this dose, induced 31-57% resistance to cecarial challenge. Cercariae irradiated at 35.2 mW min cm^{-2} afforded significantly less resistance in mice.

1.9.2.3.2. Magnitude of immunizing inoculation.

The majority of vaccination studies have used approximately 500 larvae in a single inoculation. However, the number of immunising organisms does not seem to be crucial to induction of immunity. Minard et al (1978a) obtained 56% protection against challenge with an immunizing dose of only 69 gamma-irradiated cercariae, while Miller and Smithers (1980) observed 40% and 71% resistance with 50 and 100 gamma-irradiated cercariae, respectively. 400 irradiated cercariae induced 60-80% resistance in this experiment. Hsü et al (1981) induced 69% resistance in CF1 mice with a total of 100 48 krad X-irradiated cercariae, administered as 5 doses of 20 organisms at 4-week intervals, while 500 cercariae subjected to the same irradiation dose, again divided into 5 equal inoculations, induced only 55.5% immunity. Nor did raising the number of immunising cercariae, from 500 to 1000 or 1240, increase levels of resistance in the studies of Dean et al (1981 b, c).

Minard et al (1978a) found that increasing the number of immunisations (up to as many as 6, at 1-week intervals) did not further enhance resistance when the irradiation dose was optimal for inducing resistance with a single immunisation (56 krad in this case). However, when the irradiation dose was suboptimal (16 krad), levels of protection did increase with number of inoculations. Hsü et al (1979, 1981, 1982) observed a progressive increase in resistance, from 65.8% to > 90%, when the number of exposures to 48 krad X-irradiated cercariae was increased from 1 to 5. These workers allowed 4 weeks between successive exposures, as opposed to 1-week intervals in the study by Minard et al (1978a).

Taken together, these somewhat contradictory results suggest that low numbers of irradiated cercariae are as likely to generate substantial immunity as many. However, regardless of whether individual inoculations are with few or many organisms, a single exposure may not generate maximum immunity. Levels of protection may then be boosted by repeated immunisations.

1.9.2.3.3. Mouse strains.

Several mouse strains have been successfully immunized with gamma-irradiated cercariae of S. mansoni. From the comprehensive review by Dean (1983), outbred Swiss albinos, Bagg, Nmri, C57BL/6N, C57BL/10N, C58/J, SJL, C3H/HeJ, LAF/J, TAC, CF1, ICR, CBA/J, CBA/Ca, BALB/c, C57BL/6J, B10.D2 strains have all displayed greater than 30% resistance to challenge in at least one study.

Most reports agree that NIH/Nmri and C57BL mice consistently develop high levels of immunity in response to gamma-irradiated cercariae, while CBA mice are moderate to low responders (Murrell et al, 1979a; Lewis and Wilson, 1982; James et al, 1981). P-strain and A/J mice generally show no immunity in the gamma-irradiated vaccine

model, or else only marginal resistance (Murrell et al, 1979a; James and Sher, 1983). Sher et al (1982) showed that the MHC complex exerted some influence over vaccine immunity, in that inbred mice of b or d MHC haplotypes developed higher levels of resistance than mice with other MHC haplotypes. Correa-Oliveira et al (1986) subsequently demonstrated that defective immunity in P-strain mice is genetically controlled, being inherited in a fully recessive manner under the influence of a single locus, Rsm-1, not associated with the MHC.

However, even in supposedly responsive mouse strains, immunity may vary from below 30 to 80% in individual experiments using the same vaccination protocol. For instance, on two separate occasions within the same study (Murrell et al, 1979a), Nmri mice (usually high responders) immunized 6 weeks previously with 500 50 krad gamma-irradiated cercariae percutaneously displayed only 25% and 30% reductions in challenge worm survival. In contrast, Stek et al (1981a) obtained 65-80% resistance, using the same parasite and mouse strains in a very similar immunisation protocol. The sources of such variation in host responsiveness and/or parasite immunogenicity seem to deserve further investigation.

The responses of different mouse strains to X- or U. V.-irradiated S. mansoni vaccines have not been studied systematically. The successful immunisations with highly X-irradiated cercariae were performed in CF-1 mice (Hsü et al, 1979, 1981, 1982), while the Nmri strain was used in the most comprehensive protection study with U. V.-irradiated cercariae (Dean et al, 1983).

1.9.2.3.4. Route of immunisation with cercariae.

The majority of protection experiments with irradiated cercariae have involved percutaneous immunisation, via the abdomen, ear pinna or

tail (experiments listed by Dean, 1983). Sher and Benno (1982) obtained considerable (40-60%) protection in C57BL/6J mice with 30 krad gamma-irradiated cercariae, administered intramuscularly. However, the subcutaneous route of immunisation with irradiated cercariae seems to be ineffective; Savage and Colley (1980) were unable to induce any protection in either CF-1 or CBA/J mice exposed to gamma-irradiated cercariae by this route. Immunisation with later developmental stages of the parasite gave further information on the relative efficiencies of different immunisation routes (see below).

1.9.2.3.5. Immunizing parasite stage in the irradiated vaccine model.

Autoradiographic tracking studies with ^{75}Se-labelled, gamma-irradiated cercariae in C57BL mice have shown that, despite being retained in the skin for 24-48 hours longer than normal ones, almost all irradiated parasites go on to reach the lungs. The majority of irradiated schistosomula persist in the lungs until up to 3 weeks post-infection, and die in this location. A very small proportion dies en route from lungs to liver (Dean et al, 1981b; Mastin et al, 1983; Mangold and Dean, 1984; Mastin et al, 1985b).

Immunisation protocols which eliminate either the skin or the lung phase of migration by gamma-irradiated parasites stimulate at most half of the total potential immunity induced by attenuated cercariae which accomplish both stages of development. For instance, Mangold and Dean (1984) demonstrated that highly-irradiated cercariae (90 krad), which did not migrate out of the skin, induced approximately half the resistance to challenge afforded by parasites irradiated at the optimal 20 krad dose which allowed survival to the lung stage. In this study, however, the reduced levels of protection could be due, not only to the inhibition of migration, but also to the direct effects of such a high radiation dose on parasite antigenicity. A second approach to the

question of the relationship between development of irradiated larvae and the level of resistance stimulated has been to treat immunising parasites with schistosomulicidal drugs, or to excise the site of infection at various times after exposure to irradiated cercariae. Resistance to challenge infection is then assessed. Bickle (1982) showed that eliminating gamma-irradiated parasites by either of these methods at one week after vaccination, just as arrival in the lungs begins, allowed 26-37% protection against challenge - about half of that induced by irradiated organisms whose development and migration were not interrupted (51-71%).

Other workers have avoided the early, skin-associated stage of development and migration by immunising laboratory hosts with irradiated schistosomula at different stages after transformation. Bickle et al (1979) used 20 krad gamma-irradiated, 1 hour-old schistosomula in intramuscular, intradermal and intravenous immunisation of mice. By all 3 routes, the schistosomula induced 20-40% resistance, compared to 60-80% after percutaneous exposure to irradiated cercariae. Sher and Benno (1982) also concluded that exposure of the host to irradiated cercariae transforming in the skin was essential for optimal induction of immunity. In this study, 30 krad - irradiated cercariae, applied percutaneously, induced on average 63.0% resistance to challenge, while irradiated day 0 skin penetrants and irradiated 4-5 day lung schistosomula, injected intramuscularly, induced 42.8% and 24.8% immunity, respectively. Dean et al (1981b) were able to induce significant resistance to challenge with day 6 lung-stage schistosomula, exposed to 50 krad of gamma-irradiation, and administered by intravenous, intramuscular or intraperitoneal routes. In one experiment, irradiated cercariae by percutaneous exposure induced significantly greater resistance than the older schistosomula, but in a second experiment, both larval stages induced similar levels of immunity.

Overall, these results suggest that both skin and lung phases of development by the irradiated larvae are generally required for induction of optimal immunity.

It seems that, at both developmental stages, the attenuated parasites present antigens in such a way as to render them specially immunogenic. The mere presence of large numbers of dead schistosomula in either the skin or the lungs does not necessarily stimulate immunity. For instance, cryopreserved irradiated schistosomula administered intradermally do not induce resistance unless the cryopreservation protocol permits a high percentage viability among the immunising organisms (Lewis et al, 1984). Presumably only the living (though attenuated) schistosomula are able to stimulate a protective host immune response. As regards the immunogenicity of parasites dying in the lungs, autoradiographic tracking studies demonstrate that schistosomula treated with oxamniquine at 24 hours after infection of mice show an identical pattern of migration to optimally-irradiated cercariae - delayed exit from the skin, followed by persistence and death in the lungs. Yet these oxamniquine-treated schistosomula do not elicit protective immunity against challenge infection (Mastin et al, 1985a). Thus, it does seem that some subtle complexities of antigen presentation, quite distinct from premature parasite death in the skin or lungs, are essential to irradiated vaccine immunity.

It is noteworthy that the subcutaneous route of immunisation is unsuccessful in inducing immunity with irradiated schistosomula as well as with cercariae (section 1.9.2.3.4). Bickle et al (1979), using 1 hour old 20 krad gamma-irradiated schistosomula and Eveland and Morse (1978) using schistosomula X-irradiated at much lower doses, 3-6 krad, were both unable to stimulate resistance by this route.

1.9.2.3.6. Sites and targets of challenge attrition in the irradiated vaccine model.

Studies of challenge attrition in hosts immunized with irradiated schistosome vaccines have implicated both skin- and lung-stage challenge schistosomula as the principal targets of the protective immune response.

Earlier workers used tissue mincing and recovery techniques, or histology of tissue sections, to assess elimination of challenge worms at the different stages in their migration and development - skin, lung and liver stages. Some investigators concluded that the skin was the major site of challenge attrition, while others, using the same techniques, showed that lung-stage elimination was predominant. Miller and Smithers (1980) found that the peak recovery of challenge worms from the lungs of gamma-irradiated cercariae-vaccinated mice was severely reduced in comparison with those from control mice, and also reported a reduction in the number of schistosomula recovered from the skin during the first two days after challenge. Using the same tissue mincing and incubation methods, Hsü et al (1979) obtained similar results after challenge of mice vaccinated with X-irradiated cercariae. Ghandour and Majid (1978) also observed a significant reduction in recovery of lung schistosomula at day 6 after challenge of U.V.-irradiated cercariae-vaccinated mice. A different approach - histological examination of sections from skin, lungs and liver - also indicated to Hsü et al (1983) that the skin was the major site of challenge attrition in X-irradiated cercariae - immunized mice.

However, the tissue recovery experiments of Minard et al (1978b) and Stek et al (1981a) led these workers to conclude that the lung was the major site of vaccine-based elimination of challenge parasites. Although it was not excluded that some attrition could occur in the skin, this could account for at most half of the reduction in challenge

worm recovery. Mastin et al (1983) observed from serial sectioning techniques that virtually all challenge schistosomula reached the lungs of gamma-irradiated cercariae-immunized mice. It was concluded that the skin was not likely to be an important site of attrition in this instance.

Autoradiographic tracking experiments have provided the clearest evidence for the site of death of challenge parasites in vaccinated hosts. Again, however, individual investigators have demonstrated challenge elimination at different sites.

Tracking of radiolabelled challenge cercariae in vaccinated CBA/Ca mice (Kamiya et al, 1987) pointed to challenge elimination in the skin; 58.5% of challenge larvae failed to reach the lungs. Lung phase attrition was estimated as only 25%. The same technique produced contradictory results when vaccinated C57BL/6J mice were used as hosts. Dean et al (1984) and Wilson et al (1986) observed only 27% and 10% challenge parasite loss in the skin of this mouse strain. Although schistosomula were retarded some 2-3 days longer in the skin of vaccinated mice compared with naive hosts, it was clearly demonstrated that the majority of challenge parasites dies in the lungs.

The targets of immune attrition have also been investigated in stage- and site- elimination experiments, in which challenge parasites of different ages are injected intravenously, or intrahepatically, to bypass the skin and lungs, respectively. McLaren et al (1985) performed a comprehensive study of site-elimination in 3 animal hosts - mice, rats, and guinea-pigs. Vaccinated rats eliminated challenge both in the form of cercariae administered percutaneously and lung-stage parasites introduced directly into the lung vasculature. Vaccinated guinea-pigs were resistant, not only to challenge with skin and lung forms, but also to older worms introduced directly into the liver. In agreement with the autoradiographic tracking data, however, CBA mice immunized with gamma-irradiated cercariae were only significantly

resistant to percutaneously applied cercariae. Miller et al (1981) obtained very similar results. In this study, vaccinated mice were 43-54% resistant to cercarial challenge, but showed only 15-26% protection against 5-day lung worms. On the other hand, in the very similar system of Dean et al (1981b), vaccinated mice were 45-60% resistant to percutaneously applied cercariae, and also significantly, though slightly less, resistant (32-44%) to challenge infection by 6- or 12- day old lung worms.

Any conclusions on the mechanisms of induction and expression of irradiated vaccine immunity are complicated at present by these contradictions within the literature. Studies on the mechanisms of immunity in the irradiated vaccine model may help to clarify and reconcile these apparently conflicting results.

1.9.2.3.7. Immune mechanisms in the irradiated vaccine model.

Experiments with immunodeficient mice have demonstrated that the resistance induced by irradiated cercariae requires participation by both T- and B-lymphocytes, but not complement (Sher et al, 1982). Athymic nude mice, congenitally deficient in T-lymphocytes, and mice depleted of B-lymphocytes by treatment from birth with anti-μ antibody failed to develop significant resistance following immunisation. Immunocompetent mice of the same strains showed 47 to 67% reduction in challenge worm recovery. Resistance was partially restored in nude mice that received a thymus graft. Mice congenitally deficient in the C5 component of complement, and mice depleted of C3 during the first few days after challenge by treatment with cobra venom factor, developed levels of resistance (69 to 74%) comparable to that of counterparts with normal complement levels.

Similar studies performed in mice deficient in mast cells, IgE or IgM demonstrated that these components of the immune system are not

essential to the development of irradiated vaccine immunity (Sher *et al*, 1983; Correa-Oliveira *et al*, 1984; James *et al*, 1984).

Transfer of resistance between parabiotic mouse partners sharing a common circulation was reported by Dean *et al* (1981b). Mice immunized with irradiated cercariae 4 weeks previously were joined surgically by the skin and scapulas to normal mice, and both sets of partners challenged with cercariae percutaneously. In comparison with parabiotic controls, the adult worm counts of the unimmunized mice were reduced to at least the same extent as those of their immunized partners. However, no conclusions could be drawn about the factors responsible for transferring resistance, since exchange of both humoral and cellular blood elements could be demonstrated. Subsequent investigations by Mangold and Dean (1986) showed that the IgG fraction of serum from twice-vaccinated mice could transfer approximately half the level of protection manifested by the donors. Taken together, these observations suggest that antibodies do play some role in irradiated vaccine immunity.

Omer-ali *et al* (1986, 1988) characterised the antibodies involved in gamma-irradiated vaccine immunity in some detail. It was shown that, under conditions of antibody excess, sera from vaccinated mice bound to the surfaces of schistosomula of *S. mansoni* at levels 2- to 3- fold lower than chronic infection sera. Treatment of target schistosomula with reagents that selectively removed or modified carbohydrate epitopes had little effect on vaccine serum binding, but abolished most (approximately 90%) of the binding by antibodies in chronic serum. These carbohydrate moieties were shown to be present principally on schistosomular surface antigens of molecular weight >200 000, 38 000, 17 000 (see section 1.7). Binding studies with monoclonal antibodies indicated that these carbohydrate epitopes on the schistosomular surface were also expressed on other species of schistosomes, and were shared with the eggs. It was suggested that

these carbohydrate antigens are the targets of concomitant immunity, in accordance with the lack of species specificity of this form of protection, and its correlation with the presence of eggs in host tissues. The less abundant species-specific polypeptide epitopes, not associated with the eggs, were postulated to form the basis of vaccine immunity.

Some of the IgM anti-carbohydrate antibodies in the serum of chronically-infected mice inhibit the protective capacity of the serum as judged by antibody-dependent cellular cytotoxicity activity against schistosomula *in vitro.* Such blocking antibodies are not present in irradiated vaccine sera, and do not interfere with the expression of vaccine immunity (Yi *et al,* 1986a, b).

As regards cellular involvement in irradiated vaccine immunity, various mechanisms have been postulated by which challenge schistosomula may be damaged and destroyed. Vaccine sera have been successfully employed in *in vitro* antibody-dependent cellular cytotoxicity (ADCC) assays with eosinophils and macrophages as effector cells (Capron *et al,* 1980; Yi *et al,* 1986a, b). *In vivo,* however, typical ADCC reactions involving cellular adherence to the schistosomula, and degranulation directly onto the parasite surface as described *in vitro,* have been recorded in the epidermis of vaccinated hosts, but are very rare in the dermis, subcutaneous or pulmonary tissues (Smithers *et al,* 1987). Moreover, *in vitro* studies indicate that schistosomula are susceptible to ADCC mechanisms only within the first 24 hours (at most) of their development (McLaren, 1980). Beyond this time, the parasites develop an intrinsic resistance to ADCC, lung stage schistosomula in particular being completely refractory in such systems, and generally failing to bind antibody at all (section 1.6). In contrast, irradiated vaccine immunity clearly acts against skin-stage schistosomula older than 24 hours, also against lung and post-lung forms (section 1.9.2.3.6).

Cellular inflammatory responses, both antibody-dependent and independent, have been implicated in this later-stage attrition in both the skin and lung. It is proposed that cellular infiltration and inflammation occur in response to antigens released by migrating or dying challenge schistosomula. The parasites are trapped and immobilised in the inflammatory foci, and damaged by toxins released locally by immune effector cells, without necessitating the direct schistosomulum/cell contact typical of ADCC systems (McLaren and Smithers, 1987).

An important role for inflammatory responses in mediating parasite attrition was suggested by the study of Smith et al (1975). These workers showed that induction of an inflammatory response in the lung by immunisation with E. coli afforded partial protection against cercarial challenge. Presumably, non-specific inflammatory reactions were responsible for resistance in this model.

Inflammatory responses in the skin of challenged, vaccinated CBA/Ca mice were described by Ward and McLaren (1988). Challenge schistosomula were trapped in subdermal foci comprising chiefly eosinophils and macrophages. Since a number of the trapped parasites had apparently accomplished the morphological transition from skin- to lung-stage worms, these authors concluded that blocking of parasite migration was as important in vaccine-attrition as directly damaging the challenge worms. Further evidence for the importance of the cutaneous inflammatory response in irradiated vaccine immunity came from experiments in which a rat monoclonal antibody directed against mouse neutrophils was administered to immunized mice at the time of challenge. This treatment significantly depleted the cutaneous reaction around the challenge parasites, and reduced immunity by some 67% compared with untreated controls (McLaren et al, 1987).

Observations on the pulmonary reactions of vaccinated mice that effect lung phase challenge elimination revealed an inflammatory

response, consisting chiefly of macrophages and other monocytes, which disrupted the vascular epithelium in the vicinity of migrating schistosomula, thereby allowing the parasites to enter the alveoli (Crabtree and Wilson, 1986). Neither dead nor immune cell-damaged schistosomula were identified in this study; once again it seemed that death of challenge schistosomula was chiefly the result of blocking parasite migration.

These inflammatory responses could represent type I (IgE-type hypersensitivity), type III (immune complex hypersensitivity) or type IV (delayed-type hypersensitivity - cell-mediated; no antibody involvement) reactions (see Smithers et al, 1987). Figure 1.5 illustrates these different types of reaction.

Both type I and type III cellular reactions involve antibody. In both cases, however, local inflammatory responses to schistosomular released products could cause trapping and damage of challenge parasites without necessitating direct cellular adherence to the schistosomular target. In type I mechanisms, it is envisaged that IgE antibodies adhering to various effector cells via their Fc receptors are cross-linked on encountering antigens secreted from schistosomula, causing degranulation and release of mediators. Fc^E receptor-bearing cells which might participate in type I reactions against schistosomula include mast cells, basophils, macrophages, eosinophils and platelets.

Type III reactions would be initiated by deposition, in the skin, blood vessel walls or lungs, of antigen-antibody complexes. The antigens involved are again postulated to be material released by migrating or dying challenge schistosomula. Immune complexes trigger a variety of inflammatory responses. They can interact with complement, leading to the generation of C3a and C5a components, which have anaphylatoxic and chemotactic properties. These cause release of vasoactive amines from mast cells and basophils, increasing vascular permeability, and attracting eosinophils and neutrophils. Immune complexes can also

interact with platelets and macrophages through their Fc receptors. Release of lytic enzymes, cationic proteins and oxygen radicals will damage any schistosomula trapped locally by this inflammatory response.

Both these mechanisms have the advantage that they provide a role for antibody in irradiated vaccine immunity. As described above, transfer of vaccine serum is partially protective in mice, and if ADCC is excluded as a major effector mechanism, some other role for antibody must be postulated. Adoptive transfer of immune serum, but not lymphoid cells, has also been reported to induce both accelerated lung inflammation and resistance in mice and rats (Olveda et al, 1981; Oshman et al, 1986). This observation is compatible with involvement of antibody-based, cellular inflammatory responses (hypersensitivity types I and III) in vaccine immunity. However, the experiments in immunodeficient mice described above argue against a major role for IgE or mast cells in protection in the irradiated vaccine model.

Experimental evidence in favour of a wholly cell-mediated response is much stronger than for antibody-dependent mechanisms. Activated macrophages have been implicated as effector cells in this arm of irradiated vaccine immunity.

Macrophages activated by lymphokine exposure can kill both 3-hour schistosomula and 2-week old juvenile worms in vitro (Pearce and James, 1986). The susceptibility of these two parasite stages to macrophage effector cells may coincide with the early and late phases of resistance described in vivo in vaccinated hosts (see section 1.6). Mice immunized with radiation-attenuated larvae manifest antigen-specific cell-mediated immunity upon secondary exposure. Lymphocytes from vaccinated mice respond to in vitro culture with schistosomula by proliferating and producing the macrophage chemotactic and activating lymphokines IL-2 and IL-3 (James, 1986; James et al, 1984). In vivo, specific antigen challenge elicits delayed hypersensitivity and recruits activated macrophages to the site (James

et al, 1984). Based on these observations, it is postulated that attrition of challenge schistosomula in vaccinated hosts involves secondary antigen recognition by sensitized T-cells, which produce macrophage-activating lymphokines and cause infiltration of macrophages around challenge parasites.

This hypothesis was strengthened by in vivo studies in animal models, which confirmed a relationship between levels of protection and cell-mediated immunity. For instance, inbred strains of mice with characterized defects in macrophage activation and delayed hypersensitivity, such as P and A strains, failed to become resistant to S. mansoni infection after immunisation with irradiated cercariae (James et al, 1984). A correlation was also observed between cell-mediated immunity and resistance in the cryopreserved irradiated vaccine model. Schistosomula with the least freezing-associated damage were most capable of inducing protective immunity in the form of cell-mediated immunity, whereas animals immunised with parasites that were wholly non- viable after freezing showed minimal cell-mediated immunity and no resistance (Lewis et al, 1984).

In vitro studies indicated that activated macrophages need attach only transiently to target schistosomula, and that the toxins they release damage the muscle layers, rather than the surface, of the parasites (McLaren and James, 1985). McLaren and Smithers (1987) suggested that, in this way, macrophages effectively paralyse the parasites, preventing their migration and development.

Whatever the mechanisms behind cellular effector mechanisms in irradiated vaccine immunity, the inflammatory process is likely to be cumulative, as schistosomula trapped and damaged in the cellular foci release antigens which attract more inflammatory cells. Smithers et al (1987) suggested that, in the concomitant immunity model, the presence of IgM anti-carbohydrate ("blocking") antibodies in infection serum might prevent or modulate such inflammation, as already described

for antibody-mediated suppression of granuloma around eggs injected intravenously in mice (Pelley and Warren, 1978). Such modulation would not occur in vaccinated animals which lack "blocking" antibodies. Hence, the immune attrition associated with cellular inflammation would be highly effective.

Table 1.4 summarises the various mechanisms which have been postulated to operate in the concomitant and irradiated vaccine models of immunity to schistosomiasis mansoni.

The consistent success of irradiated schistosome vaccines in inducing protective immunity in a wide range of animal models urges investigation of the molecular basis for the immunogenicity of irradiated cercariae. This project therefore examines the effects of irradiation on metabolism and antigen presentation by developing schistosomula. The results are interpreted in the light of biochemical effects of irradiation established in other systems.

1.10 Biochemical effects of irradiation.

1.10.1 Gamma and U.V. radiation.

This project is concerned mainly with the mechanism by which gamma- and U.V.-irradiation (peak wavelength 254 nm) attenuate *S. mansoni* larvae and render them immunogenic for vaccine purposes. Since the energy per photon is considerably higher for gamma than for U.V. radiation, gamma-irradiation can cause ionization of molecular species, whereas 254 nm U.V. rays are generally considered to cause excitation rather than ionization of molecular targets. In solution, however, the effects of the two types of radiation are in practice very similar. Firstly, since ionization potentials can be lowered by up to 3eV in solution, ionization can occur on exposure to U.V. radiation (Bernas *et al,* 1980). Secondly, whether ionization takes place or

Table 1.4. Components of concomitant and irradiated vaccine immunity to challenge with cercariae of *S. mansoni*.

Table 1.4

Component of resistance.	Involvement in rodent models of: Concomitant Immunity	Irradiated Vaccine Immunity
1. Contribution of egg-associated pathology to resistance.	Yes. Resistance is clearly correlated with the presence of eggs in the tissues, or with other factors related to the presence of eggs, such as portal hypertension. The resulting vascular changes block migration of schistosomula, and cause parasite death (1,2). Non-specific component of protection, associated with egg-induced pathology, accounts for resistance to heterologous species in concomitant immunity (3)	No. Irradiated parasites die before reaching the mature, egg-laying stage. Irradiated vaccine immunity is species-specific (4,5)
2. Humoral immunity: 2a) B-cells	No: depletion of B-lymphocytes with anti-μ serum did not abrogate resistance (6,7)	Yes: B-lymphocytes required for immunity (8).
2b) Protective antibody.	? unclear (a) Yes (b) No (a) Yes: successful attempts to transfer resistance by (continued on next page)	Yes: IgG fraction of serum from twice-vaccinated mice transfers approximately half (continued on next page)

Table 1.4. continued

Component of resistance.	Involvement in rodent models of: Concomitant Immunity	Irradiated Vaccine Immunity
2 b) Protective antibody (continued)	i.v. injection of chronic infection serum (9,10,11,12, 13).	the level of protection of the donors (20)
	IgG_1 fraction from infection serum transfers resistance (14)	Serum from 3x vaccinated mice transfers immunity (21).
	(b) No: unsuccessful serum transfer experiments (15,16, 17).	Parabiotic transfer successful (19).
	: parabiosis experiments (transfer of serum and cells) unsuccessful (18,19)	
2 c) Blocking antibody.	Yes: blocking activity is associated with IgM anti-carbohydrate antibodies, which react with both schistosomular surface antigens and egg polysaccharide antigens. Protective activity associated chiefly with IgG anti-polypeptide antibodies (22,23,24). (continued on next page)	No: no blocking antibodies in vaccine serum (22,23,24). (continued on next page)

Table 1.4., continued.

Component of resistance.	Involvement in rodent models of: Concomitant Immunity	Irradiated Vaccine Immunity
2 c) Blocking antibody (continued)	Protective antibodies are postulated to be involved in both direct ADCC and mediating cellular inflammatory responses against migrating schistosomula. Blocking antibodies are believed to modulate or inhibit both ADCC and cellular inflammatory responses.	
3. Complement	?unclear a) Yes b) No (a) Yes: resistance abrogated in mice deficient in C5 (25) or depleted of C3 by treatment with CVF (26). (b) No: CVF had no effect on resistance (16)	No: mice congenitally different in C5 or depleted of C3 by CVF showed expected levels of resistance (8).
4. T-cells.	Yes: T-lymphocyte depletion abrogates resistance (16)	Yes: athymic nude mice do not develop resistance (8).

NOTE: CVF = Cobra Venom Factor.

Table 1.4., continued.

5. Effector cells: It is postulated that the same effector cell mechanisms contribute to vaccine immunity and the specific component of concomitant immunity. (Smithers et al, 1987; McLaren and Smithers, 1987; James et al, 1987).

Cellular response.	Mechanism	Postulated effector cells.	Evidence for this mechanism	
			In vitro	In vivo
5.1. Antibody-dependent or complement-dependent cellular cytotoxicity (ADCC)	Cells adhere to schistosomular target, via Fc or complement receptors, and release toxic mediators onto the parasite surface.	Eosinophils	Yes: most effective cell in in vitro ADCC (27,28,29,30).	From histology: ADCC reactions occur around challenge schistosomula in the epidermis. But ADCC is rarely detected against >24h.-old schistosomula in the dermis, subcutaneous or pulmonary tissues of infected or vaccinate animals (37).
		Neutrophils	Yes: (31,32)	
		Mast cells	Yes: Adhere to parasite surface, and enhance eosinophil-mediated damage (33, 34,35).	
		Macrophages	Yes: rat system (36)	
		Effective against schistosomula less than 24 hours old in vitro (35)		
5.2. Inflammatory reactions	Cellular inflammation in response to released schistosomular antigens impedes parasite migration. Local release of toxic mediators causes parasite damage and death. Parasite/cell contact not essential.			

Table 1.4., continued.

5.2. Inflammatory reactions comprise:	Mechanism	Postulated effector cells.	Evidence for this mechanism	
			In vitro	In vivo
5.2.1 Type I and Type III hypersensitivity	Antibody involvement (IgE-mediated=type I. Immune complex-mediated=type III).	Eosinophils Neutrophils Mast cells Macrophages Platelets.	/	(1) Histology of skin and lungs of vaccinated, or infected, and challenged hosts (30,37,38). (2) Depletion of cutaneous inflammation reduced both concomitant and vaccine resistance (39). (3) Transfer of infection serum to rodents induced cellular inflammation in the lung, and resistance to challenge (40,41).
5.2.2 Type IV hypersensitivity.	Wholly cell-mediated; no antibody involvement ("Delayed-type hypersensitivity")	Macrophages	Lymphokine-activated macrophages, or macrophages from infected or vaccinated mice kill newly-transformed and 2-week schistosomula in vitro (42,43,44).	P and A mouse strains with defects in macrophage activation and cell-mediated immunity fail to become resistant to challenge after normal infection or vaccination (45,46,47).

Table 1.4., continued.

5.3. Other studies on effector cell involvement in resistance. Participation in ADCC or inflammatory reactions not defined	Concomitant Immunity model.	Irradiated Vaccine model
	<u>Eosinophils:</u> Depletion of eosinophils with rabbit antiserum abrogated resistance to challenge (12) <u>Mast cells:</u> Mast cell disrupting agent 48/80 abolished protection against challenge in chronically infected mice (48)	Mice deficient in IgE or mast cells still developed resistance in response to the irradiated vaccine (49)

References.

1. Wilson *et al* (1983)

2. Dean (1983)

3. Smithers and Doenhoff (1982)

4. Bickle *et al* (1985)

5. Moloney and Webbe (1987)

6. Maddison *et al* (1980)

7. Maddison *et al* (1981)

8. Sher *et al* (1982)

9. Sher *et al* (1975)

10. Hillyer *et al* (1975)

11. Rombert and Trinca (1979)

12. Mahmoud *et al* (1975)

13. Smith *et al* (1982)

14. Sher *et al* (1977)

15. Maddison and Kagan (1978)

16. Doenhoff and Long (1979)

17. Harrison *et al* (1982)

18. Hunter *et al* (1967)

19. Dean *et al* (1981b)

20. Mangold and Dean (1986)

21. Reported by Smithers *et al* (1987)

22. Omer-ali *et al* (1986)

23. Omer-ali *et al* (1988)

24. Yi *et al* (1986b)

25. Work described by Dean (1983). Reference reported as Sher *et al* (1975a) in Dean (1983).

26. Tavares *et al* (1978a)

27. Butterworth *et al* (1975)

28. McLaren and Ramalho-Pinto (1979)

29. McLaren (1982)

30. McLaren and Smithers (1987)

31. Anwar *et al* (1979)

32. Incani and McLaren (1984)

33. Sher (1976)

34. Capron *et al* (1978)

35. McLaren (1980)

36. Capron *et al* (1975)

37. Ward and McLaren (1987)

38. Crabtree and Wilson (1986)

39. McLaren *et al* (1987)

40. Oshman *et al* (1986)

41. Olveda *et al* (1981)

42. Pearce and James (1986)

43. James *et al* (1982)

44. Bout *et al* (1981)

45. James and Cheever (1985)

46. James (1986)

47. James *et al* (1984)

48. Dean *et al* (1976)

49. Sher *et al* (1983)

not, U.V.- and gamma-irradiation both also cause excitation of water molecules to produce the same primary radical species. Thus, much of the radiation damage finally suffered by macromolecular species occurs by similar routes for both radiation sources. The biochemical effects of U.V. and gamma radiations will therefore be considered together.

Traditionally, radiation damage is ascribed to both "direct" and "indirect" effects of excited or ionized species. Direct effects describe the immediate interaction of radiation energy with molecular targets. U.V. radiation is absorbed much more specifically than gamma, according to the radiation absorption spectrum of various cellular constituents. The peak wavelength of the U.V. radiation employed in the experiments presented here, 254 nm, is very close to the absorption maximum of purines and pyrimidines, hence much of the U.V. radiation damage will be concentrated in DNA and RNA.

Indirect effects result from the interaction of original reactive species, formed by radiation, with new molecular targets. Since the environment of a living cell contains a wide variety of molecular species - organic compounds, inorganic ions and water - a wide variety of potential sources of reactive species can exist as excited molecules, ion radicals or free radicals, which are ultimately converted into chemically stable products by subsequent decay reactions. This multiplicity of reaction mechanisms and potential reactants creates the possibility for a broad spectrum of radiation-damaged products within the cell. However, the predominance of water in biological systems means that species formed by radiolysis of water are the major potential sources of damage. These species have been well-characterised, and are described below.

1.10.2 Radiation chemistry of aqueous solutions.

1.10.2.1. The radiolysis of water.

Three major free radicals are formed in the radiolysis of water - the OH radical, the solvated electron (e^-_{aq}) and the H atom.

As mentioned above, the two major processes in the interaction of radiation with water are ionization and electronic excitation. Equations (1) and (2) describe these reactions:

$$H_2O \xrightarrow{h\nu} H_2O^+ + e^- \qquad \text{ionization} \qquad (1)$$

$$H_2O \xrightarrow{h\nu} H_2O^* \qquad \text{excitation} \qquad (2)$$

The water radical cation in (1) is a strong acid, which rapidly loses a proton to the surrounding water molecules:

$$H_2O^+ + H_2O \longrightarrow H_3O^+ + \cdot OH \qquad (3)$$

The electron from (1) is rapidly solvated:

$$e^- + nH_2O \longrightarrow e^-_{aq} \qquad (4)$$

The excited water molecules formed in the second primary process (reaction (2)) can break up into H-atoms and OH radicals:

$$H_2O^* \longrightarrow H\cdot + \cdot OH \qquad (5)$$

These radical species, from reactions (3) to (5), may react with one another, or diffuse into the bulk of the solution. Hence, by the reaction of solvated electrons with protons, further H-atoms are produced:

$$e^-_{aq} + H^+ \longrightarrow H\cdot \qquad (6)$$

Reactions (7) to (9) yield the molecular products H_2 and H_2O_2:

$$H\cdot + H\cdot \longrightarrow H_2 \qquad (7)$$

$$e^-_{aq} + e^-_{aq} \longrightarrow H_2 + 2OH^- \qquad (8)$$

$$\cdot OH + \cdot OH \longrightarrow H_2O_2 \qquad (9)$$

A considerable part of the radicals is converted back to water:

$$H\cdot + \cdot OH \longrightarrow H_2O \qquad (10)$$

Solvated electrons, hydroxy radicals and protons also eventually recombine to neutralise one another (reactions 11 and 12):

$$e^-_{aq} + \cdot OH \longrightarrow OH^- \qquad (11)$$

$$H^+ + OH^- \longrightarrow H_2O \qquad (12)$$

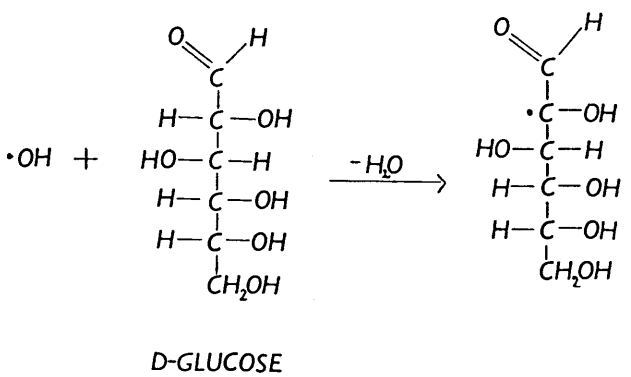
(A fuller account of these reactions is provided by von Sonntag, 1987).

1.10.2.2. Reactions of water radicals with substrates.

a) Hydroxy radicals.

The OH radical is a strongly oxidising species and converts metal ions to higher oxidation states (reviewed by Buxton and Sellers, 1977).

OH radicals also often abstract carbon-bound hydrogen atoms, more or less non-selectively, eg. from D-glucose:



(13)

Addition to double bonds by OH radicals also occurs at a very rapid rate in solution (von Sonntag, 1987).

$$\cdot OH + CH_2 = CH_2 \longrightarrow HO - CH_2 - CH_2\cdot \qquad (14)$$

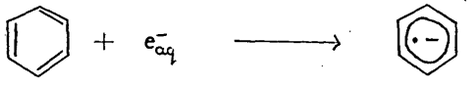
b) Hydrogen atoms and solvated electrons.

The H-atom and the solvated electron are both reducing species. The solvated electron is the strongest reducing agent known, but the reducing power of the H-atom is considerably lower. Thus, many metal ions are reduced to lower oxidation states by these species (reviewed by Buxton and Sellers, 1977).

Similarly to OH radicals, H-atoms readily add to C-C and C-N double bonds:

$$H\cdot + CH_2 = CH_2 \longrightarrow CH_3 - CH_2\cdot \qquad (15)$$

Conjugated olefins and aromatic compounds react with solvated electrons. The resulting radical anions are often very unstable, and react rapidly with water (Gordon et al, 1977):



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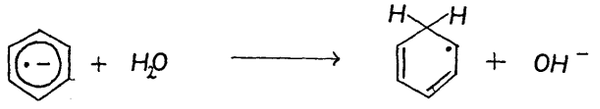
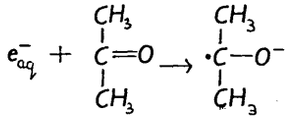


(17)

Another typical reaction of the solvated electron is addition to C=O double bonds:



(18)

1.10.2.3. Radiation chemistry of oxygenated solutions: peroxyl radicals.

Because of the near omnipresence of oxygen under natural

conditions, peroxyl radicals play an important role whenever free radicals are generated in biological systems.

From the primary radicals generated by radiolysis of water (see above), the H-atom and hydrated electron e_{aq} react readily with O_2. The OH radical alone does not react with O_2, but the secondary radicals produced by its reactions - abstraction of carbon-bound H-atoms, or addition to C=C and C=N double bonds of organic compounds - will react with O_2 to give corresponding peroxy radical species. Studies on the reactions of peroxyl radicals in solution are reviewed by Hunter and Simic (1983).

a) <u>The superoxide radical $HO_2\cdot/O_2^{\cdot-}$</u>

The superoxide radical ion $O_2^{\cdot-}$ and its conjugated acid, the hydroperoxyl radical $HO_2\cdot$, play a major role in many radiolytic systems containing oxygen, and are also generated in normal metabolic processes.

Oxygen readily scavenges the solvated electrons and H-atoms generated by radiolysis of water:

$$e^-_{aq} + O_2 \longrightarrow O_2^{\cdot-} \qquad (19)$$

$$H\cdot + O_2 \longrightarrow HO_2\cdot \qquad (20)$$

The radicals $HO_2^{\cdot}$ and $O_2^{\cdot-}$ are in equilibrium:

$$HO_2^{\cdot} \rightleftharpoons H^+ + O_2^{\cdot-} \qquad (21)$$

The bimolecular decay of $HO_2^{\cdot}/O_2^{\cdot-}$ produces hydrogen peroxide and oxygen:

$$HO_2\cdot + HO_2\cdot \longrightarrow H_2O_2 + O_2 \qquad (22)$$

b) <u>Reactions of $HO_2\cdot$ and $O_2^{\cdot-}$ with substrates.</u>

The $O_2^{\cdot-}$ radical can undergo a large number of redox reactions with molecular species, including transition metal ions (and enzymes

such as superoxide dismutases which contain transition metal ions):

$$Cu^{2+} + O_2^{\cdot-} \longrightarrow Cu^{+} + O_2 \qquad (23)$$

$$Cu^{+} + O_2^{\cdot-} + 2H^{+} \longrightarrow Cu^{2+} + H_2O_2 \qquad (24)$$

It is proposed that superoxide dismutases help protect the cell against radiation damage by removing superoxide radicals in this way (see chapter 8).

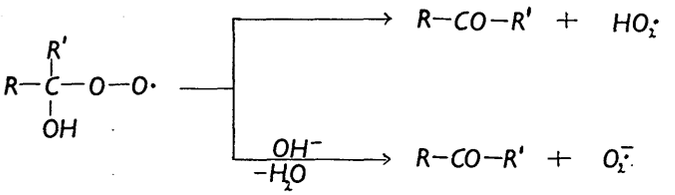
$O_2^{\cdot-}$ also participates in redox reactions with various amino acids, and compounds containing aromatic rings, e.g. quinones, adrenaline.

The $HO_2\cdot$ radical does not undergo electron transfer reactions as readily as $O_2^{\cdot-}$, but has been shown to abstract hydrogen from a number of substrates, including several amino acids and fatty acids.

c) Organic peroxyradicals.

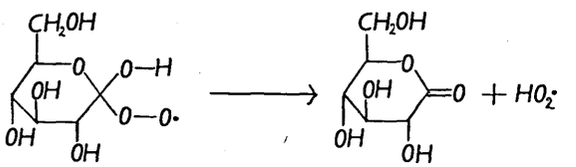
Carbon-centred radicals, formed by hydrogen abstraction or by radical addition to double bonds, react readily with oxygen.

Some peroxyl radicals decay by releasing $HO_2\cdot/O_2^{\cdot-}$:



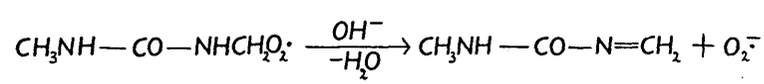
(25)

For instance, the peroxyl radicals derived from C(1) of D-glucose have half-lives of less than a few microseconds:



(26)

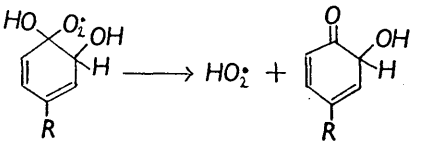
The $HO_2\cdot/O_2^{\cdot-}$ elimination is not restricted to compounds with an OH group in the α-position; NH groups also mediate this process:



(27)

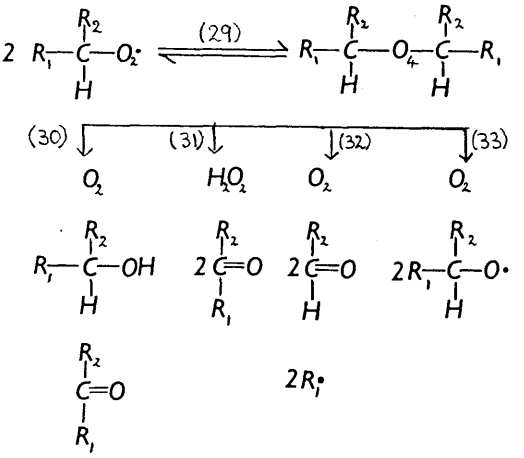
This reaction is important in radiolysis of oxygenated solutions of amino acids and pyrimidines (Abramovitch and Rabani, 1976).

$HO_2\cdot$ - elimination is also observed in some aromatic compounds:



(28)

Organic peroxyl radicals decay bimolecularly by a number of routes:



In general, peroxyl radical systems contain more than one type of peroxyl radical. Often, primary, secondary and tertiary radicals are formed simultaneously. Tertiary peroxyl radicals react very slowly with each other, but much more rapidly with primary and secondary peroxyl radicals. Thus, under steady-state conditions, tertiary peroxyl radicals generally disappear by reacting with the primary and secondary peroxyl radicals present in the system, so that the distribution of products may be biased against those of bitertiary termination (Schuchmann and von Sonntag, 1987).

Peroxyl radicals are known to undergo chain reactions. The best-known chain reaction, shown below, produces a hydroperoxide:

Initiation.

$$\cdot OH + RH \longrightarrow H_2O + R\cdot \quad (34)$$

Propagation.

$$R\cdot + O_2 \longrightarrow RO_2\cdot \quad (35)$$

$$RO_2\cdot + RH \longrightarrow RO_2H + R\cdot \quad (36)$$

Termination.

$$2RO_2\cdot \longrightarrow \text{products} \quad (37)$$

1.10.2.4. Protection against radiation damage.

In considering the biological effects of radiation, it must be borne in mind that a number of mechanisms exist for protecting cells from the damaging effects of free radicals. Thiol containing compounds - eg. cysteine, cysteamine, glutathione, and lipid-soluble anti-oxidants - vitamin C and vitamin E - act as radioprotective agents. Various enzymes also help remove the damaging radicals, e.g. catalase, superoxide dismutases, cytochrome-c oxidase, ascorbate oxidase. These mechanisms of protection against radiation damage are discussed in more detail in chapter 8.

Radiation protection can also occur at a later stage in the form of enzymatic repair of the final radiolesions fixed in target macromolecules. This type of enzymatic repair has been studied most intensely for DNA damage, and is described briefly in the following section. No DNA repair enzymes have as yet been described in schistosomes.

1.10.3 Biological targets of radiation.

1.10.3.1. The main target is DNA

When a cell is subjected to radiation, many chemical reactions are

induced, eventually leading to lesions which express themselves in a number of biologically significant changes. A variety of cellular targets may suffer radiation injury. However, it is generally accepted that levels of damage to DNA correlate directly with reproductive cell death (Gentner and Paterson, 1984). Membrane damage appears to be the second most important target for this type of biological end-point (von Sonntag, 1987).

Radiation-induced damage of DNA may result from either direct or indirect effects (see section 1.10.1). However, no qualitative differences can be distinguished in the biological damage resulting from the two routes.

a) Spectrum of radiolesions in DNA

The various radiation-induced lesions in DNA have been arranged into a hierarchy, according to decreasing frequency of occurrence, as follows (von Sonntag, 1987):

1. Base damage.
2. Single-strand break.
3. Alkali-labile site (i.e., sugar (or base) damage turning into a strand break on treatment with alkali).
4. Double-strand break.
5. Single-strand break with base damage on opposite strand.
6. Base damage on two opposite strands.
7. Alkali-labile site and another damage, eg. a strand break on the other strand.

Diagrams of some of these lesions are shown in figure 1.6.

Any of these lesions can, in principle, be lethal, but in practice, inactivation of particular cell functions and cell death are most closely related to unrepaired double-strand breaks (Roberts and Holt,

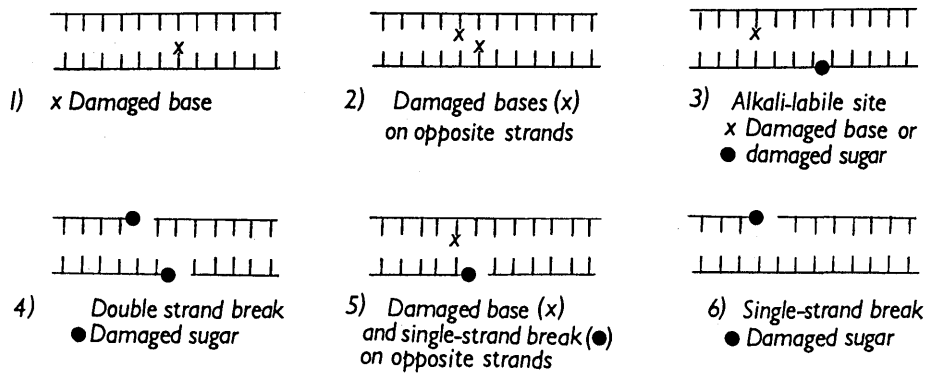


Figure 1.6. Some typical radiolesions in DNA.

1985). However, double-strand breaks need not be induced directly by radiation alone. A major source of damage appears to be enzymatically-labile sites induced by radiation damage, which are rapidly converted into single-strand breaks by endonucleases. The section excised and replaced by these enzymes may be substantial - as much as 1000-2000 nucleotides. If the radiation-damaged DNA happens to have an altered base on the opposite strand within this section, this altered base, upon restitution of the excised section, will cause a point mutation. If a single-strand break happens to be on the opposite site, it will be converted into a double-strand break. Thus, during the repair processes, many more single- and double-strand breaks are produced than were originally induced by the radiation (Bresler et al, 1984).

b) Repair of DNA and additional formation of strand breaks.

Some additional features of DNA damage and repair may be mentioned briefly. The rate at which a given damage is repaired is species-dependent. Single-strand breaks are generally quite rapidly repaired. In lymphocytes, for example, 70% of such strand breaks are repaired with a half-life of about 3 minutes, the remainder being repaired an order of magnitude more slowly (McWilliams et al, 1983). Weibezahn and Coquerelle (1981) measured the rejoining of double-strand breaks, and observed that in V79 and E. coli cells, half the breaks were rejoined within 8 minutes at 37°C, but some of the breaks rejoined more slowly. The fast process required DNA ligase, the slow one the rec^+ enzymatic repair system. Sapora et al (1977) showed that the enzymatic incision which produces strand breaks at enzyme-sensitive sites is considerably faster than the subsequent repair, at least at 25-37°C. In this temperature range, maximum strand break production was reached within 20 seconds (25°C), or even within 5 seconds (37°C).

c) Faulty repair

There are practically no radiation-induced strand breaks where only the phosphate linkage is enzymatically cleaved. In those lesions where it has been possible to identify the chemical nature of the damaged sugar, the base was lost as well (reviewed by von Sonntag, 1987). This implies that, in the strand-breaking process, the information which is supplied by the base is also lost. Among the lesions that have been recognized, there are cases where a whole altered nucleoside has been eliminated.

Base damage on opposite sites may or may not be lethal, depending on what the damaged bases code for. Repair enzymes, in coping with such damage, may convert it into a double-strand break. A similar situation may apply in the case of alkali-labile sites. In those cases where alkali-labile sites have been identified by analysis of the altered sugars, the structure of these sugars indicated that the base had also been lost, or was at least very labile.

Lesions that involve both strands at opposite sites are most likely to be lethal in haploid cells. In diploid cells, the lost information is still available in the sister chromosome. Hence, double-strand breaks and related lesions can be repaired by recombination (Resnick, 1976). The larger the number of copies that exist of the genome, the higher will be the radiation dose which can be tolerated. Micrococcus radiodurans contains several copies of its genome, which allows more efficient recombination repair. Some misrepair appears to occur in competition with recombination repair (Tobias, 1985).

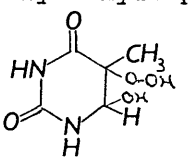
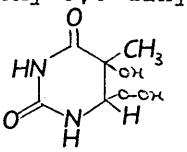
Repair processes are reviewed in detail in the book by Friedberg (1985).

1.10.3.2. Chemistry of radiation damage to DNA.

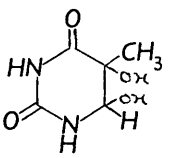
I. General

a) Radiation products of free purine and pyrimidine bases.

Thymine. The major radiolysis products of thymine in aerated aqueous solution are trans -5-hydroperoxy-6-hydroxy-5,6-dihydrothymine (I), and cis -5-hydroxy-6-hydroperoxy-5,6-dihydrothymine (II):—

(I.)   (II.)

These may in part react to form 5,6-dihydroxy-5,6-dihydrothymine on standing, or after exposure to high radiation doses (Hariharan and Cerutti, 1972):

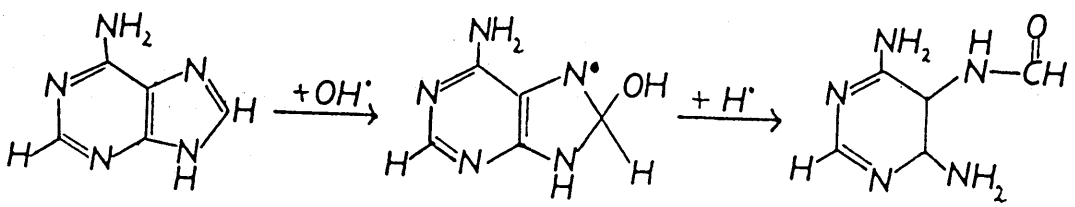


Other thymine radiation products detected in irradiated DNA include formylpyruvylurea and hydroxybarbituric acid. Formylpyruvylurea can undergo further degradation to yield urea and N-substituted urea derivatives that largely remain stably attached to deoxyribose residues in DNA (Hariharan and Cerutti, 1972).

Cytosine and the Purines.

There have been fewer studies on the effects of ionizing radiation damage to cytosine and to the purines, although it has been established with respect to cytosine that the major site for attack by hydroxyl radicals is the 5,6 double bond, as is the case with thymine (Holian and Garrison, 1966). Although hydroxyl radicals react with purines as rapidly as with pyrimidines, the rate of destruction of the former is lower (Scholer et al, 1960). Destruction of the imidazole ring of

purines to yield 5-formamidopyrimidine compounds has been observed, and the following scheme has been proposed to explain these reactions (Friedberg, 1985):



ADENINE 4,6-DIAMINO-5-FORMAMIDOPYRIMIDINE

b) Base damage in DNA caused by radiation.

Measurements of damage in DNA (as opposed to free bases or nucleosides) indicate that all four bases are subject to radiation damage (Ward, 1975). However, the macromolecular organization of DNA apparently affects the quantitative yields of base damage. For example, the total yield of base destruction depends markedly on DNA concentration, being greater at higher DNA concentrations. This may result from the relatively low rate constant for reaction of hydroxyl radicals with DNA, so that at low DNA concentrations, scavenging of hydroxyl radicals can occur, due to competing reactions.

c) Chemistry of radiation-induced strand breaks in DNA.

The majority of strand breaks are caused by breakage of phosphodiester linkages in one of the polynucleotide chains, but

destruction of the deoxyribose ring can also result in interruptions of the deoxyribose-phosphate backbone (Ward, 1975). Studies on the mechanism of production of strand breaks have relied largely on the use of deoxynucleotides and alkylphosphates as model substrates in which the equivalent of a strand break-producing reaction liberates inorganic phosphate, which can be readily quantitated (Ward, 1975).

Studies with nucleotides suggest that deoxyribose-phosphate bond breakage occurs via a β-elimination reaction. However, the mechanism by which the alkali-labile deoxyribose moiety is derived is dependent on the presence or absence of oxygen (Ward, 1975). Thus, the ratio of the yield of immediate phosphate release to labile phosphate esters in thymidylate is lower in the absence of oxygen than in its presence.

Like base damage, DNA strand breaks can result from direct or indirect effects of ionizing radiation. In the former case, the deposition of radiation energy is sufficient to break the deoxyribose-phosphate backbone (Youngs and Smith, 1976). As regards indirect effects, hydroxyl radicals appear to be of primary importance in the hydrolysis of phosphodiester bonds (Bonura and Smith, 1976). The precise chemical mechanisms of DNA strand breakage induced by ionizing radiation are varied and complex. They may involve direct ionization of the phosphodiester bond, or fragmentation reactions involving the bases or sugars, coupled with electron rearrangements that ultimately result in hydrolysis of labilized phosphodiester bonds.

II. DNA damage specific to 254 nm U.V. radiation.

a) Pyrimidine Dimers.

When DNA is exposed to U.V. radiation at wavelengths approaching its absorption maximum (about 254 nm), adjacent pyrimidines become covalently linked by the formation of a four-membered ring structure

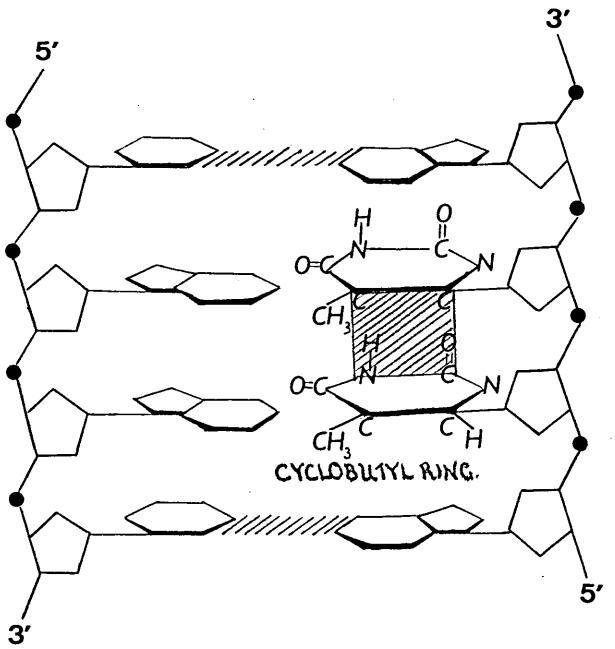


Figure 1.7. The cyclobutyl pyrimidine dimer is formed in DNA by the covalent interaction of two adjacent pyrimidines in the same polynucleotide chain. Saturation of their respective 5,6 double bonds results in formation of a 4-membered cyclobutyl ring (red area), linking the two pyrimidines.

resulting from the saturation of their respective 5,6 double bonds. The structure formed by this photochemical cycloaddition is described as a cyclobutane-type dipyrimidine, or pyrimidine dimer (figure 1.7).

The formation of pyrimidine dimers during U.V.-irradiation of DNA is a reversible process that can be represented as:

$$Py + Py \underset{}{\overset{U.V.}{\rightleftharpoons}} Py\langle\rangle Py.$$

Since, under normal conditions, the equilibrium is shifted far to the right, dimer formation is favoured over dimer reversal. However, if E. coli DNA radiolabelled in thymine is continuously irradiated at 254 nm, the thymine-containing pyrimidine dimer content (thymine-thymine plus thymine-cytosine dimers) of the DNA does not increase beyond about 7% of the total thymine content (Radany et al, 1981). This steady state reflects a dynamic equilibrium in which the rates of dimer formation and reversal are equal.

The radiation dose at which maximum dimer formation is reached varies for different types of dimers. The level of C<>C plateaus at doses of approximately 500 J/m², whereas for T<>T, doses of greater than 2 000 J/m² are required. This result is presumably related to the fact that the quantum yields for product formation differ for different pyrimidine dimers (Setlow, 1968). The absolute level of dimerization also varies at individual dimer sites. For example, different sites of potential T<>T attain different steady-state levels that vary between 4 and 16% (Gordon and Haseltine, 1982). The steady-state level of dimer formation is also influenced by the nature of the nucleotides flanking potential dimer sites. In general, the equilibrium level of dimers is greater for TT sites flanked on both sides by A than for TT sites flanked on the 5' side by A and on the 3' side by G (Gordon and Haseltine, 1982).

b) Non-cyclobutane-type Pyrimidine Adducts.

In irradiated solutions of free bases and of nucleosides, formation of non-cyclobutane-type pyrimidine lesions referred to as pyrimidine adducts can be detected (Kittler and Löber, 1977). Figure 1.8 shows the structure of 5-thyminyl-5,6-dihydrothymine, a major photoproduct produced in the U.V.-irradiated spores of B. subtilis (Varghese, 1970). As much as 30% of the thymine in spore DNA can be converted into this product following exposure to very high doses of U.V. radiation.

c) The pyrimidine-pyrimidine (6-4) lesion.

Alkali-labile lesions at positions of cytosine (and, much less frequently, thymine), 3' to pyrimidine nucleosides are also present in U.V.-irradiated DNA (Lippke et al, 1981). These photoproducts are referred to as pyrimidine-pyrimidine (6-4) lesions (figure 1.9) The (6-4) adducts of TC, CC and TT sequences are observed in U.V.-irradiated DNA, but CT is not.

As is true for pyrimidine dimers, the number of (6-4) lesions formed is proportional to the incident U.V. dose in the dose range of 100 to 500 J/m^2 (Lippke et al, 1981). The incidence of (6-4) TC lesions in the E. coli lacI gene was greater than that of CC lesions, while TT lesions were only detected at very high U.V. doses (greater than 5kJ/m^2; Brash and Haseltine, 1982).

A good correlation exists between the frequency of nonsense mutations and that of U.V.-induced damage at TC and CC sequences. This correlation is significantly better for (6-4) photoproducts than for pyrimidine dimers (Brash and Haseltine, 1982). Thus, the (6-4) lesions may be biologically important photoproducts in U.V-irradiated cells.

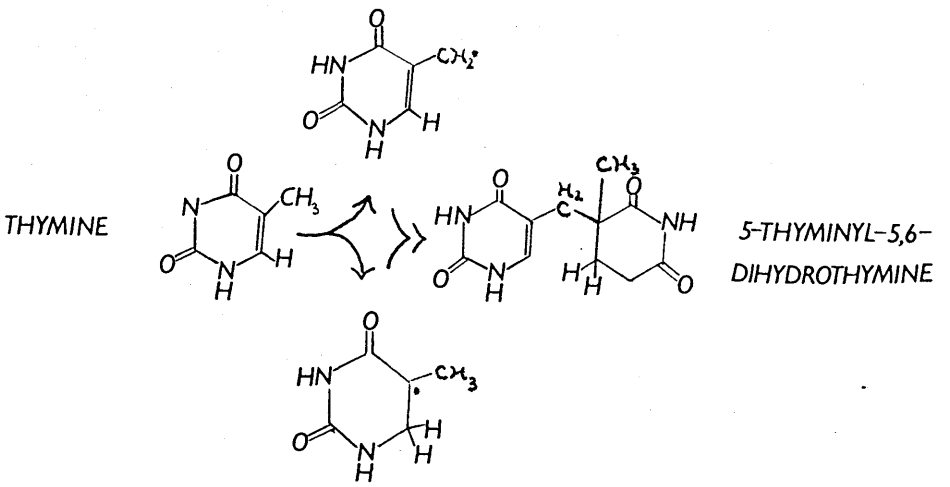


Figure 1.8. The formation of 5-thyminyl-5,6-dihydrothymine by addition of two different radicals of thymine generated by U.V. radiation.

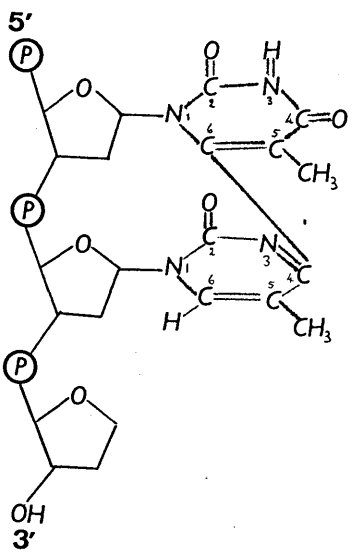
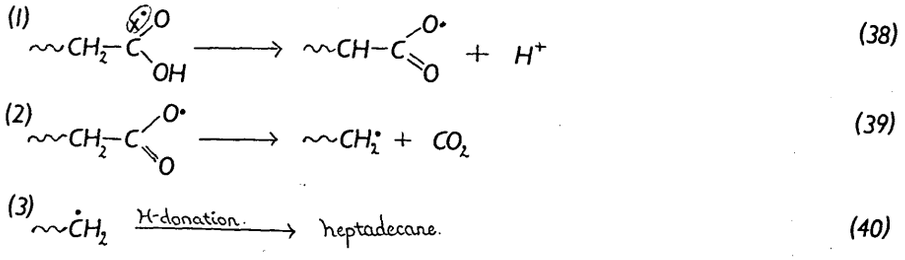


Figure 1.9. Photoproduct produced by linkage between C6 position of one thymine and the C4 position of the adjacent thymine.

1.10.3.3. Lipids and membranes

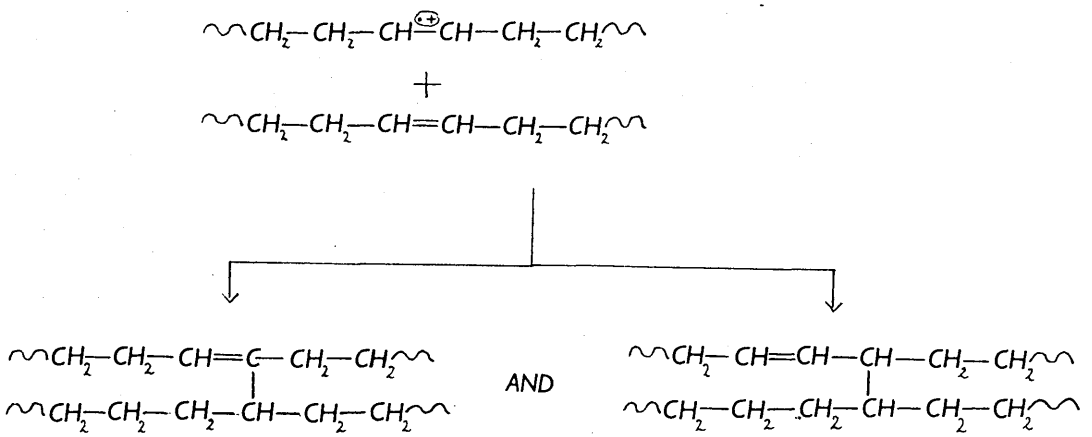
a) Fatty acids and their esters.

In the radiolysis of solid stearic acid, the major process seems to be decarboxylation of the fatty acid (Wu and Howton, 1975):



The radical cation (1) is the protonated form of an alkylcarboxyl radical (2). These radicals readily decarboxylate (reaction 39). The alkyl radical (3) formed in reaction (39) must then be reduced to heptadecane, the major product.

In oleic acid, which is unsaturated, the decarboxylation process is no longer predominant. Instead, dimeric material is the major product (von Sonntag, 1987):



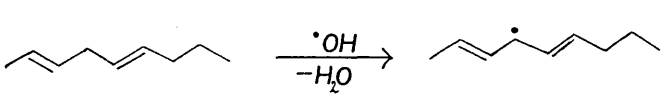
(41)

The radiation chemistry of neutral fats (triglycerides) is much more complex, and less well understood. Some radiolysis products have been identified, however (Vajdi et al, 1978). (n-1) alkanes have been observed, presumably produced from saturated fatty acids during radiolysis of the corresponding triglycerides (see above).

b) Lipid autoxidation.

In the presence of oxygen, polyunsaturated fatty acids readily undergo autoxidation. This autoxidation is a chain process which can be started by various free-radical sources (Mandal and Chatterjee, 1980). The chain reaction may occur by a number of routes, one of which is shown below:

(1) Initiation. A biallylic radical is formed in the initiation process:

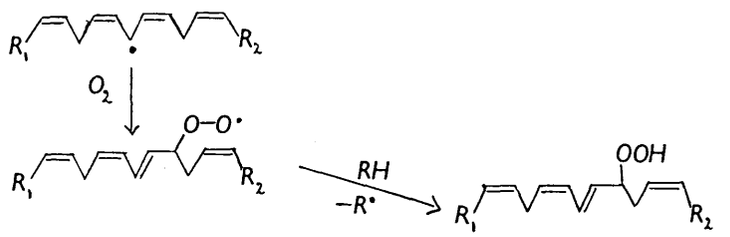


(42)

(2) Propagation.

The biallylic radical adds oxygen to form a peroxy radical (reaction 43), which in turn may react with a suitable hydrogen donor, e.g. the biallylic hydrogen atom of another polyunsaturated fatty acid, to form a cis, trans-hydroperoxide:

(43)



(44) Cis, Trans-hydroperoxide.

(3) Bimolecular termination of the chain.

In the absence of radical scavengers such as vitamin E and vitamin C, the peroxyl radicals eventually decay bimolecularly, but very little is known about the products of these bimolecular processes (von Sonntag, 1987).

(c) Effects of radiation on membrane biology.

There are a large number of biological effects which have been attributed to radiation-induced changes in membranes, but for most of the observed effects, the precise chemistry is still uncertain. Edwards et al (1984) review damage to eucaryotic cells manifested as alterations in surface charges, membrane-bound enzyme activities and receptor functions. In different cellular systems, radiation may induce an increase in permeability for potassium and sodium ions, reduction in amino acid uptake, autoxidation of the fatty acid components, and,

depending on irradiation conditions, loss or gain of sulphydryl functions, as well as disturbances in lipid metabolism.

Osmotic fragility and haemolysis of erythrocytes also increase upon irradiation. Using ghost membranes of red cells, structural modifications have been followed with the help of spin labels and fluorescent probes, and also by measuring the loss of sulphydryl groups and enzyme activity. Radiation damage of the membranes of rat-liver microsomes is thought to result in malfunction of the electron transport system. Membrane-bound enzymes may be activated, deactivated or released from the membranes, as may carbohydrate fragments.

1.10.3.4. Oligopeptides and proteins.

The majority of studies on the radiation chemistry of peptides have been performed in deoxygenated aqueous solution.

a) Reactions of the solvated electron.

The amide subunit is common to all peptides. The solvated electron reacts readily with amides:

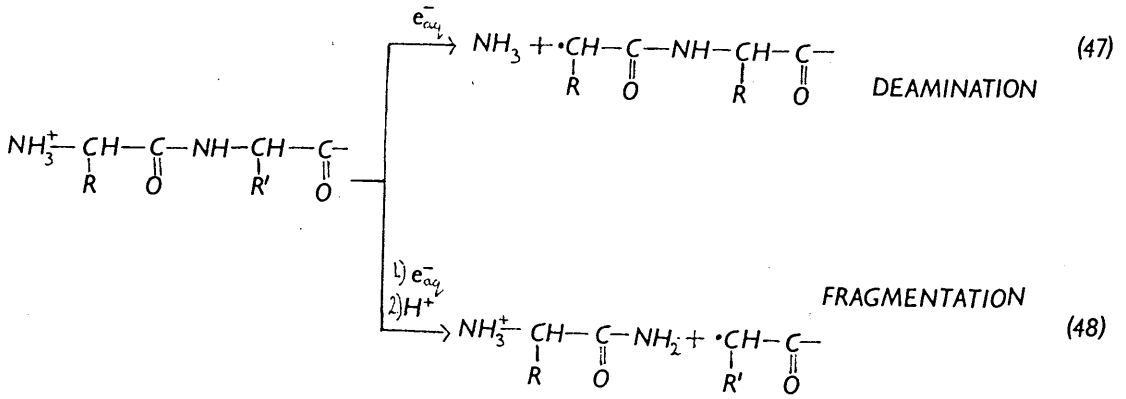
$$R - \overset{\overset{O}{\|}}{C} - NH_2 + e^-_{aq} \longrightarrow R - \overset{\overset{O^-}{|}}{C} - NH_2 \qquad (45)$$

In aqueous solution, the electron adduct is in equilibrium with its protonated form:

$$R - \overset{\overset{OH}{|}}{\dot{C}} - NH_2 \rightleftharpoons R - \overset{\overset{O^-}{|}}{\dot{C}} - NH_2 + H^+ \qquad (46)$$

In oligopeptides, the rate of reaction with the solvated electron

increases with increasing chain length, suggesting that the internal amide bonds contribute to electron capture. Deamination radicals and fragmentation radicals are observed by electron spin resonance (ESR) and spin-trapping techniques (von Sonntag, 1987). The reactions leading to these products are as follows:



These primary (internal) electron adduct radicals have been reported to transfer an electron to disulphides, thereby cleaving the disulphide bridge. A similar transfer to histidine has also been postulated (Faraggi et al, 1978). There is also evidence from low-temperature ESR that the radicals formed in the deamination reaction (47) may abstract an H-atom from within the peptide chain (Sevilla et al, 1979).

b) Reactions of the OH radical

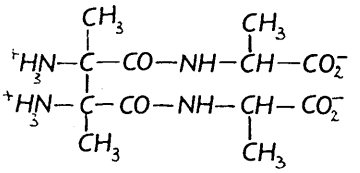
Studies of simple peptides such as glycinamide indicate that hydrogen abstraction by OH radicals occurs mainly at the carbon adjacent to the peptide nitrogen to produce peptide radicals:

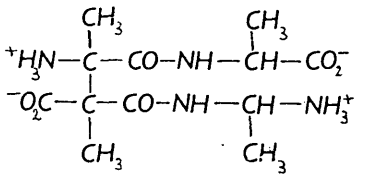
$$\overset{+}{N}H_3 - \dot{C}H - CONH_2 \xrightarrow{-H^+} NH_2 - \dot{C}H - CONH_2 \qquad (49)$$

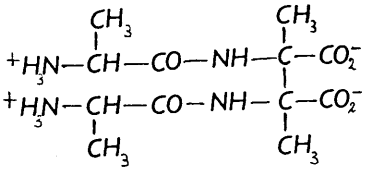
glycinamide

The peptide radicals mainly dimerize. Dizdaroglu and Simic (1983)

characterized the various crosslinked products formed in the L-Ala-L-Ala system:







Mixing of labelled phenylalanine with the 29-amino acid peptide glucagon revealed radiation-induced cross-linking with aromatic, sulphur-containing and basic aliphatic amino acids (Kim et al , 1984).

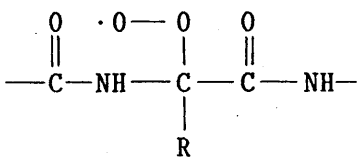
The crosslinking process also plays an important role in the free-radical chemistry of larger proteins. About 10% of the radicals in the irradiated lysozyme system lead to dimer formation (Hashimoto et al, 1981). In bovine serum albumin, some of the crosslinking is due to the formation of disulphide bridges (Schuessler and Schilling, 1984). The combination of two oxidized tyrosyl units, leading to a 2,2' - dihydroxybiphenyl derivative (detected by fluorescence), is also an important pathway in the crosslinking of proteins (von Sonntag, 1987).

The formation of 2,2'-dihydroxybiphenyl bridges is also considered to play a role in the crosslinking of histones and histone octamer complexes (Prutz et al 1983).

Other altered products in irradiated proteins have been determined. However, only those products will be detected which can withstand the severe conditions of hydrolysis (conc. HCl at 110°C for 24 hours) used in amino acid analysis. So far, α-amino-butyric acid,

allo -threonine, o - and m -tyrosine, dopamine and 2-hydroxytyrosine have been found in irradiated lysozyme (Dizdaroglu et al, 1983).

As regards the limited number of studies performed on peptides in oxygenated aqueous solutions, the reactions of OH radicals with peptides yield, among other radicals, peroxyl radicals next to the amide group:

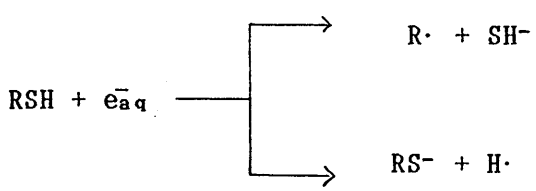


These peroxyl radicals will then undergo the types of reaction described in 1.10.2.3.

As for proteins in oxygenated solution, in the case of irradiated bovine serum albumin, a large number of discrete peaks have been observed on analysis by chromatography (Schuessler and Schilling, 1984). It is concluded that in this protein, there must be a small number of sites which undergo reactions leading to strand scission.

c) Sulphur-containing amino acids

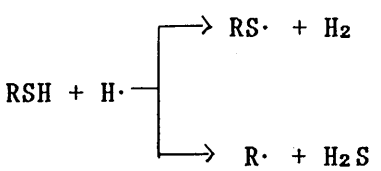
Thiol groups in proteins may react with the solvated electron to form either carbon- or sulphur-centred radicals, as follows:



$$RSH + e^-_{aq} \rightarrow R\cdot + SH^- \quad (50)$$

$$RSH + e^-_{aq} \rightarrow RS^- + H\cdot \quad (51)$$

The H-atom also undergoes two types of reaction:



$$RSH + H\cdot \rightarrow RS\cdot + H_2 \quad (52)$$

$$RSH + H\cdot \rightarrow R\cdot + H_2S \quad (53)$$

Hydroxyl radicals also attack SH groups:

$$RSH + \cdot OH \longrightarrow RS\cdot + H_2O \quad (54)$$

The thiyl radicals formed in (52) and (54) can perform H-abstraction:

$$RS\cdot + HCO_2^- \longrightarrow RSH + CO_2^{-\cdot} \quad (55)$$

$$RS\cdot + HPO_3^{2-} \longrightarrow RSH + PO_3^{2-\cdot} \quad (56)$$

These radicals also readily form radical anion complexes:

$$R'S\cdot + RS^- \overset{(57)}{\rightleftharpoons} RSSR'^{-\cdot} \overset{(58)}{\rightleftharpoons} R'S^- + RS\cdot$$

Finally, like other radicals, thiyl can add to double bonds:

$$RS\cdot + \rangle C = N\langle \longrightarrow RS - \overset{|}{\underset{|}{C}} - \overset{|}{\underset{|}{N}}\cdot \quad (59)$$

This type of reaction can lead to crosslinking of polypeptide chains.

With regard to disulphides, these compounds can react with the solvated electron to yield a disulphide radical anion, which may then undergo a chain reaction, resulting in reduction of the disulphide bond. For instance, for the disulphide-containing compound lipoamide, the following reaction occurs:

$$\overline{S - S - CH_2 - CH} - (CH_2)_4 - CONH_2$$

Lipoamide (Lip SS)

$$Lip\ SS + e_{aq}^- \longrightarrow Lip\ SS^{-\cdot} \quad (60)$$

$$Lip\ SS^{-\cdot} + H_2O \longrightarrow Lip\ (SH)\ S\cdot + OH^- \quad (61)$$

$$Lip\ (SH)S\cdot + HCO_2^- \longrightarrow Lip\ (SH)\ SH + CO_2^{-\cdot} \quad (62)$$

d) Enzyme inactivation.

The mechanisms of radiation inactivation of enzymes have received

considerable attention. In general, damage is inflicted on enzymes by attack of water radicals or any other radicals generated in the aqueous solution by adding an appropriate scavenger for the water radicals. Various routes are conceivable by which an enzyme might be inactivated by free-radical attack. For example, an amino acid at the active centre might be altered, and become incapable of exerting its required function. Often, the active centre is not exposed, and the substrate must penetrate to the active site. If this access is rendered impossible by a crosslinking reaction, the enzyme will again be inactivated. However, reactions often occur which only partially inactivate the enzyme, but a residual activity persists (Hashimoto et al, 1981).

Radiation inactivation has been studied for a wide range of enzymes, including α-chymotrypsin, ribonuclease, trypsin, yeast alcohol dehydrogenase (data reviewed by von Sonntag, 1987).

1.10.3.5. Carbohydrates.

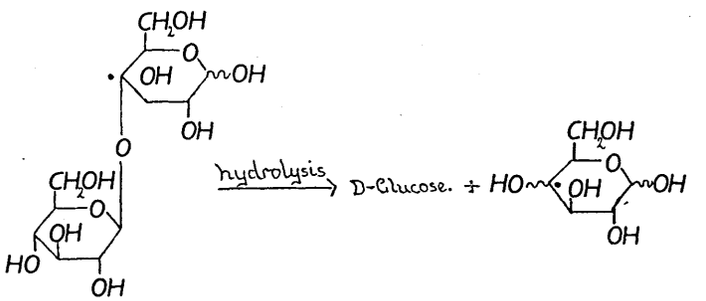
Low molecular weight carbohydrates are omnipresent in biological systems. Polymers such as cellulose, starch and chitin also play important roles, either as structural components of the cell, or as food reserves. More complex, protein- and carbohydrate-containing compounds, eg. glycoproteins and proteoglycans, are also very important biologically, and their damage will have substantial effects on cell function and survival.

Free radicals can induce scission of glycosidic bonds, and degradation of polysaccharides. A small number of breaks or crosslinks can seriously alter the physical properties of a carbohydrate polymer (Phillips, 1980), and hence affect cell viability.

(Alterations of the sugar backbone of DNA and RNA, resulting in strand breaks, alkali-labile sites, and base release, have been

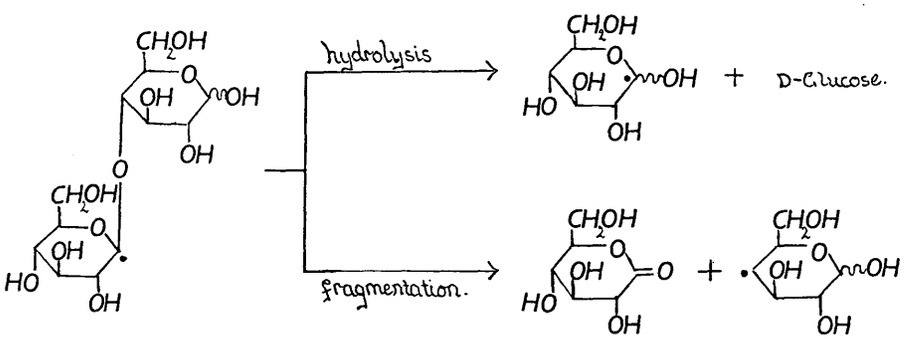
described in section 1.10.3.1.).

In the radiolysis of oligosaccharides, a number of reactions give rise to the scission of the glycosidic linkage. Reactions 63 to 68 are the key ones. They were first studied in detail with cellobiose as substrate, and can be applied to other disaccharides (von Sonntag et al, 1987).



(63)

(64)



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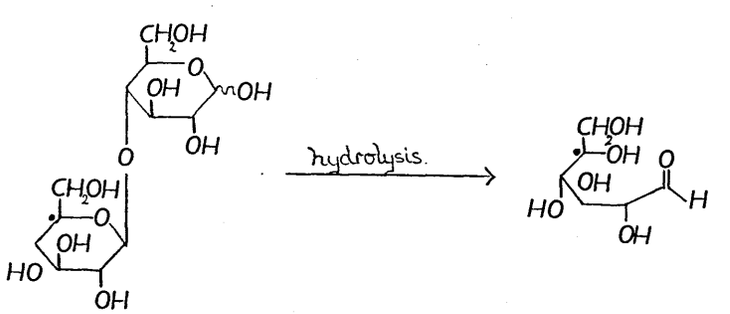

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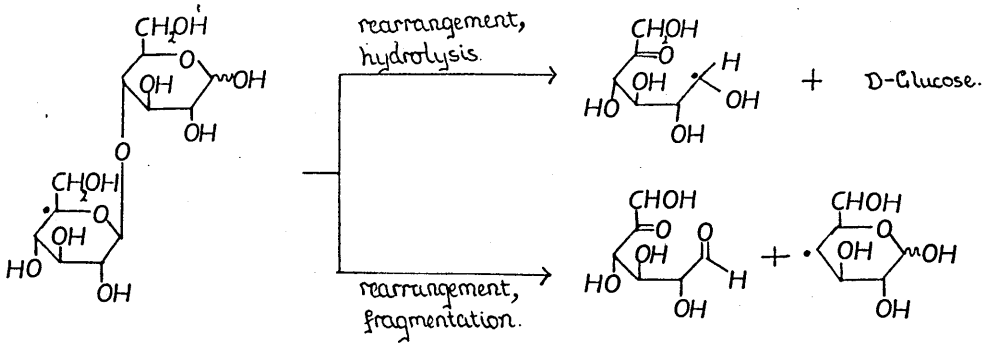
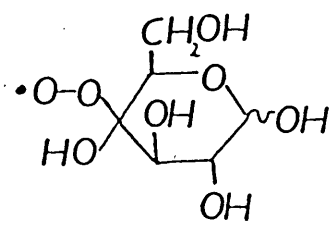
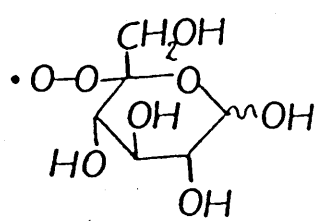


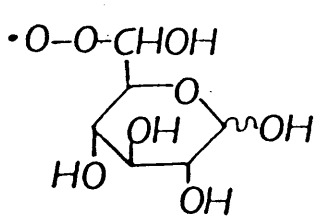
(67)

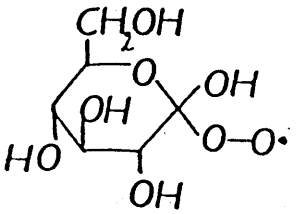
(68)

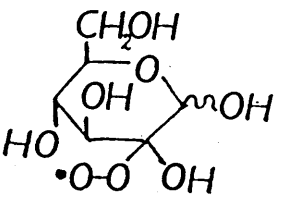
In oxygenated solutions, α-hydroxyalkylperoxyl radicals are formed by addition of oxygen to α-hydroxyalkyl radicals formed by H-abstraction from carbohydrates. The six primary peroxyl radicals derived from D-glucose have the following structures:

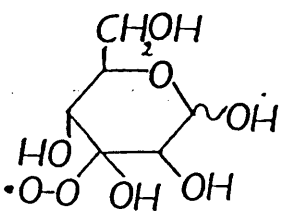












These α-hydroxyperoxyl radicals readily eliminate $HO_2^{\bullet}$ as described in section 1.10.2.3.

Aims of the project.

Irradiated cercariae stimulate a highly protective immune response. Clearly, it would be most valuable to determine the radiolesions which provoke the host immune system to respond so effectively to the attenuated schistosomula. This study therefore aimed to analyse how irradiation changes the biochemistry and metabolism of schistosome larvae to enhance their immunogenicity so effectively. With this aim in mind, the following questions were investigated:

(1) Effects of irradiation on parasite metabolism.

How do high doses of U. V.- and gamma-irradiation alter the major synthetic pathways of developing schistosomula? Metabolic labelling studies with radioactive precursors allowed comparison of synthesis of proteins and phospholipids, and protein glycosylation, by normal and irradiated larvae.

(2) Relationship between disrupted metabolism and immunogenicity.

Is it possible to relate the effects of irradiation on synthetic activity to the increased immunogenicity of irradiated parasites? This problem was investigated in two ways:

(i) By treating schistosomula with the protein synthesis inhibitor Actinomycin D to mimic the metabolic effects of irradiation. The ability of these drug-treated larvae to induce immunity was tested in animal protection experiments.

(ii) By attempting to detect changes in the nature of the antigens expressed by irradiated cercariae and schistosomula. Antigen expression was studied using a variety of techniques, including

binding by anti-schistosome antisera and several lectins, also analysis of the organisation of the parasite surface by electron microscopy, surface iodination, and reaction with fluorescein iodoacetamide.

(3) Induction and expression of immunity in skin and lung.

Can observations on the changes in metabolism and antigenicity of irradiated parasites throughout their abbreviated lifespan suggest how irradiated larvae might activate the host immune system to accomplish attrition of challenge larvae at different sites and stages in their migration and development?

(4) Variability in immunogenicity of irradiated cercariae.

Do individual populations of larvae differ in metabolism and antigen expression after irradiation? Attempts were made to investigate possible genetic or environmental causes of variation in the biochemical characteristics of irradiated cercariae, and to determine whether such diversity could contribute to the reported variability in levels of protection induced by irradiated cercariae in different experiments.

On the basis of these studies, a model is suggested for induction of immunity by irradiated cercariae of S. mansoni. The possible implications of this model for future vaccine development are discussed.

CHAPTER TWO.

MATERIALS AND METHODS.

2. Materials and Methods.

2.1. Life-cycle maintenance.

2.1.1. Parasite strain

A Puerto Rican strain of *S. mansoni*, described by Smithers and Terry (1965), is maintained in our laboratory. This *S. mansoni* strain was originally obtained from stocks at the NIMR, Mill Hill, London.

2.1.2. Maintenance of snails.

The snail host used in our laboratory is a highly susceptible albino strain of *Biomphalaria glabrata* (Newton, 1955). Original snail colonies were obtained from the NIMR, Mill Hill, London.

Stock snails were maintained in a constant temperature room at 25°C. In order to avoid contamination with copper deposits, water for snail maintenance was conveyed through plastic pipes. Chlorine was allowed to evaporate by leaving the water to stand in large plastic containers in the 25°C room for at least 48 hours before use. During this time, the water was oxygenated by bubbling air through it.

Uninfected snails were housed in plastic aquaria (volume approximately 2 litres) under a 12 hour light/12 hour dark regime. Snail food consisted of powdered nettle ("Lothian Herbs", Edinburgh), or, occasionally, dried lettuce leaf. Water was changed, and the snails fed, at regular intervals, usually 2 to 3 times weekly, although this régime varied according to snail age and size. Snail water was supplemented with calcium to encourage shell formation. Sometimes calcium carbonate was added directly in chemical form, but more frequently, a few fragments of crushed chicken egg-shell from which the vitelline membrane had been removed were used instead.

2.1.3. Infection of snails.

(1) Materials.

(i) Standard solutions:

Physiological saline: 0.15M NaCl.

Sorenson's buffer: Solution A: 0.5M KH_2PO_4

Solution B: 0.6M Na_2HPO_4.

Sorenson's buffer consists of 5% (v/v) solution A.

95% (v/v) solution B.

(ii) Fine chemicals: Trypsin - obtained from Difco Labs.

(A list of manufacturers' and suppliers' addresses is provided in section 2.9).

(2) Infection procedure.

(i) Obtaining miracidia.

The small intestines of 8-week schistosome-infected mice were used as the source of eggs to produce miracidia for infecting snails. The guts were excised, cleaned with physiological saline, and homogenized in 50ml Sorenson's buffer. The homogenate was then transferred to a conical flask, and a further 50 ml of Sorenson's buffer added. 1mg of trypsin per gut was added to the homogenate, and the suspension incubated at 37°C for 2 hours. After digestion, the homogenate was sieved through two layers of muslin cloth. The filtrate was then centrifuged for ten minutes at 800g. Centrifugation was repeated twice, washing and resuspending the pellet in physiological saline. The supernatant was decanted, and the final homogenate of packed eggs was transferred into a canister containing approximately 1.5 litres of

aquarium water per 6 guts. The eggs were allowed to hatch under a light source at room temperature. Miracidia were usually released by 20 to 30 minutes after first exposure to light.

(ii) Exposing the snails to miracidia.

Snails of 4 to 8 mm diameter were placed individually in small glass vials (3 x 2 x 2cm), and just enough aquarium water added to cover each snail. Using a Pasteur pipette, an aliquot containing 7 miracidia was collected from the canister where the eggs were hatching. Miracidia were counted either by eye, or using a hand-held magnifying glass. 7 miracidia were added to each vial. As many snails as possible were infected, until either all the miracidia were used up, or they ceased to be infective, as indicated by loss of directionality in their movements.

Newly-exposed snails were left in the vials with the miracidia overnight at 25°C. They were then pooled and transferred to the transparent plastic aquaria, at approximately 10 snails per aquarium.

(iii) Maintenance of exposed and infected snails.

All exposed snails were maintained in constant darkness. Changing of water and feeding were continued as described in section 2.1.2. At 6 to 8 weeks after exposure, when infection becomes patent (Jourdane and Théron, 1987), snails were transferred to opaque plastic containers (volume approximately 1 litre) which completely excluded all light. The snails were then maintained in the dark, with exposure to light approximately once a week to release cercariae.

(iv) Obtaining cercariae from infected snails.

A group of 20 to 25 snails, infected on the same date, were transferred to a 100ml glass beaker, rinsed briefly, and approximately 25ml of aquarium water added. The snails were then placed under a light source (140 lux) to induce shedding of cercariae.

Cercariae were collected and used to infect mice, thus continuing the life-cycle.

2.1.4. Routine infection of mice.

(1) Materials

(i) Biological materials.

The parasite was routinely maintained in BALB/c mice, bred and housed in the Biochemistry/Physiology Animal House, University of Glasgow.

ii) Anaesthetics: Sagatal - from May and Baker Ltd.
Hypnorm - Janssen Pharmaceuticals Ltd.
Hypnovel - Roche Products Ltd.

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

(2) Infection procedure

Mice were infected percutaneously, using the ring exposure technique described by Smithers and Terry (1965). Mice were anaesthetized in one of two ways:

(i) with 10% (v/v) Sagatal in distilled water: ethanol, 9:1 by volume, at a dose of 0.9 ml anaesthetic per 100g bodyweight.

(ii) with Hypnorm: Hypnovel: H_2O, 1:1:2 by volume, at a dose of 0.1 ml for a mouse of approximately 20 grams. This anaesthetic cocktail was made up freshly before use.

The anaesthetics were administered by intraperitoneal injection.

Anaesthetised mice were shaved ventrally, and laid on their backs in polystyrene trays. 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 covered with tinfoil, taking care not to disturb the rings. Lamps were shone onto the covered mice to keep them warm, since the anaesthetics cause considerable vasodilation and heat loss. The mice were left undisturbed for 20 to 30 minutes, to allow cercarial penetration. The rings were then removed, and the mice returned to their cages.

At approximately 8 weeks after exposure to cercariae, the guts of successfully parasitized mice were rich in mature eggs, and suitable for infecting snails, as described in section 2.1.3.

2.2. Irradiation of cercariae.

Cercariae were exposed to either ultraviolet- or gamma-irradiation.

2.2.1. Ultraviolet-irradiation.

(1) The source.

A high-intensity ultraviolet lamp (Mineralight and Blak Ray model UVGL-58 from UVP Ltd), delivering its peak output at 254 nm, was used as the source of U.V. radiation. The U.V. bulb in this lamp is in the form of a thin tubular strip.

The lamp was stationed in the horizontal position with metal clamps, and its output at 254 nm measured using a U.V.X. digital radiometer with a 254 nm U.V. sensor (both from UVP Ltd). A warm-up time of approximately 10 minutes was necessary to ensure a stable intensity of output. A platform was set up at 10 cm below the lamp. At this distance, the radiation intensity varied both along the length of the U.V. bulb and with distance to either side of the thin U.V. tube. The position was marked where radiation output was at a maximum. U.V. intensity at this point was 250 $\mu W\ cm^{-2}$, varying by approximately $\pm$ 7.0% with a 3 cm radius.

(2) Irradiation procedure.

The U.V. lamp was allowed to warm up for 10 minutes. Its output was routinely checked, usually at the beginning of each experiment.

Cercariae collected after a 2-hour period of shedding were counted (sections 2.1.3.(iv); 2.1.4.), and used at a concentration of 500-800/ml. If necessary, they were concentrated on a Sinterglass filter (model P40; Gallenkamp), shining a beam of light onto the cercarial suspension to prevent the parasites sinking to the base of the filter. 8ml of the cercarial suspension were then pipetted into a sterile plastic Petri dish of 6cm diameter, 1.5cm depth (Becton, Dickinson Labware), and carefully placed at the optimal position below the U.V.

lamp. Irradiation was carried out for the required time, usually 90 seconds, supplying 350-400 μW min cm^{-2} of ultraviolet energy. This radiation dose was chosen in accordance with the work of Dean et al (1983), who showed that S. mansoni cercariae exposed to U.V.-irradiation at 330-440 μW min cm^{-2} were fully attenuated, and induced optimal immunity to reinfection in NIH mice.

The irradiated cercariae were dispensed into test-tubes or universals as required for individual experiments. Any cercariae adhering to the bottom of the Petri dish were washed out by adding a further 2 ml of aquarium water, and agitating gently with a pipette. A fresh Petri dish was used for each batch of cercariae to be irradiated.

2.2.2. Gamma-irradiation.

(1) The source.

The gamma radiation source is located at the Veterinary School, Bearsden, Glasgow. The radioactive isotope, which is deep underground, is ^{60}Cobalt, emitting gamma rays of energy 1.17 and 1.33 MeV, with a half-life of 5.26 years. In the studies presented here, the dose rate was 0.45 or 0.33 krad/minute, depending on when the experiment was performed during a 2-year period.

(2) Irradiation procedure.

Cercariae were collected after the 2-hour shedding period, counted (sections 2.1.3.(iv), 2.1.4.), and adjusted to a concentration of 500-800/ml in aquarium water. Approximately 25ml of cercarial suspension were transferred into 30ml sterile plastic universals (Sterilin), capped securely, and transported by car to the gamma-irradiation source, a drive of approximately 30 minutes. The universals containing

the cercariae were placed in a plastic beaker, and wedged firmly in the upright position with tissue. The beaker was conveyed mechanically to the underground radiation source. Radiation proceeded for 45 minutes (20 krad at 0.45 krad/min), 60 minutes (20 krad at 0.33 krad/min) or 150 minutes (50 krad at 0.33 krad/min). 20 and 50 krad represent, respectively, 0.2 and 0.5 Joules of energy absorbed per gram of parasite tissue. For a comparison of the doses of gamma and U.V. radiation energy to which cercariae were exposed, see chapter 9, note 9.1.

The irradiated parasites were transported by car back to the Department of Biochemistry, and treated as required for individual experiments.

2.3. Preparation and culture of schistosomula.

2.3.1. Materials

(1) Sterile plastics.

Disposable plastic syringes Syringe needles	Becton, Dickinson Labware
25cm³ tissue culture flasks	Falcon
24-well culture plates (dimensions of wells: 1.5cm x 1.5cm)	Costar
Bottle-top filters (0.2µm and 0.45µm pore-size)	Millipore
1ml, 2ml, 5ml, 10ml pipettes	Sterilin
Universals (24mm x 90mm; capacity 30ml)	

(2) Culture media.

The most commonly used media for culturing schistosomula were GMEM and Elac. Basch's Schistosoma Culture Medium was used occasionally (section 8.2.2.4.). All media were prepared under sterile conditions in a laminar flow hood, and were stored at 4°C.

(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) Elac (GMEM plus lactalbumin hydrolysate).

Elac was made up as for GMEM, with the addition of lactalbumin hydrolysate (obtained from Sigma) at a final concentration of 0.5% (w/v).

The lactalbumin hydrolysate was dissolved in a small quantity of distilled water or medium, filter-sterilised, and added to the medium before it was pH'd and made up to volume.

(iii) Basch's Schistosoma Culture Medium-169.

This medium was prepared as described by Basch (1981). Its composition is listed in table 2.3.

Tables 2.4, 2.5 and 2.6 describe the composition of BME, MEM vitamins, and Schneider's Drosophila medium, respectively. These solutions are all components of Basch's medium.

Glucose stock solution for Basch's culture medium was made up as 100g/litre in distilled H_2O, autoclaved at 5 psi, and stored at 4°C.

Once prepared, the medium was filter-sterilized on a 0.2μm pore-size filter, and stored at 4°C.

Table 2.1. Preparation of 1 litre of GMEM (Glasgow's modification of Eagle's Medium).

Component	Stock concentration	Add stock (mls)	Working concentration
BHK-21	10X	100	1X
Glutamine	200mM	10	2mM
Sodium bicarbonate	7.5% (w/v)	30	0.225% (w/v)
Penicillin/ Streptomycin	each at 10 000 I.U./ml	10	each at 100 I.U./ml
Sodium hydroxide	5M	to pH 7.4	
distilled, de-ionized water		to 1 litre	

All stock solutions were obtained from Gibco.

Table 2.2. Composition of GMEM (Glasgow's modification of Eagle's medium). Stock solutions from Gibco.

Inorganic salts	mg/litre
$CaCl_2:H_2O$	264.00
$Fe(NO_3)_3:9H_2O$	0.10
KCl	400.00
$MgSO_4:7H_2O$	200.00
NaCl	6400.00
$NaHCO_3$	2750.00
$NaH_2PO_4:H_2O$	124.00
Other Components	
Glucose	4500.00
Phenol red	15.00
Amino acids.	
L-Arginine:HCl	42.00
L-Cystine	24.00
L-Glutamine	292.00
L-Histidine HCl:H_2O	21.00
L-Isoleucine	52.40
L-Leucine	52.40
L-Lysine HCl	73.10
L-Methionine	15.00
L-Phenylalanine	33.00
L-Threonine	47.60
L-Tryptophan	8.00

Table 2.2., continued.

L-Tyrosine	36.20
L-Valine	46.80
Vitamins	mg/litre
D-Ca pantothenate	2.00
Choline chloride	2.00
Folic acid	2.00
i -Inositol	3.60
Nicotinamide	2.00
Pyridoxal HCl	2.00
Riboflavin	0.20
Thiamine HCl	2.00
Antibiotics added:	
Penicillin	100 I.U./ml
Streptomycin	100 I.U./ml
pH'd to 7.4 with 5M NaOH.	

Component	Stock Concentration	Add Stock	Working Concentration	Supplier
BME (w/o glutamine, $NaHCO_3$ - see table 2.4)	10X	100ml	1X	Gibco
MEM Vitamins (table 2.5)	100X	5ml	0.5X	"
Schneider's medium (table 2.6)	1X	50ml	5% (v/v)	"
HEPES	100mM	100ml	10mM	"
L-Glutamine	200mM	10ml	2mM	"
Penicillin/ Streptomycin	each at 10 000 I.U./ml	10ml	100I.U./ml	"
$NaHCO_3$	7.5% (w/v)	30 ml	0.225% (w/v)	"
Lactalbumin hydrolysate		1g	1g/litre	Sigma
Hypoxanthine	10^{-3}M	0.5ml	$5x10^{-7}$M	"
Serotonin	10^{-3}M	1ml	10^{-6}M	"
Insulin (crystalline)	8mg/ml	1ml	8µg/ml	"
Hydrocortisone	10^{-3}M	1ml	10^{-6}M	"
Triiodothyronine	2×10^{-4}M	1ml	2×10^{-7}M	"
Glucose	100g/litre	10ml	11.1mM	
NaOH	5M		pH to 7.4	
distilled, de-ionized water			to 1 litre	

Table 2.3. Preparation of 1 litre of Basch's *Schistosoma* culture medium-169.

Table 2.4.

Composition of Basal Medium (Eagle's) (BME) (1X; without glutamine or $NaHCO_3$). From Gibco.

Inorganic salts.	mg/litre
$CaCl_2$ (anhydrous)	200.00
KCl	400.00
$MgSO_4.7H_2O$	200.00
NaCl	6800.00
$NaH_2PO_4.H_2O$	140.00c.
Other components.	
Glucose	1000.00
Phenol Red	10.00
Amino Acids.	
L-Arginine:HCl	21.00
L-Cystine	12.00
L-Histidine	8.00
L-Isoleucine	26.00
L-Leucine	26.00
L-Lysine	29.20
L-Methionine	7.50
L-Phenylalanine	16.50
L-Threonine	24.00
L-Tryptophan	4.00
L-Tyrosine	18.00
L-Valine	23.50

Table 2.4., Continued.

Vitamins	mg/litre
Biotin	1.00
D-Ca pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i -Inositol	1.80
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00

Table 2.5. Composition of MEM Vitamins (1X) From Gibco.

	mg/litre
D-Ca pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i -Inositol	2.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00
NaCl	85.00

Table 2.6.

Composition of Schneider's *Drosophila* medium. From Gibco.

Inorganic salts	mg/litre
$CaCl_2$ (anhydrous)	600.00
KCl	1600.00
KH_2PO_4	450.00
$MgSO_4.7H_2O$	3700.00
NaCl	2100.00
$NaHCO_3$	400.00
$Na_2HPO_4.7H_2O$	1321.00
Other components	
α-Ketoglutaric acid	200.00
Fumaric acid	100.00
Glucose	2000.00
Malic acid	100.00
Succinic acid	100.00
Trehalose	2000.00
Yeastolate	2000.00
Amino acids.	
β-Alanine	500.00
L-Arginine	400.00
L-Aspartic acid	400.00
L-Cysteine	60.00
L-Cystine	100.00
L-Glutamic acid	800.00

Table 2.6. continued.

L-Glutamine	1800.00
Glycine	250.00
L-Histidine	400.00
L-Isoleucine	150.00
L-Leucine	150.00
L-Lysine HCl	1650.00
L-Methionine	800.00
L-Phenylalanine	150.00
L-Proline	1700.00
L-Serine	250.00
L-Threonine	350.00
L-Tryptophan	100.00
L-Tyrosine	500.00
L-Valine	300.00

(3) Sera.

(i) Foetal Calf Serum (FCS) : obtained from Northumbria Biologicals Ltd.

(ii) Normal Human Serum (NHS).

Out of date human blood, collected in heparin, was obtained from Law Hospital, Carluke, ML8 5ES. Serum was separated out as described by Johnstone and Thorpe (1985). The blood was centrifuged at 200g for 10 minutes at room temperature. The supernatant, containing platelets, plasma and some lymphocytes, was decanted, and centrifuged at 3 000g for 10 minutes at 20°C. The resulting supernatant (plasma) was sterilized on a 0.45μm pore filter. To obtain serum, 1/100 volume of a solution of thrombin (Sigma) at 100 I.U./ml in 1M $CaCl_2$ was added (under sterile conditions), stirred vigorously to induce clot formation, and incubated for 10 minutes at 37°C, then for 1 hour at room temperature, and finally overnight at 4°C to allow clot formation. After centrifugation at 40 000g for 15 minutes, the supernatant was refiltered on a 0.2 μm pore-size filter, and aliquoted.

(iii) Heat-inactivation of sera.

Both FCS and NHS were depleted of complement activity by incubation at 56°C for 30 minutes. Aliquots of heat-inactivated sera (hiFCS, hiNHS) were stored at -20°C.

2.3.2. Preparation of schistosomula.

Cercariae were transformed to the schistosomulum stage by one of two methods:

(1) Mechanical transformation.

(2) Penetration of excised mouse skin.

N.B: Several hours of culture in an appropriate medium at 37°C after the mechanical trauma of syringe or skin passage are necessary before the parasite acquires all the characteristics of a true schistosomulum (Hockley and McLaren, 1973; Stirewalt et al, 1983).

(1) Mechanical transformation.

(see figure 2.1.).

The mechanical transformation procedure described by Colley and Wikel (1974) was followed, with some modification. Cercariae were collected after a 2-hour shedding period, and transferred into conical-bottomed sterile plastic universals. Cooling on ice for 45 minutes reduced cercarial motility, causing them to concentrate at the bottom of the universal. The supernatant, consisting of aquarium water, was decanted, and the sedimented cercariae gently resuspended in 5-10ml of medium warmed to 37°C. The cercariae were then passaged ten times through a 21-gauge (green) needle attached to a 10ml syringe. This shearing stress resulted in the separation of cercarial bodies and tails.

The newly-transformed schistosomula were washed 5 times by slow centrifugation (about 500g for 1 minute) in warm medium. The tail-rich supernatant was decanted, and the parasite bodies cultured as required.

For cultures which were to be kept overnight or longer, syringe passage and washing were all performed under sterile conditions. Parasites were transferred to a fresh sterile universal after the first wash.

For incubations of 5 hours or less, no special measures were taken to ensure sterility, although all plasticware and media were sterile at the beginning of the experiment.

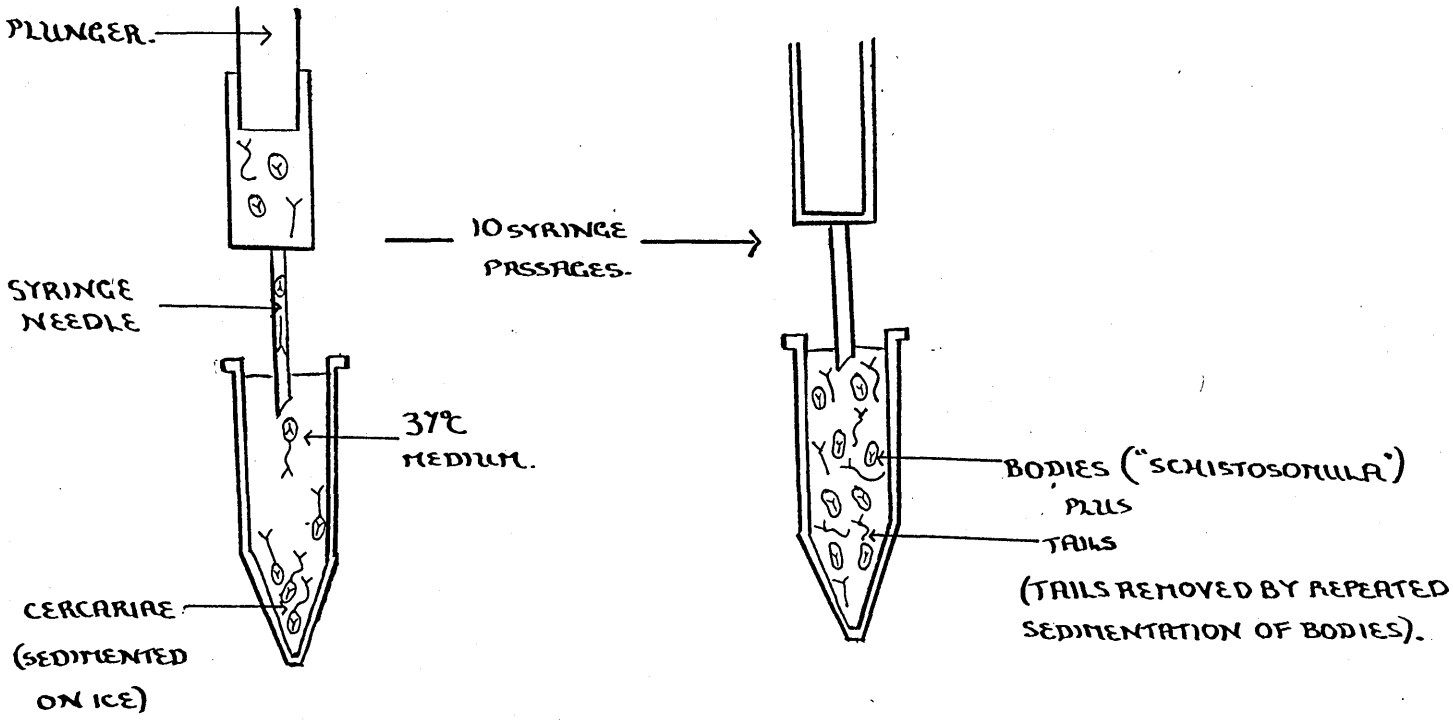


FIGURE 2.1. MECHANICAL TRANSFORMATION BY THE SYRINGE METHOD OF COLLEY AND WIKEL (1974).

(2) Transformation by skin penetration.

(see figure 2.2).

Schistosomula were collected after penetration of isolated pieces of excised mouse skin as described by Clegg and Smithers (1972). Male Parkes mice, obtained from the Biochemistry/Physiology Animal House, Glasgow University, were killed by asphyxiation with chloroform. Hair was removed from the ventral skin with clippers, and a piece of skin about 2 cm square was excised. This was placed in a sterile Petri dish, covered, and removed to the sterile hood. Here, most of the fat and dermal tissue were removed from the undersurface of the skin by rubbing with pieces of sterilized muslin soaked in warm, sterile medium.

The cleaned, thinned skin was then mounted in the glass penetration assembly shown in figure 2.2. (penetration apparatus made by Quickfit Ltd). The upper and lower joints of the assembly both had an internal diameter of 1cm. Both components of the apparatus were sterilized before use. The lower tube (B) was completely filled with warm, sterile medium, the prepared skin placed, dermal side downwards, over the medium and held in place by the upper tube (A) clamped to the lower one with a large metal clip. Tube A was filled with water to check for leakage. The cleaned skin should form a perfect seal over the sterile medium in tube B.

The upper surface of the skin was briefly washed with water to remove any medium, and the whole apparatus placed in a rack in a water bath at 37°C, so that the medium was held at this temperature, while the upper tube was at room temperature. A suspension of newly-shed cercariae (maximum 9ml, at 500-800 cercariae per ml) was then applied to tube A. Schistosomula were harvested from tube B between 1 and 3 hours after application of cercariae.

The skin-transformed schistosomula were transferred to 13.5 ml sterile centrifuge tubes in the laminar flow hood, and washed 4 times

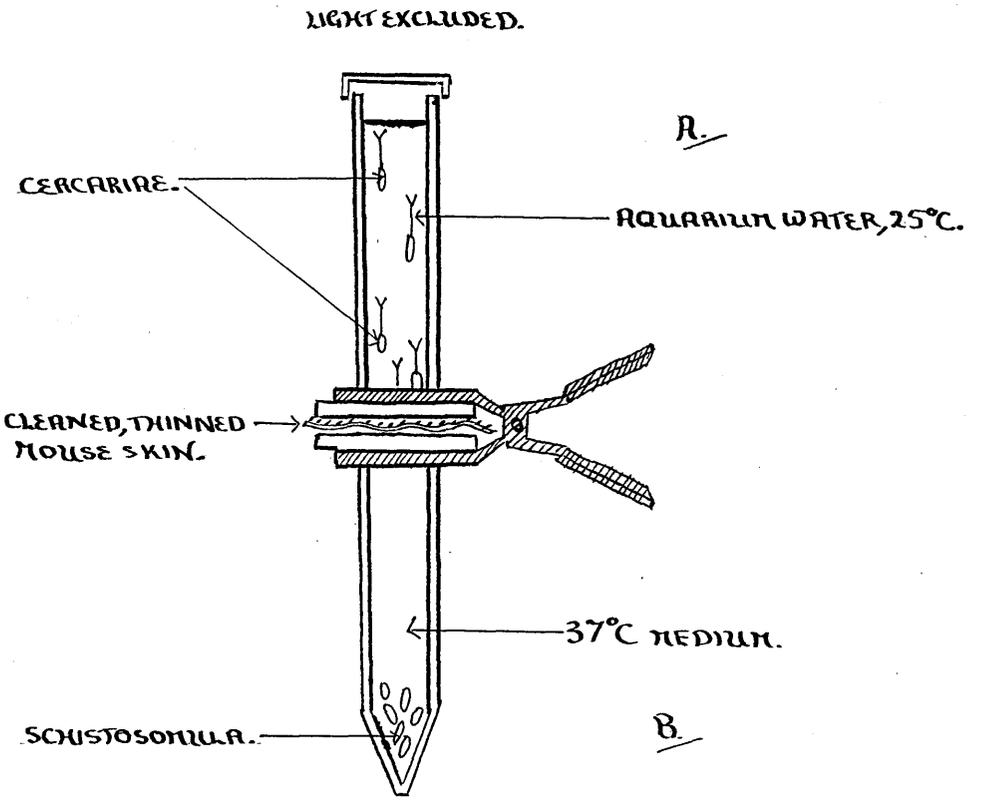


FIGURE 2.2. PENETRATION APPARATUS USED TO TRANSFORM CERCARIAE INTO SCHISTOSOMULA.

(AFTER CLEGG AND SMITHERS, 1972; CHAPPELL, 1979.)

by slow centrifugation (500g for 1 minute) in warm, sterile medium. The schistosomula were then cultured as required for individual experiments.

2.3.3. Culture of schistosomula.

(1) General conditions

Schistosomula were usually cultured in the wells of a 24-well culture plate (well dimensions 1.5cm x 1.5cm). A squared grid was etched on the bottom of each well, to assist in counting the parasites under the light microscope (Olympus, SZ-III). Schistosomula were normally cultured at a concentration of 500-1000 per ml, each well containing 1 ml of schistosomular suspension.

For incubations lasting overnight or longer, the culture medium was always supplemented with 10% serum (hiFCS or hiNHS). For shorter incubations (5 hours or less), serum was frequently omitted. The type of medium used for transformation and culture depended on the nature of the experiment. For radiolabelling studies with ^{3}H-mannose, $^{32}PO_4^{3-}$ or ^{35}S-methionine, media deficient in the radioactive constituent were frequently used (section 2.4.1.). Details of media and sera are provided for individual experiments.

(2) Actinomycin D treatment.

(i) Materials; preparation of stock solution.

Actinomycin D (from Streptomyces, approximately 98% pure by HPLC) was obtained from Sigma.

A stock solution of Actinomycin D was made up in ethanol (May and Baker Ltd; Analar Grade) at a concentration of 200 µg/ml. 2ml aliquots

were dispensed, the tubes sealed carefully, covered in foil, and stored in the dark at -20°C.

(ii) Procedure.

The routine procedure for Actinomycin D treatment was as follows.

Cercariae were transformed mechanically in Elac, and washed 5 times under sterile conditions, as described in section 2.3.2. The newly-transformed schistosomula were resuspended to approximately 500/ml in Elac/10% hiFCS, and Actinomycin D was added to give a final concentration of 2.6 µg/ml. Depending on the number of parasites and the volume of the suspension, schistosomula were transferred to a 25cm³ tissue culture flask or one well of a 24-well culture plate. The parasites were then incubated for 20 hours at 37°C in the presence of 5% CO_2. At the end of this incubation with the drug, schistosomula were transferred to 13.5ml centrifuge tubes (still under sterile conditions), and washed 4 times in warm Elac by slow centrifugation at approximately 500g for 1 minute. They were then treated as required for individual experiments.

Control schistosomula (non-Actinomycin D-treated) were subjected to the same culture and washing procedures. In initial studies, normal schistosomula received an equivalent amount of Analar ethanol to the Actinomycin D-treated ones. This treatment had no adverse effect on parasite protein synthesis or survival during the 96-hour culture period examined.

Modifications of this basic procedure, e.g. use of skin-transformed schistosomula, different media, different concentrations of Actinomycin D, are described for individual experiments.

(3) Fenfluramine treatment.

Fenfluramine (obtained from Sigma) was used to inhibit phospholipid synthesis in only one experiment. Details of the procedure are provided in section 4.2.1.

(4) Assessing viability of cultured parasites.

Schistosomula were classified as live or dead according to their appearance under the light microscope at X40 magnification. Wholly viable schistosomula were translucent and very motile. Dead parasites were highly granular, had a swollen, flattened appearance, and were non-motile. Some schistosomula showed intermediate levels of damage. Such parasites displayed granularity (sometimes quite extensive), with the beginnings of swelling and distortion, but were still alive as judged by motility and retention, at least in part, of their normal shape. These damaged, but still living, schistosomula, were scored as "live".

2.4. Metabolic radiolabelling studies.

2.4.1. Materials for radiolabelling in culture.

(1) Radioisotopes

All the following radioisotopes were obtained from Amersham:

Radioisotope (abbreviation)	Specific Activity
(i) [2-^{3}H]-adenosine (^{3}H-adenosine) (in aqueous solution; sterilized)	21 Ci (777 GBq)/mmol.
(ii) D-[2,6-^{3}H]-mannose (^{3}H-mannose) (originally made up in ethanol:water, 1:1 solution. Before use, the required volume of stock ^{3}H-mannose was dispensed, evaporated to dryness under nitrogen, and resuspended in a small volume of medium).	54 Ci (2 TBq)/mmol.
(iii) [methyl-^{3}H]-thymidine (^{3}H-thymidine) (in aqueous solution: sterilized).	42 Ci (1.55 TBq)/mmol.
(iv) [5,6-^{3}H]-uridine (^{3}H-uridine) (in aqueous solution; sterilized).	45 Ci (1.66 TBq)/mmol.
(v) L-[^{35}S]-methionine (^{35}S-methionine) (in 20mM potassium acetate solution, containing 0.1% 2-mercaptoethanol).	in different batches, from 1030 to 1476 Ci (38.1-54.6 TBq)/mmol.
(vi) $^{32}PO_4^{3-}$ (^{32}P) (as orthophosphate in dilute HCl, pH2-3; carrier-free).	1mCi (37 MBq)/ml

(2) Special media for radiolabelling studies.

All media were prepared under sterile conditions, and stored at 4°C.

(i) Methionine-free GMEM (GMEM w/o methionine).

This medium was obtained ready-prepared (minus glutamine and antibiotics) from Flow Laboratories. Glutamine, penicillin and streptomycin were added to give final concentrations of 2mM, 100 I.U./ml and 100 I.U./ml, respectively.

For metabolic labelling studies with ^{3}H-mannose and ^{32}P, Eagle's Minimal Essential Medium with Earle's salts (EMEM) was used, with the omission of phosphate, or substitution of pyruvate for glucose, as appropriate. EMEM differs from Glasgow's Modification (GMEM) chiefly in containing approximately half the concentration of vitamins found in the latter. There are also minor differences in the salt and amino acid contents of the two media. Tables 2.5, 2.8 and 2.9 describe the composition of EMEM.

(ii) EMEM with sodium pyruvate (10mM) in place of glucose.

This medium contains 10mM sodium pyruvate as an energy supply in place of glucose, since glucose is likely to compete with the uptake of ^{3}H-mannose. The composition of this medium is described in tables 2.7, 2.8 and 2.9.

(iii) EMEM without phosphate (EMEM w/o phosphate).

See tables 2.8 and 2.10.

2.4.2. Radiolabelling procedures.

The precise details of radiolabelling - amount of radioactivity, medium, serum, time of incubation, preparation of schistosomula - are described for individual experiments. A general outline of the

Table 2.7. Preparation of 1 litre of Eagle's Minimal Essential Medium with Earle's salts, with sodium pyruvate (10mM) in place of glucose.

Component	Stock concentration	Add stock (mls)	Working Concentration	Supplier
MEM Earle's salts (w/o $NaHCO_3$)	10X	100	1X	Made up as in table 2.8 (made in lab.)
MEM Vitamins (table 2.5)	100X	10	1X	Gibco
MEM Amino acids (w/o glut.) (table 2.9)	50X	20	1X	"
Sodium pyruvate	100mM	100	10mM	"
$NaHCO_3$	7.5% (w/v)	30	0.225% (w/v)	"
Penicillin/ Streptomycin	10 000 I.U./ml each	10	100 I.U./ml each	"
L-Glutamine	200mM	10	2mM	"
Phenol red	1% (w/v)	1	0.001%	Made up in lab.
NaOH	5M		pH to 7.4	
distilled, de-ionized water			to 1 litre	

Table 2.8. Composition of MEM Earle's salts (without $NaHCO_3$) - made up in the lab.

	Stock Concentration (10X) g/litre	Working Concentration (1X) mg/litre
$CaCl_2$ (anhydrous)	2.00	200.00
KCl	4.00	400.00
$MgSO_4.7H_2O$	2.00	200.00
NaCl	6.80	6800.00
$NaH_2PO_4.H_2O$	0.14c	140.00c

Table 2.9. Composition of MEM Amino acids (1X) (without glutamine) From Gibco.

	mg/litre
L-Arginine:HCl	126.00
L-Cystine	24.00
L-Histidine HCl:H_2O	42.00
L-Isoleucine	52.00
L-Leucine	52.00
L-Lysine HCl	72.50
L-Methionine	15.00
L-Phenylalanine	32.50
L-Threonine	48.00
L-Tryptophan	10.00
L-Tyrosine	36.00
L-Valine	46.00

Table 2.10. Preparation of 1 litre of Eagle's Minimal Essential Medium, with Earle's salts, minus phosphate.

Component	Stock concentration	Add Stock (mls)	Working concentration	Supplier
MEM Earle's salts (w/o $NaH_2PO_4.H_2O$ or $NaHCO_3$	10X	100	1X	as table 2.8, minus $NaH_2PO_4.H_2O$ (made in lab).
MEM Vitamins (table 2.5)	100X	10	1X	Gibco
MEM Amino acids (w/o glut.) (table 2.9)	50X	20	1X	"
$NaHCO_3$	7.5% (w/v)	30	0.225% (w/v)	"
Penicillin/ Streptomycin	10 000 I.U./ml each	10	100 I.U./ml each	
L-Glutamine	200mM	10	2mM	"
Glucose	100g/1	10	11.1mM	Made up as described in 2.3.1.(2)(iii)
Phenol red	1% (w/v)	1	0.001% (w/v)	Made up in lab
NaOH	5M		pH to 7.4	
distilled de-ionized water			TO 1 LITRE	

conditions is given below.

For experiments where only normal schistosomular metabolism was being examined, or normal and irradiated parasites were being compared, the radiolabel was usually added to the culture immediately after transformation of the normal and irradiated cercariae. When normal, irradiated and Actinomycin D-treated schistosomula were all being studied in the same experiment, normal and irradiated schistosomula were cultured, usually in Elac/10% hiFCS, at 37°C/5% CO_2 for 20 hours after transformation, while the Actinomycin D samples were drug-treated for the same length of time (section 2.3.3.(2)). The 3 groups of parasites were then washed in an appropriate medium, and radiolabel added.

Schistosomula were usually radiolabelled at a concentration of between 500 and 1000 per ml. Each radiolabelling group in a single experiment contained approximately equal numbers of schistosomula.

(1) ^{35}S-methionine radiolabelling

In separate experiments, GMEM w/o methionine, Elac, and complete GMEM, in the presence or absence of serum, were all used for ^{35}S-methionine radiolabelling.

(2) ^{3}H-adenosine, ^{3}H-thymidine, ^{3}H-uridine radiolabelling.

Labelling with these isotopes was always performed in Elac/10% hiFCS.

(3) ^{3}H-mannose radiolabelling.

For labelling with this isotope, schistosomula were cultured in EMEM with 10mM pyruvate in place of glucose, supplemented with 10% hiFCS.

(4) ^{32}P-radiolabelling.

The culture medium was EMEM w/o phosphate, supplemented with 10% hiFCS.

At the end of the labelling period, schistosomula were counted (using the grids etched on the bottom of the culture wells), and transferred to 13.5 ml sterile plastic centrifuge tubes. They were cooled on ice for 10 minutes, then washed 3 or 4 times, by centrifugation at 500g for 1 minute, in 10 ml of ice-cold medium. Complete GMEM was generally used for washing, unless labelling had been performed in Elac, when cold Elac was also used for washing.

After the final wash, all medium was removed, and the schistosomular pellet frozen at -20°C.

2.4.3. Collection of proteins released by schistosomula during culture.

(1) Materials

Dialysis tubing 8/32"	- obtained from the Scientific Instrument Centre Ltd
OR PD-10 columns pre-packed with Sephadex G-25M.	- from Pharmacia.
Bovine serum albumin (BSA)	- from Sigma
Dichlorodimethylsilane solution, approx. 2% in 1,1,1-trichloroethane ("Repelcote").	- from BDH Chemicals.

(2) Protease Inhibitors.

Phenyl methyl sulphonyl fluoride (PMSF) - obtained from Sigma. Made up as a 0.1M solution in acetone (Analar grade; May and Baker Ltd).

L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), and N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK) - both from Sigma. Made up together in acetone, each at 0.1M final concentration.

(3) Procedure.

At the end of the culture period, schistosomula were counted, transferred to sterile 13.5 ml plastic centrifuge tubes, and cooled on ice for 10 minutes. The schistosomula were spun down at 1000g for 1 minute, and the supernatants, containing proteins released by the parasites during culture, were transferred to fresh centrifuge tubes. 1µl of each of the protease inhibitor solutions was added per ml of medium, and the supernatant examined under the light microscope to check that no parasite bodies were still present. Usually one or two more centrifugations (1000g for 1 minute) were necessary to remove all the schistosomula.

In order to allow analysis of the secreted proteins by SDS-PAGE, salt and low molecular weight solutes were then removed from the medium in one of two ways:

(i) Dialysis.

The culture supernatants, containing protease inhibitors, were transferred to small dialysis bags, and dialysed against 2 litres of distilled water at 4°C. The dialysis water was changed 4 times over a

24-hour period. The dialysed solutions were then decanted into 1.5ml polytubes or conical glass centrifuge tubes, both of which were pre-treated with Repelcote. After freezing in a bath of dry ice (solid CO_2) and methanol, the supernatants were freeze-dried overnight. Once in the dry form, the secreted proteins were stored at -20°C.

(ii) De-salting by gel filtration on a Sephadex G-25 column.

A PD-10 column (bed height 5cm; bed volume 9.1ml) packed with Sephadex G-25M was pre-blocked with 100µl of 10% (w/v) BSA in distilled water, then equilibrated with 25ml distilled water. The culture supernatant, with added protease inhibitors, was diluted to 2.5ml with distilled water, and added to the column. When the sample had run into the column, 3.5ml distilled water were used to elute the proteins into a glass centrifuge tube treated with Repelcote. The de-salted solution of proteins was then freeze-dried as described in (i).

2.4.4. Quantifying uptake of free radioisotope and incorporation into protein or nucleic acid: Trichloroacetic Acid (TCA) precipitation.

(1) Materials:

TCA, sodium pyrophosphate, 90% formic acid	all from BDH Chemicals (all Analar grade).
methionine (non-radioactive)	from Sigma.
Ecoscint A	from National Diagnostic
Bench centrifuge for 1.5ml polytubes (MSE Microcentaur)	from Beckman Instruments Ltd.

(2) Procedures.

(i) Estimation of ^{35}S-methionine uptake and incorporation into protein.

(Protocol based on Pratt, 1984).

The frozen schistosomular pellets, obtained, as described in section 2.4.2., from schistosomula which had been radiolabelled and thoroughly washed, were thawed and immediately removed to ice. 500µl of ice-cold 10% (w/v) TCA/0.1% (w/v) methionine was added, mixed, and the samples transferred to 1.5ml polytubes. 10µl hiFCS were added to each sample, to act as a carrier in precipitation. After mixing, the samples were left to stand for 20 minutes on ice, allowing the precipitates to form, and sink to the bottom of the tubes. The precipitates were then spun down for 10 minutes at high speed in a bench centrifuge in the 4°C room.

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

The pellets were washed twice by resuspension in 1ml 5% (w/v) TCA/0.1% (w/v) methionine, incubation on ice, and centrifugation as before. The final pellets were dissolved in 500 µl 90% formic acid, and transferred to scintillation vials. 4.5ml Ecoscint were added, and mixed well.

ii) Estimation of ^{3}H-adenosine, ^{3}H-thymidine, ^{3}H-uridine uptake and incorporation into nucleic acid.

(Protocol based on Marzluff and Huang, 1984).

Procedure was as described in (i), except that 10% (w/v) TCA/1%

(w/v) sodium pyrophosphate was used for the initial precipitation, and 5% (w/v) TCA/1% (w/v) sodium pyrophosphate for the subsequent washes.

iii) Counting procedure

Samples were counted on a Beckman LS335 scintillation counter. Each sample was usually counted for 4 minutes. However, when the level of radioactivity was very low, as for ^{3}H-thymidine incorporation by schistosomula, the counting time was increased to 20 minutes for each sample.

2.4.5. Separation of bodies and surfaces of schistosomula.

The saponin method developed by Kusel (1972) was used for extracting surface membranes from schistosomula.

Saponin was obtained from Sigma.

Pelleted schistosomula from which all medium had been removed were treated for approximately 7 minutes with 5.0 ml of 0.5% (w/v) saponin in 3% (w/v) $CaCl_2$. Examination under the light microscope showed the surfaces lifting away from the bodies. The schistosomula were spun down very gently (approximately 300g for 1 minute), the saponin-containing supernatant removed, and replaced with 0.9% (w/v) NaCl. The schistosomula were drawn 10 times through a fine Pasteur pipette - this treatment sheared the surfaces from the bodies. The bodies were then spun off as a pellet (300g for 1 minute), to leave the surface fragments in the supernatant. The supernatant was examined under the light microscope for the presence of any remaining parasite bodies. Centrifugation was repeated until all the bodies had been removed.

The final supernatant was centrifuged at 1500g for 10 minutes to spin down the surfaces as a pellet. Both pellets - bodies and surfaces - were washed 4 times in 0.9% (w/v) NaCl, by centrifugation as before,

and stored at -20°C. Figure 2.3 describes this protocol as a flow diagram.

2.5 Analysis of metabolically-labelled proteins and phospholipids.

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

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

(1) Gel Preparation.

(i) Materials

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

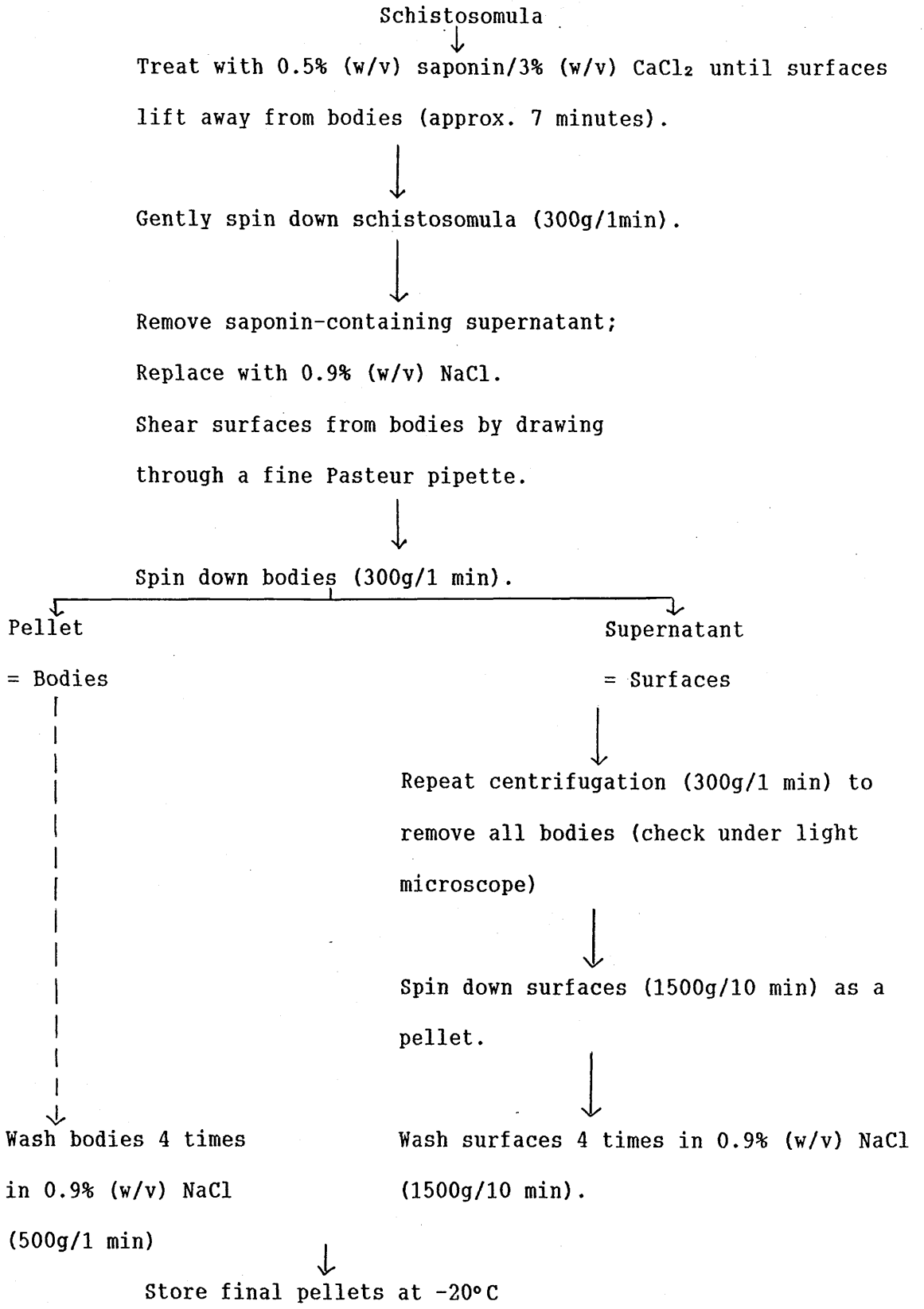
All chemicals were maximum purity grade.

(ii) Stock solutions

Solution A: 30% (w/v) Acrylamide.

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

Figure 2.3. Separation of schistosomular bodies and surfaces (after Kusel, 1972).



Solution B: 1.5M Tris-HCl buffer, pH 8.8.

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

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

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

(iii) 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 2.11 gives the composition of the different gels. In the text, the expression "3% gel" refers to the percentage content of acrylamide.

(iv) Preparing the gels

Materials: (per gel)

- 2 glass plates for electrophoresis tank model V16 (Bethesda Research Labs). Small plate: dimensions 7.75 x 6.31 in; large plate 7.75 x 7.5 in.
- Spacer set, 1.5mm width. Includes 2 side spacers with neoprene blocks and 1 notched bottom spacer.
- 1 20-well comb, 1.5mm 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 plates were placed on clean tissue paper, with the sides which were to be in contact with the gel uppermost, and the ethanol allowed to evaporate.

The three spacers were assembled on the larger glass plate, and the smaller one 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 sandwich just over the spacer positions. The assembly was checked for leakage at the bottom corners using a small volume of water. These

Table 2.11 Composition of SDS-polyacrylamide gels.

Solution	Separating gel - volume (ml) to make 40 mls.		Stacking gel: volume (ml) to make 20 mls.
	Final acrylamide concentration		Final acrylamide concentration
	7.5%	10%	3%
A	10.0	13.3	2.5
B	5.0	5.0	/
C	/	/	5.0
D	0.4	0.4	0.2
E	2.0	2.0	1.0
Distilled H_2O	22.6	19.3	11.3
TEMED	Mix gently; de-gas if necessary; add		
	50 µl	50 µl	15 µl

spacers usually gave a perfect seal. The water was then poured off.

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 3.5cm was left above the resolving gel for the stacking gel to be poured later. The top of the resolving gel was gently overlaid with a small volume of 70% ethanol, to give a smooth surface. After polymerisation (20 to 30 minutes), the ethanol overlay was poured off, the surface of the resolving gel rinsed with a small volume of stacking gel buffer, and blotted dry with a piece of filter paper. The remaining space between the gel plates was filled with stacking gel mixture immediately after adding the TEMED. The comb was then inserted into the stacking gel mixture, being careful to avoid trapping any air bubbles beneath it. The assembly was left undisturbed while the stacking gel polymerised (40 to 60 minutes).

After polymerisation, the comb was carefully removed to expose the sample wells. Gel fragments and fluid were removed from the wells with a syringe. The wells were filled with electrophoresis buffer (section (3) below) until the samples were ready for application.

(2) Sample preparation

(i) Determination of protein concentration (Lowry method).

Protein concentration was determined following the method of Lowry et al (1951), with some modification. Bovine serum albumin (BSA) was used as the standard protein.

Materials

Folin and Ciocalteu's phenol reagent - from BDH Chemicals.

BSA - from Sigma.

Solutions: 2% (w/v) $CuSO_4.5H_2O$.

4% (w/v) sodium potassium tartrate.

3% (w/v) sodium carbonate in 0.2N NaOH.

PBS (Phosphate buffered saline): 0.14 M NaCl

2.7 mM KCl

1.5 mM KH_2PO_4

8.1 mM Na_2HPO_4.

Spectrophotometer: Ultrospec II UV/Vis - from LKB Instruments Ltd.

A 1mg/ml solution of BSA in PBS was made up. 0-100µl of this solution were pipetted into separate tubes, in duplicate or triplicate, giving 0-100 µg of protein per tube.

A known number of schistosomula were washed 3 times in ice-cold PBS, resuspended to 1ml PBS, protease inhibitors added as described in 2.4.3., and the samples homogenized on ice. Aliquots of the homogenate (less than 100µl) were dispensed into separate tubes, in duplicate or triplicate. The contents of each tube were brought up to 100µl with PBS.

1ml of copper sulphate solution and 1ml of tartrate solution were mixed with 48 ml of carbonate solution. 1ml of this mixture was added to each tube, mixed, and incubated for 10 minutes at room temperature. 50µl of Folin reagent were added to each tube, mixed immediately, and incubated for 25 minutes. After a second mix, and standing for 5 minutes, the absorbance of each tube was read at 750 nm, using a PBS blank (0 µl BSA, 100 µl PBS) to zero the spectrophotometer. Absorbance was plotted against protein content for the standard solution, and the amount of protein in the schistosomular homogenate read off from this graph.

Initial Lowry assays showed that 3500 schistosomula correspond to

approximately 100 µg of protein. (1 adult worm pair also contains 100 µg of protein). Having established this equivalence, in subsequent experiments, schistosomula were counted, and approximately equal numbers applied to each well of a gel. Usually, between 500 and 1000 schistosomula were used per well, giving 14 to 28 µg of protein.

(ii) Sample preparation.

Protein samples to be electrophoresed were dissolved in 20 µl of sample buffer, and placed in a boiling water bath for 10 minutes. Sample buffer consisted of 2% (w/v) SDS, 10% (w/v) sucrose (Koch-light Labs.), 5% (v/v) 2-mercaptoethanol (Koch-light Labs.), 0.001% bromophenol blue (BDH Chemicals) in 0.1M Tris-HCl, pH 6.8.

After cooling to room temperature, samples were ready for electrophoresis.

(3) Electrophoresis.

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

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

The gel assembly was clipped into position in the electrophoresis tank, and buffer added to the upper and lower reservoirs. Air bubbles were removed from the bottom of the gel using a syringe with a bent needle. After checking for leaks, the samples were carefully loaded onto the gel surface using a micropipette.

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

bromophenol blue tracking dye was approximately 5mm from the bottom of the gel.

(4) Fixing and staining gels.

(i) Kenacid Blue staining.

Gels were fixed and stained for 2 hours at 37°C in 500 ml of 0.1% (w/v) Kenacid Blue (BDH Chemicals) in 50% methanol, 5% acetic acid.

Excess stain was removed with several changes of destain solution: 25% methanol, 10% acetic acid. Destaining was continued until the background of the gel was clear.

The Kenacid Blue stain detects 0.2 to 0.5µg of protein in a single band (Hames, 1981). For greater sensitivity, silver-staining was used.

(ii) Silver-staining

The silver-stain of Merril et al (1980) was used, with some modification. This stain is claimed to detect 0.01 ng of protein per square mm.

Solutions: Oxidiser: 3.4mM potassium dichromate (Fisons) in 3mM nitric acid (May and Baker Ltd)

Silver reagent: 12mM silver nitrate (Johnson Matthey Chemicals).

Developer: 0.28M sodium carbonate (BDH Chemicals) plus 0.5ml paraformaldehyde (May and Baker Ltd). per litre.

The gel was fixed for 1 hour at room temperature in 400ml 40% methanol/10% acetic acid, then for 1 hour in 10% ethanol/5% acetic acid, changing the solution once. 200 ml of oxidiser were added, with

shaking, for 10 minutes. The gel then received 3 10 minute washes in de-ionized water. This was followed by incubation for 30 minutes, with shaking, in 200 ml of silver reagent. After a 2-minute wash in de-ionized water, 200 ml of developer were added. This first rinse of developer turned brownish-yellow within 30 seconds, and was immediately discarded. The gel was then gently agitated in 2 further portions of developer solution until the image had reached the desired intensity (usually about 10 minutes in total). Development was stopped by discarding the developer, and adding 200 ml of 5% acetic acid. After 10 minutes in acetic acid, the gel was washed well in distilled water, before storage in 2% acetic acid.

(5) Determination of protein molecular weights.

(i) Preparation of protein standards.

High and low molecular weight protein calibration kits were obtained from Pharmacia.

Table 2.12 lists the proteins contained in each kit.

Low molecular weight protein mixtures were boiled in 100µl sample buffer, as described in (3)(ii) above. For the high molecular weight standards, the sample buffer described in (3)(ii) was diluted 1:1 with 0.1M Tris-HCl, pH 6.8/10% sucrose to give half the usual concentrations of SDS and 2-mercaptoethanol (final concentrations 1% and 2.5%, respectively). The protein mixture was incubated at 60°C for 15 minutes in 100µl of this modified sample buffer. This treatment resulted in a mixture of ferritin half-units and subunits (see table 2.12). After preparation, the protein standards were aliquoted, and stored at -20°C.

For Kenacid Blue staining, 5µl of the low molecular weight protein standards, and 10µl of the high molecular weight ones were used per

Table 2.12. Protein standards used for SDS-PAGE.

Protein	Mol. Wt. ($x10^{-3}$)	Subunit Mol. Wt. ($x10^{-3}$)
High Molecular Weight Proteins.		
Thyroglobulin	669	330
Ferritin	440	half-unit: 220 subunit: 18.5
Catalase	332	60
Lactate Dehydrogenase	140	36
Albumin	67	67
Low Molecular Weight Proteins		
Phosphorylase b.	94	94
Albumin	67	67
Ovalbumin	43	43
Carbonic Anhydrase	30	30
Trypsin Inhibitor	20.1	20.1
α-Lactalbumin	14.4	14.4

well. For silver-staining, an aliquot of protein standards was diluted 20-fold with sample buffer or modified sample buffer (high mol. wt. standards), and the same volumes used per well.

(ii) Preparation of the calibration curve.

The migration distance of each protein standard was measured, and the relative migration values (R_f) calculated. R_f was defined as:

$$R_f = \frac{\text{distance protein has migrated from top of resolving gel.}}{\text{distance from top of resolving gel to bromophenol blue dye-front.}}$$

A calibration curve was constructed by plotting the R_f values of the standard proteins versus the log of their corresponding molecular weights.

To determine the molecular weight of a protein of interest, its R_f value was calculated, and located on the calibration curve. The value on the log scale which corresponded to this point was the estimated molecular weight of the protein.

A new calibration curve was produced for each gel. Between Mr 67 000 and 14 400, the calibration curve for 10% gels was linear, and molecular weights of unknown proteins were readily determined. Above Mr 67 000, the curve started to become non-linear. It was more difficult to determine accurately the molecular weights of proteins above this value. However, with repeated electrophoresis of samples of the same type, drawing new calibration curves for each gel, it was possible to establish the molecular weights of these proteins within a range of about 2 000 Daltons.

2.5.2. Identification of radiolabelled proteins

(1) ^{35}S-methionine-labelled proteins: fluorography.

Materials: sodium salicylate - from Sigma.

glycerol - from BDH Chemicals.

X-Omat X-ray film (18cm x 24cm) - Siemans Ltd.

Film cassettes (18cm x 24cm) with intensifying screens:

Metal - Ilford Ltd.

Plastic - Sky Plastics Ltd.

Fixer and Developer - Kodak.

The basic fluorographic and autoradiographic techniques are described by Bonner and Laskey (1974) and Laskey and Mills (1975, 1977).

The sodium salicylate method for fluorography of ^{35}S-labelled material described by Chamberlain (1979) was used. Stained and destained gels were washed for 2 hours 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 soaked for 5 minutes in 2% (w/v) glycerol, to prevent cracking during drying, and dried onto Whatman 3MM filter paper under vacuum. The gel was then labelled, removed to a dark room with safe-light illumination, and placed in contact with X-ray film in a cassette backed by an intensifying screen. The cassette was wrapped in a black polythene bag to ensure all light was excluded, and the film exposed at -70°C until the bands developed.

(2) ^{32}P-labelled proteins: autoradiography.

For ^{32}P-labelled proteins, immersion in sodium salicylate was not required, but drying and exposure of the gel to X-ray film, with an intensifying screen, were as for ^{35}S-labelled proteins.

(3) ^{3}H-labelled proteins: gel-slicing and liquid scintillation counting.

When schistosomular glycoproteins were labelled with ^{3}H-mannose, the amount of radioactivity incorporated into individual bands was too low for detection by fluorography. Gel slices were therefore prepared for liquid scintillation counting as described by Gray and Steffensen (1968) and Tishler and Epstein (1968).

After drying the gel onto filter paper, each track was cut into slices 5mm in length and of equal width. A few slices were cut from regions of the gel containing no protein, to give background values. 500 µl of 30% hydrogen peroxide (Sigma) were added to each slice in a scintillation vial. Vials were capped, and incubated overnight at 37°C, to allow solubilization of the protein. The vials were then cooled to room temperature, and 9 ml of Ecoscint added to each. Scintillation counting (see section 2.4.4.(2)(iii)) was performed for 20 minutes for each vial.

R_f values were calculated for each slice, and molecular weights estimated by comparison with the migration of proteins standards, as usual.

2.5.3. Analysis of radiolabelled phospholipids.

(1) Extraction of phospholipids.

Phospholipids were extracted according to the method of Folch et al (1957), with modifications.

(i) Materials

All solvents were Analar grade, and obtained from May and Baker Ltd.

Extraction solvents mixture:

chloroform: methanol, 2:1.

"Upper phase solvent" mixture:

chloroform: methanol: water, 3:48:47.

(ii) Procedure

The pellet of labelled, washed schistosomula was resuspended to 100 µl PBS in a graduated glass centrifuge tube. 2 ml of the extraction solvents mixture were added, and more as necessary to produce one clear phase. The extract was vortexed, and allowed to stand for 1 hour at room temperature under a gentle stream of nitrogen. The precipitated protein was then removed by filtering the extract through a small wad of glass wool placed in a micropipette tip (1ml capacity). The precipitated protein adhered to the glass wool, and was stored at -20°C for future use. The protein-free, crude lipid extract was filtered into a fresh glass centrifuge tube. To this extract, 800 µl of distilled water were added. Solvent and water were mixed, then allowed to separate into two phases. The upper, aqueous phase was discarded. To the solvent phase, approximately 300 µl of "upper phase solvent" were gently added, then removed. This was repeated 3 times, to wash the interface. Since the phospholipid extract now had a little "upper phase solvent" on the top, enough methanol was added to return the extract to one phase. The final washed extract was then blow-dried with a jet of nitrogen, and the dried pellet stored in a sealed tube under nitrogen at -20°C.

(2) Two-dimensional thin-layer chromatography (TLC) of phospholipids.

The method used was based on that described by Yavin and Zutra (1977).

(i) Materials: 3MM filter paper - Whatman Separation Ltd.
20 x 20cm TLC plastic sheets with 0.2 mm thick silica gel coating - Camlab Ltd.
3 Chromatography tanks - Camlab Ltd.

Solvents (Analar grade: May and Baker Ltd.)

1st Dimension solvent:

Chloroform: methanol: 40% methylamine, 13:6:1.5.

Intermediate solvent:

Diethyl ether: acetic acid, 19:1.

2nd Dimension solvent:

Chloroform: acetone: methanol: acetic acid: water, 10:4:2:3:1.

Phospholipid standards: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) were all obtained from Sigma.

(ii) Procedure

The three TLC tanks (for 1st, intermediate and 2nd dimensions) were lined at each end with 3MM filter paper as wicks. The appropriate solvent mixtures were added to the tanks (approximately 80 ml). The lids were greased well with vacuum grease, and the tanks left to equilibrate for 2 hours.

The TLC plates were baked for 2 hours at 60°C. They were then cut into 10cm x 10cm squares, pre-run in the 1st dimension tank for 25 minutes, and hot-air-dried before use. The origin was marked lightly in pencil at one corner, 2 cm from each edge of the plate (see figure 2.4).

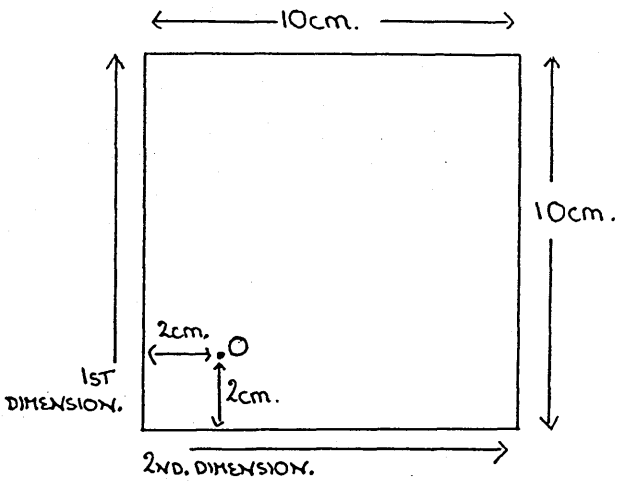


FIGURE 2.4 TLC PLATE: POSITION OF ORIGIN, AND ORIENTATION FOR CHROMATOGRAPHY IN TWO DIMENSIONS.

Samples were removed from -20°C, and allowed to warm to room temperature for over an hour. Each sample was dissolved in 100 µl chloroform: methanol, 2:1. Samples were applied in small spots to the origin of each plate with a 50µl capillary tube, and dried with hot air between applications. Plates with standards were run at the same time as the samples.

The plates were placed in the 1st dimension tank, with the origin on the bottom left hand side. Chromatograms were run until the solvent front was 1 cm from the top. The chromatograms were removed, and dried thoroughly with hot air.

The TLC plates were then placed over glass dishes filled with concentrated hydrochloric acid. The conc. HCl surface was 1 cm below the plates, so that the chromatograms were exposed to the acid fumes. After 5 minutes of exposure, the chromatograms were removed, and hot-air-dried to clear the acid fumes.

When all traces of HCl had been removed, the chromatograms were placed in the intermediate tank. This time, the plates were placed in the 2nd direction, with the origin at the bottom right hand side.

Chromatograms were run for 15 minutes, and dried thoroughly.

Finally, the chromatograms were placed in the 2nd dimension tank, again with the origin at the right hand side, and run until the solvent front reached 1cm from the top.

After hot-air-drying as before, the chromatograms were exposed to iodine vapour to stain the phospholipid spots. These were circled in pencil. The chromatograms with standards were stored for reference, and the chromatograms with radioactive phospholipids exposed to X-ray film as described in 2.5.2.(2).

(3) Quantifying incorporation of ^{32}P into each phospholipid class.

After exposure to X-ray film for 3 to 6 days, the positions of radioactive phospholipids on the TLC plates were clear. (In most cases, there was not enough material to detect by iodine staining). The different phospholipid classes were identified by comparison with migration of the phospholipid standards. The areas of silica gel representing the different phospholipids were outlined, scraped from the plate, and transferred to separate scintillation vials. 4.5 ml of Ecoscint were added, mixed thoroughly, and the vials counted for 4 minutes on a Beckman LS335 scintillation counter.

2.6 Analysis of antigen expression by schistosomula.

2.6.1. Surface iodination.

A modification of the Iodogen iodination method described by Markwell and Fox (1978) was used.

(1) Materials

$Na^{125}I$ (carrier-free) was supplied by the Western Infirmary, Glasgow.

Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) was supplied by Sigma.

(2) Procedure

(i) Preparation of Iodogen vials.

1mg Iodogen was dissolved in 5ml chloroform. 100µl aliquots were dispensed into small glass vials. The vials were rotated while the chloroform was blown off gently with N_2 gas or a blow-drier, until the bottom of each vial was coated with a fine white film of Iodogen. The vials were either used immediately or stored in a dessicator at 4°C.

ii) Iodination procedure.

Before the iodination was performed, 50µl of 0.5M sodium phosphate, pH 7.4, and 2µl of 10mM potassium iodide were added to the Iodogen tube.

3 000-5 000 schistosomula were removed from culture at the required time, washed 5 times in warm medium to ensure that all FCS was removed, and introduced into the Iodogen vial in 200 µl of medium. 100 µCi of $Na^{125}I$ were added to the vial, and the schistosomula allowed to stand for 10 minutes at room temperature with occasional mixing. After this time, reaction was stopped by removing the schistosomula from the Iodogen vial to a 13.5 ml centrifuge tube. The parasites were washed 7 times by centrifugation at approximately 500g for 1 minute in 10 ml of

GMEM, to remove unreacted ^{125}I, then either resuspended to the volume required for further culture, or frozen as a pellet.

2.6.2. Culturing iodinated parasites with mouse peritoneal macrophages.

Methods for obtaining and culturing mouse peritoneal macrophages were obtained from Stuart et al (1978).

0.5 ml of hiFCS were injected intraperitoneally in several Parkes mice to induce production of macrophages. (Parkes mice were bred and maintained in the departmental Animal House). 3 to 4 days after injection, each mouse was killed by asphyxiation, immersed in 70% methanol, and removed to the sterile hood. The abdominal skin was reflected, and 2ml of warm Elac/10% hiFCS, containing 10 I.U. heparin/ml, were injected into the peritoneum at midline, using a 25G (orange) needle. The medium was washed through the peritoneum by bending and straightening the mouse. A 21G (green) needle on a 2ml syringe was inserted behind the spleen, with the eye of the needle facing the peritoneum. The syringe was pulled out and down to make a pocket for the fluid to collect in, and the peritoneal exudate was slowly withdrawn into the syringe. The needle was then removed from the syringe, the peritoneal exudate expelled into a sterile universal, and stored on ice to prevent macrophages from adhering to the walls of the vessel.

Peritoneal exudates from 3 mice were pooled, the cells stained with 0.1 mg/litre methylene blue in 1.0% (v/v) acetic acid, and counted in a Neubauer counting chamber. The cells were adjusted to a concentration of 1.5×10^6/ml in Elac/10% hiFCS, dispensed into wells of a 24-well culture plate at 1ml per well, and incubated at 37°C/5% CO_2.

After 3 to 4 hours of incubation, non-adherent cells were removed,

leaving the macrophages adhering to the culture well. The adherent cells were rinsed 4 times with warm Elac (without serum).

1 000 newly-iodinated schistosomula in 1ml Elac without serum were then added to each well containing macrophages. Cells and parasites were cultured together at 37°C for the required period of time.

At the end of the culture period, schistosomula were carefully decanted off into 13.5 ml centrifuge tubes, and removed to ice. Most of the macrophages remained adhering to the culture well. The macrophages were gently rinsed 4 to 5 times in warm medium, and checked under the microscope to make sure that all schistosomula had been removed. 1ml of ice-cold medium was then added to each culture well, protease inhibitors added (section 2.4.3.), and the culture plate removed to ice for 20 minutes.

Meanwhile, the schistosomula were washed 5 times in ice-cold Elac. Microscopic examination showed that a few macrophages were still present at the end of the washes. The schistosomula were frozen as a pellet at -20°C.

After their incubation on ice, macrophages were dislodged from the wells using a small glass rod, transferred to 1.5 ml polytubes and stored on ice. The cells were washed 3 times in ice-cold medium by centrifugation at high speed in a bench centrifuge for 10 minutes at 4°C, and stored at -20°C as a pellet.

2.6.3. Fluorescence studies of the parasite surface.

2.6.3.1. Immunofluorescence studies.

(1) Reagents

(i) General:

Fluorescein-conjugated goat anti-rabbit IgG (whole molecule)

Fluorescein-conjugated goat anti-mouse IgG (whole molecule)

Fluorescein-conjugated goat anti-human polyvalent immunoglobulins (IgG, IgA, IgM).

These FITC-conjugated second antibodies were all obtained from Sigma.

(ii) Preparation of antisera.

(a) Rabbit anti-coat 1 and anti-coat 2 antisera.

Preparation of coat 1 and coat 2 fractions of the cercarial glycocalyx was performed by Leda Vieira as described by Vieira et al (1986). Briefly, the procedure was as follows.

Cercariae were cooled on ice for 20 minutes, and centrifuged at 240g for 2 minutes at 4°C. Packed cercariae were resuspended with 0.5 to 1.0 ml ice-cold Elac, transferred to 15ml glass conical tubes, agitated for 45 seconds on a Vortex mixer, and centrifuged for 15 to 30 seconds at low speed. Supernatants were decanted, re-centrifuged at 240g, collected, frozen, and lyophilized, to await further extraction. The material obtained at this stage was a crude extract of coat 1.

Cercarial bodies and tails were resuspended in 1 ml of Elac, and added to a 15ml glass conical tube containing 6ml of Elac. After standing for 7 minutes, the tail-rich supernatant was decanted, the bodies washed twice in 6 ml of Elac and transferred to a 15ml vial. The cercarial bodies (maximum 200 000 per vial in 5 ml Elac) were incubated with continual agitation at 37°C, for 90 minutes. After this period, the supernatant fluid was collected, centrifuged at 240g to remove particulate matter, frozen, and lyophilized to await further extraction. This material was a crude extract of coat 2.

The crude extracts of coat 1 and coat 2 were each resuspended in small volumes of distilled water, dialysed against distilled water for 60 hours, and lyophilized again. The extracts were then each extracted

3 times with PBS by homogenization, and centrifuged at 12 000 g for 15 minutes. The supernatants were collected, and the pellets re-extracted twice. Combined supernatants of each type were prepared separately, and sterilized by filtration to give the final coat 1 and coat 2 material.

Injection of these fractions in rabbits (see (d) below) gave anti-coat 1 and anti-coat 2 antisera.

(b) Rabbit anti-cercarial membrane antigen (anti-CMAG).

Cercariae were transformed mechanically by syringe passage (section 2.3.2.(1)), and incubated for 90 minutes at 37°C in GMEM. Parasite bodies and tails were removed by centrifugation, and the supernatant dialysed and freeze-dried. The lyophilized material, described as "cercarial membrane antigen" (CMAG), should thus contain a mixture of the coat 1 and coat 2 fractions described in (a).

Anti-CMAG antiserum was produced by immunising a rabbit with this material (see (d) below).

(c) Rabbit anti-snail haemolymph.

Snail haemolymph was obtained by bleeding uninfected Biomphalaria glabrata by heart puncture. The haemolymph was centrifuged at 1 000g at 4°C for 1 hour to remove large tissue debris.

Once again, specific antiserum was obtained by immunisation of a New Zealand rabbit (see (d) below).

(d) Production of rabbit antisera.

Rabbit anti-coat 1, anti-coat 2, anti-CMAG and anti-snail haemolymph antisera were produced in New Zealand rabbits, maintained in the departmental Animal House. Immunisation procedures were as

described by Johnstone and Thorpe (1985). Before immunisation, rabbits were bled from the ear, in order to obtain their pre-immunisation sera (normal rabbit serum, NRS). The rabbits then received, by subcutaneous injection, 500 mg of protein of the antigens (protein determined by Lowry method, section 2.5.1.(2)(i)), in 1 ml of saline with 1 ml of Freund's Complete Adjuvant (Difco Labs). After 10 days, the rabbits were boosted with the same amount of antigen in Freund's Incomplete Adjuvant (Difco Labs), and bled from the ear 2 weeks after boosting.

(e) Infected human serum (IHS).

Infected human serum, obtained from infected Brazilian patients, was a gift from Professor G. Gazzinelli, Centro de Pesquisas René Rachou, Belo Horizonte, M. G. Brazil.

The normal human serum used as a control was prepared from out-of-date human blood, obtained from Law Hospital, as described in section 2.3.1.(3).

(f) Monoclonal antibodies.

A3: A3 monoclonal antibody was a gift from S.R. Smithers, NIMR, Mill Hill, London.

M7B3A: This mouse IgG3 monoclonal was described by Bickle et al (1986). It recognises a schistosomulum surface antigen of molecular weight 16 000 and confers 28-70% immunity to mice by passive transfer.

M7B3A was a gift from Q. Bickle.

FITC-conjugated goat anti-mouse IgG (whole molecule) was used as second antibody in studies with the A3 and M7B3A monoclonals.

Normal mouse serum (NMS) obtained by pooling sera from several uninfected BALB/c mice was used as a control in experiments involving

mouse monoclonal antibody labelling.

(2) Procedure for immunofluorescence.

(i) Immobilisation of parasites.

Cercariae and schistosomula were immobilised in one of two ways, either by formaldehyde-fixation before labelling began, or by treatment with carbamyl choline ("carbachol"; obtained from Sigma), after labelling and washing were finished.

For fixation with formaldehyde, schistosomula were removed from culture at the required time, washed 4 to 5 times by centrifugation at 500g for 1 minute, to remove all traces of FCS, and resuspended to 10 ml GMEM. Formaldehyde was added to give a final concentration of 0.1%. The schistosomula were incubated in the presence of formaldehyde for 15 minutes at 37°C, with occasional agitation. They were then washed by centrifugation 5 times in cold GMEM, and resuspended to the volume required for individual experiments.

Alternatively, after fluorescent labelling and washing were complete, each preparation was resuspended to the required volume in GMEM, and carbachol solution made up in GMEM was added to give a final concentration of 1mg/ml. The labelled parasites were examined under the fluorescent microscope in the presence of this immobilising agent.

(ii) Protocol for immunofluorescence.

Fixed or live schistosomula were incubated for 1 hour at 37°C, in a total volume of 200 µl GMEM, in conical, plastic centrifuge tubes, in the presence of antiserum or control serum at a final dilution of 1/10, or monoclonal antibody at a dilution of 1/2. After 3 washes in 10 ml of warm GMEM, the parasites were resuspended to 200 µl of medium in the

presence of the appropriate fluorescein-conjugated second antibody at a final concentration of 1/40. After incubation for 40 minutes at 37°C in the dark, the parasites were again washed 3 times in warm GMEM, and resuspended to 20 to 100 µl, the volume depending on the number of schistosomula present. Carbachol was added if necessary, and 10-20 µl of the final suspension were mounted on microscope slides under coverslips supported on small ridges of silicone grease.

When the labelling experiment included cercariae, whether live or formaldehyde-fixed, it was thought advisable to perform all incubations at room temperature rather than at 37°C. Incubation with first antibody proceeded for 90 minutes, and with second antibody for 1 hour, with continual agitation. For live cercariae, aquarium water, rather than GMEM, was used for labelling and washing.

The parasites were examined with a Leitz Ortholux microscope, and the intensity of fluorescence quantitated using a Leitz MPV compact attachment. A blue filter was used to measure FITC fluorescence. The average background reading from each slide was subtracted from the readings taken from the parasites.

(3) Detection of blood group antigens on the schistosomulum surface by immunofluorescence.

Two mouse monoclonal antibodies, specific for human blood group A and B antigens, respectively, were obtained from the National Blood Transfusion Service, via the Western Infirmary, Glasgow.

Mechanically-transformed schistosomula were cultured for 72 hours in 10 ml Elac/50% A^+ normal human plasma/1% A^+ washed, packed human red blood cells at 37°C/5% CO_2 (culture conditions described by Clegg, 1965). At the end of this culture period, schistosomula were washed by repeated slow centrifugation (300g for 1 minute), and sedimentation, until examination under the light microscope showed that

nearly all the red blood cells had been removed.

The schistosomula were then incubated in the presence of anti-A or anti-B (control) monoclonals at 1/2 final concentration, washed, and labelled with fluorescein-conjugated anti-mouse IgG (whole molecule) at 1/40 final concentration, according to the protocol outlined in (2)(ii) above. After the final wash, carbachol was added, and fluorescence intensity measured as usual.

(4) Expression of carbohydrate and polypeptide epitopes by normal and attenuated schistosomula. Detection by periodate-treatment and immunofluorescence

(see chapter 7).

(i) Antisera

The sera used in these immunofluorescence studies were obtained from the NIH and CBA/Ca mice used in the protection experiments described in chapter 9. The immunisation and infection protocols to which these mice were subjected are outlined in table 2.13. Mice were bled from the eye vein (by Dr. Janet Jones) at the end of the protection experiment (section 2.8 (4)).

The fluorescein-conjugated second antibody for these protection experiments was FITC-rabbit anti-mouse IgG (whole molecule), obtained from Sigma.

(ii) Periodate-treatment and addition of competing sugars.

Sodium meta -periodate was obtained from Sigma. The procedure for periodate-treatment of schistosomula was adapted from the methods described by Omer-ali et al (1986) and Boswell et al (1987).

Schistosomula were formaldehyde-fixed, and washed thoroughly before treatment (see section 2.6.3.1.(2)(i)). Each sample of

Table 2.13 Source of sera for immunofluorescence assays distinguishing carbohydrate and polypeptide epitopes.

Table 2.13.

Serum obtained from	Mouse strain(s)	Initial Infection or Immunisation	Challenge (6 weeks after initial infection)	Perfusion and bleeding (5-6 weeks after challenge)	Abbreviation for serum.
chronically-infected mice	NIH	150 normal cercariae p.c.	—	✓	CMS
mice immunised with U.V.-irradiated cercariae	NIH	— 500 U.V.-irradiated (400 μW min cm^{-2}) cercariae p.c.	—	✓	UVMS
mice immunised with gamma-irradiated cercariae	NIH	— 500 gamma-irradiated (20 krad) cercariae p.c.	—	✓	GMS
mice immunised with Actinomycin D-treated schistosomula	NIH and CBA/Ca	— 500 Actinomycin D-treated schistosomula s.c.	—	✓	AMS
normally infected and challenged mice	NIH and CBA/Ca	150 normal cercariae p.c.	100-150 normal cercariae p.c.	✓	N/C
mice immunised with Actinomycin D-treated sla. and challenged	NIH and CBA/Ca	— 500 Actinomycin D-treated schistosomula s.c.	100-150 normal cercariae p.c.	✓	A/C
normal mouse serum	NIH	Obtained from a pool of uninfected NIH mice. This serum was also used as the control in experiments with CBA/Ca serum.			NMS

schistosomula was then divided in two. One lot was treated with 10 ml of a freshly made-up solution of 0.05M sodium periodate in 0.05M sodium acetate, pH 5.8. The second batch of schistosomula received only 0.05M sodium acetate, pH 5.8. The parasites were incubated for 1 hour in the dark at room temperature. The reaction was stopped by adding glycerol (stock solution made up in PBS) to give a final concentration of 0.1M. After incubation in the presence of glycerol in the dark for 20 minutes, the schistosomula were pelleted by centrifugation, and washed 5 times in ice-cold GMEM.

In some experiments, immunofluorescence proceeded as usual, immediately after periodate-treatment and washing. In others, both the periodate-treated and untreated samples were divided into two aliquots of 50µl, each containing approximately equal numbers of schistosomula. To one member of each pair, 100 µl of GMEM containing methyl α-D-manno-pyranoside, D(+)-galactose and N-acetyl-D-glucosamine (all from Sigma), each at a concentration of 0.4M, were added. The other member of each pair received 100 µl of GMEM only. After mixing, 50 µl of the appropriate mouse serum was added, made up in GMEM to give a concentration of 1/10 in the final 200 µl volume.

After incubation for 1 hour at 37°C, the parasites were washed 3 times in GMEM. FITC-conjugated rabbit anti-mouse IgG (whole molecule) was added to 1/40 final concentration, and the remaining steps in the immunofluorescence protocol proceeded as described above.

Figure 2.5 outlines these procedures in the form of a flow-chart.

2.6.3.2. Fluorescent lectin binding studies.

(1) Reagents

The fluorescein isothiocyanate-conjugated lectins used, and their

Figure 2.5. Distinguishing between carbohydrate and polypeptide epitopes on the schistosomular surface by periodate (PI) treatment, addition of competing sugars, and immunofluorescence.

Formaldehyde-fixed schistosomula.

↓

Divide in two

(1)	(2)
Non PI-treated:	PI-treated
0.05M sodium acetate, pH5.8	0.005M sodium periodate/ 0.05M sodium acetate, pH5.8.

Incubate 1 hour in the dark at room temp.

Stop reaction with 0.1M glycerol.

Wash 5x in cold GMEM ——→ (a) Immunofluorescence as usual.

OR ↓

(b)

Addition of competing sugars.

(1)	(2)
Non PI-treated	PI-treated

Divide each group in 2:

1A	1B	2A	2B
+ sugars	- sugars	+ sugars	- sugars

Add: i) 50 µl of schistosomula to each.

ii) 100 µl of 0.4M competing sugars - to 1A, 2A.

OR 100 µl of GMEM - - to 1B, 2B.

iii) 50 µl of serum in GMEM (1/10 final dilution) to each.

↓

Incubate 1h at 37°C

↓

Wash 3x in GMEM

↓

Add FITC-second antibody as usual.

Fluorescein-conjugated lectin from	Common name	Abbreviation	Sugar specificities (in order of decreasing affinity).
Arachis hypogea	Peanut agglutinin	PNA	D(+)-Galactose
Canavalia ensiformis	Concanavalin A	Con A	(1) Methyl-α-D-mannose, (2) D-(+)-mannose (3) N-Acetyl-D-glucosamine, and D-(+)-glucose.
Triticum vulgaris	Wheatgerm agglutinin	WGA	(1) N,N',N"-Acetylchitotriose (Goldstein et al, 1975). (2) N-Acetyl-D-glucosamine
Tetragonolobus purpureas	Fucose-binding protein	FBP	L(+)-Fucose
Ulex europaeus	Fucose-binding protein	FBP	L(+)-Fucose

Table 2.14. Fluorescein-conjugated lectins and their specificities.

specificities, are shown in table 2.14.

All the fluorescent lectins were obtained from Sigma. Unless otherwise stated, the sugar specificities assigned to each lectin are those listed on the Sigma information sheet accompanying each lectin.

The following inhibitory sugars, all obtained from Sigma, were used to inhibit lectin binding:

Lectin	Inhibitory sugar(s)
Con A	(1) Methyl-α-D-mannopyranoside
	(2) N-acetyl-D-glucosamine
FBP, UEA	L(+)-fucose.
PNA	D(+)-galactose
WGA	(1) N, N', N"-acetylchitotriose
	(2) N-acetyl-D-glucosamine.

Each inhibitory sugar was made up at a concentration of 0.4M in GMEM, and used at a final concentration of 0.2M.

(2) Protocol

The protocol for fluorescent lectin binding was based on the methods outlined by Simpson et al (1983a) and Murrell et al (1978).

As for immunofluorescence studies (section 2.6.3.1.), parasites were immobilised either by formaldehyde-fixation before lectin binding, or by addition of carbachol after lectin binding and washing.

Each sample of schistosomula was divided into 2 100 µl aliquots in GMEM, and placed in conical plastic centrifuge tubes. To half of the schistosomula, 200 µl of the appropriate inhibitory sugar (stock concentration 0.4M in GMEM) were added. The second lot of schistosomula received 200 µl of GMEM only. After mixing, 100 µl of fluorescein-

conjugated lectin (stock concentration 200 µg/ml in GMEM) were added to both samples. The final suspensions of schistosomula were thus 400 µl in volume, containing fluorescent lectin at 50 µg/ml, with or without 0.2M inhibitory sugar.

The parasites were incubated in the dark at 37°C for 1 hour, with occasional agitation. After 3 washes in GMEM, they were resuspended to between 20 and 60 µl, treated with carbachol if necessary, and examined by fluorescence microscopy as described in 2.6.3.1.

(3) Fluorescent lectin binding after neuraminidase-treatment of schistosomula.

The possibility of sialic acid being present on the parasite surface was investigated by testing for increased exposure of galactose residues (assessed by FITC-PNA binding) after neuraminidase-treatment of schistosomula. Neuraminidase-treatment was performed as described by Simpson et al (1983a). Formaldehyde-fixed schistosomula were incubated for 2 hours at room temperature with neuraminidase from Vibrio cholerae (Sigma) at 0.1 I.U./ml in GMEM. The schistosomula were then washed 5 times in ice-cold GMEM, and binding of FITC-PNA assessed as usual.

2.6.3.3. Binding of 6-iodoacetamidofluorescein (6-IAF).

Exposure of SH groups by cercariae and schistosomula was examined by measuring the binding of 6-IAF (obtained from Molecular Probes Inc.) to the parasites.

Cercariae and schistosomula were formaldehyde-fixed before labelling. A saturated solution of 6-IAF in 0.01N NaOH was made up, and filtered several times to remove undissolved chemical. 20µl of this solution was added to the formaldehyde-fixed parasites, suspended in

200µl of GMEM in conical plastic centrifuge tubes. After incubation for 1 hour in the dark at room temperature with continual agitation, the parasites were washed 5 times by centrifugation in GMEM. They were resuspended to approximately 100µl of GMEM, and fluorescence measured as usual.

2.6.3.4. Measurement of surface damage by uptake of Hoechst 33258.

Hoechst 33258 (H33258; Sigma) shows increased fluorescence in mammalian cells when their plasma membranes are damaged. High fluorescence values in schistosomula are taken to indicate that the surface membrane is damaged (see Jones et al, 1988).

Schistosomula were incubated in the dark at 37°C in 200µl GMEM containing Hoechst 33258 at a final concentration of 1µg/ml, for 20 minutes. The parasites were then washed 3 times in warm GMEM, and reduced to a volume of 100µl or less. Fluorescence was quantitated in the usual way, but with an ultra-violet filter in place of the blue one used to measure FITC.

2.6.3.5. Statistical analysis of fluorescence results.

In all these studies investigating binding of fluorescent reagents to the parasite surface, Student's two-sample t-test was used to compare the mean fluorescence measurements obtained for normal and attenuated (irradiated or Actinomycin D-treated) parasites.

The Mann Whitney U-test was also sometimes used to compare the median values of the different groups. However, in all the cases examined, both tests gave the same significance levels. Only the t-test results are therefore reported.

2.6.4. Immunoblotting

Immunoblotting with the rabbit antisera described in section 2.6.3.1.(ii) was performed using iodinated protein A and autoradiography to detect antibody binding, as described by Johnstone and Thorpe (1985).

(1) Iodination of protein A

Protein A was iodinated by the Iodogen method, based on the procedures described by Markwell and Fox (1978) and Johnstone and Thorpe (1985).

(i) Materials

Protein A from Staphylococcus aureus was obtained from Sigma.
Iodogen and $Na^{125}I$ - see section 2.6.1.

Phosphate Buffered Saline (PBS) - for composition, see section 2.5.1.(2)(i).

PD-10 columns, pre-packed with Sephadex G-25M. - obtained from Pharmacia.

(ii) Procedure

Iodogen vials were prepared as described in section 2.6.1.

50 µl of sodium phosphate, pH 7.4, and 2µl of 10mM potassium iodide were added to an Iodogen vial, followed by 200µl of protein A at a concentration of 1mg/ml in PBS. 200 µCi of $Na^{125}I$ were dispensed into the tube, and the reaction allowed to proceed for 10 minutes at

room temperature. The mixture was then removed from the Iodogen vial, and applied to the top of a PD-10 de-salting column. The column had previously been blocked with 100µl of 10% (w/v) BSA in PBS, washed through with 25 ml of PBS. The mixture was allowed to enter the gel, and the eluant discarded. The iodinated protein was then separated from free iodide by applying 7 0.5 ml aliquots of PBS directly to the top of the Sephadex bed, allowing each aliquot to enter the gel before applying the next. Each 0.5ml of eluant was collected in a separate polystyrene tube. The iodinated protein was normally contained in fractions 4 to 6. This was confirmed with the gamma monitor, the fractions pooled, and incorporation of radioactivity into protein determined by counting 3 10µl aliquots in a gamma counter. The iodinated protein A was divided into aliquots containing 2 x 10^6 cpm, and stored under a lead shield at -20°C.

(2) Protocol for immunoblotting SDS-gels with ^{125}I-protein A.

(i) Materials: Samples and protein standards electrophoresed on an SDS-polyacrylamide gel, as described in 2.5.1.

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

Nitrocellulose sheets, 0.45µm pore-size - from Anderman and Co Ltd.

3MM filter paper - Whatman.

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

0.02% (w/v) SDS

pH was 8.3

Incubation/ washing buffer:	3% (v/v) hiFCS in 20mM Tris-HCl, pH 7.2.
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.

(ii) Protocol

2 pieces of filter paper and 1 piece of nitrocellulose were cut to the size of the immunoblotting cassette. Scotchbrite pads, filter paper and nitrocellulose were all soaked thoroughly in a tray containing transfer buffer. A Scotchbrite pad was placed on one half of the opened cassette, and a piece of filter paper laid on top. The SDS-polyacrylamide gel, removed from its glass plates immediately after electrophoresis, was placed on top of this first sheet of filter paper, and covered with the nitrocellulose sheet, expelling any air bubbles. Using a razor blade, the nitrocellulose sheet was cut to exactly the size of the gel beneath it, and notches cut to indicate the orientation of the gel and position of protein standards. 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 with rubber bands.

The immunoblotting tank was filled with transfer buffer, and the whole cassette slotted into place, with the gel facing the cathode, and the nitrocellulose facing the anode. Figure 2.6 illustrates the set-up.

Electrophoresis was carried out for 3 to 4 hours at 400mA constant current, or for 16 hours at 40 mA.

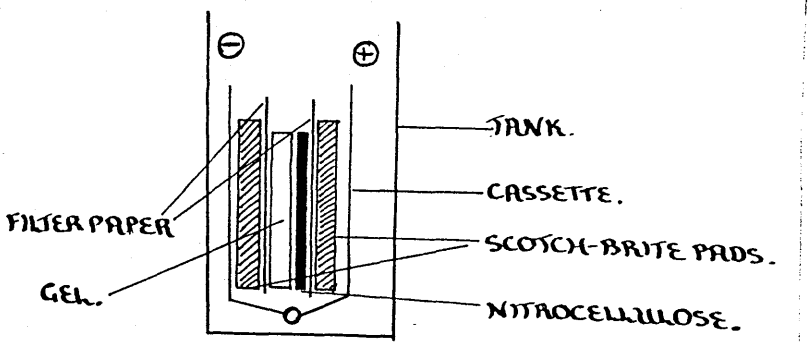


FIGURE 2.6. ELECTROBLOT TRANSFER SYSTEM.

The gel and nitrocellulose sheet were then removed from the cassette. The gel was stained with Kenacid Blue to check for transfer efficiency. 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. This procedure allowed a second assessment of transfer efficiency, and also permitted later determination of the molecular weights of proteins of interest.

The rest of the nitrocellulose sheet was incubated on a gel shaker for 2 hours at room temperature in 200 ml of incubation buffer. This buffer was then discarded, and the nitrocellulose immersed in 50 ml of incubation buffer, containing 1-2 ml of rabbit antiserum, or normal rabbit serum. The nitrocellulose was incubated in this solution for 2 hours at room temperature, with shaking. This was followed by 2 5-10 minute washes in 200 ml incubation buffer. The nitrocellulose was then incubated for 2 hours at room temperature, with shaking, in 60 ml incubation buffer containing 2×10^6 cpm of ^{125}I-protein A. Finally, the nitrocellulose was given 6 to 8 washes (5 to 10 minutes each) in 20mM Tris-HCl, pH 7.2, until the radioactivity in washings returned to

background level (indicated by a gamma monitor). The nitrocellulose was allowed to dry at room temperature between 2 sheets of filter paper, then exposed to X-ray film as described in section 2.5.2.(2).

Two controls for non-specific antibody binding were always included in immunoblotting studies. Firstly, a duplicate gel transferred to nitrocellulose was always incubated with normal rabbit serum in place of anti-schistosome antiserum. Secondly, protein standards were always included in the gel to highlight antibody binding to non-schistosome proteins.

2.6.5. Electron microscopy

(1) Materials

sodium cacodylate, glutaraldehyde	from BDH Chemicals
osmium tetroxide	from Sigma.

(2) Procedure

For electron microscopy of the schistosomular surface, specimens were exposed to 3 consecutive fixatives, according to the system described by Hockley and McLaren (1973). The 3 fixatives were:

(i) 2% (v/v) glutaraldehyde in the cacodylate buffer system of Lewis and Shute (1966), consisting of 0.05M sodium cacodylate/0.002M calcium acetate, pH 7.4. Glutaraldehyde fixation proceeded for 4 hours at 4°C, followed by 3 washes in ice-cold Lewis/Shute buffer.

(ii) 1% (w/v) osmium tetroxide in Lewis/Shute buffer for 2 hours at 4°C, followed by 10 washes in ice-cold distilled, de-ionized water.

(iii) A 0.5% aqueous solution of uranyl acetate, pH 5.0, containing

45 mg/ml sucrose, for 1.5 hours at 4°C in total darkness (system developed by de Harven, 1967). After 3 washes in cold, distilled, de-ionized water, the specimens were dehydrated by a series of incubations in increasing concentrations of ethanol, as follows:

(1) 25% ethanol for 10 minutes.

(2) 50% ethanol for 10 minutes.

(3) 75% ethanol for 10 minutes.

The specimens were then resuspended in 100% ethanol, and taken to the Electron Microscopy Unit in the Western Infirmary, Glasgow. Here, they were embedded in Araldite, sections cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined under the electron microscope. I am grateful to T. Downie of the Electron Microscopy Unit for his expertise in sectioning and examining the specimens at this stage.

2.6.6. ELISA assays (with periodate-treatment).

(1) Materials

(i) Antisera

The sera used for ELISA assays were obtained from the NIH mice used in the protection experiments described in chapter 9. The immunisation/infection procedures to which the serum donors were subjected are outlined in table 2.15. Mice were bled from the eye vein (by Dr. Janet Jones) 5 to 6 weeks after challenge infection.

(ii) Second and third antibodies.

These were obtained from Sigma:

Serum obtained from	Initial infection/ immunisation (p.c.)	Challenge (p.c.) (6 weeks after initial infection)	Perfusion and bleeding (5-6 weeks after challenge)	Abbreviation for serum.
mice receiving challenge only	/	150 normal cercariae	✓	Ch
mice immunised with gamma-irradiated cercariae	500 gamma-irradiated (20 krad) cercariae	/	✓	G
mice immunised with gamma-irradiated cercariae, then challenged.	500 gamma-irradiated (20 krad) cercariae	150 normal cercariae	✓	G/C
mice immunised with U.V.-irradiated cercariae	500 U.V.-irradiated (400 μW min cm^{-2}) cercariae	/	✓	U
mice immunised with U.V.-irradiated cercariae, then challenged	500 U.V.-irradiated (400 μW min cm^{-2}) cercariae	150 normal cercariae	✓	U/C
normal mice	serum obtained from a pool of uninfected, unimmunised NIH mice.			NMS

Table 2.15. Sources of the sera for ELISA assays. All mice were of the NIH strain.

Second antibodies

Goat anti-mouse IgM (μ-chain specific)

Goat anti-mouse IgG1 (heavy-chain specific)

Goat anti-mouse IgG2a (heavy-chain specific)

Goat anti-mouse IgG2b (heavy-chain specific)

Goat anti-mouse IgG3 (heavy-chain specific)

Third antibody

Horseradish peroxidase-conjugated rabbit anti-goat IgG (whole molecule).

(iii) Other materials

Microelisa plates - from Dynatech.

Phosphate-buffered saline (PBS) - see section 2.5.1.(2)(i).

BSA (bovine serum albumin) } all from Sigma

Polyoxyethylene sorbitan monolaurate (=Tween 20) } all from Sigma

Sodium *meta* -periodate } all from Sigma

Normal goat serum - from Scottish Antibody Production Unit (SAPU)

Substrate for horseradish peroxidase:

For 1 ELISA plate: require

4mg *ortho* -phenylene diamine (OPD) - from Sigma - dissolved in 10 ml freshly made-up citrate phosphate buffer, plus 33 μl of 30% H_2O_2 (from Sigma).

Citrate phosphate buffer: 17.9 ml 0.1M citric acid plus

32.1 ml 0.2M Na_2HPO_4

pH'd to 6.0; diluted to 100 ml with H_2O.

(2) Procedure

The protocol for these ELISA assays was based on that described by Johnstone and Thorpe (1985), modified by periodate-treatment and by the use of an intermediate antibody layer before the enzyme-conjugated third antibody was added.

(i) Preparation of antigen, and coating the plates.

Cercariae were transformed mechanically, and cultured for 3 hours in GMEM at 37°C. They were then washed 3 times in ice-cold PBS, resuspended to 1ml cold PBS, protease inhibitors added (section 2.4.3.), and homogenized on ice until a non-particulate suspension was obtained.

Aliquots of the homogenate were used to determine protein concentration by the Lowry method (section 2.5.1.(2)). The homogenate was then resuspended to 2µg protein/ml in 0.05M sodium carbonate, pH 9.6. 100 ul of the antigen solution (ie. 200ng of protein) were dispensed into as many wells of a microelisa plate as required, and incubated overnight at 4°C.

Unbound antigen was then discarded, and the wells washed 3 times with PBS/0.5% (v/v) Tween 20. Any remaining binding sites on the wells were blocked by incubation with 100 µl of PBS/2% (w/v) BSA/3% (v/v) normal goat serum for 1 hour. The blocking solution was then thrown out, and the wells washed 3 times with PBS/0.5% (v/v) Tween 20.

(ii) Periodate-treatment

Duplicate ELISA plates were set up. One plate was treated with periodate as described in figure 2.5. Briefly, each well received 100 µl of 0.05M sodium periodate in 0.05M sodium acetate, pH 5.8. In

the second plate, each well received 100 µl of acetate buffer only. The plates were incubated in the dark for 1 hour at room temperature. 100 µl of 0.2M glycerol made up in PBS were then added to each well, and incubated, in the dark, for a further 20 minutes. The contents of each well were then discarded, and the plates washed 4 times with PBS.

(iii) Antibody binding

Doubling dilutions of antiserum in PBS/0.5% (v/v) Tween 20 were prepared, starting from 1/50. 100 µl of each dilution were added, in duplicate, to separate wells, and incubated at room temperature for 2 hours. The wells were then washed 3 times with PBS/0.5% (v/v) Tween 20.

Depending on the antibody isotype of interest, goat anti-mouse IgM, IgG1, IgG2a, IgG2b or IgG3 was made up at a concentration of 1/100 in PBS/0.05% (v/v) Tween 20. 100 µl of this solution were added to each well, and incubated for 90 minutes at room temperature. The plate was then washed 3 times as in (i).

Finally, a 1/500 dilution of horseradish peroxidase-conjugated rabbit anti-goat IgG (whole molecule) was made up in PBS/0.05% (v/v) Tween 20. 100 µl were added to each well, and incubated for 90 minutes at room temperature. The wells were then washed 3 times as before.

(iv) Developing the plates

100 µl of freshly prepared OPD substrate were added to each well. The plate was developed in the dark for 10 minutes, and the reaction stopped by adding 50 µl of 4N H_2SO_4 to each well. The absorbance of each well was then read at 492 nm in a microelisa reader.

(v) Controls

Each plate contained one row of wells to which no antigen was added, and another row to which no antiserum (first antibody) was added. The other steps in the ELISA procedure were carried out as normal.

2.7 Studies of parasite variability

2.7.1. Production of clones of cercariae.

Clones of parasites were obtained by infecting snails with a single miracidium each. Each miracidium divides asexually within a snail to produce mother and daugher sporocysts, which in turn, develop into mature cercariae. Hence, the parasites derived from a monomiracidial infection should be genetically identical.

Schreiber and Schubert (1949) and Stirewalt (1954) were among the earliest workers to employ this approach to produce clones of parasites.

Miracidia were obtained as described in section 2.1.3. For monomiracidial infections, 24-well culture plates were used (well dimensions 1.5 cm x 1.5 cm). A drop of aquarium water containing a single miracidium was placed in each well, using a Pasteur pipette. The number of miracidia in each well was then checked under a dissecting microscope. Individual snails, 4 to 8 mm diameter, were placed in each well containing a single miracidium, and enough aquarium water added to cover the snail. The infection trays were covered, and the snails left in the infection wells at 25°C overnight. The exposed snails were then pooled, transferred to plastic aquaria, and maintained in the dark as described in section 2.1.3.

6 to 8 weeks after exposure, snails were transferred individually

into glass universals (25 ml capacity), capped with muslin to allow aeration. Each universal was numbered, and the cercariae derived from each snail were described as members of clones 1, 2, 3 or A, B, C, etc. The water in the universals was changed at least twice weekly, and the snails were fed 2 to 3 times per week. Cercariae were obtained by exposing individual snails to light, as described in section 2.1.3. Snails which yielded no cercariae after exposure to light on two to three occasions were regarded as uninfected, and were discarded.

Cloned cercariae from several snails were counted, and resuspended to equal concentrations. They were U.V.-irradiated in 8 ml of aquarium water, transformed, then cultured and metabolically-labelled at equal concentrations, as described for mixed populations.

2.7.2. Radioprotective agents. Manipulation of the metabolic response to irradiation.

(see chapter 8).

Materials: All the following reagents were obtained from Sigma.

(i) Studies on the role of SH groups

L-cysteine (free base, cell culture-tested)

Glutathione (γ-glu-cys-gly) (Free acid, reduced form; cell culture-tested).

L-cysteine ethyl ester (hydrochloride) (cell culture-tested).

N-ethylmaleimide.

(ii) d-α-tocopherol acetate (d-vitamin E acetate).

In different experiments, the parasites were incubated with the protective agents before, during or after U.V.-irradiation. Details of the procedures for individual experiments are provided in chapter 8.

2.7.3. Use of hydrogen peroxide to generate oxygen radicals.

(see section 8.2.3.3.)

Materials: H_2O_2 and ascorbic acid (cell culture-tested) were obtained from Sigma.

$CuSO_4$ and $MgSO_4$ were obtained from BDH Chemicals, and were of maximum purity grade.

Details of the treatment of cercariae are provided in section 8.2.3.3.

2.8. Protection studies in mice.

(1) Mouse strains

NIH/Ola and CBA/Ca/Ola mice were obtained from Herlan Olac Ltd. BALB/c mice were bred and maintained in the Physiology/Biochemistry Animal House, University of Glasgow.

(2) Infection procedures

Mice were infected either percutaneously or subcutaneously. Percutaneous exposure was by the ring method of Smithers and Terry (1965), described in section 2.1.4.

In our protection experiments, the subcutaneous infections always involved schistosomula which had already been cultured for 20 hours in Elac/10% hiFCS at 37°C/5% CO_2, in presence or absence of Actinomycin D (section 2.3.3.(ii)). The schistosomula were transferred to 13.5 ml plastic centrifuge tubes, under sterile conditions. They were then washed 4 times in 10 ml of warm Elac, and resuspended to the required concentration in Elac/2% hiFCS. The required number of schistosomula

was then injected subcutaneously in mice using a 25G (orange) needle. Mice received no anaesthetic for these subcutaneous infections.

(3) Perfusion of mice for recovery of adult worms.

(i) Materials

heparin, 1000 I.U./ml - from Flow Labs.

citrate saline: 0.05M trisodium citrate.
0.145M sodium chloride
(both from Formachem)

Sagatal anaesthetic: May and Baker Ltd.

(ii) Procedure

Mice were perfused 5 to 6 weeks after challenge infection. The perfusion method was essentially that of Smithers and Terry (1965). A lethal dose of undiluted Sagatal (1ml/100g body weight) was administered intraperitoneally. Mice were then bled from the eye vein (by Dr. Janet Jones), yielding 100 to 200 µl of blood. 500 units of heparin were then injected intraperitoneally, to prevent blood clotting during perfusion.

The abdominal and thoracic cavities of the dead animal were opened. Rubber bands were then 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 into a 50 ml conical centrifuge tube positioned directly below the dead mouse.

The worms were allowed to sediment for 5 to 6 minutes, then washed 2 to 3 times with citrate saline. After the final wash, the worms were resuspended to approximately 5 ml, and tipped into a Petri dish. They were counted chiefly by eye, with the assistance of a dissecting microscope when necessary. To ensure maximum accuracy, each worm was transferred to a second Petri dish as soon as it had been counted.

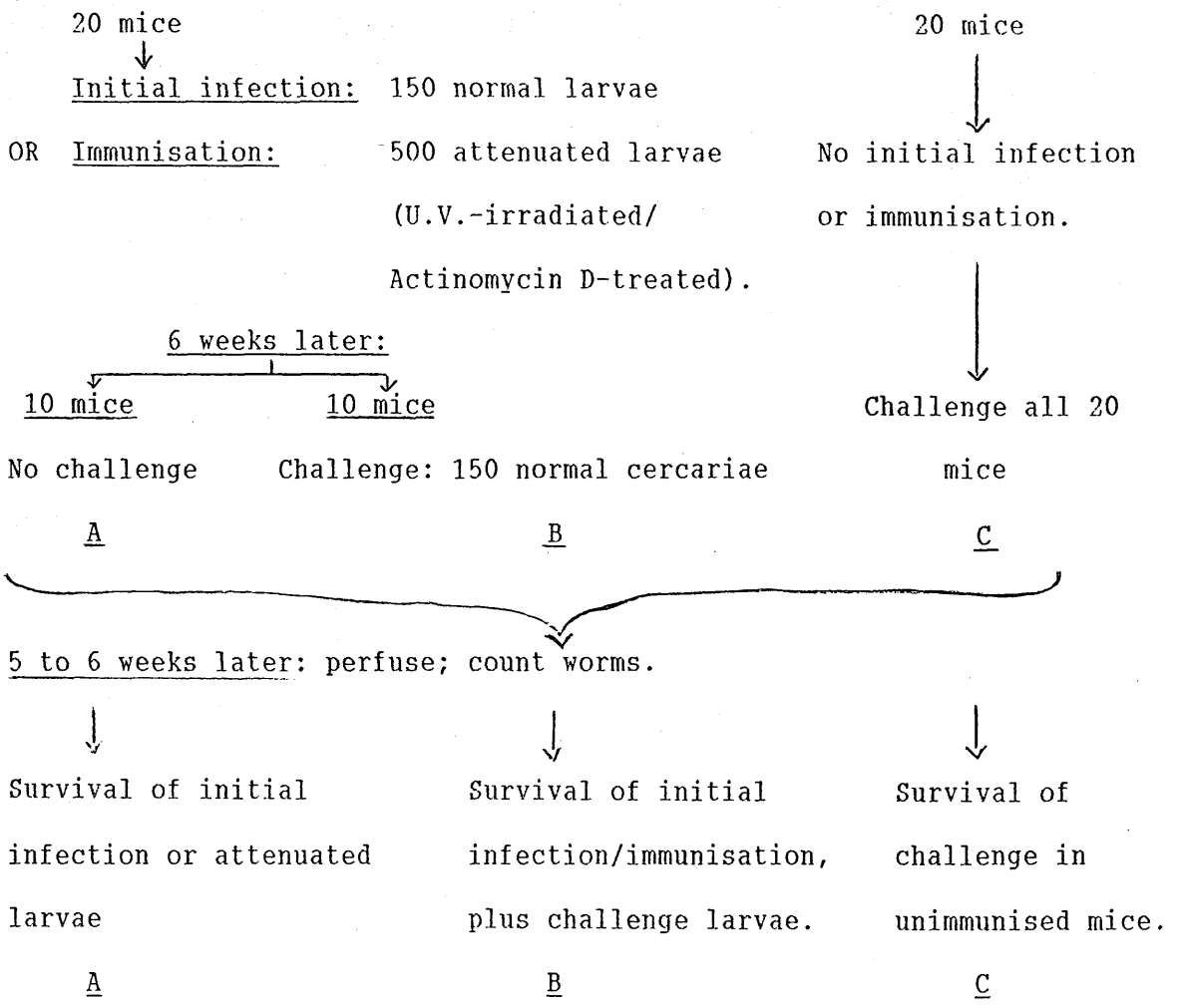
(4) Isolation of mouse serum from blood

Mouse blood was collected into 1.5ml polytubes, each containing a 1.5 cm length of wooden cocktail stick. The blood was allowed to clot around this stick for 1-2 hours at room temperature, then was left overnight at 4°C, causing the clot to contract. The cocktail stick, with the clot adhering to it, was then discarded, and the remaining cells separated from the serum by centrifugation for 2 to 3 minutes at high speed in a microfuge in the 4°C room. The serum was decanted into fresh polytubes, and stored at -20°C.

(5) Determining levels of protection conferred by immunisation.

A group of 10 or 20 8-week old mice was infected with 150 normal larvae, or 500 attenuated larvae (U.V.-irradiated, gamma-irradiated, or Actinomycin D-treated). 6 weeks later, half of each group was challenged percutaneously with 100 or 150 normal cercariae. At the same time, a new group of naive mice received the same percutaneous challenge. 5 to 6 weeks later, worms were recovered by perfusion, and counted. The degree of protection conferred by the initial infection with normal or attenuated larvae was then assessed as follows:

Standard Protocol for Protection Experiments.



The number of worms surviving in immunised hosts (group B) is corrected for the survival of worms from the initial infection (group A):

Mean challenge worm survival in immunised host = (Mean no. of worms/mouse in group B) *minus* (Mean no. of worms/mouse in group A).

$$= \bar{X}_B - \bar{X}_A .$$

The percentage protection against challenge infection afforded by the initial immunisation is then given by:

Continued on next page.

Continued.

$$\frac{\bar{X}_C - (\bar{X}_B - \bar{X}_A)}{\bar{X}_C} \times \frac{100\ \%}{1}$$, i.e.

$$\frac{\text{(Mean no. of challenge worms in unimmunised host) minus (mean no. of challenge worms in immunised host)} \times \frac{100\ \%}{1}}{\text{(Mean no. of challenge worms in unimmunised host)}}$$

Gamma-irradiated and Actinomycin D-treated larvae did not survive to maturity in any of the protection experiments, hence $\bar{X}_A = 0$ in these studies. A few U.V.-irradiated larvae occasionally survived to adulthood, giving an $\bar{X}_A$ of $\ll 1$. When the initial infection was with normal larvae, substantial numbers reached maturity. A concomitant immunity model of this type (group of mice receiving initial normal infection plus challenge) was included in every protection experiment, as a positive control to check that at least some of the predicted mechanisms of resistance were operative.

Details of infection/challenge procedures for each protection experiment are provided in chapter 9.

(6) Statistical analysis of protection experiments

The mean recovery of challenge worms from each group of immunised mice ($\bar{X}_B - \bar{X}_A$) was compared with the mean recovery of challenge worms from unimmunised mice ($\bar{X}c$) by Student's two-sample t-test.

When the Mann Whitney U-test was used to compare the median values from the same groups, similar levels of significance were obtained.

2.9. Manufacturers 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.

Becton, Dickinson & Co., Oxnard, California 93030. U.K. suppliers:
Scientific Supplies (see below).

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

Bio-Rad Labs Ltd., Caxton Way, Watford, Herts.

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

Camlab Ltd., Nuffield Road, Cambridge, CB4 1TH.

Costar; U.K. distributor: L. H. Engineering, Bells Hill, Bucks.

Difco Labs., Central Avenue, West Molesey, Surrey.

Dynatech Labs Ltd., Billinghurst, Sussex, RM14 9SJ.

Eppendorf, Gerateban, Netheler and Hinz, GmbH; U.K. suppliers: Anderman and Co. (see above).

Falcon: see Becton, Dickinson and Co; U.K. suppliers: Scientific supplies (see below).

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

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

Formachem (Research International) Ltd., 80 Kirk Street, Strathaven, Scotland.

Gallenkamp and Co Ltd., P.O. Box 290, Christopher Street, London.

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

Herlan Olac Ltd., Shaws Farm, Blackthorn, Bicester, Oxon, OX6 OTP.

Ilford Ltd: P.O. 21, Mobberley, Knutsford, Cheshire.

Janssen Pharmaceuticals Ltd., Grove, Oxford, OX12 ODQ.

Johnson Matthey Chemicals Ltd., Orchard Road, Royston, Hertfordshire, England.

Koch-light Labs, Colnbrook, Berks.

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

Law Hospital, Carluke, ML8 5ES, Lanarkshire, Scotland.

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

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

"Lothian Herbs", Peffermill Road, Edinburgh.

May and Baker Ltd., Dagenham, England.

Millipore Corporation, Harrow, Middlesex, HA1 2YH.

Molecular Probes Inc., P.O. Box 22010, 48-49 Pitchford Avenue, Eugene, OR 97402, U.S.A.

National Blood Transfusion Service, Harkness Building, Radcliffe Infirmary, Woodstock Road, Oxford.

National Diagnostic, Manville, New Jersey.

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

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

Roche Products Ltd., Welwyn Garden City, England.

The Scientific Instrument Centre Ltd., No. 1, Leake Street, London, WC1.

Scientific Supplies, Vine Hill, London, EC1 R5EB.

Scottish Antibody Production Unit (SAPU) - Law Hospital (see above).

Siemans Ltd., 26-28 Napier Court, Wardpark North, Cumbernauld.

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.

UVP Ltd., Science Park, Milton Road, Cambridge CB4 4BN.

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

Outline of chapters 3 to 10.

In the following chapters, some effects of irradiation on synthetic activity and antigen expression by schistosomula are described. The relevance of these observations to stimulation of protective immunity against schistosomes is then discussed. Chapter 3 compares protein synthesis and secretion by normal and U.V.-irradiated parasites during the initial four days following transformation from cercaria to schistosomulum. Synthesis of RNA, phospholipids and glycoproteins during this period are examined in chapter 4. Actinomycin D treatment is shown to mimic the effects of U.V.-irradiation on all these aspects of parasite metabolism. Chapter 5 analyses how gamma-, as opposed to U.V.-irradiation, affects schistosomular synthetic activity. Antigen expression by normal and attenuated schistosomula is examined by a variety of techniques, including immunofluorescence, immunoblotting, ELISA assays, and electron microscopy, in chapters 6 and 7. Chapter 7 concentrates on comparing expression of carbohydrate and polypeptide antigenic determinants by normal, irradiated, and Actinomycin D-treated parasites. Considerable variability was observed in the responses of cercariae and schistosomula to irradiation. Possible sources of this variability, and its influence on the degree of protective immunity induced in experimental hosts, are discussed in chapter 8. Chapter 9 describes a number of mouse protection experiments, comparing as immunising agents larvae attenuated by U.V.-irradiation, gamma-irradiation, or Actinomycin D treatment. Finally, chapter 10 proposes a model which aims to relate the observations on irradiation-induced modification of parasite metabolism and antigen expression to stimulation of effective anti-schistosome immunity.

CHAPTER THREE

PROTEIN SYNTHESIS AND SECRETION BY NORMAL, U.V.-IRRADIATED AND ACTINOMYCIN D-TREATED LARVAE.

3. Protein synthesis and secretion by normal, U.V.-irradiated and Actinomycin D-treated larvae.

3.1. Transformation of normal cercariae: effects on amino acid uptake and protein synthesis.

3.1.1. Amino acid uptake and incorporation by normal cercariae and newly-transformed schistosomula.

Initially, changes in amino acid uptake and protein synthesis during transformation of normal cercariae to schistosomula were analysed.

In the experiment illustrated in figure 3.1., ^{35}S-methionine was added to aquarium water in which newly-shed cercariae were swimming, or to methionine-free GMEM containing either cercariae or newly-transformed schistosomula. Uptake of radioactive methionine into the internal pool of free amino acids by cercariae in water was observed to be an order of magnitude greater than for their counterparts in methionine-free medium.

Interestingly, transfer of cercariae to aquarium water supplemented with NaCl at 6.4g/litre, $NaHCO_3$ at 2.75g/litre, and NaH_2PO_4 at 0.124g/litre (the concentrations found in GMEM) and pH'd to 7.2, altered the characteristics of amino acid uptake in the same way as complete GMEM.

While ^{35}S-methionine uptake by cercariae in water always far exceeded that by schistosomula in medium, it was extremely variable. In identical experiments, uptake after a three-hour incubation could vary from 20 000 to 80 000 cpm per 100 cercariae.

The very high uptake of free methionine by cercariae in aquarium water meant that their TCA-precipitable protein was correspondingly heavily labelled. Some indication of the rate of protein synthesis by

Figure 3.1. Uptake of free (non-TCA-precipitable) ^{35}S-methionine by cercariae and schistosomula under different conditions.

Each point is the mean of duplicate samples.

Deviation of duplicates from mean = ±15.2%

PROTOCOL.

Newly-shed cercariae

↓

Divide in four

(1) (2) (3) (4)

Concentrate each on Sinterglass filter

↓

Make volumes up to

(1)	(2)	(3)	(4)
9 mls aquarium water	9 mls GMEM w/o methionine	9 mls aquarium water/NaCl 6.4gl^{-1} $NaHCO_3$ 2.75gl^{-1} NaH_2PO_4 0.124gl^{-1}	9 mls GMEM w/o methionine

↓

Transform (2) mechanically (Retain tails)

Divide each of (1)-(4) into 6 samples of 1.5 mls. Add 20 µCi (740 kBq) ^{35}S-methionine each.

(1)	(2)		(3)	(4)
23°C	37°C	Incubate at:	23°C	23°C

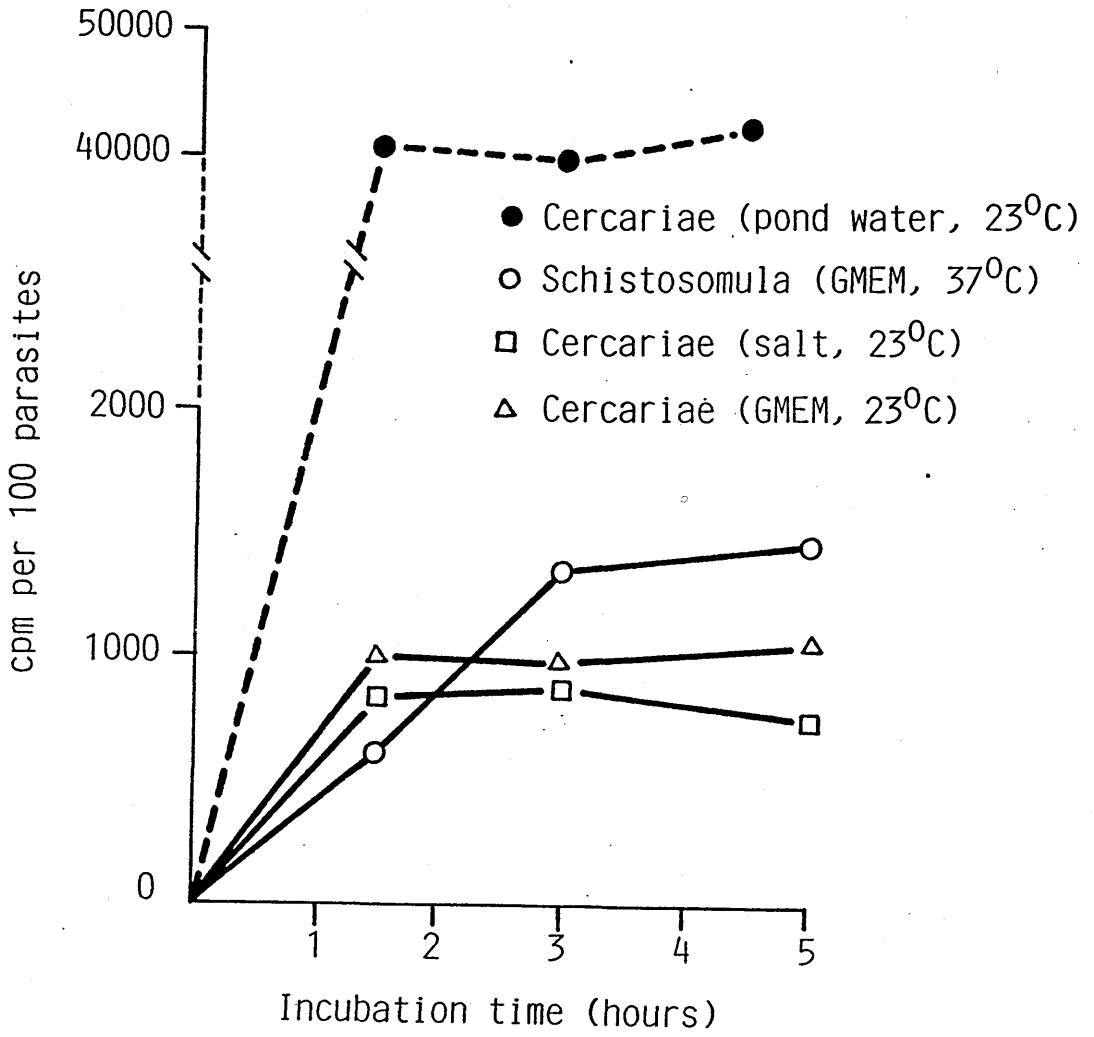
↓

At 90 mins, 3h, 5h - remove 2 duplicate samples from each of (1) - (4) to ice.

↓

Wash three times with ice-cold GMEM; freeze for TCA-precipitation

Uptake of free (Non-TCA-precipitable) ^{35}S-Methionine by Cercariae and Schistosomula under different conditions.



cercariae, as opposed to schistosomula, may be obtained from the ratio of TCA-precipitable to free ^{35}S-methionine at each time-point. Figure 3.2 shows that this ratio was three to four times greater for cercariae in water than for parasites in medium or salt solution.

3.1.2. Comparison of proteins synthesized before and after transformation.

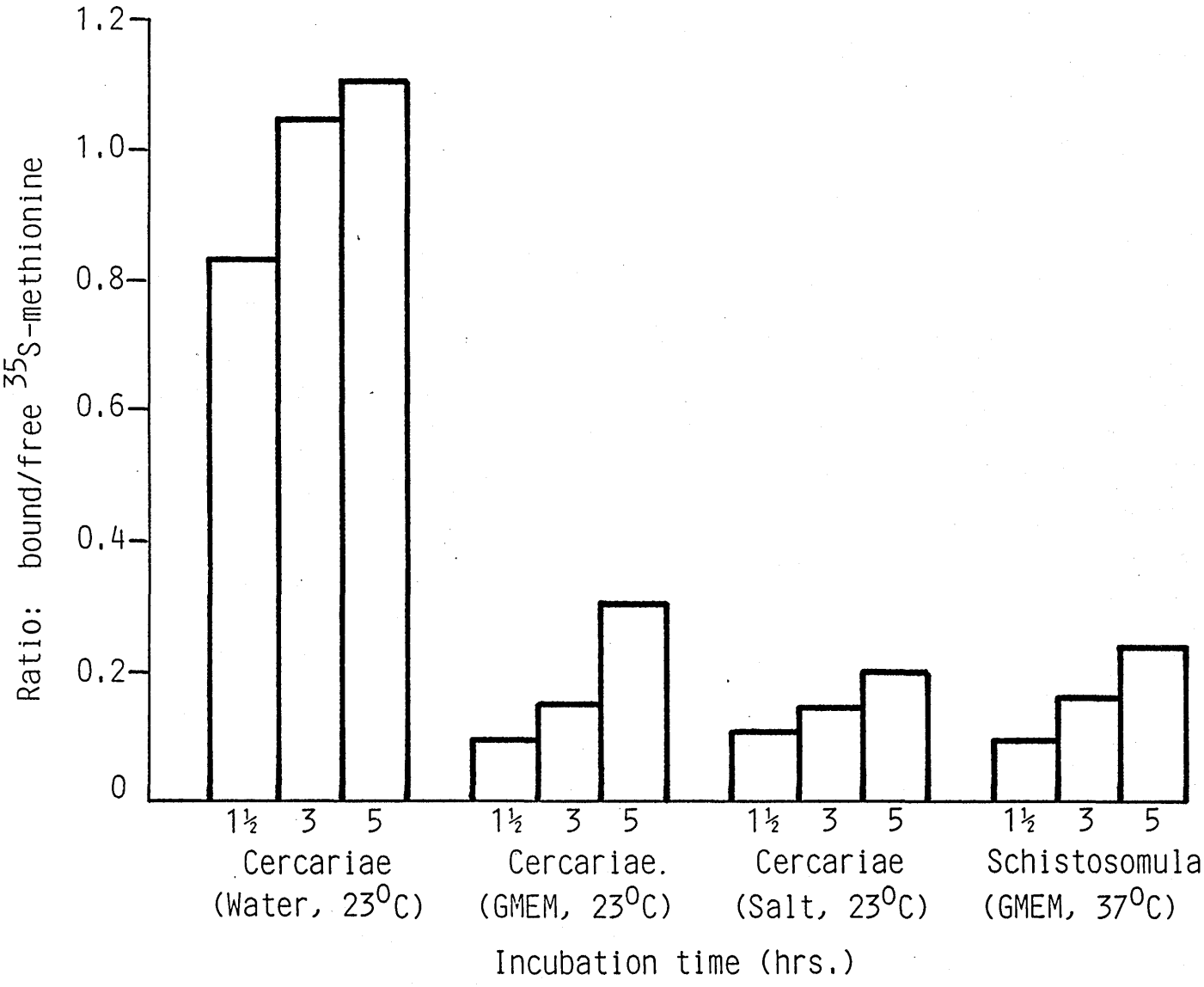
SDS-PAGE of radiolabelled proteins from cercariae and newly-transformed schistosomula allowed us to identify differences in the proteins synthesized by each.

Figure 3.3 a) underlines the striking difference between cercariae and schistosomula in capacity for free amino acid uptake, and hence in the radioactive pool available for incorporation into protein - the cercarial track is intensely blackened, while, for equal numbers of schistosomula, labelled bands are just faintly visible.

Schistosomula immediately after transformation appear to synthesize predominantly proteins of molecular weight 46 000 and 70 000. Yuckenberg *et al* (1987) and Blanton *et al* (1987) demonstrated that synthesis of a 70 000 molecular weight protein was induced in schistosomula immediately after transformation. This protein was identified as a member of the Mr 70 000 heat-shock protein (hsp 70) family. We would suggest that the protein at Mr approximately 70 000 synthesized by newly transformed schistosomula in our experiments represents this heat-shock protein. This observation will be discussed more fully in section 3.10.3.

By exposing the cercarial track to X-ray film for a shorter time (figure 3.3b), it is possible to identify the proteins synthesized by free-living cercariae prior to transformation. Although not the predominant band as it is for schistosomula, the 70 000 molecular weight protein does appear to be constitutively synthesized by

Figure 3.2. Ratio of TCA-precipitable to non-TCA-precipitable ^{35}S-methionine (bound/free ratio) at successive time points for cercariae and schistosomula treated as in the protocol for figure 3.1.


1.2
1.0
0.8
0.6
0.4
0.2
0
Ratio: bound/free ^{35}S-methionine
1½ 3 5
Cercariae
(Water, 23°C)
1½ 3 5
Cercariae.
(GMEM, 23°C)
1½ 3 5
Cercariae
(Salt, 23°C)
1½ 3 5
Schistosomula
(GMEM, 37°C)
Incubation time (hrs.)

Figures 3.3a and 3.3b

Proteins synthesized by cercariae prior to transformation, and by normal schistosomula during the 5 hours following mechanical transformation.

Figure 3.3b is a shorter exposure of the cercariae track in figure 3.3a.

C = cercariae

S = schistosomula. 200 parasites in each track.

10% resolving gel, 3% stacking gel.

Protocol.

Freshly-shed cercariae.

Aliquot of cercariae;

label in 1 ml aquarium water with 20 µCi (740 kBq) ^{35}S-methionine for 3h at room temperature.

Remainder:

Transform mechanically in GMEM w/o methionine; retain tails.

Divide into 5 samples of 1ml GMEM w/o methionine.

Add 20 µCi (740 kBq) ^{35}S-methionine to each.

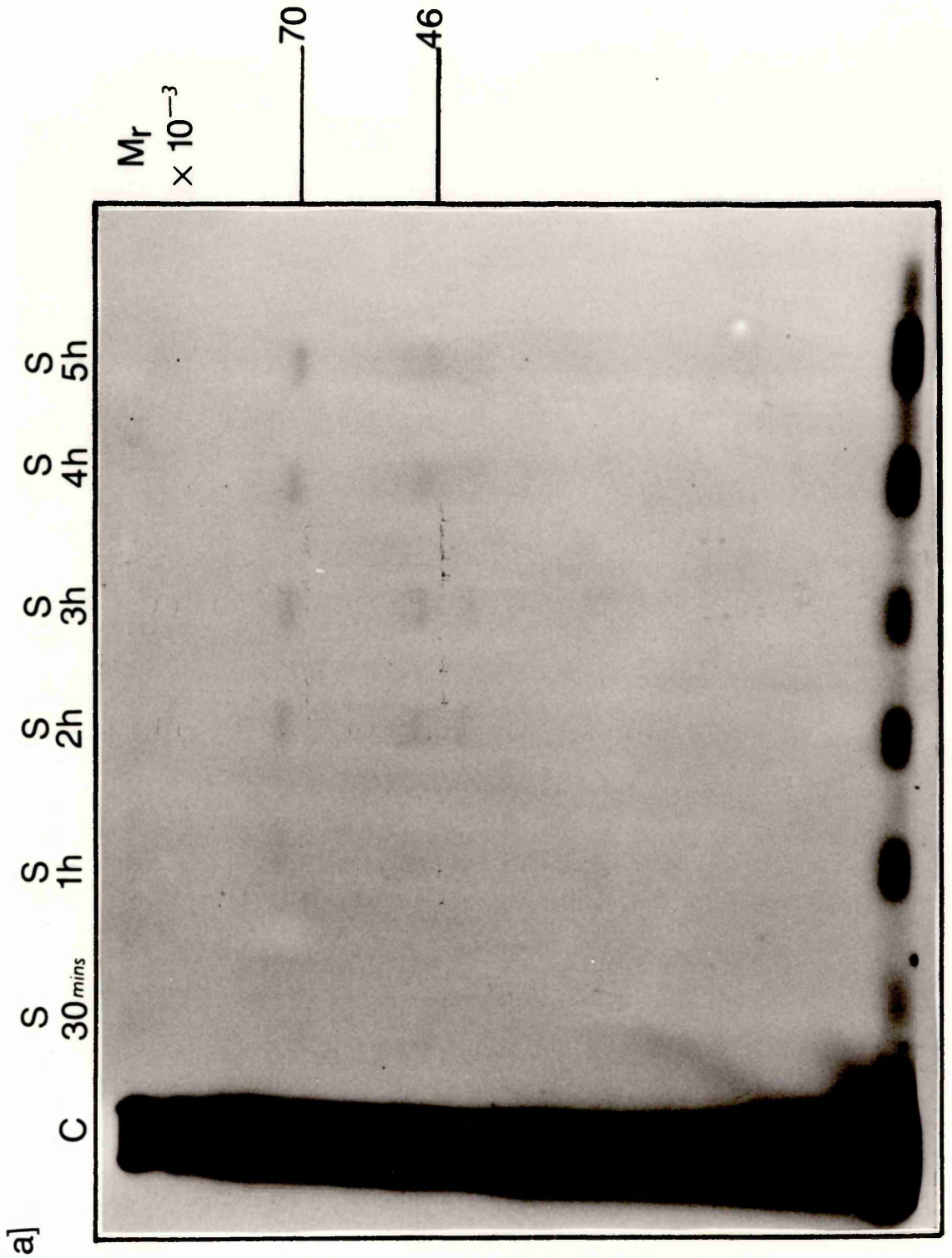
Incubate at 37°C

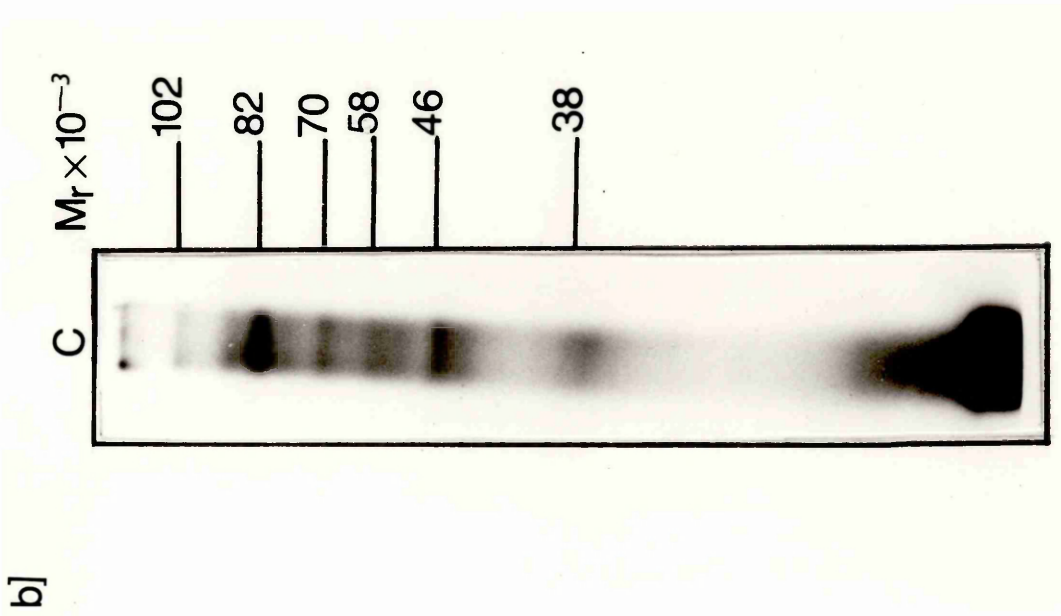
Sample at 30 mins, 1h, 2h, 3h, 4h, 5h.

Remove samples to ice at each time-point.

Wash three times in ice-cold GMEM.

Freeze for SDS-PAGE


a]
C
S 30 mins
S 1h
S 2h
S 3h
S 4h
S 5h
M_r × 10^{-3}
70
46


b]
C
$M_r \times 10^{-3}$
102
82
70
58
46
38

cercariae. The 46 000 Mr band is also evident. Also heavily labelled by cercariae in water are proteins at molecular weights of 102 000, 82 000, a fainter one at 58 000, and a diffuse band around Mr 38 000.

For both cercariae and schistosomula, low molecular weight material which migrates with the marker dye is very heavily labelled. Using higher amounts of radioactive methionine, and larger numbers of parasites, more minor bands, not detectable in gel 3.3 a), can be distinguished for schistosomula. In the experiment of figure 3.4, cercariae were labelled, at room temperature, in GMEM w/o methionine, rather than in water. This incubation procedure was more convenient, in that the resulting bands on SDS-PAGE were sharper, and the level of radioactivity incorporated was much more comparable to that of schistosomula, facilitating comparison of proteins synthesised by the two life-cycle stages. Moreover, this approach allowed us to examine the effect of a change in temperature on parasite protein synthesis independently of a change in medium. At the end of their 3-hour incubation in GMEM, cercariae were fully motile, and more than 95% retained their tails.

In this experiment, schistosomula could be seen to synthesize a broad range of proteins, many differing from the cercarial pattern. Induced synthesis of a band at Mr 72 000 was apparent, as well as a fainter band at Mr 78 000. Both these proteins were heavily synthesized by newly-transformed schistosomula, but are scarcely detectable in the cercarial track. Proteins of molecular weight 62 000, 43 000, and 28 000 also become heavily labelled after transformation. Particularly noticeable in both cercariae and schistosomula are strongly labelled proteins at molecular weights 17 000 and 19 500. A 15 000 molecular weight protein, just detectable above the marker dye, and low molecular weight material that migrates with the dye front, as previously seen in figure 3.3, are also heavily labelled by both cercariae and schistosomula.

Figure 3.4. Proteins synthesized by cercariae (in GMEM w/o methionine) and mechanically-transformed schistosomula after labelling with high amounts of ^{35}S-methionine.

C = cercariae.

S = schistosomula

2000 parasites per track

3% stacking gel, 10% resolving gel.

Protocol

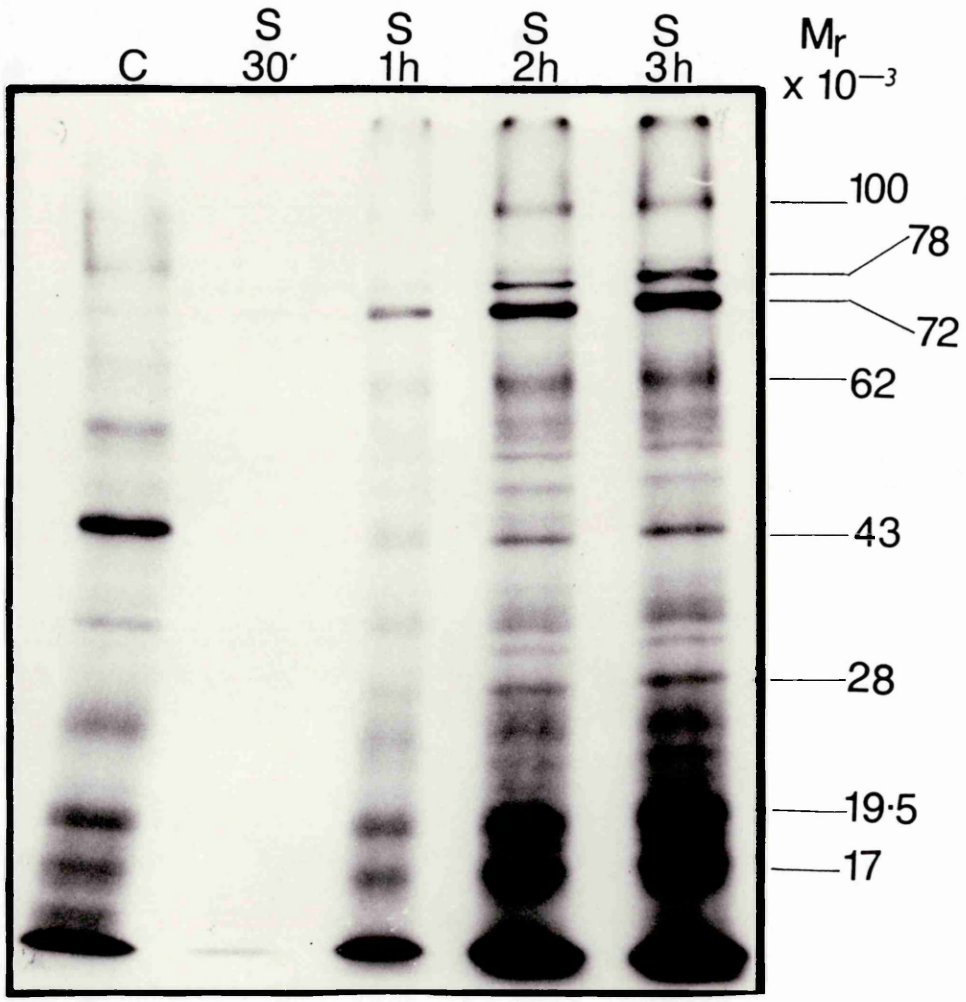
Freshly-shed cercariae.

Aliquot of cercariae	Remainder
↓	↓
Filter-concentrate.	Transform mechanically in
Wash through filter with	GMEM w/o methionine.
GMEM w/o methionine.	↓
Concentrate to 1ml GMEM w/o	Divide into 4 samples of 1 ml
methionine.	GMEM w/o methionine.
↓	Add 100 µCi (3.7 MBq) ^{35}S-
Label for 3h at room temperature	methionine.
with 100 µCi (3.7 MBq) ^{35}S-methionine	Incubate at 37°C
	↓
	Take one sample at 30 mins,
	1h, 2h, 3h.

Remove samples to ice at each time-point.

Wash three times in ice-cold GMEM.

Freeze for SDS-PAGE.


C
S 30′
S 1h
S 2h
S 3h
Mr x 10⁻³
100
78
72
62
43
28
19·5
17

Certain proteins seem, in this experiment, to be uniquely synthesized by cercariae - at molecular weights of 81 000 (as in gel 3.3a) - and 50 000. Molecules at Mr 46 000 and 26 000, though detectable in schistosomula, are more intensely labelled by cercariae.

Table 3.1 summarises these observations.

3.1.3. Further investigations of protein synthesis in response to temperature stress in cercariae and schistosomula.

The altered pattern of protein synthesis as cercariae transform to schistosomula could be a response to a number of external influences - for instance, increase in temperature, change of medium, or mechanical stress. In order to define more clearly the contribution of each of these factors to the changes in protein synthesis induced by transformation, cercariae and schistosomula were labelled in:

(1) GMEM without methionine;

(2) haemolymph from *B. glabrata* snails;

(3) aquarium water (cercariae only).

Labelling was performed in all 3 media at both room temperature and 37°C. These culture conditions aimed to represent *in vitro* counterparts of the environments of migrating cercariae within the snail (haemolymph), of free-living cercariae after release from the snail host (aquarium water), and of newly-transformed schistosomula in the mammalian host (GMEM). Figures 3.5a and 3.5b show the results of this experiment.

As expected, incorporation of ^{35}S-methionine into protein was much greater for parasites in water than in either haemolymph or GMEM (compare intensity of tracks 1A, B with 2 to 5A, B). Constitutive synthesis of the putative heat-shock protein at molecular weight approximately 72 000 was always evident at room temperature, whatever the surrounding medium. In GMEM at 37°C, mechanically-transformed

Table 3.1. Comparison of proteins synthesized by normal cercariae, labelled for 3 hours, and by schistosomula labelled for 3 hours after mechanical transformation.

(see figure 3.4 and accompanying protocol).

Mr of proteins synthesized by:	
Cercariae (GMEM, room temp).	Schistosomula (GMEM, 37°C)
82 000	100 000
50 000	78 000
46 000	72 000
26 000	46 000
19 500	28 000
17 000	19 500
15 000	17 000
	15 000
Low molecular weight material at dye-front	Low molecular weight material at dye-front

Figures 3.5a and 3.5b.

Effects of haemolymph, water and GMEM on protein synthesis by cercariae and schistosomula at room temperature and 37°C.

C = cercariae

S = schistosomula

R.T. = room temperature

H.L. = snail haemolymph

3% stacking gel, 10% resolving gel

400 parasites per track

Figure 3.5b shows tracks 1A and 1B - cercariae in aquarium water at room temperature and 37°C - after a shorter exposure time.

PROTOCOL

Freshly-shed cercariae

↓

Divide equally in ten.

↓

Concentrate on ice.

↓

Resuspend to 100 µl of

Aquarium water	H.L.	H.L.	GMEM w/o met.	GMEM w/o met.
(1) A,B.	(2) A,B.	(3) A,B.	(4) A,B.	(5) A,B.

Passage (3) and (5) 10 times through 21G x 1-1/2" syringe needle.

Add 2 µCi (74 kBq) ^{35}S-methionine to each.

↓

"A" samples: incubate at room temperature

"B" samples: incubate at 37°C

↓

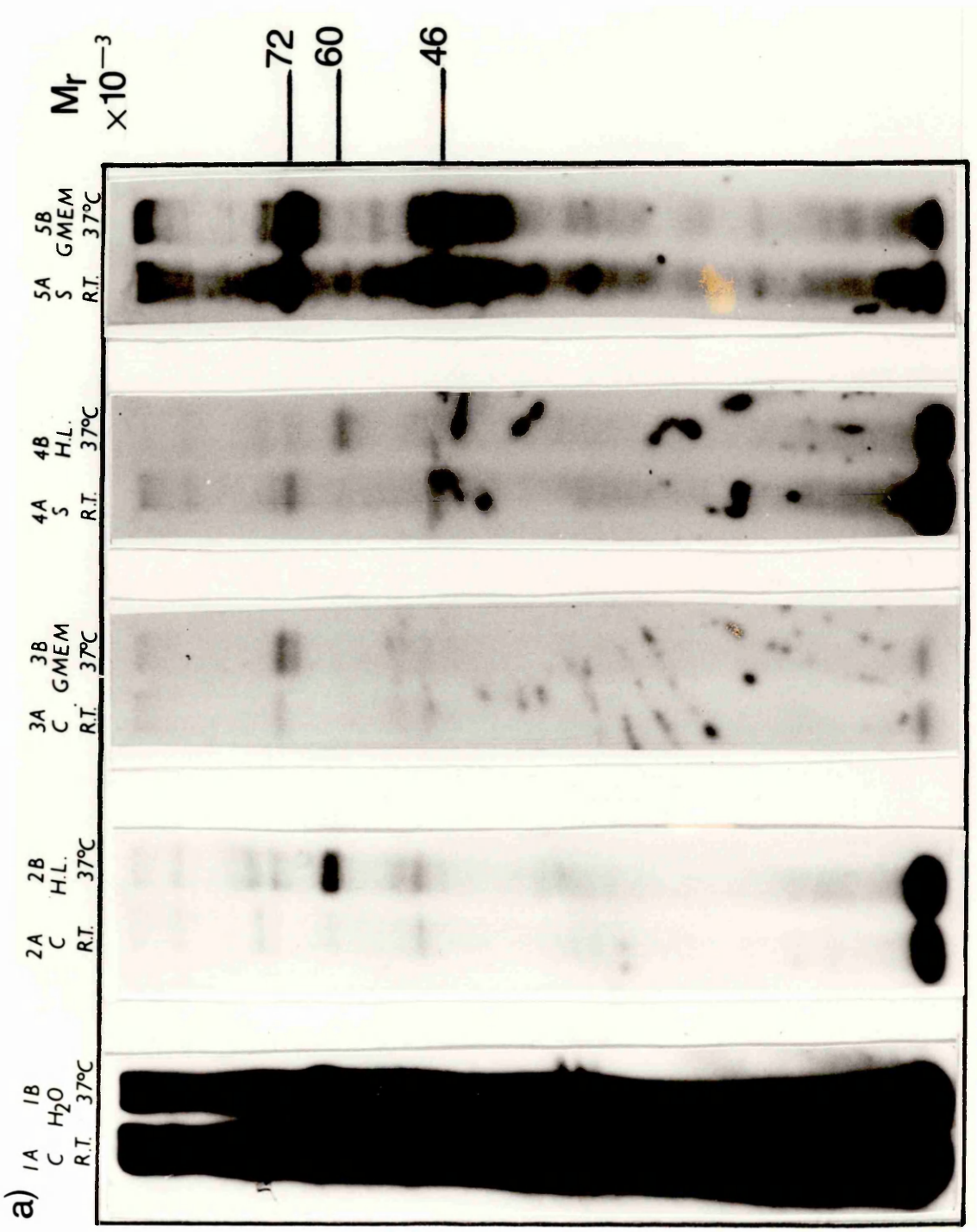
At 5 hours: remove samples to ice.

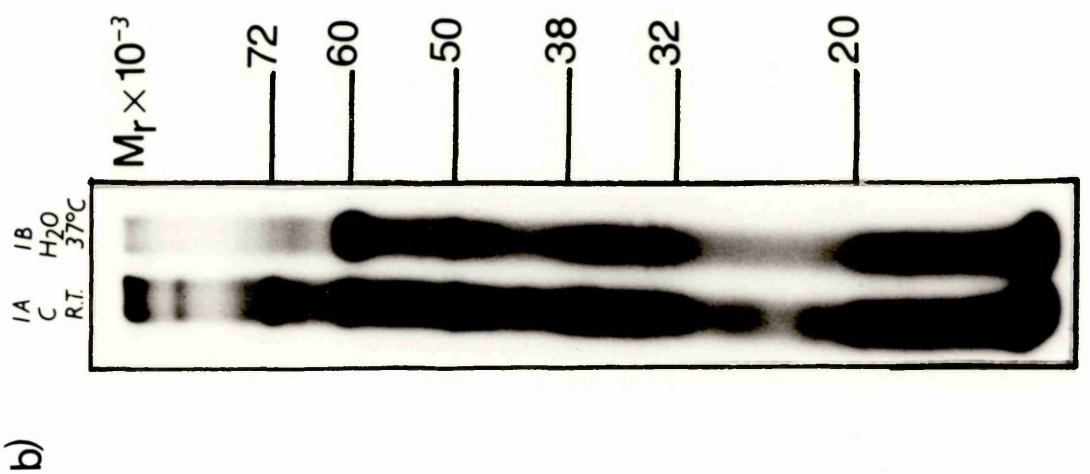
↓

Wash three times in ice-cold GMEM.

↓

Freeze for SDS-PAGE.


a)
1A C R.T.
1B H2O 37°C
2A C R.T.
2B H.L. 37°C
3A C R.T.
3B GMEM 37°C
4A S R.T.
4B H.L. 37°C
5A S R.T.
5B GMEM 37°C
Mr ×10⁻³
72
60
46


b)
1A
C
R.T.
1B
H2O
37°C
Mr×10-3
72
60
50
38
32
20

schistosomula showed the predicted pattern of protein synthesis: proteins at Mr 72 000 and 43 000 are predominant (track 5B). In fact, this typical heat-shock pattern, though not quite so heavily labelled, was also seen in syringe-passaged schistosomula in GMEM at room temperature (track 5A). In cercariae, not subjected to mechanical stress, but cultured in GMEM at room temperature, synthesis of the Mr 72 000 band was quite marked, but upon raising the temperature to 37°C, it became much more intensely labelled (tracks 4A,B). However, in water and in haemolymph, an increase in temperature induced enhanced synthesis of a 60 000 molecular weight protein, rather than the 72 000 Mr species seen on raising the temperature of parasites in GMEM (tracks 1A,B and 2A,B). Even when the cercariae in haemolymph were subjected to syringe passage (as in mechanical transformation) before incubation at 37°C, the presence of haemolymph caused their heat-shock response to remain characteristic of cercariae, rather than schistosomula, in that the 60 000 Mr protein, rather than the 72 000 Mr species, was preferentially synthesized (tracks 3A,B).

To summarise, it seems that, in water and haemolymph, the schistosome larvae show induced synthesis of 60 000 Mr protein in response to an increase in temperature from 23°C to 37°C. In contrast, larvae at the same developmental stage, incubated in GMEM, preferentially synthesize a 70 000-72 000 Mr species when the temperature is raised. It is interesting to speculate as to how the heat-shock response in the different environments might be controlled. Any of a number of factors in snail body fluid - levels of inorganic or organic nutrients, or binding by specific hormones - might ensure expression of the Mr 60 000 heat-shock protein response, and concomitant repression of the Mr 70 000 one. This Mr 60 000 response would be maintained in free-living cercariae in water, until the parasite encountered the mammalian environment, when some factor or combination of factors - for instance, levels of organic or inorganic nutrients; changes in pH -

would activate the Mr 70 000 heat-shock protein response. Changes in the physical, rather than the chemical, nature of the parasite's environment might also play a role in controlling protein synthesis in response to temperature stress. Snail haemolymph and fresh water both have much lower osmolarities than mammalian body fluid (represented by GMEM); osmotic pressure of fresh water = -0.01°C; of molluscan body fluid = -0.08 to -0.22°C; of mammalian body fluid = -0.5 to -0.58°C (see Barrett, 1981). The Mr 70 000 heat-shock protein response may be induced by an osmolarity above a certain threshold level. Further research, to analyse the contributions of these different factors in controlling parasite protein synthesis during the transition from cercaria to schistosomulum, would be valuable. This hypothesis will be discussed more fully in section 3.10.3.4.

3.2. Protein synthesis by normal schistosomula during the 48 hours following mechanical transformation.

Since U.V.-irradiated schistosomula survived, on average, for only 96 hours in culture in Elac/10% serum, attention was concentrated on protein synthesis during the first three to four days following transformation. As a preliminary to examining protein synthesis in irradiated forms, it was important to establish the pattern of protein synthesis by normal schistosomula during this period. Figure 3.6 shows three separate experiments where normal schistosomula were incubated with ^{35}S-methionine for 48 hours following mechanical transformation. Although certain proteins are common to all three experiments, there are striking differences.

Gel 3.6 a) shows the most frequently observed pattern. The 70 000 molecular weight heat-shock protein and a Mr 45 000 band are very prominent. An 82 000 Mr protein is also heavily labelled. Further down the gel is a series of proteins ranging from Mr 26 000 to 32 000, then

Figures 3.6a,b,c.

Protein synthesis by three batches of normal schistosomula during 48 hours following transformation.

500 normal schistosomula per well in each case. 3% stacking gel, 10% resolving gel.

PROTOCOL FOR EACH BATCH OF CERCARIAE:

Freshly-shed cercariae

↓

Transform mechanically in GMEM w/o methionine.

↓

Divide into 2 or 3 replicate samples in 1 ml GMEM w/o methionine/10% hiFCS.

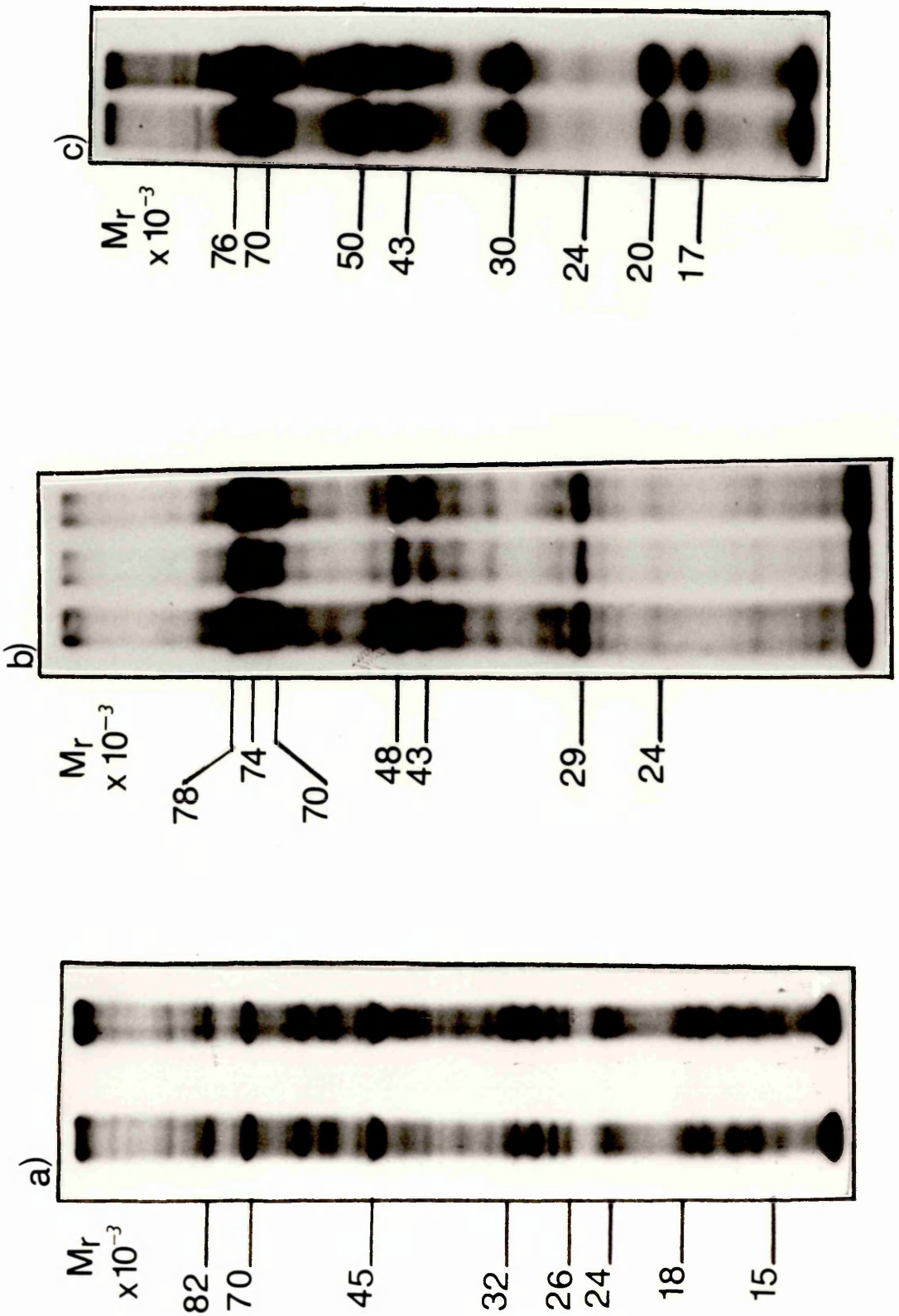
Add 50 µCi (1.85 MBq) ^{35}S-methionine.

↓

Culture at 37°C, 5% CO_2 for 48 hours.

↓

Remove to ice; wash three times in ice-cold GMEM. Freeze for SDS-PAGE.


a)
M_r x 10^{-3}
82
70
45
32
26
24
18
15
b)
M_r x 10^{-3}
78
74
70
48
43
29
24
c)
M_r x 10^{-3}
76
70
50
43
30
24
20
17

a more diffuse band at Mr 24 000. In the low molecular weight zone, five bands between Mr 15 000 and 18 000 can be clearly distinguished.

Turning to 3.6 b), however, the pattern is quite different. Bands at Mr 43 000 and 48 000 are quite pronounced. We also see, not the single heat-shock protein at Mr 70 000, but a series of 3 proteins ranging from Mr 70 000 to 78 000, all intensely labelled. In the lower half of the gel, the most striking difference from 3.6 a) is the very prominent band at Mr 29 000. Only traces can be seen of the group of proteins between Mr 26 000 and 32 000, which are heavily labelled in a). In this case, there are very few radioactive proteins above Mr 85 000, or below Mr 30 000, although most of the proteins which are intensely labelled in this zone in 3.6 a) are still faintly detectable.

The overall pattern in 3.6 c) is very similar to 3.6 b), although there appear to be only two, rather than three, heavily labelled proteins around Mr 70 000. The proteins at Mr 43 000 and 29 000-30 000 are again very noticeable. However, the notable prominence of bands at Mr 50 000, 20 000 and 17 000 differs from 3.6b).

Intense labelling at the marker dye is again evident in all three experiments. This may be due, in part, to low molecular weight peptides derived from proteolysis, or possibly to small proteins such as ubiquitin (Mr 7 000-8 000) which are not resolved on these 10% acrylamide gels.

These observations are summarised in Table 3.2.

3.3. The transformation process in U.V.-irradiated schistosomula.

Having examined amino acid uptake and protein synthesis by normal schistosomula during the initial few days following transformation, we went on to investigate how U.V.-irradiation affected parasite protein synthesis during the same period.

Mr	Are these proteins synthesized in experiments		
	3.6a)	3.6b)	3.6c)
82 000	strong	faint	faint
70 000-78 000	1 strong band (70 000)	3 strong bands (78 000, 74 000, 70 000)	2 strong bands (76 000, 70 000)
43 000-45 000	strong	strong	strong
26 000-32 000	4 strong bands	1 strong band (29 000); others faint	1 strong band (30 000)
24 000	strong, but diffuse band.	faint	faint
15 000-20 000	several sharp, strong bands	several faint bands	strong bands at 20 000 and 17 000.

Table 3.2. Summary of proteins synthesized in experiments a),b),c) of figure 3.6.

3.3.1. Uptake and incorporation of ^{35}S-methionine by normal and U.V.-irradiated cercariae, and schistosomula during the 5 hours following mechanical transformation.

Since irradiation is known to alter membrane transport characteristics in a number of biological systems (reviewed by Edwards et al, 1984), it was important to determine, initially, whether uptake of amino acids by cercariae or schistosomula was affected by irradiation.

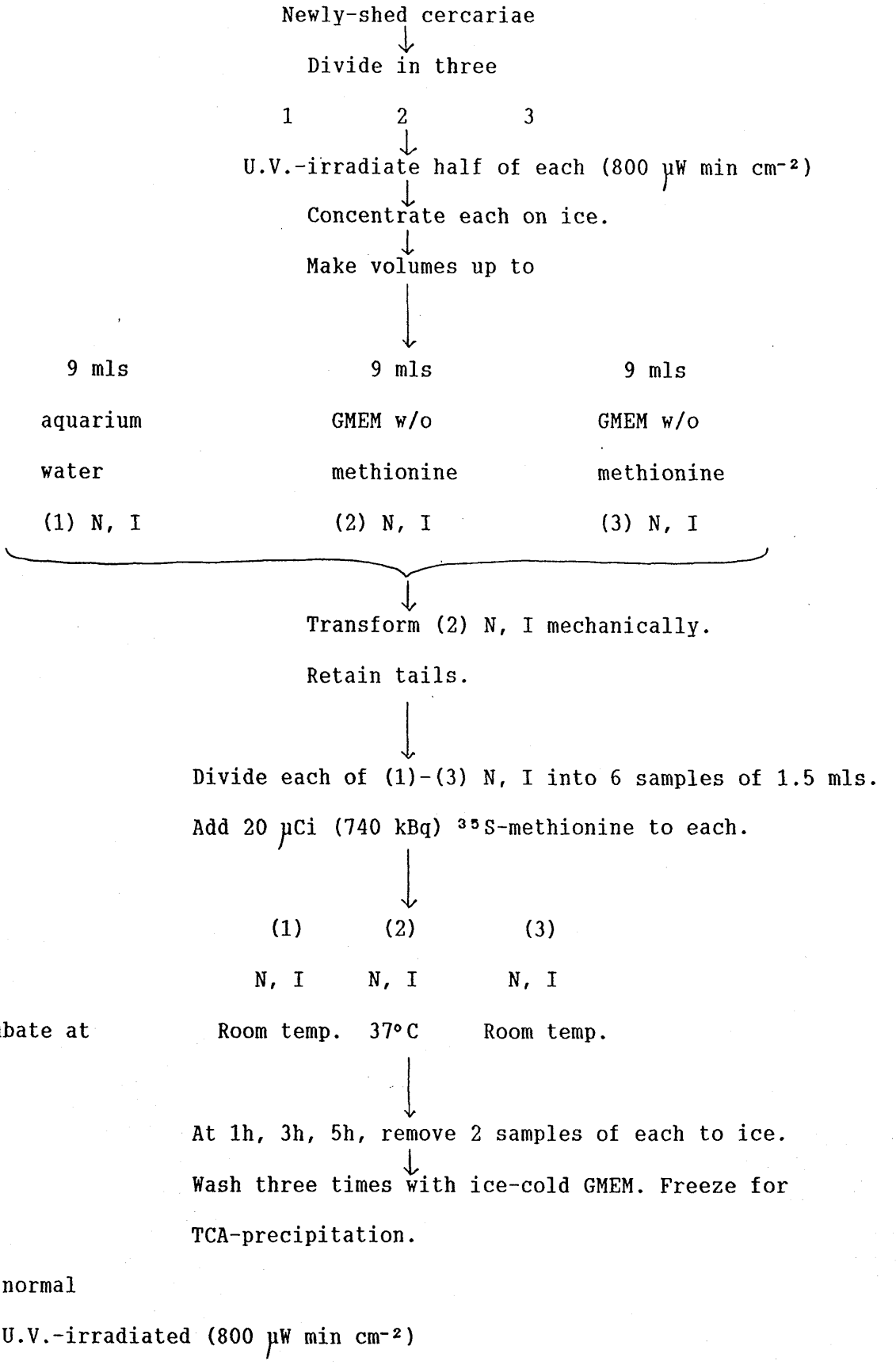
Figure 3.7 demonstrates that uptake of free ^{35}S-methionine by cercariae, and schistosomula over the 5-hour period following mechanical transformation, is not inhibited by U.V.-irradiation at 800 μW min cm^{-2}. In this instance, cercariae appeared to show an increase in permeability immediately after transformation. However, this did not occur in every experiment.

From figure 3.8, U.V.-irradiation inhibits protein synthesis by schistosomula, and cercariae, regardless of whether they are incubated in water or GMEM. During the first 3 hours following transformation, however, incorporation of radiolabel into protein by normal controls is so low that it is difficult to quantify inhibition in irradiated forms accurately, using the liquid scintillation counting technique. SDS-PAGE and fluorography allow a more detailed analysis of the effect of irradiation on protein synthesis.

3.2.2. Proteins synthesized by U.V.-irradiated schistosomula after mechanical transformation.

A typical example of the contrast between patterns of protein synthesis by normal and U.V.-irradiated (800 μW min cm^{-2}) schistosomula during the initial 5 hours after transformation is shown in

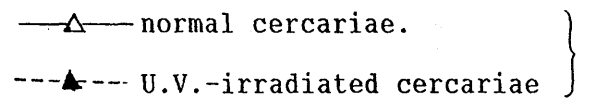
Protocol for figures 3.7 and 3.8



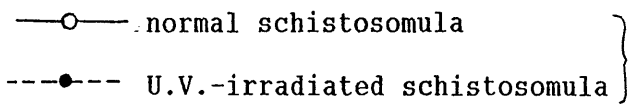
N = normal

I = U.V.-irradiated (800 µW min cm⁻²)

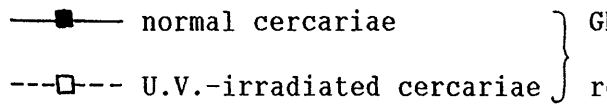
Figure 3.7. Uptake of free (non-TCA-precipitable) ^{35}S-methionine by normal and U.V.-irradiated cercariae and newly-transformed schistosomula.



Aquarium water; room temperature



GMEM w/o methionine; 37°C



GMEM w/o methionine; room temperature

U.V.-irradiation dose was 800 μW min cm^{-2}

Each point represents the mean of duplicate samples.

Deviation of duplicates from mean = ±14.8%

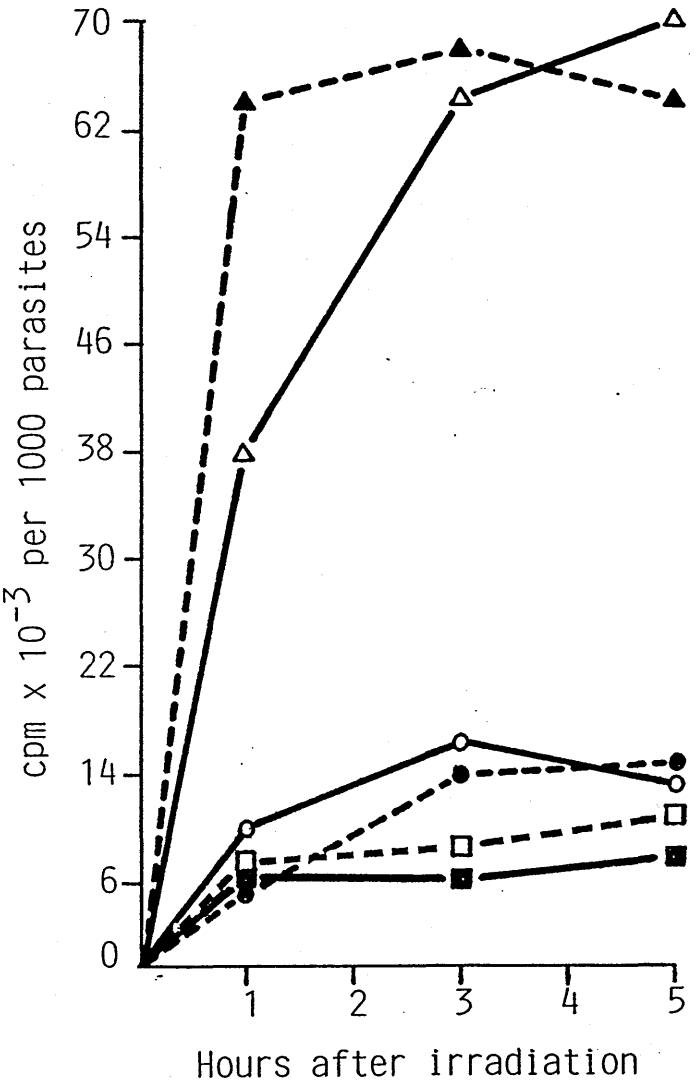
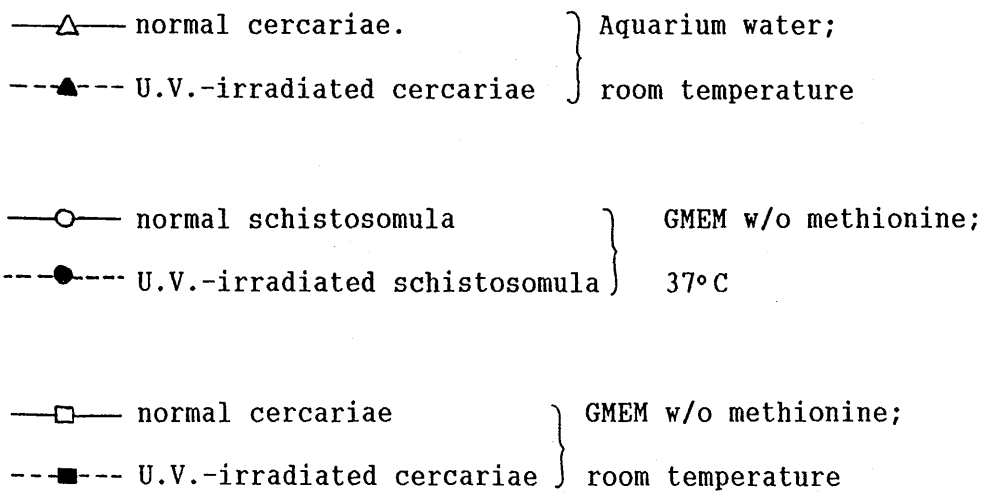

70
62
54
46
38
30
22
14
6
0
cpm x 10^{-3} per 1000 parasites
1
2
3
4
5
Hours after irradiation

Figure 3.8 Incorporation of ^{35}S-methionine into TCA-precipitable protein by normal and U.V.-irradiated cercariae and newly-transformed schistosomula.



U.V.-irradiation dose was 800 μW min cm^{-2}

Each point represents the mean of duplicate samples.

Deviation of duplicates from mean = $\pm 12.3\%$

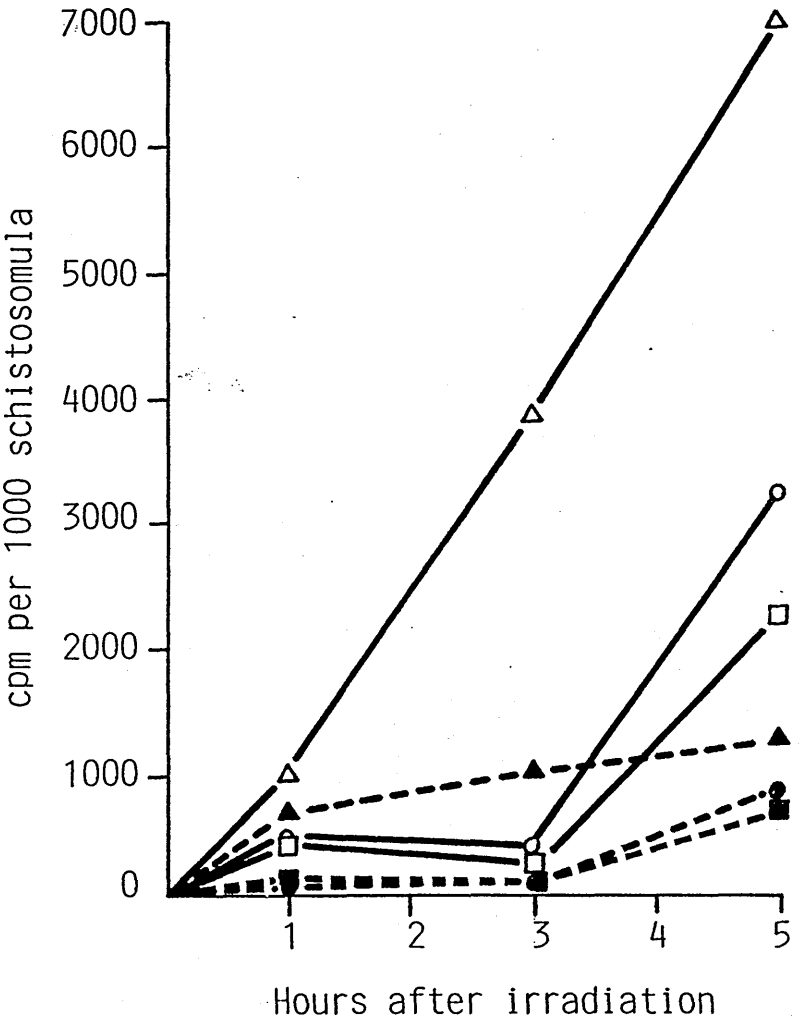

7000
6000
5000
4000
3000
2000
1000
0
cpm per 1000 schistosomula
1
2
3
4
5
Hours after irradiation

figure 3.9.

Normal schistosomula responded to transformation in this experiment by inducing especially pronounced synthesis of a 78 000 molecular weight protein during the first 5 hours following transformation. It is proposed that this 78 000 Mr protein might be a member of a family of heat-shock proteins between Mr 70 000 and 78 000 (see later discussion). This band was evident as early as 30 minutes after syringe passage. Proteins of Mr 65 000, 48 000, 43 000 and 15 000 were intensely labelled by normal larvae. Low molecular weight material below the dye-front was again very heavily labelled.

Protein synthesis by U.V.-irradiated schistosomula, on the other hand, was scarcely detectable until 5 hours after transformation. Interestingly, the most prominent band at this time was not the 78 000 molecular weight protein, as in the normal parasites, but one at Mr 65 000. Even this "predominant" band is extremely faint compared with the normal labelling pattern. The 43 000 and 15 000 Mr proteins were also just evident by 5 hours of labelling.

However, the pattern of protein synthesis by U.V.-irradiated schistosomula varies just as much as for the normal parasites (see figures 3.6a, b, c). This variability in response to irradiation and transformation will be discussed in more detail in chapter 8. Figure 3.10 exemplifies the point here. In this instance, the most intensely labelled proteins after transformation of normal cercariae occur at Mr 75 000 and 47 000. Proteins at Mr 70 000 and 43 000 are also evident by 15 hours after transformation of normal cercariae. Synthesis of the same 4 proteins is detectable, at greatly reduced levels, in irradiated schistosomula. In neither normal nor irradiated parasites are the Mr 65 000 or 15 000 proteins labelled as prominently as in figure 3.9.

Figure 3.9. Incorporation of ^{35}S-methionine into protein by normal and U.V.-irradiated schistosomula during the 5 hours following transformation.

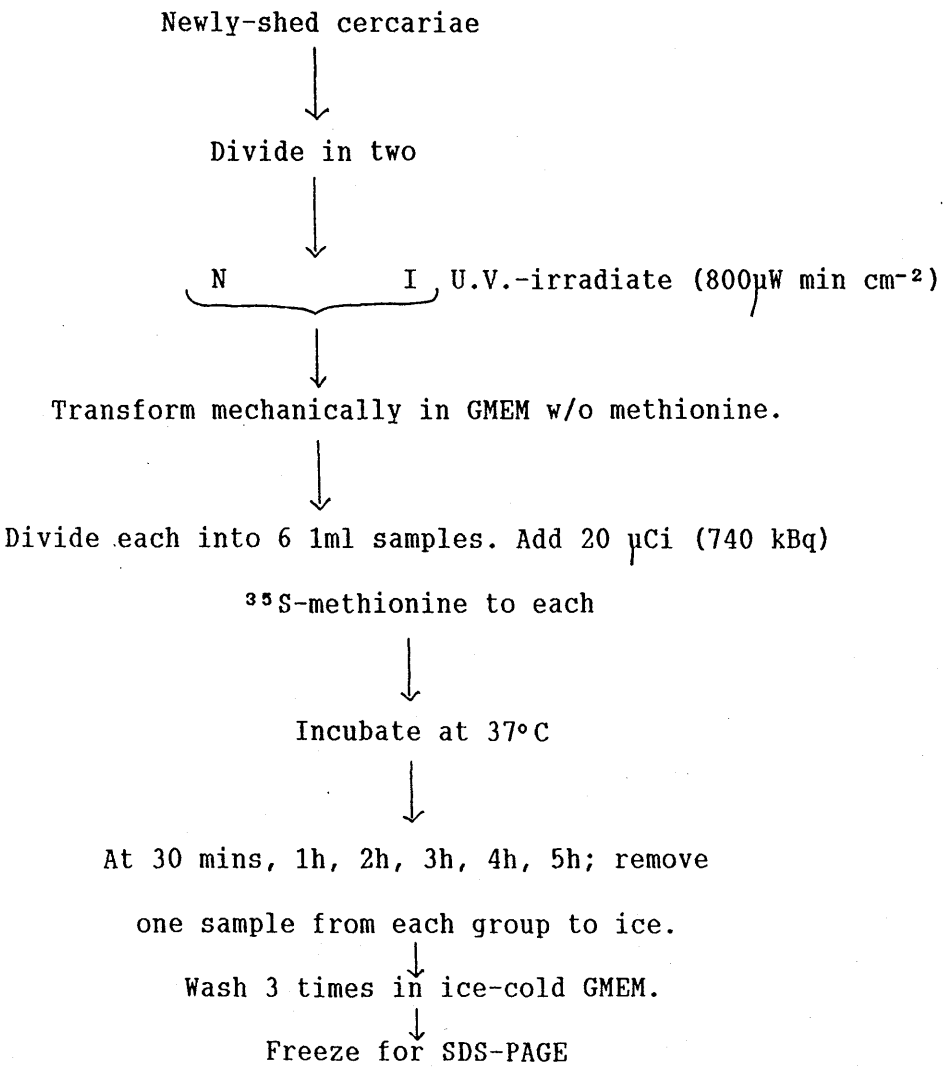
N = Normal

I = U.V.-irradiated (800 μW min cm^{-2})

1000 schistosomula per track.

3% stacking gel, 7.5% resolving gel.

PROTOCOL



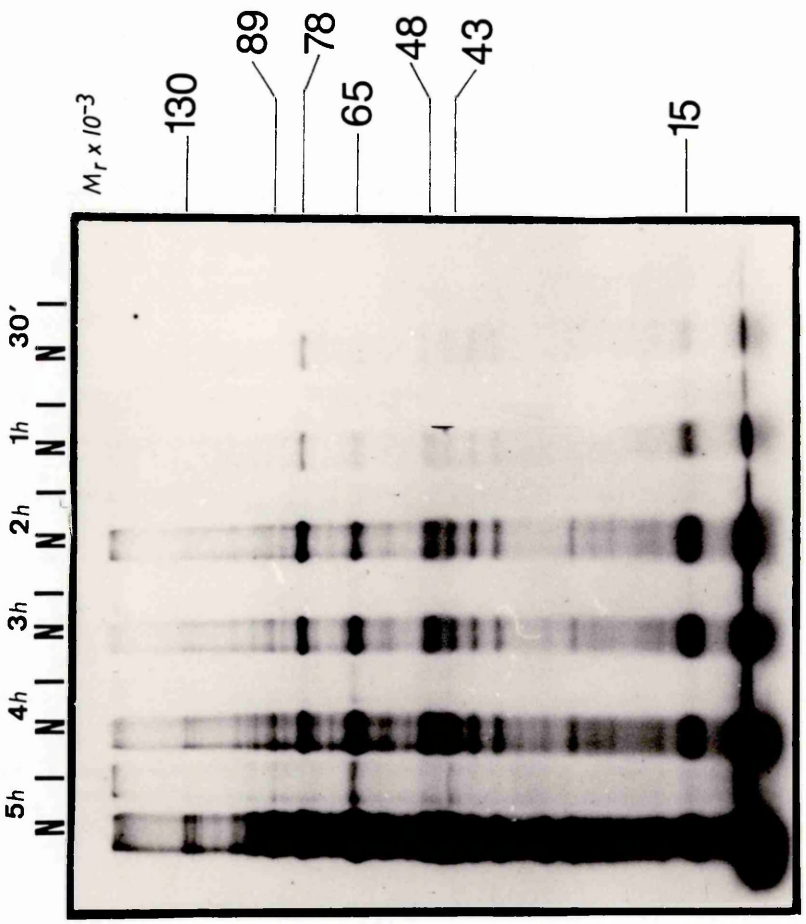

$M_r \times 10^{-3}$
130
89
78
65
48
43
15
30'
1h
2h
3h
4h
5h
N
I

Figure 3.10 Protein synthesis by normal and U.V.-irradiated schistosomula during 15 hours following mechanical transformation.

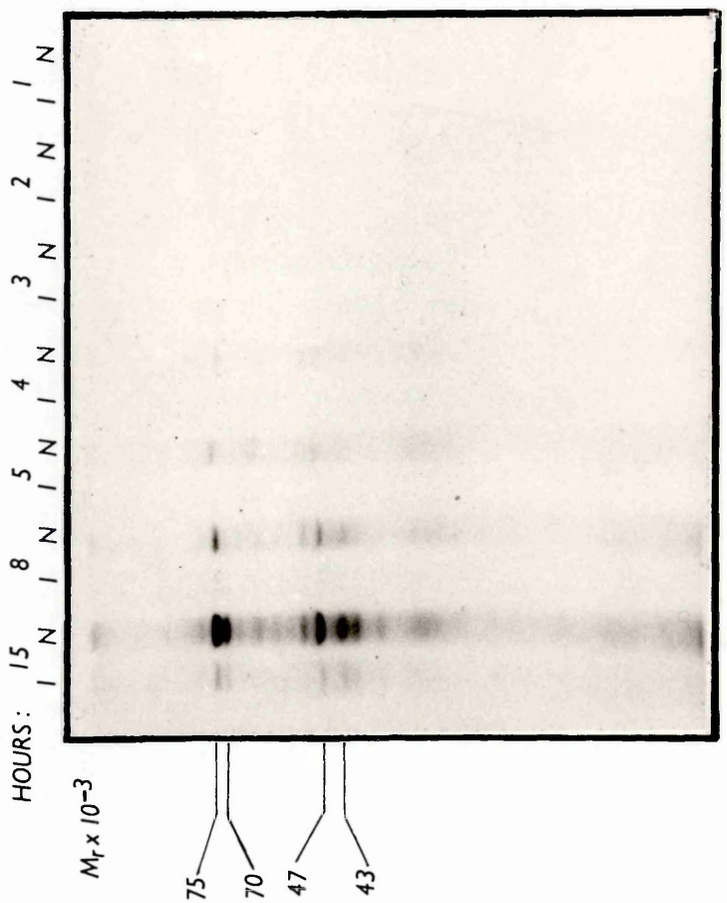
N = normal

I = U.V.-irradiated (800 μW min cm^{-2})

200 parasites per track

3% stacking gel, 10% resolving gel

Protocol as for figure 3.9, but 7 samples taken: at 1, 2, 3, 4, 5, 8, 15 hours after transformation.


HOURS:
15
8
5
4
3
2
1
I N I N I N I N I N I N I N
$M_r \times 10^{-3}$
75
70
47
43

3.4. Inhibition of protein synthesis is maintained throughout the lifetime of U.V.-irradiated schistosomula in culture.

In subsequent experiments, the U.V.-irradiation dose was normally 400, rather than 800, μW min cm^{-2}, since cercariae irradiated at this level were better able to protect mice against challenge infection (see chapter 9). Pronounced inhibition of protein synthesis was also observed after this irradiation dose.

U.V.-irradiated (400 μW min cm^{-2}), mechanically-transformed schistosomula survived some 96 hours in culture with Elac/10% hiFCS or Elac/10% hiNHS. For the higher irradiation dose (800 μW min cm^{-2}), the survival time was slightly reduced. The mean survivals at 24-hour intervals, drawn from three separate experiments, are shown in table 3.3.

By 48 hours of culture, schistosomula subjected to either radiation dose were showing clear evidence of damage: loss of shape, motility and translucency. These signs of damage became increasingly pronounced, and the rate of death of irradiated parasites increased, with time in culture.

The inhibition of protein synthesis observed just after transformation was generally maintained throughout the life-time of U.V.-irradiated schistosomula (see figure 3.11). Table 3.4 shows the level of inhibition at each time point in this experiment.

Figure 3.12 illustrates this sustained inhibition of protein synthesis in U.V.-irradiated forms, pointing out some of the proteins typically synthesized at greatly reduced levels by irradiated schistosomula. In this case, the irradiated parasites seem to concentrate their energies on producing proteins of Mr 81 000 and 75 000 up to 48 hours of culture. Bands at Mr 43 000 and 48 000 become quite heavily labelled by irradiated parasites by 72 to 96 hours after transformation and a 57 000 Mr band is quite prominent by 96 hours.

Table 3.3. Survival in culture of normal and U.V.-irradiated, mechanically-transformed schistosomula.

Schistosomula were cultured at 500/ml in 10 mls Elac/10% hiFCS, at 37°C, in the presence of 5% CO_2.

U.V.-IRRADIATION DOSE (μW min cm^{-2})	NORMAL	400	800
	%age OF SCHISTOSOMULA DEAD.		
Hours post-transformation			
3	2.0±0.6	2.5±1.8	3.2±1.4
24	4.5±1.9	5.1±1.5	14.5±1.4
48	7.5±2.7	7.2±1.2	32.1±4.4
72	10.7±4.7	33.5±8.4	78.7±3.8
96	12.7±3.7	98±0.6	100±0

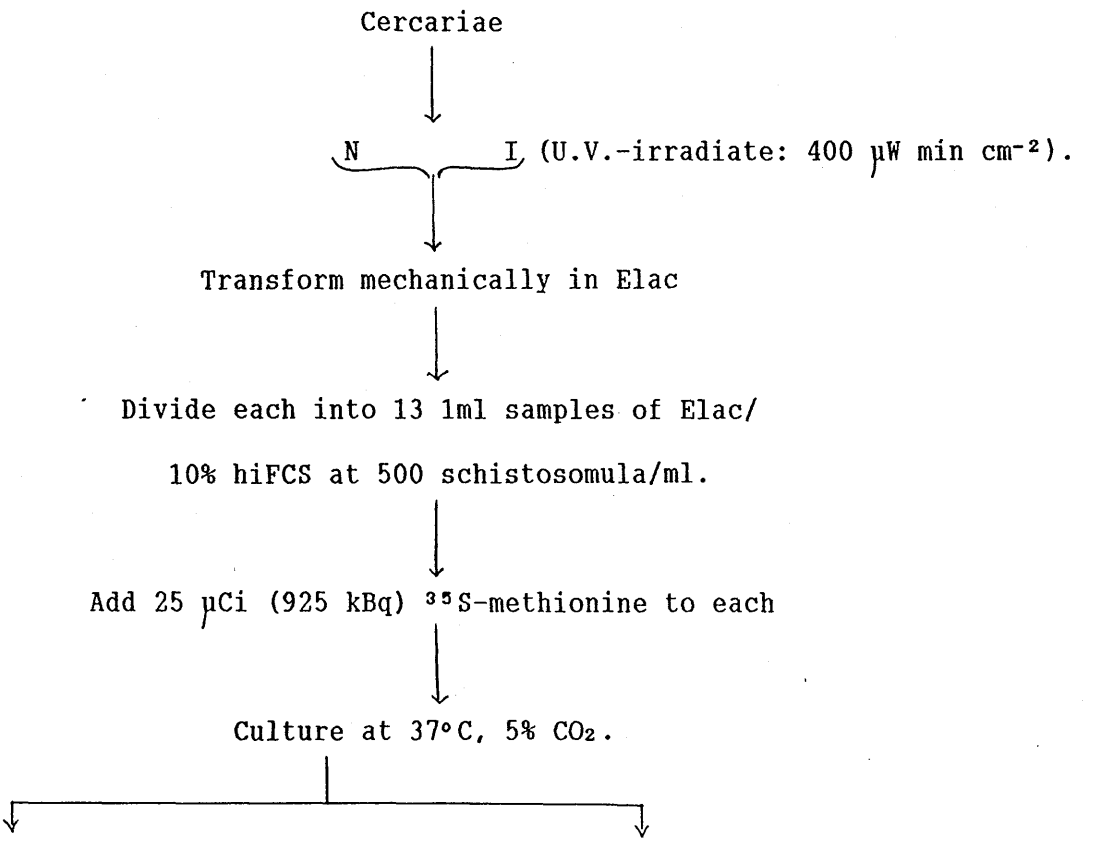
Each value is the mean ± S.E. of 3 separate experiments.
The criteria for classification of "dead" and "live" schistosomula are outlined in section 2.3.3.

Figure 3.11. Incorporation of ^{35}S-methionine into TCA-precipitable protein by normal and U.V.-irradiated schistosomula during the 96 hours following mechanical transformation.

——O—— = Normal

– – –O– – – = U.V.-irradiated (400 μW min cm^{-2})

Protocol



Each point represents the mean of duplicate samples

Deviation of duplicates from mean = ±8.2%

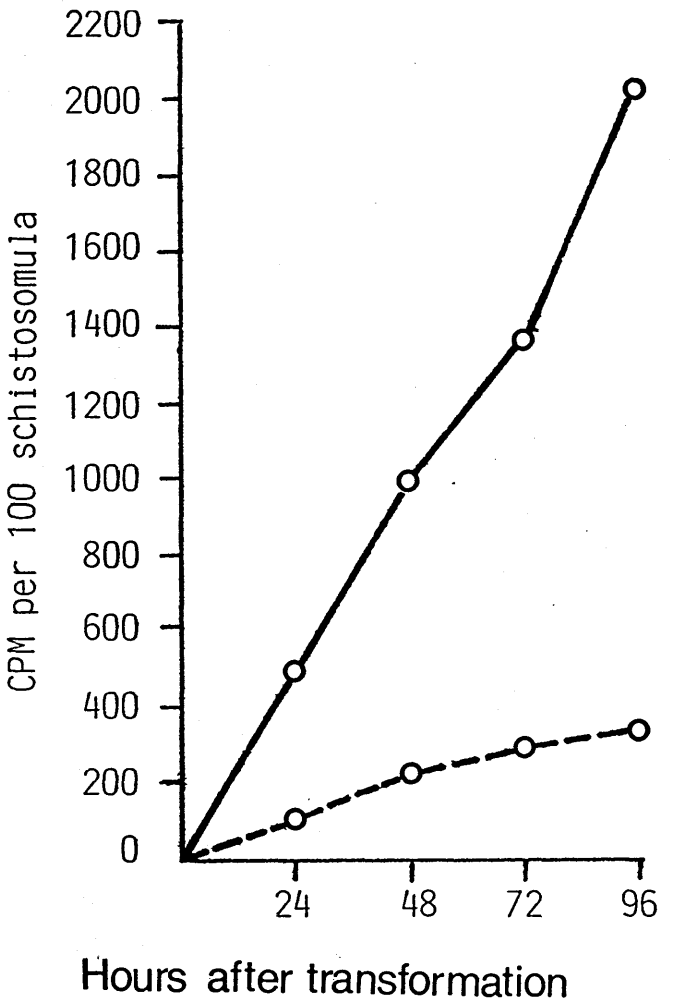

2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
CPM per 100 schistosomula
24
48
72
96
Hours after transformation

Table 3.4. Percentage inhibition of protein synthesis at each time-point in figure 3.11.

Hours after transformation	24	48	72	96
% inhibition of protein synthesis	81.4	78.6	79.5	84.6

Each value represents the mean of paired samples

Deviation of duplicates from mean = ±8.2%

Figure 3.12. Proteins synthesized by normal and U.V.-irradiated schistosomula during the 96 hours following transformation.

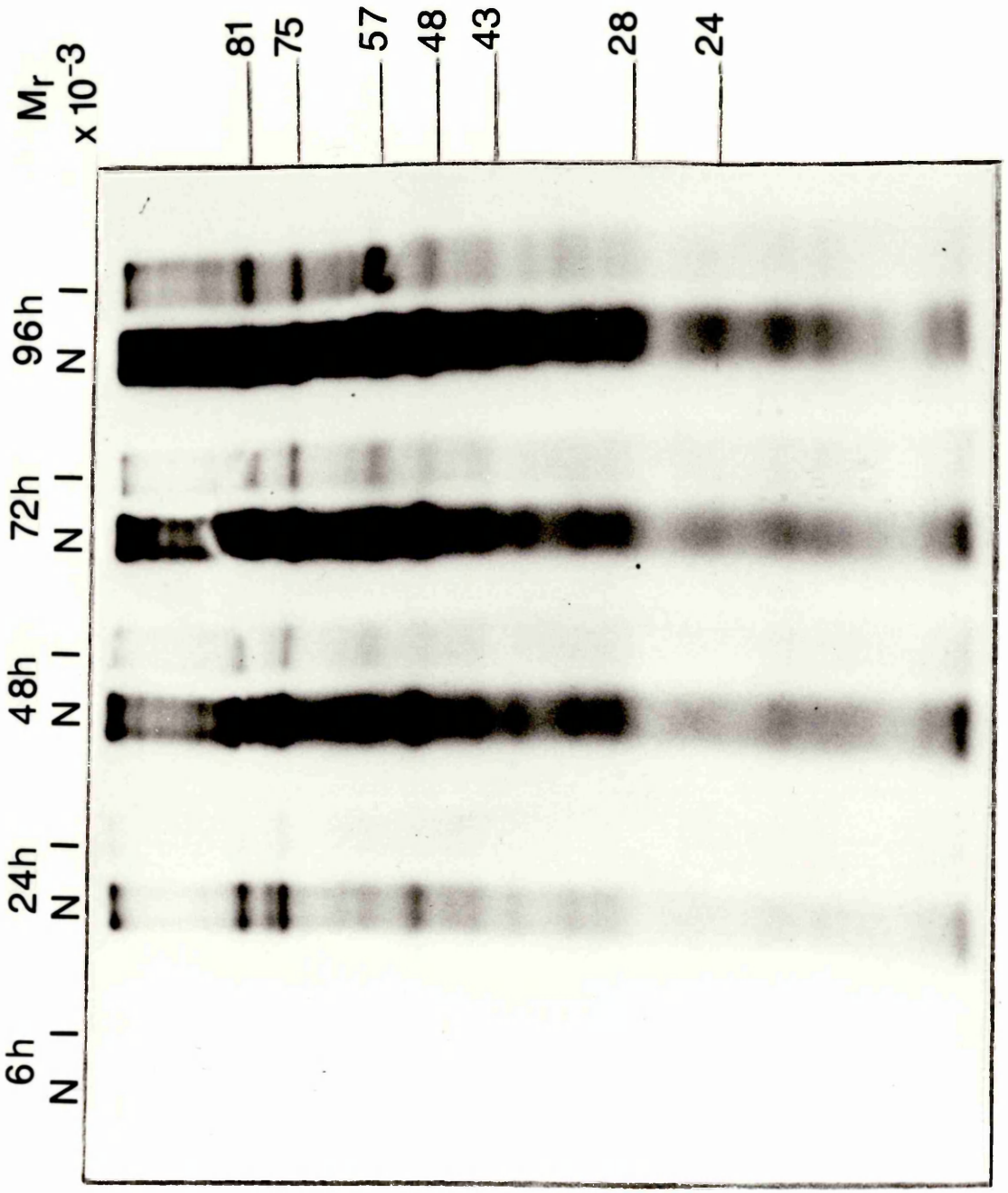
N = normal schistosomula

I = U.V.-irradiated schistosomula (400 μW min cm^{-2})

500 schistosomula per track

3% stacking gel, 10% resolving gel

This gel accompanies the graph of figure 3.11, and the same protocol applies, except that the culture medium was GMEM w/o methionine, rather than Elac.


M_r x 10^{-3}
81
75
57
48
43
28
24
96h
72h
48h
24h
6h
N
I

3.5 Inhibition of protein synthesis depends on irradiation dose.

Figure 3.13 shows that the extent of inhibition of protein synthesis is directly related to the dose of U.V.-irradiation. In this experiment, it is especially interesting to note that, as the radiation dose increases, higher molecular weight members of the Mr 70 000 to 78 000 series of proteins become more intensely labelled, while the synthesis of lower molecular weight members of this family ceases to be detectable. Thus, normal schistosomula, and schistosomula subjected to the lowest dose of U.V.-irradiation - 50 μW min cm^{-2} - show a heavily labelled band at Mr 70 000. At doses of 100 and 200 μW min cm^{-2}, 76 000 and 72 000 Mr species are prominent, while the 70 000 Mr protein is no longer evident. At 400 μW min cm^{-2}, the 76 000 Mr band is much more heavily labelled than the 72 000 species. At the highest radiation dose - 800 μW min cm^{-2} - only the highest molecular weight member of the series, at Mr 76 000, is labelled. It is tempting to speculate that there may be precursor-product relationships between these three molecules. Thus, normal schistosomula might process the 76 000 Mr precursor completely to give the 70 000 Mr protein. With increasing doses of U.V.- irradiation, the enzymes involved in processing of the 72 000 Mr intermediate form, or of the 76 000 Mr original precursor, may be inactivated, or no longer synthesized in adequate concentrations.

Dose/effect experiments showed that, as regards survival, schistosomula were remarkably resistant to U.V.-irradiation. Even after irradiation at 6.4 mW min cm^{-2} (16 times the normal dose), approximately 70% of mechanically-transformed schistosomula were motile, though very granular, and distorted in shape, after 24 hours in culture.

Figures 3.13a and b.

Incorporation of ^{35}S-methionine into protein, during the 24-hour period following mechanical transformation, by schistosomula subjected to increasing doses of irradiation.

In (b), the gel is over-exposed to show the proteins synthesized at higher doses of irradiation.

1000 schistosomula per track.

3% stacking gel/10% resolving gel. (Marker dye run off the gel).

Protocol

Cercariae

↓

Divide in 6:

↓

N,50,100,200,400,800 μW min cm^{-2}

↓

U.V.-irradiate at appropriate dose.

↓

Transform mechanically in GMEM

w/o methionine.

Resuspend each to 1 ml GMEM w/o methionine/

10% hiFCS at 1000/ml.

↓

Add 50 μCi (1.85 MBq) ^{35}S-methionine.

Culture at 37°C, 5% CO_2, for 24 hours.

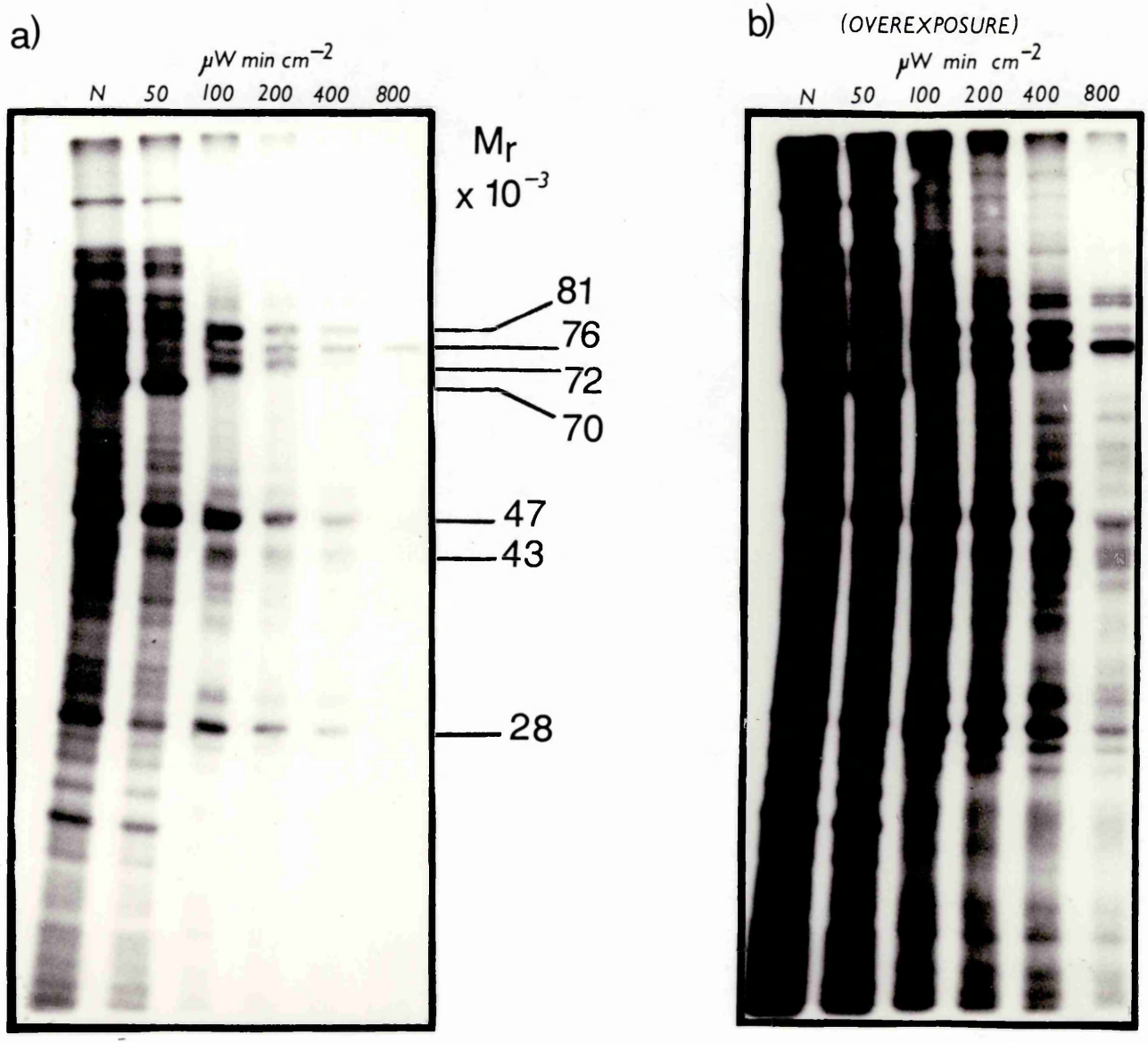
↓

Remove all samples to ice.

↓

Wash three times in ice-cold GMEM;

freeze for SDS-PAGE.


a)
μW min cm^{-2}
N 50 100 200 400 800
M_r
x 10^{-3}
81
76
72
70
47
43
28
b)
(OVEREXPOSURE)
μW min cm^{-2}
N 50 100 200 400 800

3.6. Effect of U.V.-irradiation on protein synthesis by skin-transformed schistosomula.

Although the morphological and physiological changes which occur in schistosomula *in vivo* do seem to take place, albeit at a slower rate, in mechanically-transformed parasites (Stirewalt *et al*, 1983; Wilson, 1987; section 1.5), there must still be reservations as to the *in vivo* relevance of experiments with these artificially-produced forms. It was therefore necessary to confirm that U.V.-irradiation also inhibited protein synthesis by schistosomula produced by penetration of excised mouse skin. Figure 3.14 shows that such inhibition did occur.

U.V.-irradiated schistosomula after skin transformation seemed to conserve synthesis of the same proteins as mechanical ones: labelling of proteins at Mr 81 000 and 70 000 can be detected in the 1.5 minute irradiated (400 μW min cm^{-2}) parasites. The absence of any other bands is most likely a consequence of the short labelling time - only 5 hours at each time point.

3.7. Comparison of inhibition of protein synthesis in U.V.-irradiated schistosomula after skin, or mechanical, transformation.

However, it was found that, in general, schistosomula derived from irradiated cercariae by skin penetration showed some 20 to 30% less inhibition of protein synthesis than counterparts obtained by mechanical transformation from the same pool of irradiated cercariae (see figure 3.15). Possible explanations for this observation are discussed in section 8.3.2.4.

Irradiated parasites transformed by skin penetration survived in culture some 6 to 12 hours less than their mechanical counterparts (compare tables 3.3 and 3.5).

Figure 3.14. Incorporation of ^{35}S-methionine into protein, by normal and U.V.-irradiated, skin-transformed schistosomula.

U.V.-irradiation was for 1.5 mins (400 μW min cm^{-2})

or 3 mins (800 μW min cm^{-2})

300 schistosomula per track.

3% stacking gel/10% resolving gel.

PROTOCOL

Cercariae

↓

N	1.5 minutes U.V.-irradiation (400 μW min cm^{-2})	3 minutes U.V.-irradiation (800 μW min cm^{-2})

Transform by penetration of excised mouse skin for 3 hours (see section 2.3.2).

↓

At 3 hours: sample from each washed and transferred to 1 ml GMEM w/o methionine.

Add 20 μCi (740 kBq) ^{35}S-methionine.

Label at 37°C for 5 hours.

↓

Remaining schistosomula: wash and culture in 10 ml Elac/10% O^+ hiNHS at 37°C, 5% CO_2.

↓

At 24h, 48h, 72h, 96h;

Remove sample; wash in GMEM w/o methionine. Label for 5 hours at 37°C as above.

↓

After labelling: wash schistosomula three times in GMEM.

↓

Freeze for SDS-PAGE.

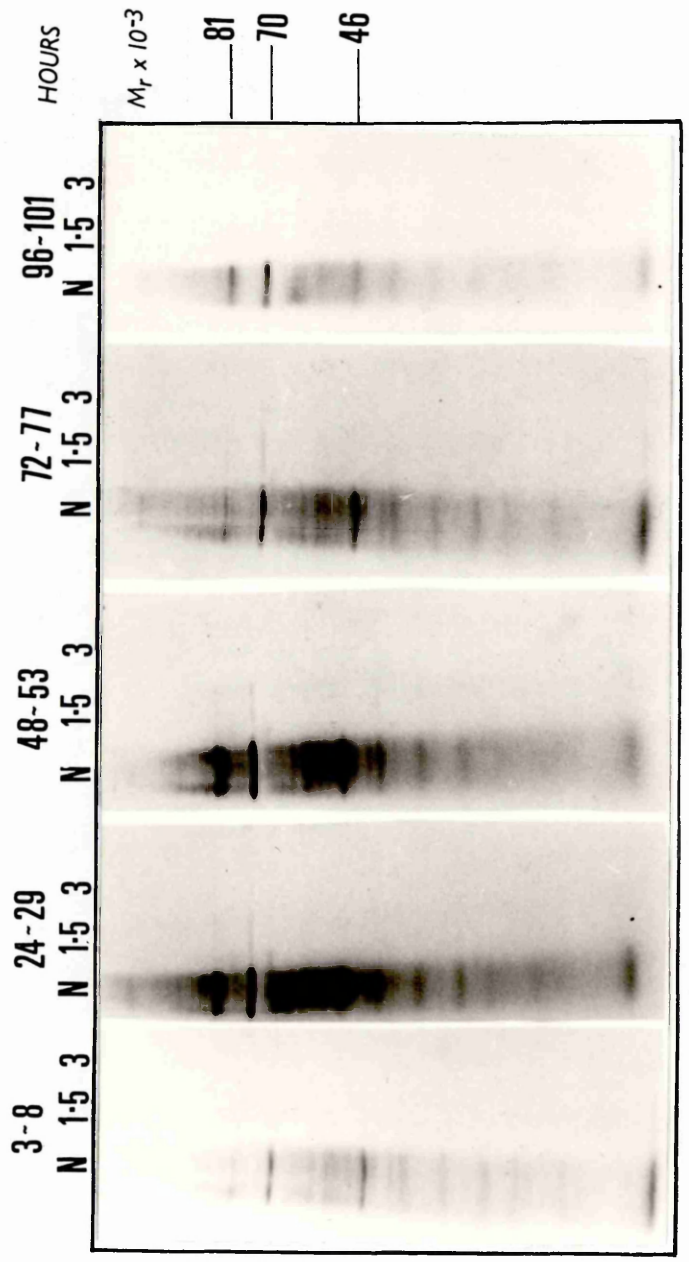
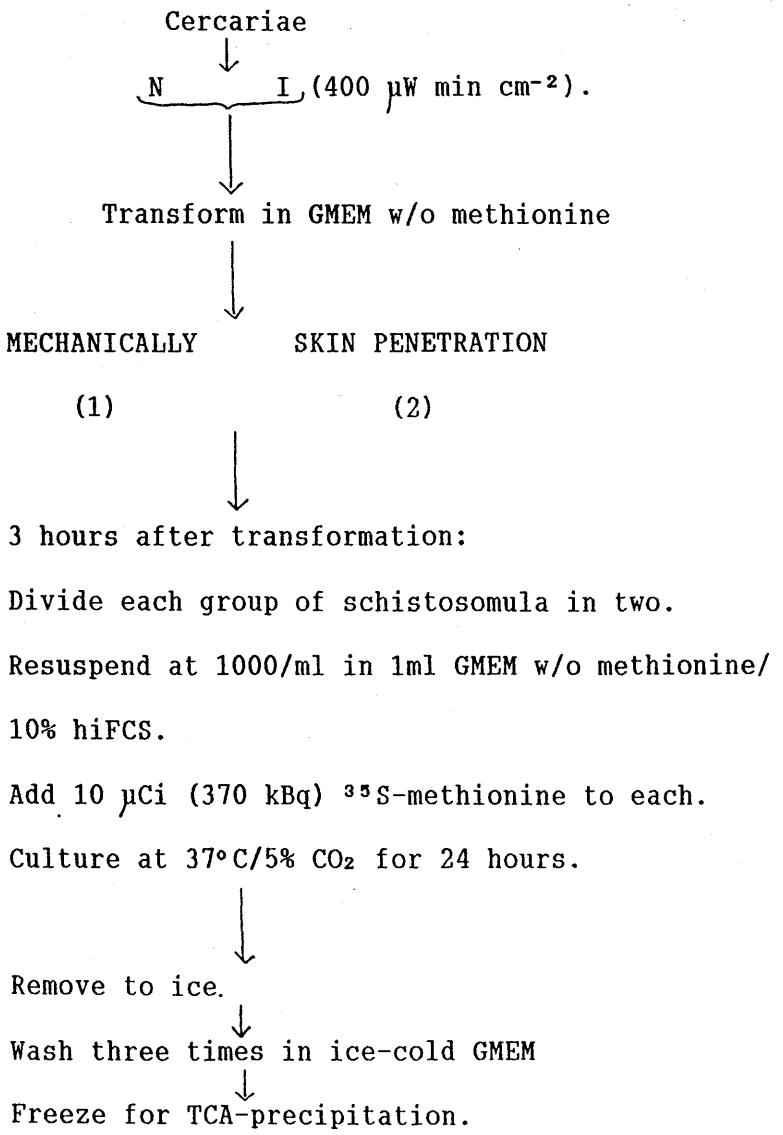

HOURS
$M_r \times 10^{-3}$
81
70
46
3-8
24-29
48-53
72-77
96-101
N 1·5 3

Figure 3.15. Incorporation of ^{35}S-methionine into protein by normal and U.V.-irradiated schistosomula after skin or mechanical transformation.

Protocol



Each column represents the mean of duplicate samples

Deviation of duplicates from mean = ±9.2%

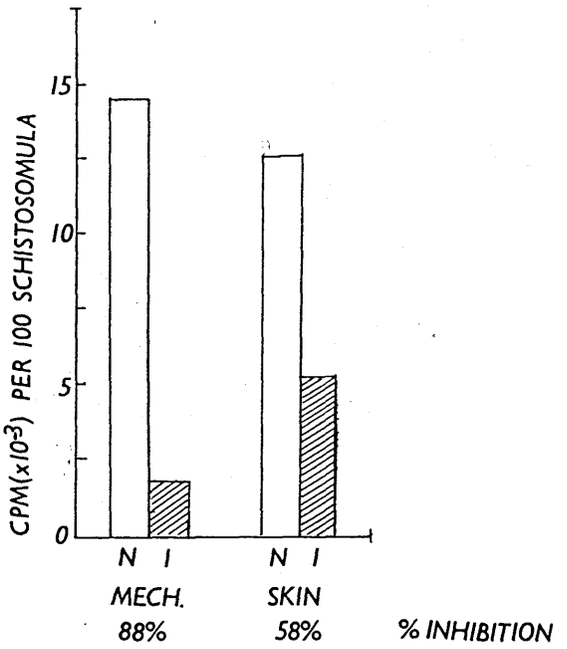

CPM(x10³) PER 100 SCHISTOSOMULA
15
10
5
0
N I
N I
MECH.
SKIN
88%
58%
% INHIBITION

Table 3.5. Survival of normal and U.V.-irradiated, skin-transformed parasites.

Schistosomula were cultured in 10 mls Elac/10% hiFCS at 37°C/5% CO_2, at 500/ml (means ± S.E. of 3 separate experiments).

U.V.-irradiation dose (μW min cm^{-2})	NORMAL	400	800
	%age OF SCHISTOSOMULA DEAD.		
Hours after trans-formation			
3	4.5±0.9	5.2±1.2	4.7±0.7
24	8.5±2.0	6.9±1.2	15.2±1.4
48	12.2±0.9	26.1±2.3	45.6±3.3
72	18.9±2.0	72.5±2.8	89.4±1.8
96	20.8±1.7	100±0	100±0

Comparing the survival of normal parasites after mechanical and skin transformation (tables 3.3; 3.5), it seems that more normal schistosomula were classified as dead at each time point after skin penetration. Salafsky et al (1988) also observed that a high proportion of skin schistosomula were lethally damaged by the stresses of skin penetration. However, it may be noted that the schistosomula which were classified as "live" by 96 hours after skin transformation were in much better condition than their mechanical counterparts. 96-hour live skin forms were translucent, very motile, and normally-shaped, whereas a high proportion of "live" mechanical schistosomula at 96 hours showed considerable granularity and distortion of shape. (see criteria for classification of live and dead schistosomula, section 2.3.3.(4)).

3.8 Effect of Actinomycin D on protein synthesis by schistosomula.

It was felt that the inhibition of protein synthesis described in the preceding sections might be important in generating the enhanced immunogenicity of irradiated larvae. In order to investigate this possibility, we used the drug Actinomycin D to mimic the metabolic effects of U.V.-irradiation. Like U.V.-irradiation, Actinomycin D disrupts the organisation of DNA, inhibiting the transcription and translation of genetic messages. The following experiments discuss the effects of Actinomycin D treatment on protein synthesis by schistosomula. Chapter 4 compares synthesis of RNA, phospholipids and glycoproteins by U.V.-irradiated and Actinomycin D-treated larvae. The role of this metabolic inhibition in rendering the schistosomula immunogenic was tested in the animal protection experiments described in chapter 9.

3.8.1 Actinomycin D dose/effect relationship.

Actinomycin D was chosen to mimic the damage inflicted by U.V.-irradiation on DNA, with its attendant consequences for parasite protein synthesis and other aspects of metabolism. The usual dose and treatment schedule - 2.6 μg/ml for 20 hours - are fully described in section 2.3.3. Unless otherwise stated, the label "Actinomycin D-treated schistosomula" refers to parasites treated in this way.

The experiment of figure 3.16 showed that Actinomycin D-induced inhibition of protein synthesis reached a plateau when the drug was at a concentration of approximately 2.6 μg/ml in the culture medium. Our choice of Actinomycin D concentration, and the corresponding inhibition of protein synthesis were in reasonable agreement with the study of Nagai et al (1977), who observed a 60% inhibition of protein synthesis in schistosomula treated with Actinomycin D at 1.3 μg/ml for 18 hours.

The labelling pattern for schistosomula treated with 2.6 μg/ml Actinomycin D is very similar to that typically seen in 400 μW min cm^{-2} U.V.-irradiated forms; in this experiment, synthesis of Mr 78 000, 70 000, 48 000, 44 000 and 29 000 molecules is maintained, though at severely reduced levels.

3.8.2. Actinomycin D-induced inhibition of protein synthesis is maintained throughout the lifetime of the parasites in culture.

Survival of Actinomycin D-treated schistosomula was protracted slightly beyond that of U.V.-irradiated parasites, as shown in table 3.6

Inhibition of protein synthesis was maintained throughout the period spent in culture after Actinomycin D treatment - see figure

Figure 3.16. Exposure of newly-transformed schistosomula to increasing doses of Actinomycin D for 20 hours. Effect on protein synthesis during the subsequent 24 hours.

Protocol

Cercariae

↓

Transform mechanically in Elac

↓

Divide into 8 1ml samples of schistosomula at 1000/ml in Elac/10% hiFCS.

Add Actinomycin D at appropriate concentrations.

Culture for 20 hours at 37°C/5% CO_2.

↓

Wash in GMEM w/o methionine 4 times.

Resuspend each to 1ml GMEM w/o methionine/ 10% hiFCS.

Add 20 µCi (740 kBq) ^{35}S-methionine. Label for 24 hours at 37°C/5% CO_2.

↓

Remove samples to ice.

↓

Wash three times in ice-cold GMEM.

↓

Freeze for SDS-PAGE.

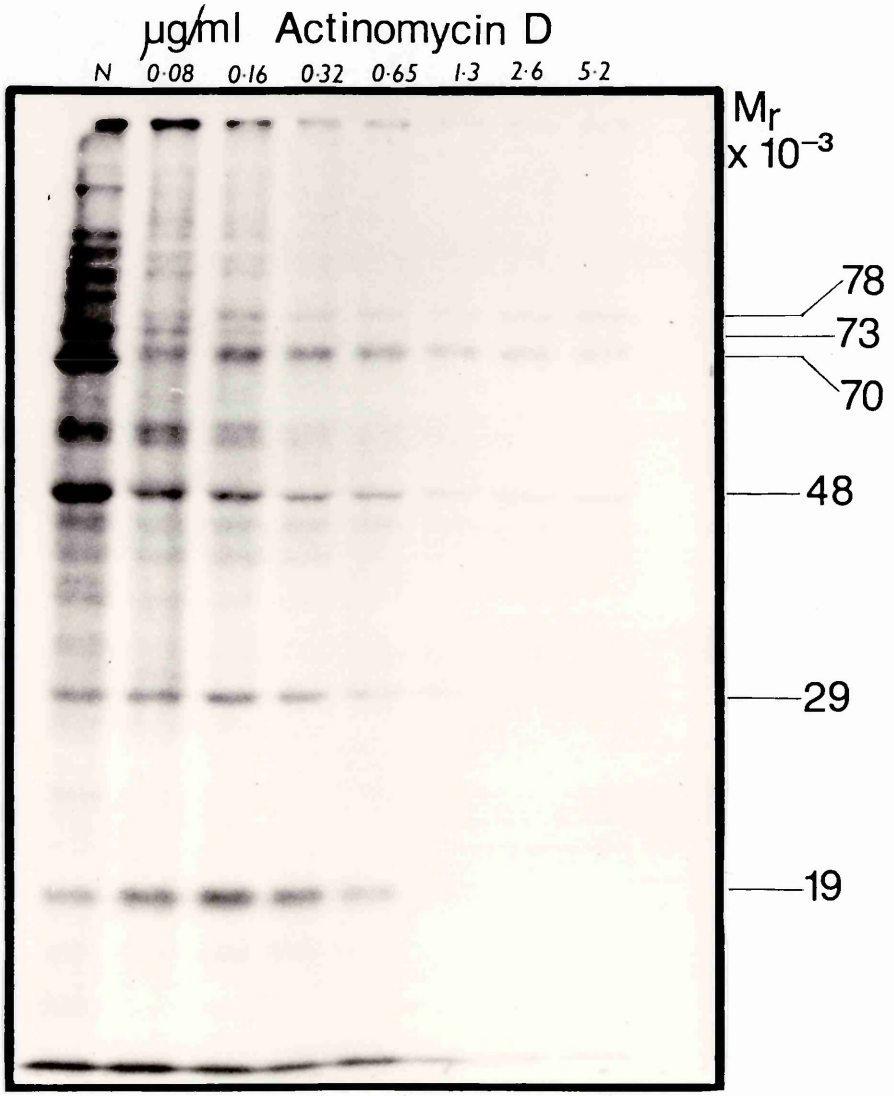

μg/ml Actinomycin D
N
0·08
0·16
0·32
0·65
1·3
2·6
5·2
Mr
x 10⁻³
78
73
70
48
29
19

Table 3.6. Survival of normal and Actinomycin D-treated, mechanically-transformed parasites.

Schistosomula were cultured in 10 mls Elac/10% hiFCS at 37°C/5% CO_2, at 500/ml. (means ± S.E. of 3 separate experiments).

TREATMENT	NORMAL	ACTINOMYCIN D
Hours after transformation	%age OF PARASITES DEAD.	
24	5.4+1.3	4.2+0.6
48	7.9+1.0	6.8+2.9
72	11.3+1.2	22.5+1.0
96	13.1+1.0	96.7+3.2

Figure 3.17. Incorporation of ^{35}S-methionine into TCA-precipitable protein by normal and Actinomycin D-treated schistosomula, over the 72-hour period following Actinomycin D treatment.

Protocol

Cercariae

↓

Divide in 3

↓

N	I	A
normal	U.V.-irradiate: 400 µW min cm^{-2}	Actinomycin D-treated.

↓

Transform mechanically in Elac.

Resuspend to 5 mls Elac/10% hiFCS at 1000 schistosomula/ml

Add Actinomycin D at 2.6 µg/ml to "A" culture.

Culture all three at 37°C/5% CO_2 for 20 hours

↓

Wash well in Elac. Divide each of N, I, A into 9 1ml samples of Elac/10% hiFCS.

Add 25 µCi (925 kBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2.

↓

After labelling for 24h, 48h, 72h, remove 3 samples of each to ice.

↓

Wash three times in ice-cold Elac.

↓

Freeze for TCA-precipitation.

Each point represents the mean of duplicate samples.

Deviation of duplicates from mean = ±8.6%

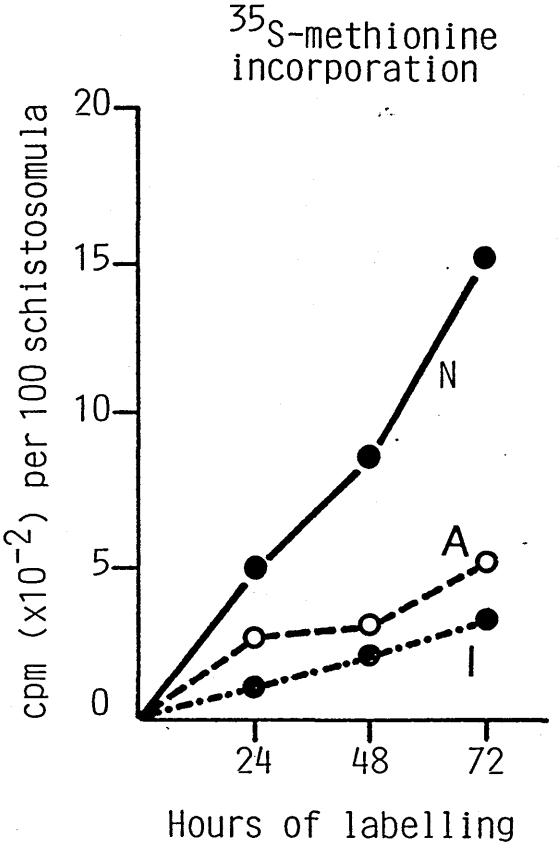

^{35}S-methionine incorporation
cpm (x10^{-2}) per 100 schistosomula
20
15
10
5
0
N
A
I
24
48
72
Hours of labelling

Table 3.7. Percentage inhibition of protein synthesis in U.V.-irradiated and Actinomycin D-treated schistosomula at each time-point in figure 3.16.

Each value represents the mean of duplicate samples.

TREATMENT	24	48	72	HOURS OF LABELLING
400 μW min cm^{-2} U.V.-IRRADIATED	80	76.7	78.7	% INHIBITION
ACTINOMYCIN D-TREATED	50	64.7	67.2	

Figure 3.18. ^{35}S-methionine incorporation into protein by normal, U.V.-irradiated (400 μW min cm^{-2}) and Actinomycin D-treated schistosomula during the 40-hour period following skin transformation.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

A = Actinomycin D-treated.

1000 schistosomula/track.

Protocol

Cercariae

↓

Divide in three:

N I A

(U.V.-irradiate:

400 μW min cm^{-2})

↓

Allow to penetrate excised mouse skin for 3 hours. Collect into GMEM w/o methionine.

↓

Resuspend each to four 1 ml samples of GMEM w/o methionine/10% hiFCS. Schistosomula at 1500/ml. Add Actinomycin D to "A".

↓

At 0h, 10h - add 50 μCi (1.85 MBq) ^{35}S-methionine to one sample of each of N, I, A. Label for 10h at 37°C, CO_2.

Continued on next page.

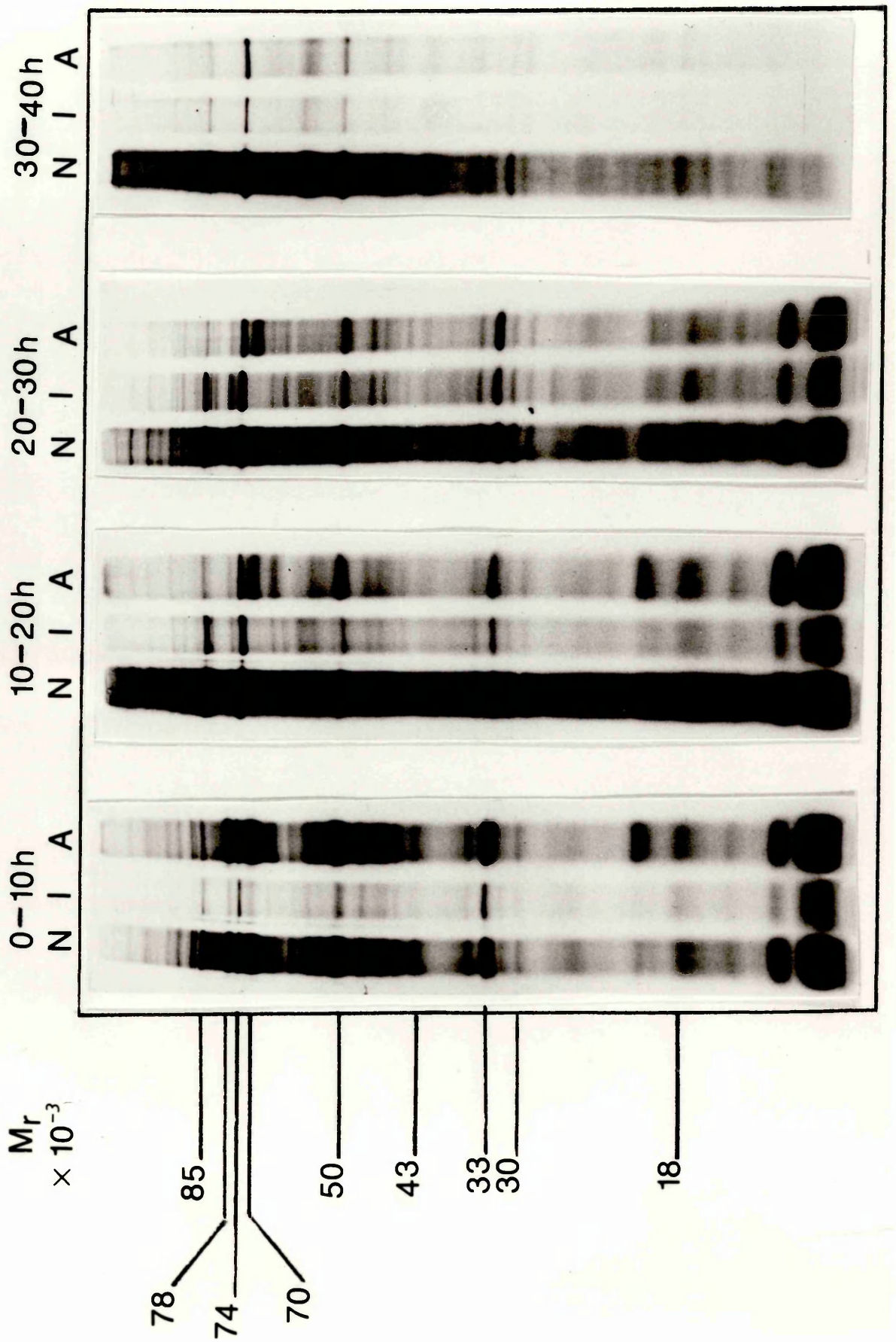

0–10h
N I A
10–20h
N I A
20–30h
N I A
30–40h
N I A
M_r
$\times 10^{-3}$
85
78
74
70
50
43
33
30
18

Figure 3.18. cont.

At 20h, wash remaining samples 4 times with GMEM w/o methionine, and resuspend as before. Then label one sample of each of N, I, A for the subsequent 10 hours with 50 µCi (1.85 MBq) ^{35}S-methionine. At 30h, add 50 µCi (1.85 MBq) ^{35}S-methionine to remaining one sample of N, I and A; label for a further 10 hours.

↓

After each 10 hour labelling period, wash each sample 3 times in ice-cold GMEM; freeze for gel.

3.17. Table 3.7 shows that the percentage inhibition at each time point was comparable to that of irradiated forms.

Actinomycin D inhibited protein synthesis, not only in mechanically-transformed schistosomula, but also in skin forms - see figure 3.18. This gel illustrates a number of important points. Firstly, during the 0 to 10 hour period, when the schistosomula are still being treated with Actinomycin D, there seems to be no inhibition of protein synthesis. In contrast, the U.V.-irradiated schistosomula show extensive inhibition at this time. Inhibition only becomes apparent during the second half of the drug-treatment period (10 to 20 hours). This agrees with the findings of Nagai et al (1977), who could detect the inhibition of protein synthesis after 18 hours, but not after 5 hours, of treatment with Actinomycin D.

A second point concerns the nature of the proteins synthesized by each group of schistosomula. From 0 to 10 hours after transformation, normal and Actinomycin D-treated parasites both show intense labelling of the series of 3 proteins between Mr 70 000 and 78 000 (Mr 78 000, 74 000, 70 000). In the track with irradiated parasites, the band at Mr 74 000 is the most intensely labelled of the three, with the 78 000 and 70 000 species faintly detectable above and below it. From 10 hours onwards, the 74 000 Mr protein is predominantly synthesized by all 3 groups of schistosomula, while the 78 000 Mr band becomes very faint. For the normal and Actinomycin D-treated schistosomula, the 70 000 Mr protein is still quite heavily labelled from 10 to 30 hours, but is not detectable at all the irradiated track. In the 30 to 40-hour samples, the 70 000 Mr band is not detectable in any of the tracks. It is also noticeable that, from 10 to 30 hours, the U.V.-irradiated parasites show relatively intense labelling of a Mr 85 000 band which is only faintly labelled in the Actinomycin D-treated forms.

The pattern of protein synthesis by both normal and Actinomycin D-treated parasites in this experiment clearly differs from that of

figure 3.16. This was not due to the fact that schistosomula in one experiment were mechanically-transformed, whereas in the second they were derived by skin penetration, but seemed rather to be a result of the general variability in pattern of protein synthesis by newly-transformed schistosomula. (see figure 3.6. and chapter 8 for a fuller discussion of variability in parasite metabolism).

3.8.3. Cycloheximide does not inhibit protein synthesis by schistosomula.

In contrast to Actinomycin D, Cycloheximide (from Sigma) did not appear to inhibit schistosomular protein synthesis, even when milligram concentrations, and a 20-hour exposure time, were used (see figure 3.19).

3.9 Synthesis of surface and secretory proteins by cercariae, normal U.V.-irradiated and Actinomycin D-treated schistosomula.

3.9.1. Loss of metabolically labelled proteins during mechanical or skin transformation of radiolabelled cercariae.

Cercariae labelled metabolically with ^{35}S-methionine in aquarium water apparently lost all detectable radioactive proteins within 90 minutes of mechanical transformation (see figure 3.20). Indeed, most of the radioactivity was lost during the first 30 minutes after syringe passage, while 3-hour schistosomula seemed to have lost all the proteins they synthesized as free-living cercariae. Both normal and irradiated parasites showed identical secretion patterns. Nor were any differences detected in the patterns of labelled proteins released before and after the 30-minute mark.

Some of the most prominently-labelled proteins released by

Figure 3.19. Effect of Cycloheximide on protein synthesis by schistosomula.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

1500 schistosomula per track.

Protocol

Cercariae

↓

Divide in 4

N	I	0.5mg/ml	2mg/ml
	U.V.-irradiate (400 μW min cm^{-2})	Cycloheximide	Cycloheximide

↓

Transform mechanically in Elac.

Resuspend each to 2 1ml samples;1500 schistosomula/ml in Elac/10% hiFCS. Add required concentrations of Cycloheximide to appropriate samples.

Culture at 37°C/5% CO_2

↓

At 3h and 20h; wash one sample from each 4 times in GMEM w/o methionine.

Resuspend to 1ml GMEM w/o methionine.

Add 20 μCi (740 kBq) ^{35}S-methionine.

Culture at 37°C/5% CO_2 for 24 hours.

↓

Remove samples to ice; wash 3 times in ice-cold GMEM; freeze for SDS-PAGE.

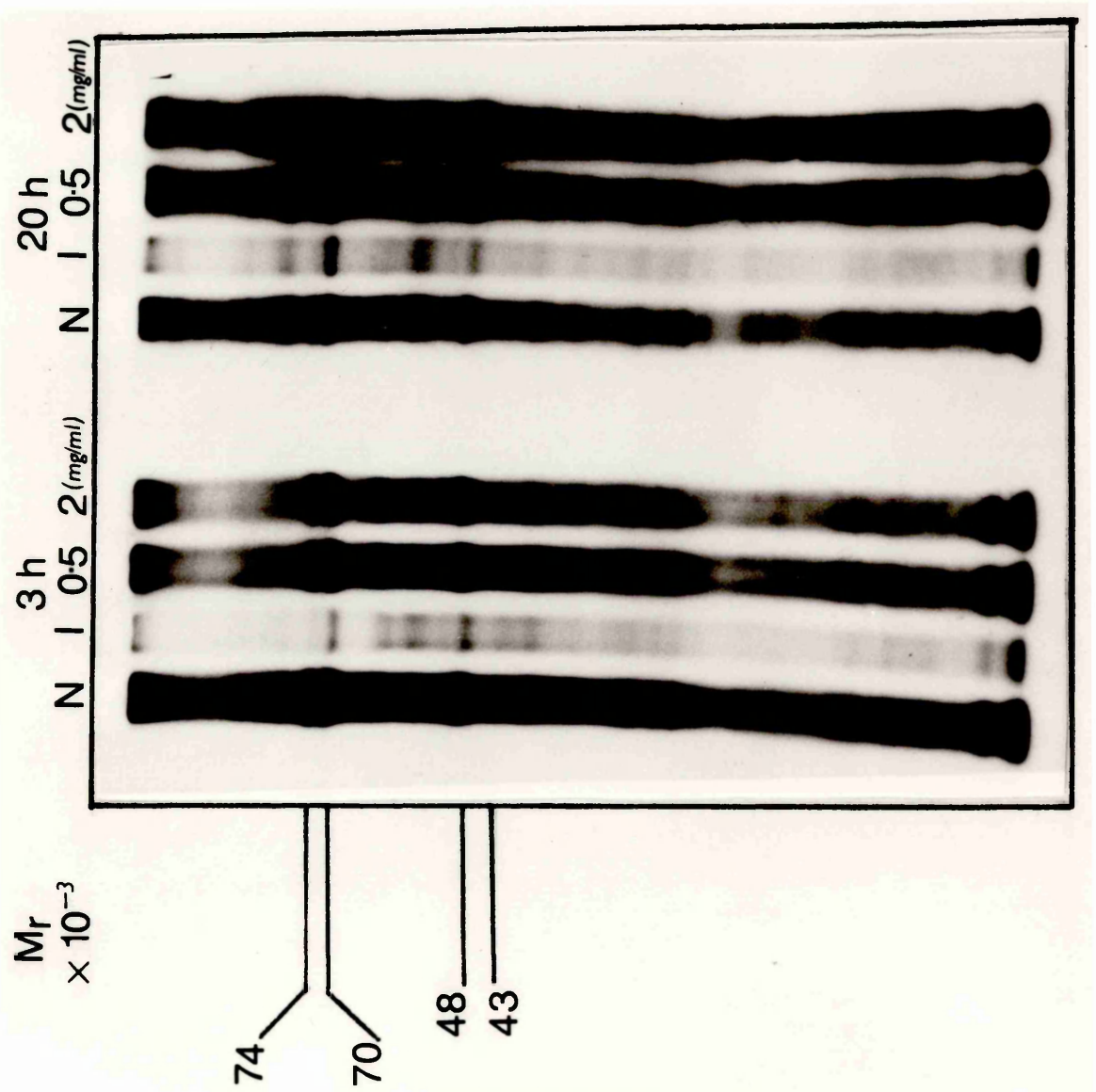

Mr
× 10⁻³
3 h
N I 0·5 2 (mg/ml)
20 h
N I 0·5 2 (mg/ml)
74
70
48
43

Figure 3.20 Loss of metabolically-labelled proteins during mechanical transformation of prelabelled cercariae.

C = cercariae N = normal

S = schistosomula I = U.V.-irradiated (400 µW min cm^{-2})

500 parasites per track.

3% stacking gel/10% resolving gel.

Protocol

Label cercariae for 3 hours at room temperature in 20 mls aquarium water with 200 µCi (7.4 MBq) ^{35}S-methionine

↓

Then: Divide into 2 lots of 10 mls

N I (U.V. irradiate after labelling: 400 µW min cm^{-2})

2.5 mls from each; sediment on ice, wash once in cold GMEM; freeze for SDS-PAGE (CN, CI samples)

Transform rest mechanically in 3 mls GMEM. Retain tails.

Divide each into 3 1ml samples

Incubate at 37°C.

↓

At 30 mins: Spin down one N and one I sample.

Wash pellet once in GMEM; freeze.

Supernatant: Remove tails by sedimenting on ice and centrifuging. Add protease inhibitors, remove salt and freeze-dry.

Continued on next page.

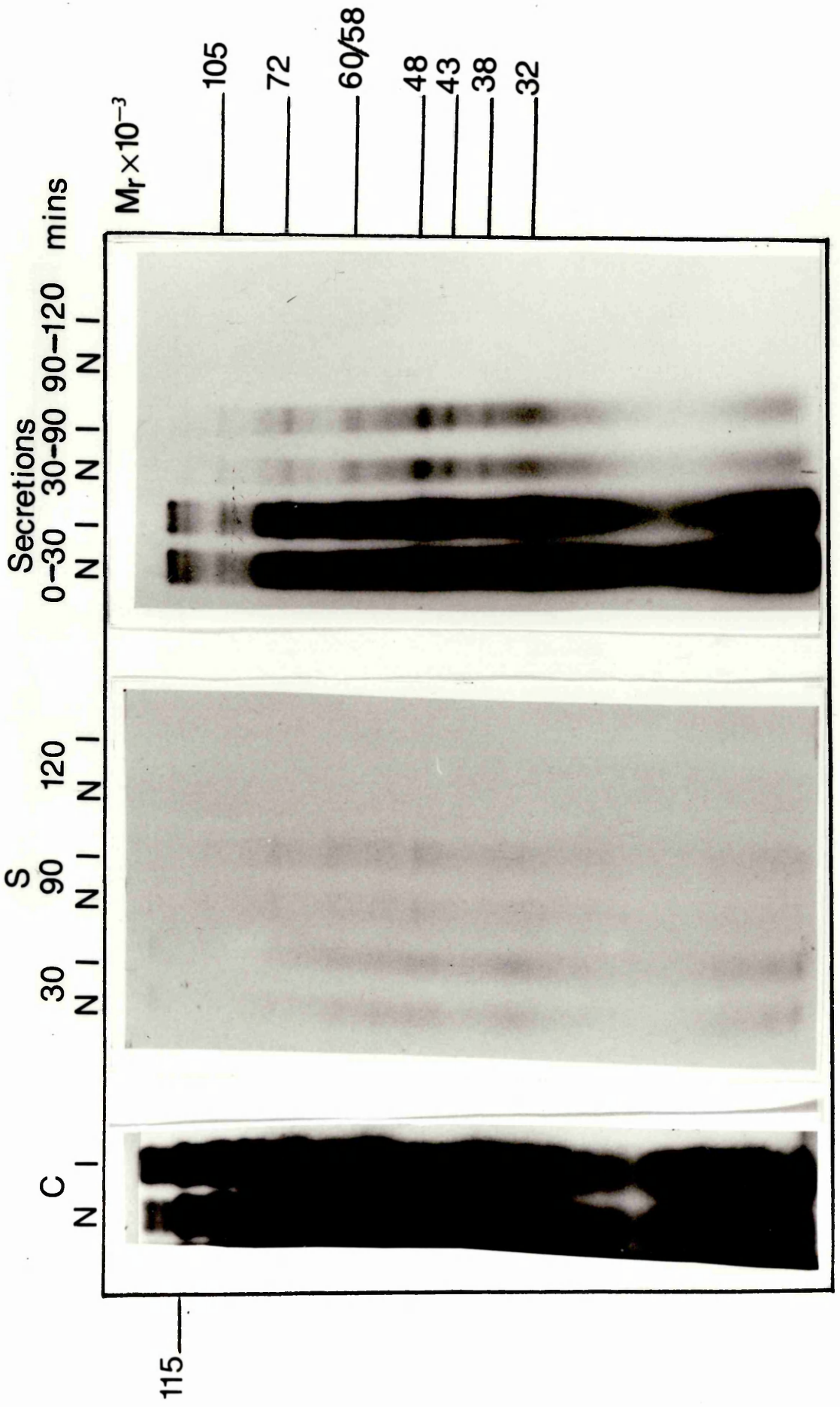

C
N I
S
30 90 120
N I N I N I
Secretions
0–30 30–90 90–120 mins
N I N I N I
$M_r \times 10^{-3}$
105
72
60/58
48
43
38
32
115

Figure 3.20 continued.

Also at 30 mins: Spin down a second normal and irradiated sample.

Replace medium with a fresh 1ml GMEM.

At 90 mins: take these samples as described above.

Also at 90 mins: Spin down third N and I samples.

Replace medium with a fresh 1ml GMEM.

At 120 mins, take these samples as described above.

cercariae had Mr 105 000, 72 000, 60 000, 58 000, 48 000, 43 000, 38, 000, 32 000. Interestingly, the band at Mr 115 000, which was so heavily labelled by cercariae before transformation, did not appear in the track for either the schistosomula or their secretions. It may have been degraded to some lower molecular weight form.

(Note that, in this experiment, the cercariae were metabolically labelled before irradiation. Hence, the samples labelled "normal" and "irradiated" both contained equal amounts of free and TCA-precipitable ^{35}S-methionine at the start of the experiment).

Labelled cercariae transformed by skin penetration lost even more of their newly-synthesized proteins than did mechanical forms (see table 3.8). Again, however, normal and irradiated schistosomula secreted equal amounts of labelled proteins.

3.9.2. Protein secretion by normal and U.V.-irradiated schistosomula radiolabelled immediately after transformation

By iodinating the surfaces of newly-transformed schistosomula (section 2.6.1.), then collecting the iodinated material which these parasites released into culture, we were able to compare the secretion of surface proteins by normal and U.V.-irradiated schistosomula during the first few hours of transformation. Figure 3.21 shows that there are no striking differences between normal and U.V.-irradiated schistosomula as regards either the antigens exposed at the surface for Iodogen labelling, or the release of these antigens during culture.

In both normal and U.V.-irradiated forms, surface proteins available for iodination are observed at Mr 70 000, 50 000, as a diffuse band between 40 000 and 32 000, and at 19 000. In this experiment, an extra labelled band appears at Mr 18 000 in the irradiated track. However, this was not consistently observed in other experiments.

Protocol for Table 3.8.

Label cercariae for 1 hour at room temperature in 20 mls aquarium water with 200 µCi (7.4 MBq) ^{35}S-methionine.

Then: Divide into 2 lots of 10 mls.

N I (U.V.-irradiate: 400 µW min cm^{-2})

2 1ml portions from each; sediment on ice; wash twice in GMEM; freeze

Remaining 6 mls: Allow to penetrate excised mouse skin. Collect schistosomula in GMEM.

↓

At 1h; remove schistosomula; replace skin over fresh GMEM. Collect schistosomula again after a further 2 hours.

↓

Remove schistosomula to ice. Count and wash twice in cold GMEM. Freeze.

2 mls from each; Transform mechanically in GMEM; resuspend each to 4 1ml samples

↓

At 1h, 3h: Remove 2N and 2I samples to ice. Wash twice in GMEM; freeze.

Table 3.8. Loss of ^{35}S-methionine-labelled protein from normal (N) and irradiated (I) cercariae transformed mechanically or by skin penetration.

		TCA-PRECIPITABLE cpm PER 100 PARASITES	% LOSS OF RADIOACTIVITY
PRE-LABELLED CERCARIAE	N	9064	
	I	8742	
SKIN SCHISTOSOMULA	1h N	28	99.7
	I	25	99.7
	3h N	16	99.8
	I	10	99.9
MECHANICAL SCHISTOSOMULA	1h N	1063	88.3
	I	1263	85.6
	3h N	319	96.4
	I	260	97.0

For cercariae and mechanical schistosomula, each value represents the mean of duplicate samples.

Figure 3.21. Iodination of antigens exposed at the surface of normal and U.V.-irradiated, newly-transformed schistosomula, and release of these antigens during a 5-hour culture period.

N = normal I = U.V.-irradiated (400 μW min cm^{-2})

500 schistosomula per well.

3% stacking gel, 10% resolving gel.

Protocol

Cercariae

↓

Divide equally in 2

N I (U.V.-irradiate at 400 μW min cm^{-2})

↓

Transform mechanically in GMEM.

Spin down schistosomula; resuspend

to 200 μl for Iodogen iodination (section 2.6.1.)

↓

Culture iodinated schistosomula in 1ml

GMEM for 37°C for 5 hours

↓

Remove samples to ice.

Spin down schistosomula.

Schistosomula	Supernatants
Wash 3 times in ice-cold GMEM; freeze for SDS-PAGE.	Add protease inhibitors: Desalt on PD-10 column and freeze-dry.

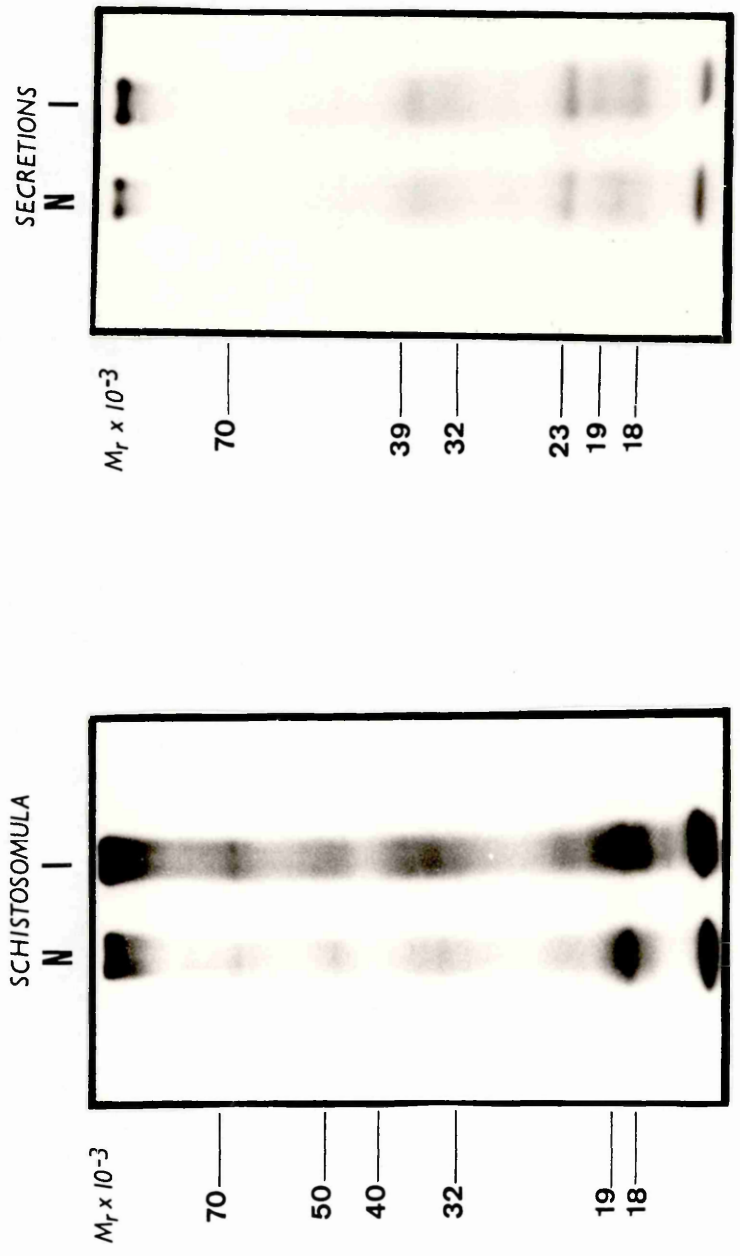

SECRETIONS
N
I
$M_r \times 10^{-3}$
70
39
32
23
19
18
SCHISTOSOMULA
N
I
$M_r \times 10^{-3}$
70
50
40
32
19
18

Table 3.9. Release of iodinated surface antigens by normal and U.V.-irradiated (400 μW min cm^{-2}) schistosomula during the 5-hour period following mechanical transformation.

(see figure 3.21 and accompanying protocol).

Mr of protein	Is this protein detected in:			
	Bodies		Secretions	
	N	I	N	I
70 000	✓	✓	faint	faint
50 000	✓	✓	X	X
32 000-40 000 (diffuse)	✓	✓	✓	✓
23 000	faint	faint	✓	✓
19 000	✓	✓	✓	✓
18 000	X	✓	faint	✓

The surface proteins released during the 5 hours following transformation have molecular weights 39 000-32 000 (a diffuse band), 23 000, 19 000 and 18 000. A faint band at Mr 70 000 is also detectable. Table 3.9 summarises these results.

Thus, it would appear that a proportion of almost every protein detectable on the surface of newly-transformed schistosomula was released during the 5-hour culture period. The additional low molecular weight bands present in the secretions but not in the whole parasites may arise from proteolytic degradation of higher molecular weight proteins. Alternatively, they may represent proteins present on the parasite surface immediately after transformation, but completely released into culture during the following 5 hours, so that none were left at the surface.

A second approach to comparing protein secretion by normal and irradiated schistosomula involved metabolic labelling of the parasites with ^{35}S-methionine, then analysing the radioactive proteins released into culture. Figure 3.22 shows the ^{35}S-methionine- labelled proteins secreted by normal and irradiated schistosomula during the 5-hour period following mechanical transformation. The labelled proteins present in the intact schistosomula in this experiment are shown in figure 3.9. The most intensely labelled proteins which are secreted by normal schistosomula have molecular weights 65 000, 43 000, 26 000 and 15 000. These bands are also very prominent in figure 3.9, showing proteins synthesized by the whole schistosomula. The Mr 78 000 postulated heat-shock protein, which is heavily labelled by whole schistosomula, is also quite prominent in the secreted material. For irradiated schistosomula, metabolically labelled, secreted proteins at the same molecular weights as for normal larvae are only detectable after 5 hours of culture.

Secretion of newly-synthesized proteins by skin schistosomula during the first 4 hours after transformation was also examined (figure

Figure 3.22. Secretion of metabolically-labelled proteins by normal and U.V.-irradiated schistosomula during the 5 hours following mechanical transformation.

Secretions from 1000 schistosomula/well. N = normal

3% stacking gel/ I = U.V.-irradiated

7.5% resolving gel. (800 μW min cm^{-2})

Protocol

Cercariae

↓

N I (U.V.-irradiate: 800 μW min cm^{-2})

↓

Transform mechanically in GMEM w/o methionine.

↓

Divide each into 5 1ml samples.

Add 20 μCi (740 kBq) ^{35}S-methionine to each.

Incubate at 37°C.

↓

At 1h, 2h, 3h, 4h, 5h: Remove one sample of each to ice. Spin down and remove supernatant.

↓

Remove any tails from supernatant by sedimenting on ice and centrifuging.

↓

Remove salt from supernatants by dialysis; freeze-dry for gel.

This gel shows the proteins secreted by the schistosomula whose metabolic labelling pattern is shown in figure 3.9.

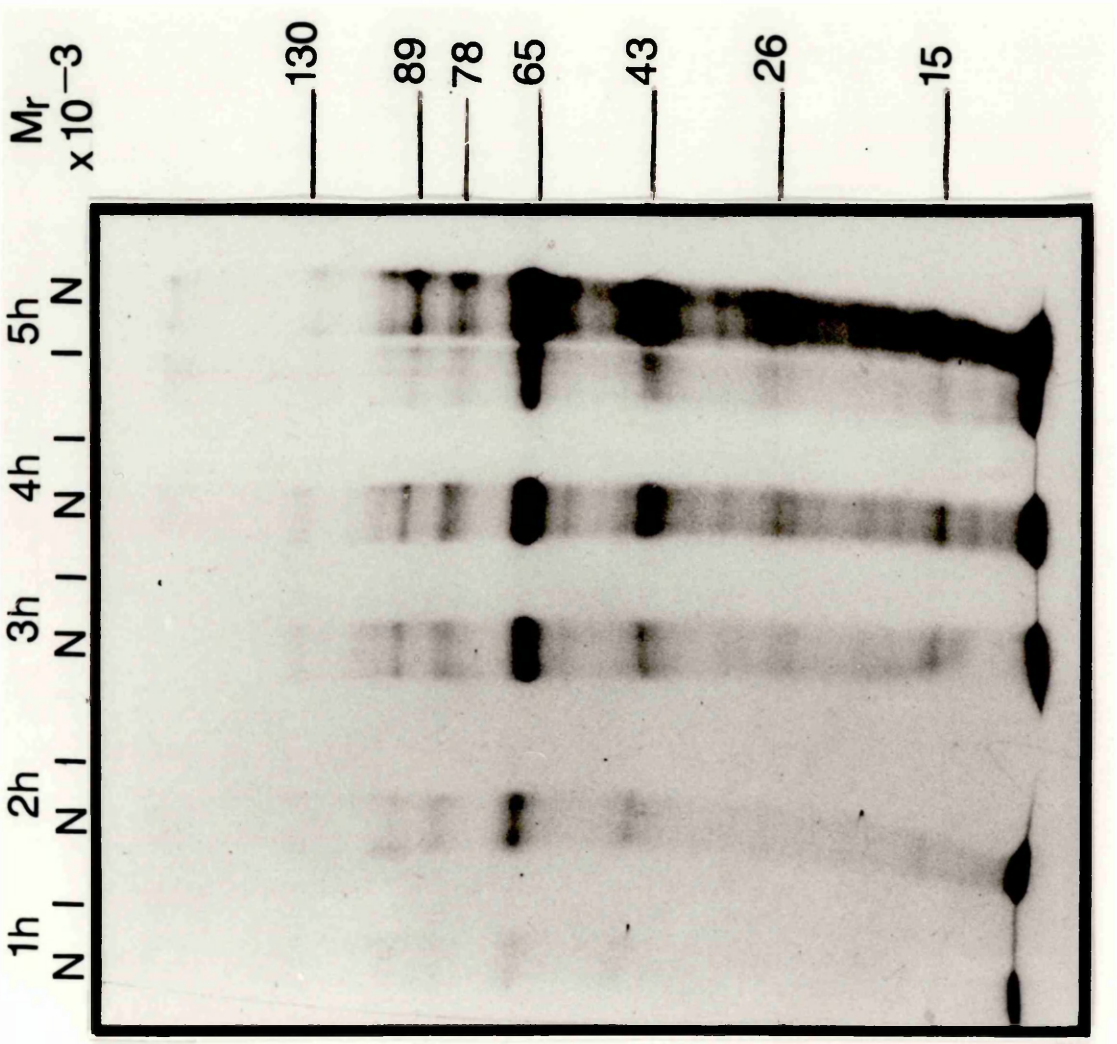

Mr
x10−3
130
89
78
65
43
26
15
1h
2h
3h
4h
5h
N
I

Figure 3.23. Release of metabolically-labelled proteins by normal and U.V.-irradiated schistosomula during the 4 hours following skin transformation.

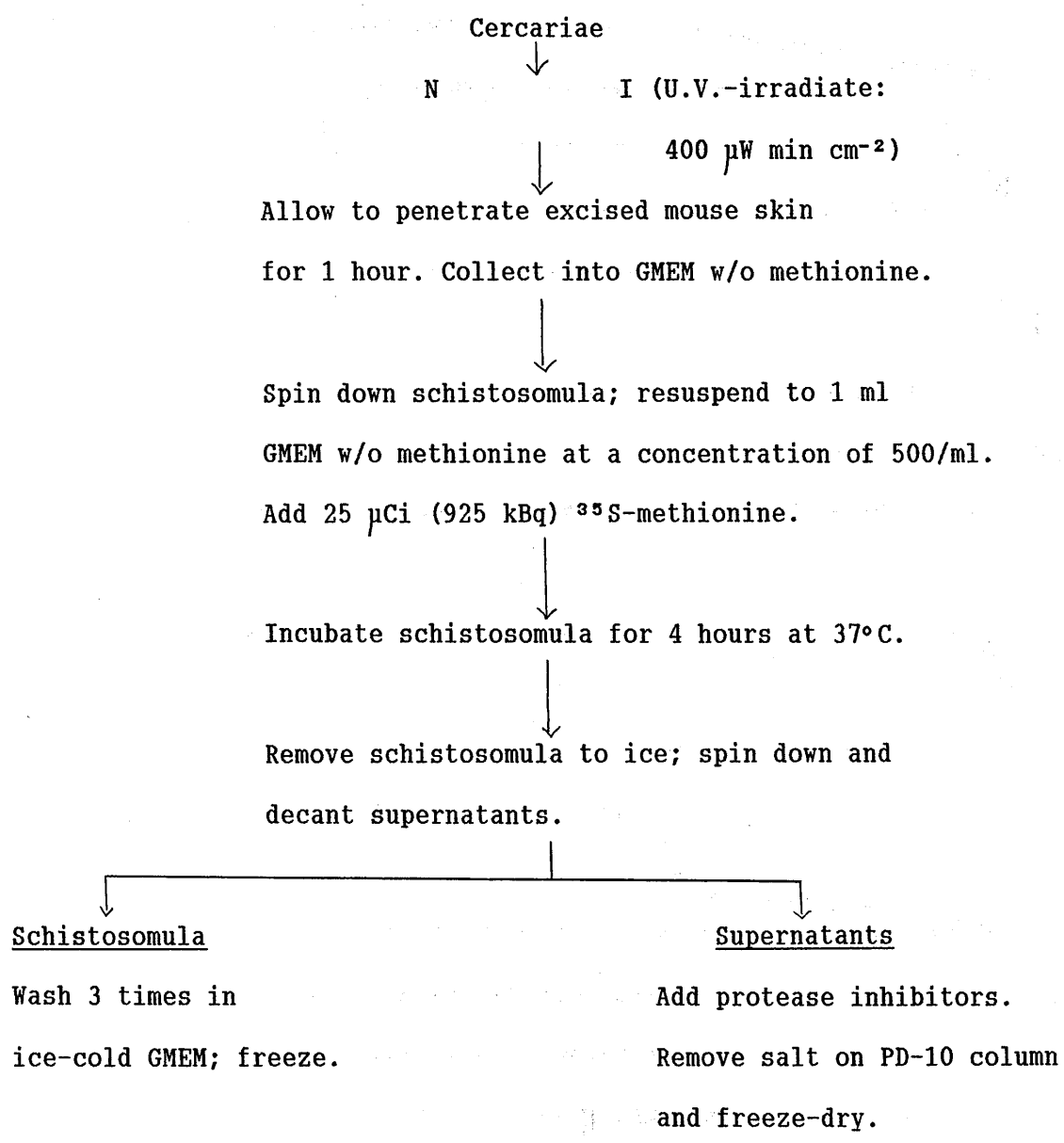
N = normal

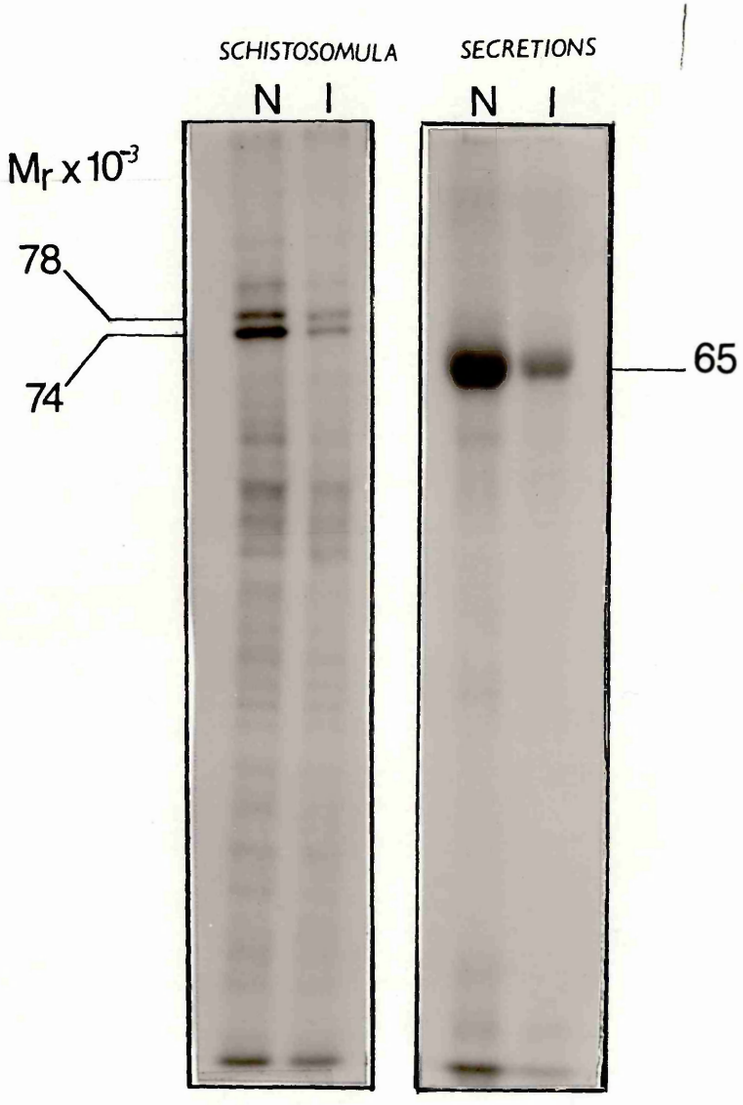
I = U.V.-irradiated (400 μW min cm^{-2})

500 schistosomula/track.

3% stacking gel/10% resolving gel.

Protocol




SCHISTOSOMULA
SECRETIONS
N I
N I
Mr x 10⁻³
78
74
65

3.23.). Schistosomula were allowed to penetrate excised mouse skin for one hour, were collected into GMEM w/o methionine, and labelled with ^{35}S-methionine for the following 4 hours.

A 65 000 Mr band was again especially prominent in the secretions. Proteins at Mr 60 000 and 14 000 were just detectable in the original fluorograph. None of these species was correspondingly heavily labelled in the tracks of whole schistosomula.

In this experiment, it seems that the irradiated schistosomula do not show as severe an inhibition of protein synthesis as in figures 3.9 and 3.22. This may be due, in part, to variability in the parasite response to irradiation, and in part to the fact that irradiated schistosomula collected after skin penetration generally did show less inhibition of protein synthesis than mechanical forms of the same age (see figure 3.15). The significance of these differences in response to irradiation will be discussed in chapter 8.

3.9.3. Synthesis of body and surface proteins by normal, U.V.-irradiated and Actinomycin D-treated schistosomula.

The saponin/$CaCl_2$ method of Kusel (1972) was used to differentiate synthesis of body and surface proteins by schistosomula (see section 2.4.5.). Figures 3.24 and 3.25 show the electrophoresed body and surface proteins of normal, U.V.-irradiated and Actinomycin D-treated schistosomula, and the corresponding fluorographs.

Silver-staining of the schistosomular bodies indicated a highly complex mixture of proteins (figure 3.24a). In contrast, the schistosomular surfaces contained relatively few, discrete bands. The major surface proteins revealed by silver-staining had molecular weights 65 000, 47 000, 38 000, 35 000, 32 000, 18 000 (figure 3.25a).

However, fluorography of the normal parasite bodies and surfaces demonstrated that the surface actually contained a greater variety of

Figures 3.24 and 3.25. Incorporation of ^{35}S-methionine into body and surface proteins by normal, U.V.-irradiated and Actinomycin D-treated schistosomula from 24 to 48 hours after transformation.

N = normal schistosomula.

I = U.V.-irradiated schistosomula (400 µW min cm^{-2})

A = Actinomycin D-treated schistosomula.

500 schistosomula per track.

3% stacking gels/10% resolving gels.

Silver-stained gels and the corresponding fluorographs are shown.

Protocol

Cercariae

↓

N I A

(U.V.-irradiate: 400 µW min cm^{-2})

↓

Transform mechanically in Elac. Resuspend to 5 mls Elac/10% hiFCS. Add Actinomycin D to "A".

↓

Culture for 20 hours at 37°C/5% CO_2.

↓

Wash 4 times in GMEM w/o methionine; resuspend to 1ml GMEM w/o methionine/ 10% hiFCS. Add 20 µCi (740 kBq) ^{35}S-methionine to each.

↓

Culture for 24 hours at 37°C/5% CO_2

Continued on next page.

Figures 3.24 & 3.25 continued.

↓

Remove to ice; wash 3 times in

ice-cold GMEM.

↓

Extract surfaces by saponin method (section 2.4.5.)

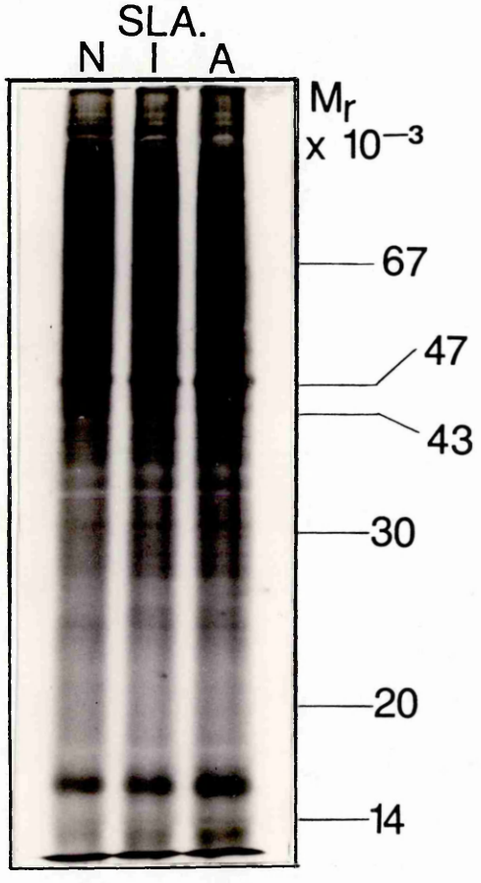
3.24 a) = silver-stain of schistosomular bodies.

3.24 b) = fluorograph of a)

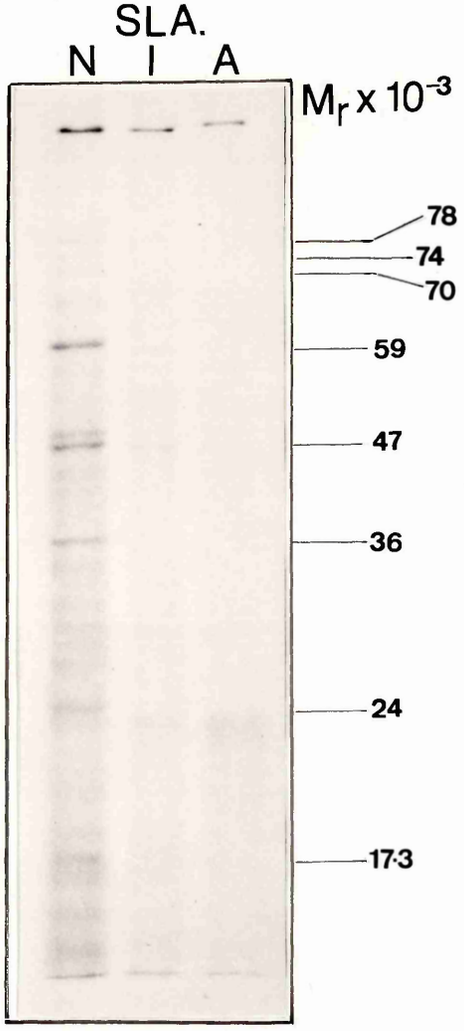
3.25 a) = silver-stain of schistosomular surfaces.

3.25 b) = fluorograph of a).

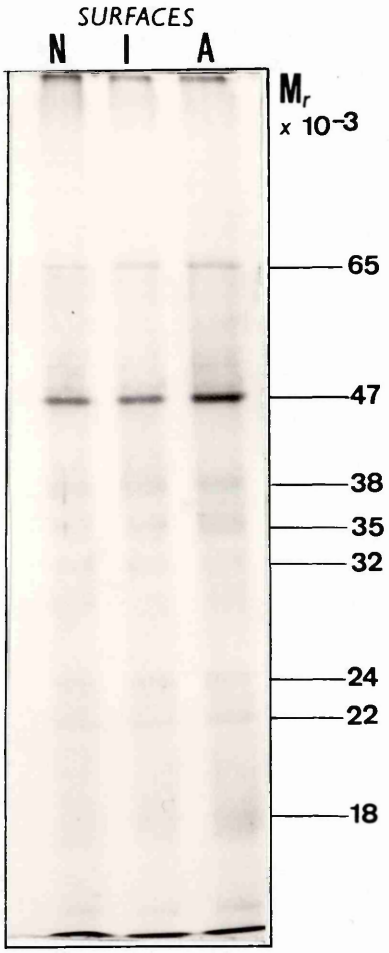
3.24 a) SILVER STAIN

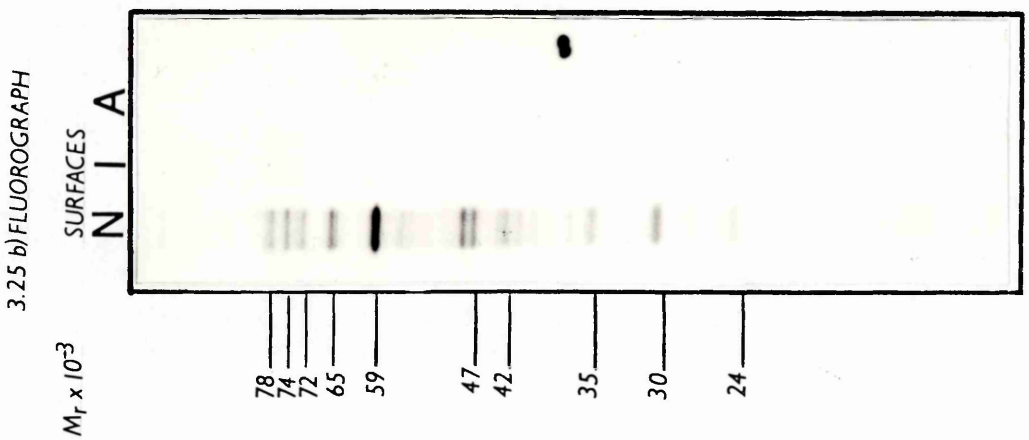


3.2.4 b) FLUOROGRAPH



3.25 a) SILVER STAIN




3.25 b) FLUOROGRAPH
SURFACES
N I A
$M_r \times 10^{-3}$
78
74
72
65
59
47
42
35
30
24

newly-synthesized proteins than the body (figures 3.24b, 3.25b). Especially prominently labelled bands in the surface extracts were found at Mr 78 000, 74 000, 70 000, 65 000, 59 000, 47 000, 35 000, 30 000, 24 000. Many other, less heavily labelled bands were also present in the normal schistosomular surface extract. The many bands detectable by fluorography of the surface proteins, but not by silver-staining, must represent molecules which are present at the surface in very minute amounts (limit of silver-stain detection is 0.01 mg protein per mm^2), but, nevertheless, are very heavily labelled. For both the U.V.- irradiated and Actinomycin D-treated schistosomula, incorporation of radioactivity into surface proteins appeared to be completely inhibited.

Although silver-staining indicates that the amount and variety of proteins in the parasite body are much greater than on the surface, the corresponding fluorographs show that comparatively little of the body protein is metabolically labelled in normal parasites. The normal schistosomula seem to have devoted most of their synthetic activity to production of surface proteins. The most prominent metabolically labelled proteins located in the bodies of normal parasites have Mr 59 000, 47 000, 36 000, 24 000. Inhibition of protein synthesis in the bodies of irradiated and Actinomycin D-treated schistosomula was not as complete as observed for the surfaces of attenuated larvae. In this experiment, faint bands are detectable at Mr 47 000 and 24 000 in the bodies of attenuated parasites. Thus, it seems that the low level of synthesis which is maintained by irradiated and drug-treated parasites is largely limited to body proteins. Few, if any, newly-synthesized proteins seem to be inserted into the surface membranes of the attenuated schistosomula.

It is especially interesting to note that the Mr 78 000, 74 000, and 70 000 bands are just faintly detectable in the bodies of normal larvae, but are much more heavily labelled in the surface extracts.

Accepting the hypothesis that these 3 species represent members of a heat-shock protein family, then it would seem that newly-synthesized heat-shock proteins associate preferentially with the schistosomular surface. This point will be discussed more fully in section 3.10.3.

3.10. Discussion

3.10.1. Amino acid uptake by cercariae and schistosomula.

Cercariae are frequently described as impermeable to both solutes and water (Nagai et al, 1977; Parra et al, 1986; Rumjanek, 1987). Experimental evidence to support this claim comes mainly from an observation by Ramalho-Pinto et al (1974), who were unable to demonstrate significant uptake or incorporation of radioactive precursors by cercariae. More recently, Salafsky et al (1988) have confirmed this result. Such difficulties in radiolabelling have led to the more general assumption that cercariae are metabolically inactive (Chappell, 1979). However, both Ramalho-Pinto et al (1974) and Salafsky et al (1988) incubated the cercariae in a nutrient-rich, salt-containing medium - Hank's basal salt solution, or Elac - rather than in the aquarium water which forms their natural environment. The results presented here (figure 3.1.) agree that uptake of ^{35}S-methionine by cercariae in salt solution or GMEM is very low. In contrast, free-living cercariae in water appear to be highly permeable to solutes, and to perform considerable protein synthesis.

The cross-linked network of carbohydrate polymers which forms the cercarial glycocalyx (Caulfield et al, 1987) does appear ideally suited to its function as a water-impermeable barrier. It is well-established that such complex carbohydrate structures, for instance, the extracellular proteoglycans of mammalian tissues, or the cell wall peptidoglycans of bacteria, have an immense capacity for hydration

which prevents the entry of water molecules into the cell (Sharon, 1975). Miller et al (1986) indicated a role for various oligosaccharide structures, other than peptidoglycans, in osmotic adaptation in various bacterial systems. In a similar way, the cercarial glycocalyx seems to play an important role in osmoregulation. Thus, within 30 minutes of disruption and partial removal of the glycocalyx by transformation, the parasites become intolerant to fresh water, and undergo osmotic lysis if returned to such an environment (Stirewalt, 1974). However, although the large solvent domain occupied by carbohydrate polymers excludes water from cells, the matrix they form does normally allow entry of small inorganic and organic solutes such as ions and amino acids (Sharon, 1975). Indeed, this permeability to nutrients is essential, in view of the ubiquitous distribution of extracellular carbohydrate networks, including tissues largely concerned with solute uptake, such as the kidney and intestinal mucosa. Thus, there is no precedent for expecting that the cercarial glycocalyx should be impermeable to small organic molecules. Moreover, fully-developed cercariae must presumably be able to assimilate nutrients during their residence in the snail host.

Our data indicate that uptake of methionine by cercariae is low in defined medium (GMEM w/o methionine), salt solution, or haemolymph (see figure 3.1; also compare intensity of tracks 1A,2A in figure 3.3.a)), but is enhanced at least ten-fold in unsupplemented water. It would be most valuable to understand the mechanism underlying this increase in permeability, since the nature and functioning of the cercarial surface is at present poorly understood.

Chappell (1974) showed that 3-hour schistosomula absorbed radioactive methionine chiefly by simple diffusion. A very minor contribution to total uptake was made by a Na^+-dependent, saturable transport system, shared by other amino acids. Podesta et al (1983), discussing solute uptake by adult schistosomes, also distinguished

a number of saturable, Na^+-dependent amino acid transport systems from simple diffusion. It seems reasonable to assume that absorption of methionine by cercariae, like the later life-cycle stages, will be predominantly by simple diffusion. If Na^+-dependent transport mechanisms are also present in cercariae, then Na^+-mediated uptake may also make its minor contribution to overall absorption of methionine in haemolymph or GMEM. However, the negligible concentration of Na^+ in aquarium water is unlikely to account for the very high uptake of methionine in this system.

It would appear that the diffusion component of solute uptake by cercariae is markedly enhanced in fresh water, but immediately decreases upon transfer to salt solution or GMEM. A rapid change in the organisation of the parasite surface membrane must generate this striking alteration in permeability to solutes.

The cercarial membrane is known to contain a high proportion of negatively-charged (acidic) phospholipids: phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylinositol (PI) (one negative charge each). (See Young and Podesta, 1984; see also chapter 4). In an environment of low ionic strength, such as fresh water, repulsion between these negatively-charged head groups might be predicted to tend to increase the area each phospholipid occupies, in order to decrease lateral repulsion between adjacent molecules, and make the surface charge more diffuse (Houslay and Stanley, 1982). This situation might favour a lateral expansion of the bilayer. We might speculate that this organisation of the membrane of free-living cercariae in fresh water might render it very "leaky" to solutes. In haemolymph, salt solution or GMEM, however, the increase in ionic strength and cation concentration could tend to reduce the bilayer surface charge, promoting a closer association of the phospholipids, so that they form a better seal, making the membrane much less permeable to solutes. We would predict that the weak intermolecular associations among

phospholipids in the surface of cercariae in fresh water might create fluid phospholipid domains, which, upon transfer to GMEM or the mammalian environment, "crystallise" into a much more rigid organisation.

According to this model, the parasite surface membrane would be predicted to exist in four quite distinct states during the process of transformation from cercaria to schistosomulum. Each state would be characterized by changes in permeability and lipid organisation. As described above, the mature cercaria prior to its release from the snail should have a comparatively impermeable surface membrane, with closely associated phospholipids in a compact organisation. On emergence into fresh water, the absence of cations which neutralise the electrostatic repulsion between acidic phospholipids would cause lateral expansion of the bilayer lipids, creating a looser organisation, and a much more permeable membrane. When the cercaria enters the new environment of mammalian skin (or transformation medium), it retains the trilaminate membrane characteristic of cercariae for at least 30 minutes before replacement with the double bilayer characteristic of schistosomula begins (Hockley and McLaren, 1973). In the high ionic strength of the mammalian environment, the cercarial membrane, retained during this interval, should again be comparatively rigid and impermeable. Over some 3 hours in the new environment, the cercarial membrane, rich in acidic phospholipids, is replaced with a double bilayer rich in phosphatidylcholine (PC) (zwitterionic; net charge = 0). This new schistosomular membrane should possess its own distinctive permeability characteristics.

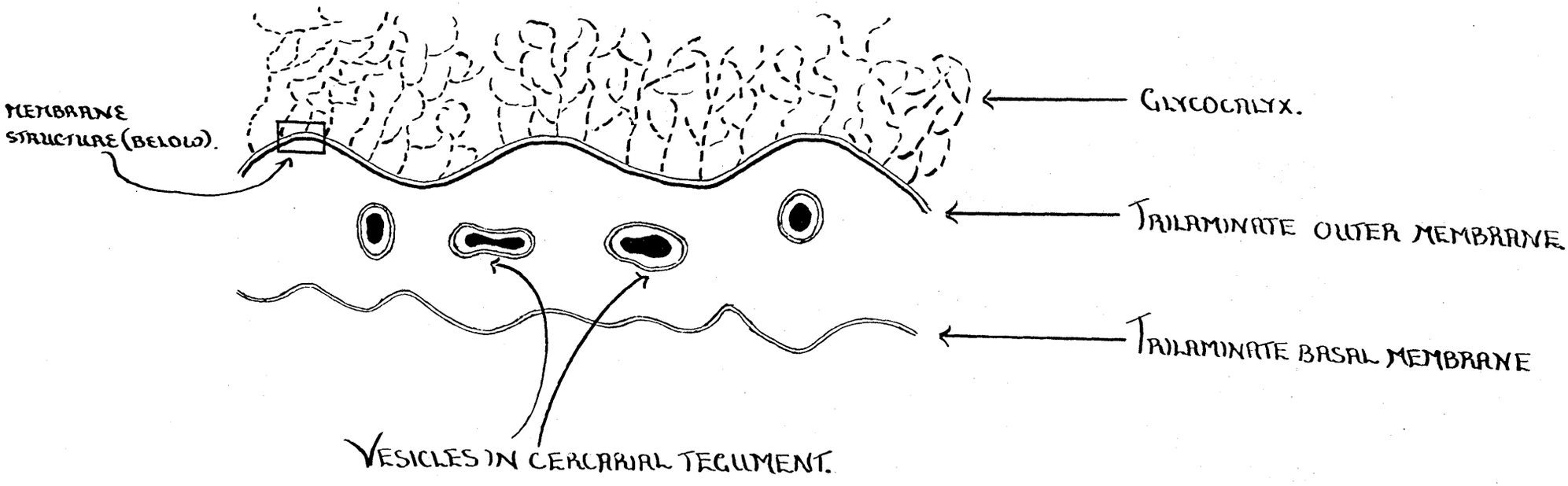
Figure 3.26 summarises these changes in membrane organisation during larval development.

The different levels of uptake of radioactive methionine observed in separate batches of cercariae and schistosomula may be due, in part,

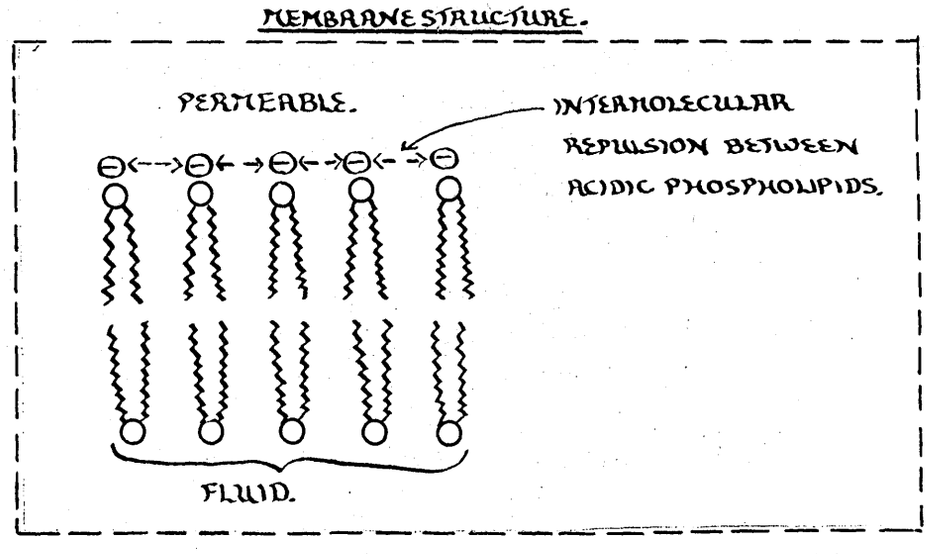
Figure 3.26. Changes in membrane organisation and permeability during the transition from cercaria to schistosomulum.

A. Free-living cercaria in water.

A high proportion of the surface membrane lipids are negatively-charged: PG, PI, PS. Repulsion between negatively-charged headgroups favours a lateral expansion of the membrane and a highly fluid state. Permeability to solutes is therefore very high.

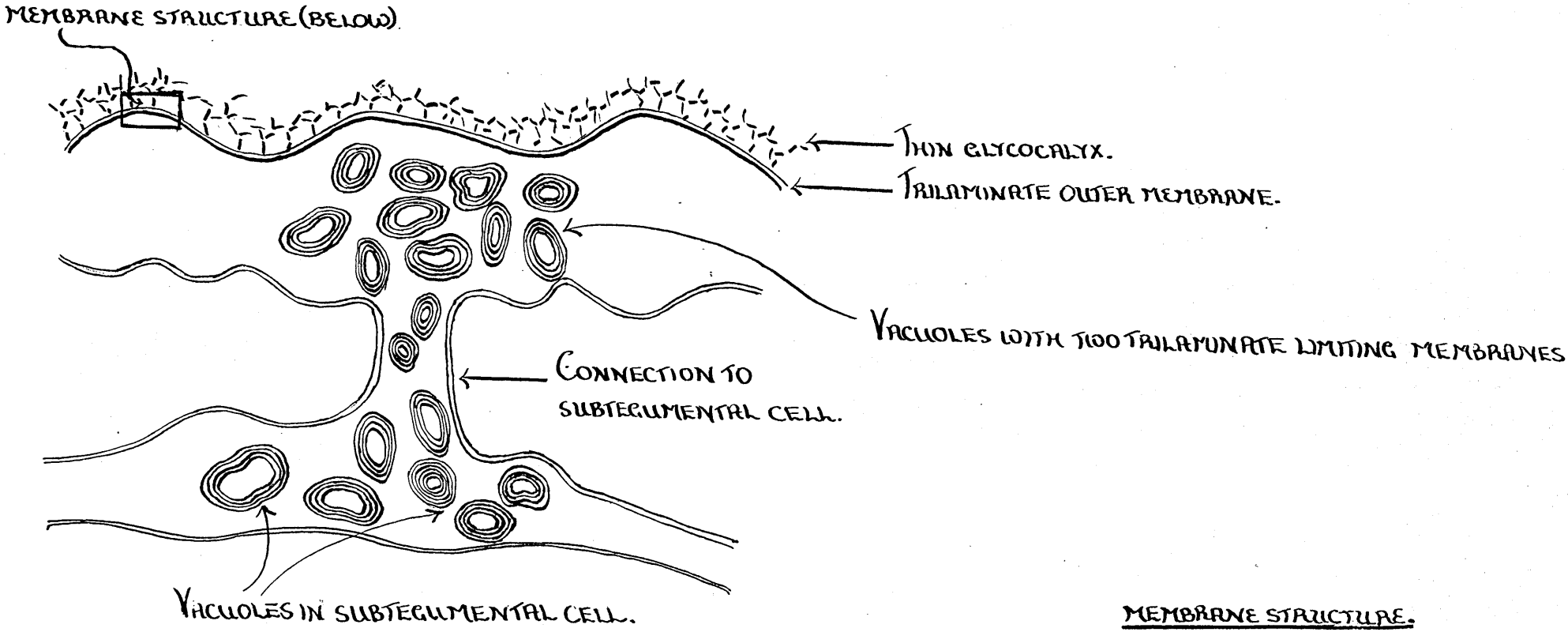


A. FREE-LIVING CERCARIA IN WATER.

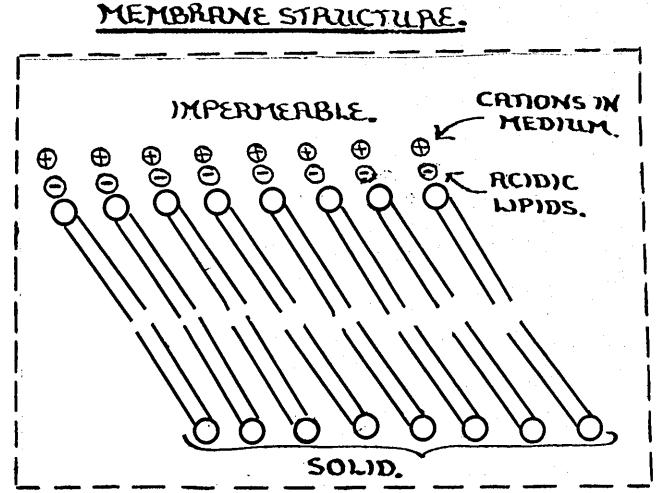


B. 0 to 40 minutes after transformation.

Parasite surface is still the cercarial trilaminate membrane. The high ionic strength and cation concentration in the mammalian environment reduce the charge on acidic phospholipids, and decrease intermolecular repulsion, producing a rigid membrane structure. Permeability of this rigid surface is low.



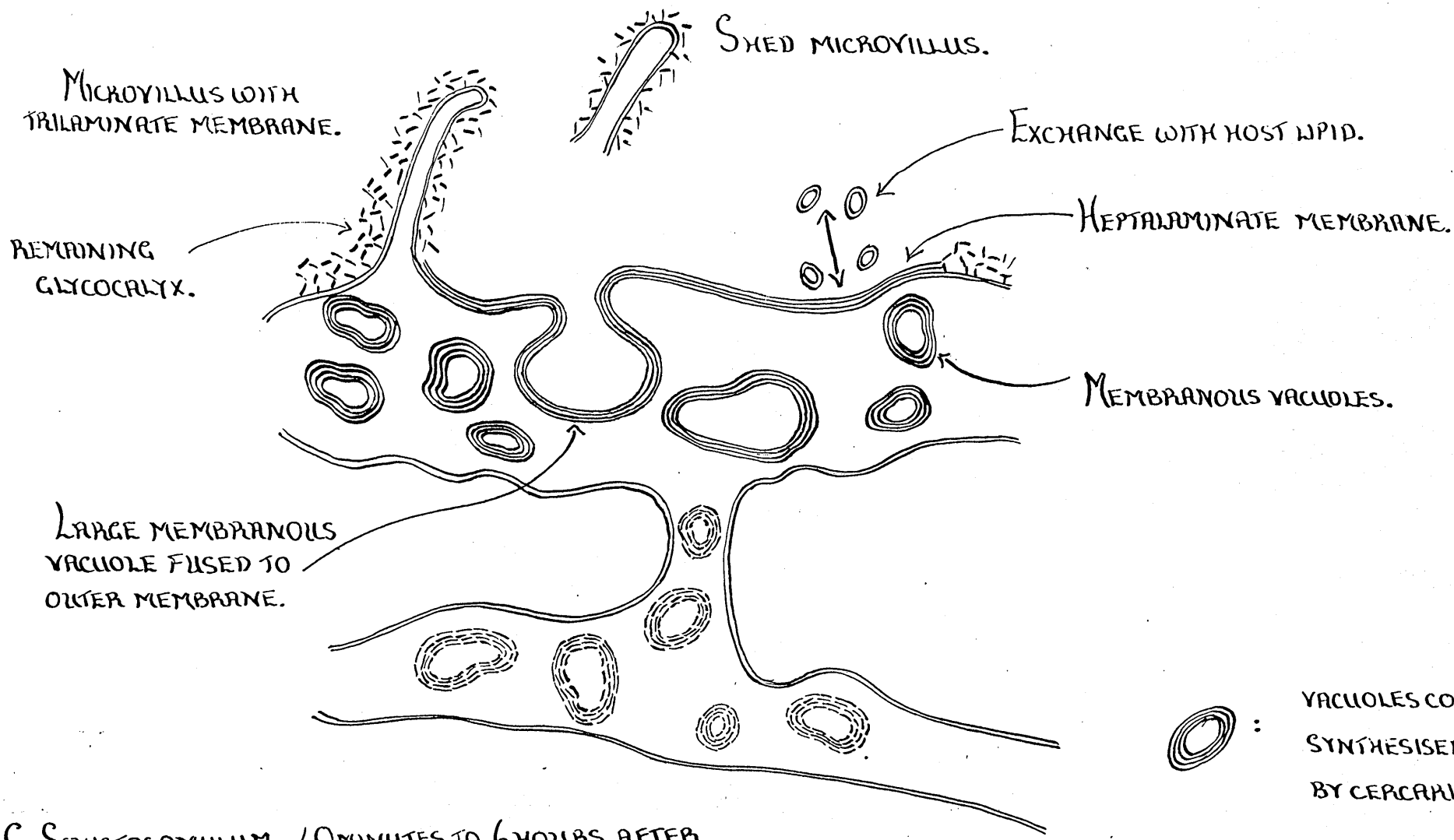
B. SCHISTOSOMULUM, 0 TO 40 MINUTES AFTER MECHANICAL TRANSFORMATION.



C. From 40 minutes to 6 hours after transformation.

Acidic phospholipids of the cercarial membrane are discarded, and replaced with a double bilayer whose extracytoplasmic face is rich in PC (no net charge), and neutral lipids. This new membrane therefore has quite a different organisation and permeability characteristics from the cercarial one.

During this period, parasite development appears to rely largely on proteins and lipids previously synthesized and stored by cercariae (see section 3.10.3.6. and chapter 4).



C. SCHISTOSOMULUM, 40 MINUTES TO 6 HOURS AFTER MECHANICAL TRANSFORMATION.

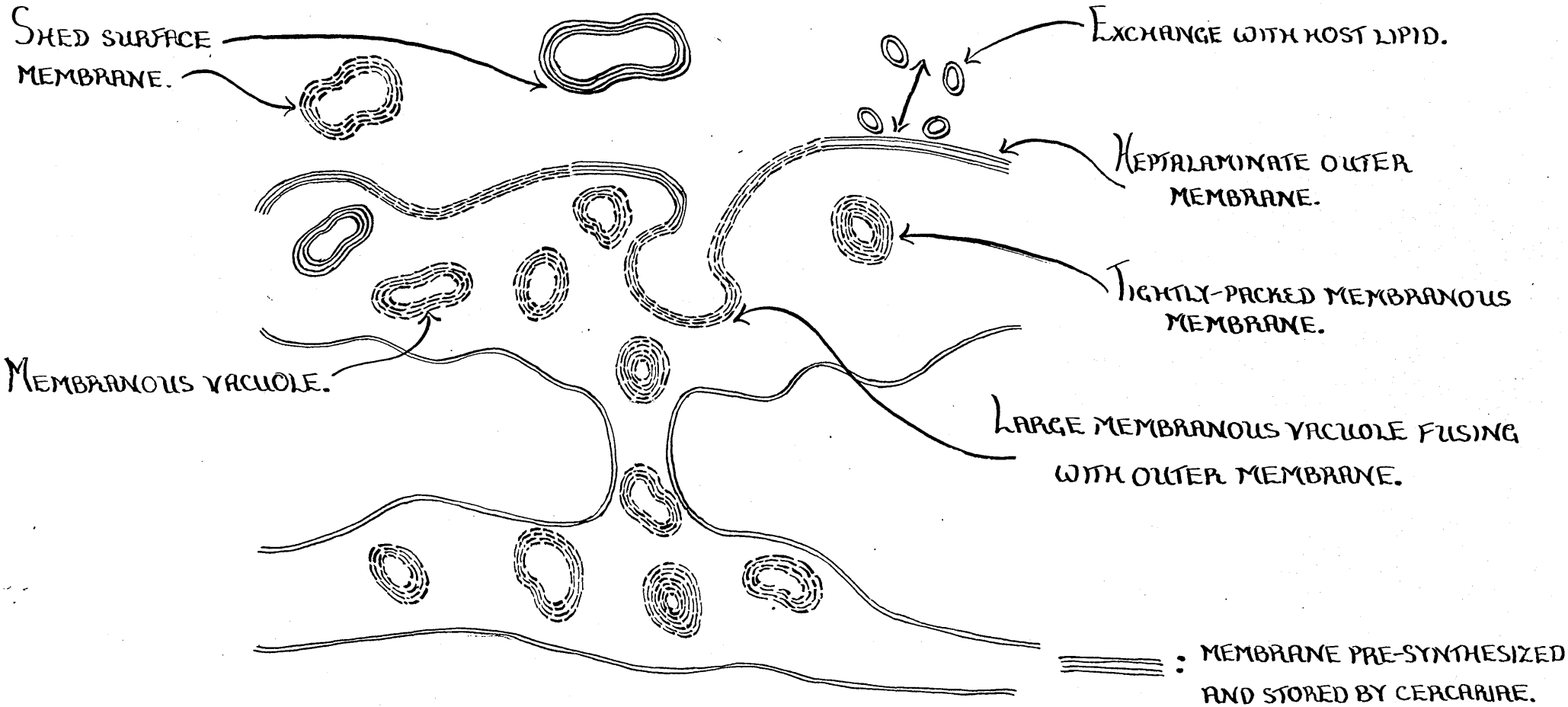
: VACUOLES CONTAINING LIPID SYNTHESISED AND STORED BY CERCARIAE.

: VACUOLES CONTAINING LIPID SYNTHESISED BY SCHISTOSOMU AFTER TRANSFORMATION.

D. From 6 hours after transformation onwards.

The store of pre-existing lipids is now used up, and the young schistosomulum must depend on newly-synthesized lipids, together with lipid exchange with host serum. These changes in lipid synthesis during schistosomular development are discussed in chapter 4.

NOTE: The term "transformation" is used here to refer to the process of syringe passage or skin penetration, not the whole series of morphological and biochemical changes which produce a fully-developed schistosomulum.



D/ SCHISTOSOMULA, FROM 6 HOURS ONWARDS.

to variation in the absorption of free amino acids during development in the molluscan host. Such variability could arise from a number of sources; for instance, snail diet and health, or location and length of residence of the larvae within the snail body. Chappell (1974) also noted a striking variation in the content of cercarial free amino acid pools. The higher the internal pool of free methionine in freshly shed cercariae, the less steep the concentration gradient for influx of the external radioactive methionine. This variability will be increased by the length of time individual cercariae spend in fresh water after shedding, since hypo-osmotic conditions cause loss of amino acids and ions from the larvae (Becker, 1971; Podesta, 1983). Parasite variability will be discussed in more detail in chapter 8.

3.10.2 Protein synthesis by cercariae and newly-transformed schistosomula - normal, U.V.-irradiated and Actinomycin D-treated.

3.10.2.1. Introduction

Nagai *et al* (1977) and Yuckenberg *et al* (1987) studied protein synthesis during the process of transformation from cercaria to schistosomulum. Both groups of workers found that protein synthesis occurred at very low levels during approximately the first 6 hours following transformation. However, morphological studies show that this same period sees the completion of the schistosomular double bilayer (Hockley and McLaren, 1973). Since construction of an entire new surface would normally be expected to demand considerable synthetic activity, it seems that the proteins (and lipids - see chapter 4) destined to contribute to the double bilayer were previously synthesized and stored by cercariae. Similar conclusions were reached by Nagai *et al* (1977) and Wilson (1987). The results presented here

also agree with this model. According to our data, not only does protein synthesis by newly-transformed normal schistosomula occur at very low levels, but when even this low level of synthesis is inhibited by some 80% after U.V.-irradiation, electron microscopy shows that formation of the double bilayer is unimpaired (see chapter 6).

As regards the nature of the proteins synthesized during this early period after transformation, Yuckenberg et al (1987) could detect synthesis of only a protein of Mr 70 000. This protein was identified as a member of the Mr 70 000 heat-shock protein (hsp) family by immunoprecipitation with antiserum raised against a bovine hsp. By analogy with bacterial and mammalian cell culture systems, these authors suggested that the stresses of transformation, for instance, higher temperature, and transfer to a new medium of higher osmotic pressure, result in a classic heat-shock response, simultaneously inducing synthesis of this Mr 70 000 hsp, and repressing transcription or translation of pre-existing messages. The studies presented here similarly show that newly-transformed schistosomula display a very low level of overall protein synthesis. In a number of experiments, we observed induced synthesis of not only one Mr 70 000 protein, but one or more of a group of proteins between Mr 70 000 and 78 000.

3.10.2.2. Protein synthesis during transition from cercariae to schistosomula.

Drawing together the results of figures 3.3, 3.4, 3.5, 3.9, 3.10, 3.22, it can be seen that free-living cercariae in water are labelled to such high specific activity that almost the whole molecular weight range of the gel incorporates some radioactivity. From figures 3.3b and 3.5b, however, some major proteins synthesized by cercariae in water at room temperature can be identified at Mr 102 000, 82 000, 70 000,

58 000-60 000, 47 000, 32 000-38 000, from 20 000 to the dye-front, also, migrating with the marker dye, some heavily labelled, low molecular weight material. Cercariae in haemolymph at room temperature (figure 3.5a, track 2A) show a much lower incorporation of radioactivity into protein than cercariae in water, but the pattern of protein synthesis appears very similar. Proteins at Mr 102 000, 70 000, 60 000, 47 000 and at the dye-front are relatively heavily labelled. Cercariae in GMEM at room temperature (figure 3.4; also figure 3.5a, track 3A) show a somewhat different pattern of protein synthesis from that seen in water and haemolymph. The most noticeable change is that synthesis of the 60 000 Mr protein is no longer detectable, although the 70 000 and 47 000 Mr species are still synthesized. In figure 3.4a, a band at Mr 50 000 is relatively heavily labelled, but this is not evident in 3.5a.

Raising the temperature of cercariae in fresh water or haemolymph to 37°C induces enhanced synthesis of the 60 000 Mr protein (figures 3.5a), b), tracks 1B, 2B), while raising the temperature of cercariae in GMEM stimulates synthesis of the 70 000 Mr band. These different heat- shock responses will be discussed in the following section.

It is especially interesting to note that cercariae at room temperature, in all the media used, do synthesize, though at greatly reduced levels, the 70 000 Mr protein which becomes so intensely labelled upon transfer to GMEM at 37°C. This observation agrees with the findings of both Yuckenberg *et al* (1987) and Blanton *et al* (1987), who found that the 70 000 Mr heat-shock protein which could be identified in newly-transformed schistosomula by immunoprecipitation or immunoblotting with anti-bovine hsp or anti- *Drosophila* hsp 70 antisera was also constitutively synthesized, at much lower levels, by cercariae at room temperature.

Figure 3.22 and table 3.8 show that most of the proteins synthesized by cercariae in fresh water are lost within the first 90

minutes of mechanical transformation or skin penetration. This observation might suggest that these proteins could form part of the cercarial membrane or glycocalyx, hence are rapidly discarded during transformation. Atkinson and Atkinson (1982) observed that proteins radiolabelled by cercariae during development in the snail underwent very rapid turnover both within the snail tissue and in fresh water, prior to stimulation by any transformation process.

Individual instances of protein synthesis by newly-transformed schistosomula vary, both during the initial 3 to 5 hours, and the 2 to 4-day period after transformation. Invariably, however, induced synthesis of one or more of a group of 3 proteins between Mr 70 000 and 78 000 is evident immediately after transformation. These proteins are found at Mr 70 000, 72 000-74 000 and 76 000-78 000. As suggested in the discussion of figure 3.13, it seems possible that there may be precursor-product relationships between these 3 species. Blanton et al (1987) and Yuckenberg et al (1987) observed that newly-transformed schistosomula immediately induced pronounced synthesis of a 70 000 molecular weight heat-shock protein. The similarity of the pattern of protein synthesis during the first 3 to 5 hours after transformation in these experiments to that observed by these two research groups strongly suggests that this series of proteins around Mr 70 000 might represent related members of a hsp 70 family. The failure of Yuckenberg et al (1987) and Blanton et al (1987) to detect any members of the 70 000-78 000 Mr series other than the 70 000 Mr protein may be due to the fact that the labelling conditions of both these workers allowed detection of only the most heavily labelled proteins. Yuckenberg et al (1987) used as their culture medium Elac, whose high concentration of unlabelled methionine and other amino acids competes with uptake of external radioactive methionine, preventing the labelling of schistosomula to high specific activity. By using methionine-free GMEM, as in the studies presented here, more extensive

incorporation of radioactive precursor into proteins is possible, permitting us to detect labelling of many proteins which may have been synthesized at levels too low to be identified under the conditions described by Yuckenberg et al (1987). Although Blanton et al (1988) did use GMEM w/o methionine, labelling proceeded for only 30 minutes at 37°C, again allowing only the most heavily synthesized proteins to be detected. The variable response to transformation observed in our own experiments, where different batches of schistosomula synthesized one, two, or all three members of the Mr 70 000-78 000 hsp family, will be discussed in chapter 8.

As regards other proteins synthesized after transformation by schistosomula after transformation, a 47 000 Mr protein is prominently labelled during the initial 5 hours, while an 82 000-83 000 Mr band is also consistently, though less intensely, labelled at this time. The subsequent 72 hours in culture see pronounced synthesis of all these proteins, as well as others at Mr 100 000, 65 000, 59 000, 43 000, 40 000, 38 000, 35 000, 32 000, 30 000, 28 000, 24 000, 19 000, 18 000, 17 000 and 15 000. Synthesis of each of these proteins was not equally pronounced in every experiment, however.

Comparison with the molecular weights of proteins described in the literature could provide some clues as to the possible identity of a number of these proteins. Atkinson and Atkinson (1982) studied protein synthesis by developing cercariae within the snail host for the 72 hours prior to shedding. When snails with a patent infection were radiolabelled in ^{14}C-leucine-containing water for 4 hours, washed, and mature cercariae released into unlabelled water, the most prominent labelled polypeptides occurred at Mr 60 000-58 000, 53 000, 35 000. The Mr 58 000-60 000 species is also strongly labelled by cercariae at room temperature in water or haemolymph in our experiments (figure 3.5a; 1A,2A). No suggestion was made by Atkinson and Atkinson (1982) as to the biological function of any of these proteins, although our

experiments suggest that the Mr 60 000 molecule may have a role in coping with heat-shock to cercariae within the snail host or in fresh water (see section 3.10.3.4.) These workers achieved extensive *in vivo* labelling of the full molecular weight range of proteins, as we see routinely after labelling in water, only when cercariae incorporated ^{14}C-leucine during ontogeny in the snail, for at least some 72 hours before reaching maturity and emerging. Under these conditions, molecules at Mr 200 000 and 43 000 were synthesized, and identified by comigration on 2-dimensional SDS-PAGE with myosin and actin. Synthesis of both these proteins by cercariae was observed in the present experiments.

Members of the series of polypeptides ranging from Mr 30 000 to 40 000 have received attention for both their biological and immunological roles (Dissous *et al,* 1986; Grzych *et al,* 1985). Apparently, these proteins share common carbohydrate determinants, which are also present in very high molecular weight proteins (Mr $>$ 200 000) of both cercariae and schistosomula. Depending on how they are presented to the immune system, these oligosaccharides may stimulate either protective immunity or a blocking antibody response (chapter 7). Since extracts from various snail species, whether schistosome hosts or not, show cross-reactivity with these carbohydrate antigens, it has been suggested that these oligosaccharide structures may play a role in osmotic adaptation in both snails and parasites. The molecule at Mr 65 000, which can be extracted with the parasite surface (figure 3.25) corresponds in molecular weight to alkaline phosphatase, which Taylor and Wells (1984) identified in the tegument of 3-hour schistosomula.

The 47 000 Mr band could represent the doublet implicated by Rumjanek (1983) as a lipid receptor. On the basis of its molecular weight, the 43 000 Mr protein might represent actin, a major component in the spines of cercariae and schistosomula.

A glutathione-S-transferase enzyme of *S. mansoni* has been

assigned a molecular weight of 28 000 by Balloul et al (1987a,b,c). A more minor S. mansoni glutathione-S-transferase has molecular weight 26 000 (Mitchell, 1989).

As for the remaining labelled bands, proteins of Mr 24 000, 22 000, 20 000, 19 000, 18 000, 17 000 and 15 000 have been identified in the tegument of newly-transformed schistosomula by various workers (Taylor et al, 1984; Samuelson and Caulfield, 1982; Simpson et al, 1983b). No function in the life of the parasite has been assigned to these proteins, although an Mr 22 000-26 000 complex apparently stimulates an IgE response in the rat host (Sher and Pearce, 1987).

Table 3.10 summarizes these observations on proteins synthesized by cercariae and schistosomula, and speculations as to their possible identity.

3.10.3. The heat-shock response in cercariae and schistosomula.

3.10.3.1. The heat-shock response.

A universal response of procaryotic and eucaryotic cells to elevated temperatures and other environmental stresses - e.g. change in osmotic pressure, toxic drugs - is the synthesis of a number of stress proteins (Lindquist, 1986; Pelham, 1986, 1988). In cells exposed to such stresses, there is a marked increase in the synthesis of a family of polypeptides referred to as heat-shock proteins (hsp's), the two most abundant of which are found around Mr 70 000 (hsp 70 family) and Mr 90 000 (hsp 90 family). Similarly, glucose starvation or inhibition of glycosylation induces synthesis of a number of "glucose regulated proteins" (grp's), which are closely related to the hsp's, but are localized to the lumen of the endoplasmic reticulum (Pelham, 1986, 1988; Deshaies et al, 1988).

Recent genetic and biochemical experiments (Pelham, 1988; Deshaies

Table 3.10 Suggested identities of some of the proteins synthesized by cercariae and schistosomula.

Table 3.10

Mr X 10^{-3}	SYNTHESIZED BY: CERCARIAE (in aquarium water)	SYNTHESIZED BY: SCHISTOSOMULA (0-96 hrs)	PROTEIN OF CORRESPONDING Mr REPORTED IN LITERATURE	REFERENCES
81-83	✓(not prominent)	✓	?	
70-78 (70, 72-74, 76-78)	✓(not prominent)	✓very strongly labelled immediately after transformation	members of hsp 70 family?	Pelham (1988); Yuckenberg et al, (1987); Blanton et al (1987).
58-60	✓especially prominent after heat-shock in water or haemolymph		cercarial hsp: tubulin?	Atkinson and Atkinson (1982) Lawrence and Robert-Gero (1985)
47	✓	✓	LDL receptor?	Rumjanek (1983)
43	✓	✓	actin?	Atkinson and Atkinson (1982)
30-40	✓	✓	The proteins in this group share carbohydrate determinants with each other, and with a >200 kDa protein. Important immunogens. May be involved in osmoregulation	Dissous et al (1982,1985,1986) Grzych et al (1984,1985)
28, 26	✓(faint)	✓	GSH transferases. Used in vaccine studies	Balloul et al (1987 a,b,c).

LDL = Low Density Lipoprotein.

(Continued on next page).

Table 3.10 continued.

Mr X 10^{-3}	SYNTHESIZED BY		PROTEIN OF CORRESPONDING Mr REPORTED IN LITERATURE	REFERENCES
	CERCARIAE (in aquarium water)	SCHISTOSOMULA (0-96 hrs)		
24,22	✓	✓	Identified on 3h schistosomular surface by several workers, but biological role unknown. Stimulate IgE production in rats.	Samuelson and Caulfield (1982); Sher and Pearce (1987).
17	✓	✓ Large amounts in body of 48h. schistoso-mulum, not on surface	Detected on surface of 3h schistosomula. Function unknown.	Simpson et al (1983b)
15	✓	✓	Identified on 3h schistosomular surface, but function unknown.	Taylor et al (1984) Simpson et al (1983b)
<14.4 (migrate with dye-front)	✓	✓	Ubiquitin? (7-8 kDa) Proteolytic degradation products? Glycolipids?	

et al, 1988) have helped to clarify some of the functions of the grp's and hsp's in normal and stressed cells. They seem to perform two related roles essential to cell viability: firstly, ensuring that individual polypeptides assume and maintain their native conformations; secondly, directing the assembly and disassembly of multimeric protein structures.

In vitro assays involving stimulation by hsp 70 of pre-pro-α factor uptake into microsomal vesicles, and an in vivo system, involving insertion of plasmids expressing hsp 70 proteins into yeast strains deficient in heat-shock genes, provided evidence for the following model of hsp action in eucaryotic cells. The Mr 70 000 hsp appears to be located in the cytosol, while a closely homologous Mr 78 000 grp is restricted by its signal sequence to the endoplasmic reticulum membrane. Both these stress proteins have a particularly high affinity for denatured and aberrant proteins, and prevent them from aggregating with each other. Hsp 70 interacts with unfolded, or incorrectly folded, proteins which are newly-synthesized, and released from free cytosolic ribosomes. Those that possess no signal sequence for membrane insertion are assisted, using the energy of ATP hydrolysis, to achieve their mature configurations. Upon recognising signal or import sequences on precursor proteins, however, hsp 70 maintains these in loosely-folded conformations until they find the appropriate receptor in the endoplasmic reticulum or mitochondrial membranes. Once the unfolded proteins have been inserted into the appropriate membrane, they are recognised by the Mr 78 000 grp, which helps membrane-associated and secretory proteins to fold into their final, native form, just as the Mr 70 000 hsp does for cytosolic proteins.

However, for severely abnormal or mutant proteins generated by aging, or environmental stresses such as heat or osmotic shock, irradiation, toxic drugs, etc., binding to heat-shock proteins appears

to be a permanent fate. Heat-shock proteins are believed to target such molecules to lysosomes for proteolytic degradation. The very low molecular weight protein ubiquitin (Mr 7 000-8 000) apparently acts in a similar way to these heat-shock proteins in recognising and removing aberrant proteins from the cell.

Figure 3.27 summarises these functions of the Mr 70 000 to 78 000 stress-induced proteins.

3.10.3.2. The heat-shock response in schistosomula

The response of schistosomula to the temperature and osmotic stresses accompanying transformation may be examined in the light of this model.

As might be expected, parasites which alternate between temperature extremes in different hosts respond to a change of environment by inducing synthesis of hsp's. This has been observed in both protozoan parasites - Leishmania (Lawrence and Robert-Gero, 1985) and trypanosomes (Van der Ploeg et al, 1985) - and metazoans such as Brugia (Snutch and Baillie, 1983). More recently, Yuckenberg et al (1987) and Blanton et al (1987) both identified a molecule, whose synthesis is induced by mechanical transformation of cercariae of S. mansoni, as a 70 000 Mr protein recognised by antisera specific for bovine and Drosophila hsp 70's. Moreover, Hedstrom et al (1987) selected, from a λgt11 expression library, schistosome cDNA clones expressing fusion peptides that bound to antibodies specific for the 70 000 Mr parasite antigen. When these cDNA clones were blotted back to genomic DNA, gene sequencing revealed considerable homology with Drosophila and human hsp 70 genes. Hsp 70 was encoded in the schistosome genome as tandemly repeated copies of closely related genes.

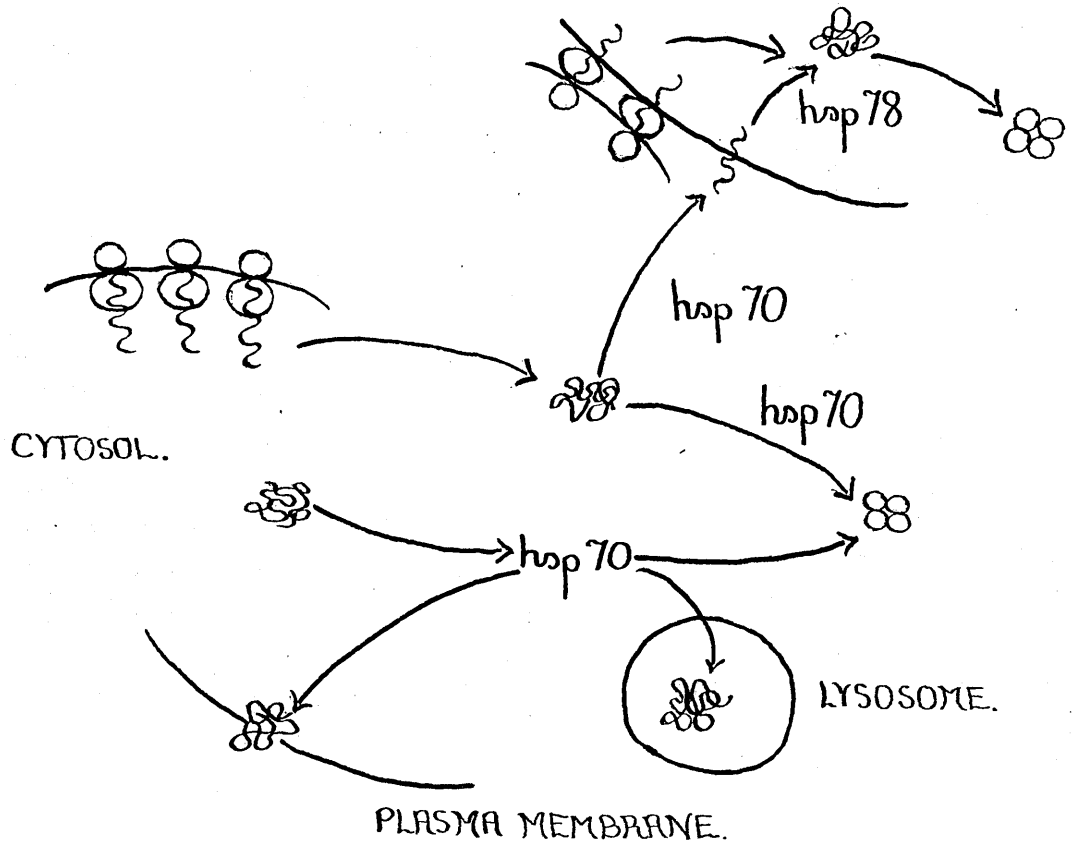
The results presented here agree with the idea that Mr 70 000

Figure 3.27. The role of heat-shock proteins (Mr 70 000-78 000) in ensuring correct protein conformations and associations.

Newly-synthesized polypeptides are released from free cytosolic or membrane-bound ribosomes in non-native conformations. Proteins destined for the cytosol surface membrane or secretion are all assisted to achieve their native conformations and associations by heat-shock proteins in the cytosol and endoplasmic reticulum.

Severely aberrant or mutant proteins generated by aging or environmental stress may be targeted to lysosomes for degradation.

(Diagram based on Pelham, 1988).


ENDOPLASMIC RETICULUM.
hsp 78
hsp 70
hsp 70
CYTOSOL.
hsp 70
LYSOSOME.
PLASMA MEMBRANE.

hsp's are induced when free-living cercariae encounter the higher temperature and osmotic pressure of the vertebrate host. The intense labelling, immediately after transformation, of one or more of a group of three proteins, at Mr 70 000, 72 000-74 000, 76 000-78 000, is consistent with the concept of a family of closely-related heat-shock proteins, perhaps corresponding to different members of the multi-gene family described by Hedstrom et al (1987). The variability in the heat-shock response by schistosomula, such that different batches of larvae synthesize one or more of the three species in the group, could possibly be due, at least in part, to preferential expression of different members of the family by separate batches of parasites. Alternatively, as previously suggested, there could be precursor-product relationships among the three proteins. Confirmation of such a close relationship between members of this complex would of course require immunoprecipitation or immunoblotting studies with antibodies raised against well-established hsp's.

It is tempting to speculate that these Mr 70 000-78 000 proteins synthesized by newly-transformed normal schistosomula might be analogous in function to the Mr 70 000-78 000 family of stress-induced proteins described by Pelham (1986, 1988). Thus, they may be essential, firstly, in removing or renaturing schistosomular proteins whose conformations are disrupted by the temperature and osmotic stresses accompanying transformation; secondly, in ensuring that the proteins inserted in the newly produced schistosomular double bilayer achieve their mature, native conformations. The newly-synthesized Mr 70 000-78 000 species appear to associate predominantly with the schistosomular surface in normal parasites, as demonstrated in figure 3.25. Indirect immunofluorescence has also detected the Mr 70 000 schistosome hsp at the surface of adult worms (reported by Hedstrom et al, 1987). This association with the surface suggests that schistosome hsp 70's could be directly involved in insertion of integral membrane proteins

at the parasite surface.

Constitutive synthesis of the postulated heat-shock proteins around Mr 70 000 is also detectable, at much lower levels, in cercariae prior to transformation. This observation is consistent with the findings of Yuckenberg et al (1987), Blanton et al (1987), and Hedstrom et al (1987) who all identified the 70 000 Mr antigen in cercariae by immunological techniques. Indeed, in a number of other eucaryotic and procaryotic systems, heat-shock proteins are constitutively synthesized at low levels at normal temperatures (Lindquist, 1986). Also consistent with established heat-shock systems is the low level of synthesis of most other proteins for at least the first five hours following transfer of newly-transformed schistosomula to 37°C.

3.10.3.3 RNA messages for heat-shock (and other) proteins are synthesized by cercariae prior to transformation.

The U.V.-irradiated or Actinomycin D-treated schistosomula generally produce these postulated heat-shock proteins in greatly reduced amounts. This decreased synthesis is presumably caused by disruption of the relevant genes. In a number of biological systems, including the protozoan parasite Leishmania (Van der Ploeg et al , 1985), and the nematode Brugia (Snutch and Baillie, 1983), the genes encoding heat-shock proteins appear to be transcribed before the increase in temperature occurs. Thus, only translation of the RNA messages is required for their expression upon change of environment, ensuring that the heat-shock response occurs as rapidly as possible. This appears to be the case for schistosomula, too, for Actinomycin D, which disrupts messages in double-helical DNA, but does not affect pre-existing RNA, has no effect on synthesis of any proteins, including the Mr 70 000-78 000 hsp's, until some 15 to 20 hours after transforma-

tion. Since inhibition of protein synthesis occurs immediately in U.V.-irradiated forms, this RNA, or the enzymes involved in translation, must be disrupted by U.V.- irradiation (discussed in 3.10.3.6).

3.10.3.4. Protein synthesis by cercariae after heat-shock in water or haemolymph.

Cercariae in water or haemolymph responded to an increase in temperature to 37°C by inducing synthesis of a protein between Mr 58 000 and 60 000, although they did produce the 70 000-78 000 Mr species at 37°C in GMEM, (figure 3.5a). Even when the parasites have been subjected to syringe passage, the presence of haemolymph ensures synthesis of the 58 000-60 000 Mr protein after heat-shock. Blanton et al (1987) also observed increased synthesis of this protein in response to increasing the temperature of cercariae in water. Atkinson and Atkinson (1982) showed that a 58 000 Mr protein was synthesized by cercariae in great amounts prior to release from the snail, but its synthesis apparently decreased after shedding at normal temperatures (23-25°C).

In L. mexicana and L. tropica (Fong and Chang, 1981; Lawrence and Robert-Gero, 1985), a stress-induced protein at Mr approximately 55 000 has been identified as tubulin. It might be tentatively suggested that the 58 000 to 60 000 Mr protein of S. mansoni larvae could also be a member of the tubulin family. α-Tubulin has molecular weight 50 000; β-tubulin, 60 000, while a lambda factor of Mr 55 000-62 000 copurifies with tubulin, and is required for its assembly into microtubules. Such an identity would be consistent both with the constitutive synthesis of the 58 000-60 000 molecule during development of cercariae in the snail, and with its induction upon heat-shock in an environment of low osmotic pressure (water or snail haemolymph), for

microtubules are important in intracellular transport, cytoplasmic contractility, and control of cell shape. Microtubules have been shown to be essential in transporting pre-synthesized membranous vacuoles to the schistosomulum surface during transformation (Wiest et al, 1988). It therefore seems reasonable to suggest that synthesis of tubulin could be necessary to allow the cercarial surface to cope with the alterations in membrane organisation caused by raising the temperature in an environment of low osmotic pressure.

An alternative identity for this cercarial heat-shock protein might be suggested by analogy with various procaryotic systems. A class of highly homologous proteins of subunit molecular weight 58 000-64 000 seem to be essential in assisting post-translational assembly of oligomeric protein structures in chloroplasts, mitochondria and procaryotes. As for the hsp 70 heat-shock protein class, synthesis of these proteins is markedly induced in response to a variety of environmental stresses, including temperature increase (reviewed by Hemmingsen et al, 1988). It is interesting to consider the possibility that the heat-inducible protein found at Mr 58 000-60 000 in cercariae might belong to this class of molecules, and hence direct protein-protein associations in cercarial mitochondria. If this were the case, then a possible explanation might be tentatively suggested for the observation that induced synthesis of the Mr 58 000-60 000 molecule in response to heat-shock is strictly limited to the pre-schistosomulum stage; newly-transformed schistosomula in GMEM at 37°C synthesize this protein at only minimal levels. Cercariae, being aerobic organisms (section 1.5.8.), are highly dependent upon the enzymes of the TCA cycle, most of which are located in the mitochondrial matrix, and upon the associated electron transport chain, whose components are integrated in the inner mitochondrial membrane. We might speculate that, when cercariae are subjected to temperature stress, induced synthesis of the Mr 60 000 hsp could be aimed at

preventing denaturation and deleterious protein-protein associations of the mitochondrial enzymes essential to cercarial viability.

Transformation to the schistosomulum stage switches parasite metabolism from aerobic to predominantly anaerobic (section 1.5.8.). Since lactic acid fermentation is a cytosolic reaction, it might be predicted that maintenance of mitochondrial enzymes in active form may no longer be so vital for parasite survival.

The observation by Coles (1973) that an increase in osmotic pressure may be the primary trigger for converting parasite metabolism from aerobic to anaerobic is consistent with the experiment illustrated in figure 3.5, where cercariae in environments of low osmolarity - fresh water or snail haemolymph - show induced synthesis of the (possibly) mitochondrial-associated protein at Mr 58 000-60 000 upon heat-shock, while cercariae in an isotonic medium such as GMEM do not.

It must be emphasized that both these possible identities are suggested only as interesting speculations, which might help to direct future investigations on the parasite heat-shock response. Confirmation of identity of the Mr 58 000-60 000 band, for instance, by immunological criteria, would of course be essential for any future work.

3.10.3.5. Effects of inhibited heat-shock protein synthesis on the nature of the antigens expressed by U.V.-irradiated and Actinomycin D-treated schistosomula.

In view of these important functions of heat-shock proteins, it seems that U.V.-irradiated and Actinomycin D-treated schistosomula, which synthesize these enzymes at greatly reduced levels, might express quite different antigenic profiles from their normal counterparts.

Firstly, the various stresses involved in transferring from fresh water to the environment of the vertebrate host: e.g., increases in temperature and osmotic pressure, protease action, and mechanical

shearing stress, will denature a proportion of parasite proteins. The striking induction of heat-shock protein synthesis by newly-transformed normal schistosomula should allow them to renature or remove molecules which have lost their native conformation. Irradiated and Actinomycin D-treated schistosomula, however, synthesize such low levels of heat-shock proteins that they will be unable to remedy the situation. Production of aberrant forms will be aggravated in the attenuated schistosomula, initially by direct irradiation-induced disruption of molecular structure (see section 1.10), then by failure to replace these non-native structures because synthetic activity is inhibited. Changes in the organisation of parasite glycoproteins and phospholipids, also induced by irradiation or Actinomycin D treatment (see chapter 4) will cause further aberrations in antigen conformation. The severe drug- or radiation- induced disruption of DNA is predicted to result in discrepancies in messages encoding those proteins which continue to be transcribed and translated. In the absence of the clearing function of heat-shock proteins, these modified structures are predicted to persist and accumulate.

Thus, upon exposure to irradiated or Actinomycin D-treated schistosomula, the immune system will be presented with antigens in non-native conformations, exposing new determinants which are normally hidden in the interior of the native protein. These denatured antigens will be present among the parasite secreted material, at the surfaces of the attenuated schistosomula, or within the parasite body. Of the proteins which are produced <u>de novo</u> by attenuated schistosomula, many of which may be presumed to be synthesized on damaged or altered mRNA, only a small percentage appear to insert into the parasite membrane as permanent components of the surface. An abnormally high proportion remain in the body (figures 3.24, 3.25), perhaps in consequence of the absence of hsp's, with their role in insertion of membrane proteins, However, protein secretion does not

appear to be affected by irradiation (figures 3.22, 3.23). These secreted proteins will be readily available for interaction with antigen presenting cells, and hence for presentation to the host immune system. The surface and internal proteins may be exposed for interaction with the immune system by the irradiated or drug-treated schistosomula as they die and disintegrate. We would suggest that the modified conformations of the internal, surface and secreted antigens expressed by attenuated schistosomula may alter their processing and presentation by antigen presenting cells in such a way that helper T-cells are stimulated to produce an especially effective response against challenge parasites.

Chapter 4 will discuss how irradiation or drug treatment alters membrane lipids and glycoproteins, as well as proteins, further contributing to the expression of modified antigens by attenuated parasites. Chapters 9 and 10 will discuss how these modified antigens may interact with the host immune system to stimulate potent protective immunity.

3.10.3.6. Increased dependence on de novo protein synthesis during development.

The morphological changes occurring during transformation of cercariae to schistosomula have been followed by electron microscopy (Hockley and McLaren, 1973). The cercarial glycocalyx and trilaminate membrane are shed in the form of microvilli 10-15 nm in length, and replaced by a heptalaminate membrane. Within 30 minutes after transformation, subtegumental cells form connections with the tegumental cytoplasm, vacuoles limited by two closely-apposed trilaminate membranes appear in the subtegumental cells, and pass into the tegument. Here, they fuse to form larger vacuoles, which ultimately fuse with the outer tegumental membrane.

The completion, within three hours, of a complete double bilayer surrounding the entire schistosomulum, would be predicted to demand extensive synthesis of both protein and lipid components. In fact, however, protein synthesis is minimal during the initial six hours following transformation. This was observed in the work presented here, also in the studies of Kusel and MacKenzie (1975), Nagai et al (1977), and Yuckenberg et al (1987). Our own electron microscopy results (chapter 6) showed that inhibition of protein and phospholipid synthesis in irradiated forms did not affect production of the double bilayer. It may therefore be concluded that the material inserted into the membrane during this period has been already synthesized, and stored, by cercariae.

As the stock of pre-synthesized proteins is depleted, however, the rapid turnover of membrane constituents demands their equally rapid replacement. A second class of proteins then becomes important - those whose RNA messages have, apparently, already been synthesized before transformation, so that only translation is now required. Presumably, this occurs during the initial 15-hour period (approximately) following transformation, when Actinomycin D-induced disruption of schistosomular DNA does not prevent protein synthesis, because the RNA remains undamaged (see figure 3.18). This theory, that protein synthesis during approximately the first 15 hours of the parasite's life in the mammalian host is largely dependent on translation of pre-existing mRNA, also explains the observation that, in contrast to Actinomycin D, the translational inhibitor puromycin immediately inhibits protein synthesis by schistosomula (Nagai et al, 1977).

U.V.-irradiation, unlike Actinomycin D, does appear to damage RNA and/or the enzymes involved in transcription or translation, for protein synthesis is instantly inhibited.

Finally, by 15 to 20 hours after transformation, it appears that schistosomula must rely wholly on DNA-dependent RNA synthesis. By this

time, the store of pre-existing RNA messages will have been utilized. Thus, from 20 hours after transformation until death in culture at approximately 96 hours, U.V.-irradiated and Actinomycin D-treated schistosomula, whose DNA is severely disrupted, show extensive inhibition of protein synthesis.

At each stage of synthetic activity, the action of heat-shock proteins will be important to normal schistosomula in insertion of integral membrane proteins, and in assisting membrane-associated, secretory and cytosolic proteins to achieve their final, mature conformations. In the absence of this essential function of heat-shock proteins, U.V.-irradiated and Actinomycin D-treated schistosomula would be predicted to express these proteins in aberrant, non-native conformations.

Figure 3.28 summarises these observations on changes in synthetic activity during development of schistosomula, and the consequences envisaged for the conformation of antigens expressed by normal and attenuated schistosomula at each stage.

Figure 3.28, (1)-(3). Changes in conformation of the proteins expressed by developing schistosomula - normal. U.V.-irradiated and Actinomycin D-treated: (1) 0-6 hours; (2) 6-15 hours; (3) 15-96 hours after transformation.

——— : proteins pre-synthesized by cercariae, and stored.

——— : proteins derived from RNA synthesis by cercariae, but translated after transformation.

——— : proteins derived from RNA synthesized after transformation.

 : protein in non-native conformation, or mutant protein.

 : protein in native conformation.

TEXT:

(1): Released material.

(2): Protein components of heptalaminate surface.

(3): Protein components in vacuoles of tegument, and subtegumental cells.

(4): Presence and function of heat-shock proteins.

The antigens expressed by attenuated schistosomula become increasingly abnormal as development proceeds.

1. 0-6 HOURS AFTER TRANSFORMATION.

A. Normal schistosomula.

(1) Cercarial glycocalyx and trilaminate membrane are released. Some schistosomular proteins pre-synthesized by cercariae may also be released.

(2) Proteins synthesized and stored by cercariae.

(3) Proteins synthesized and stored by cercariae; a small proportion derived from RNA synthesized by cercariae.

(4) Hsp's remove or renature proteins modified during transformation. Also establish the correct conformations of internal, surface and secretory proteins.

B. U.V.-irradiated schistosomula.

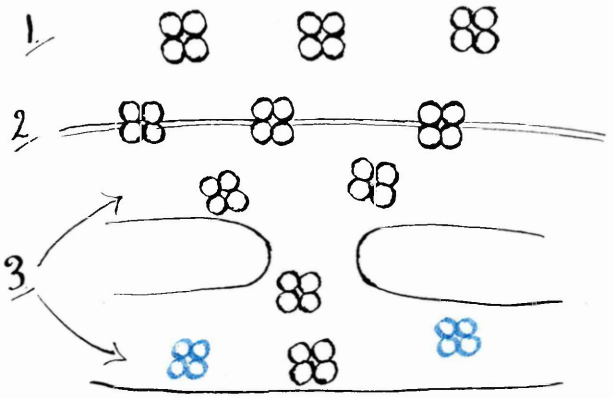
Absence of heat-shock proteins means that proteins modified by U.V.-irradiation and the stresses of transformation persist.

Translation processes are disrupted by irradiation. Any translation of RNA stored by cercariae produces aberrant proteins, which accumulate in the absence of the clearing function of hsp's.

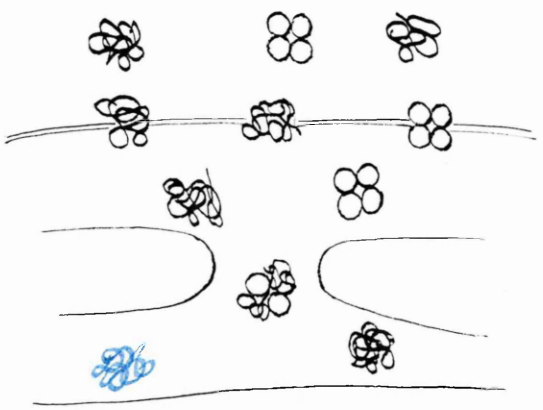
C. Actinomycin D-treated schistosomula.

Actinomycin D disrupts only double helical DNA. Hsp's are still produced, from pre-existing RNA messages. Reliance on pre-synthesized proteins and mRNA during this period means that parasite antigens are expressed normally.

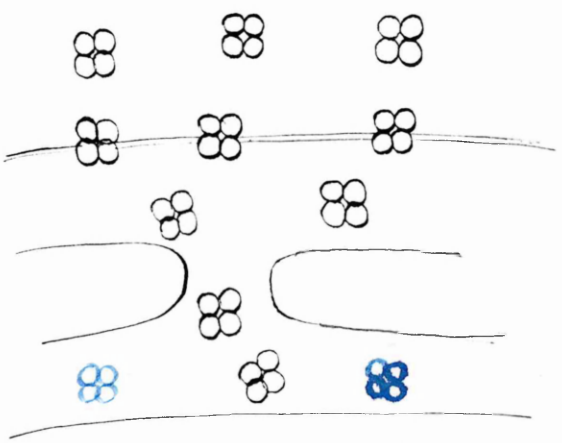
0-6 HOURS AFTER TRANSFORMATION.



A NORMAL SCHISTOSOMULA.



B U.V-IRRADIATED SCHISTOSOMULA.



C ACTINOMYCIN D-TREATED SCHISTOSOMULA.

2. 6-15 HOURS AFTER TRANSFORMATION.

A. NORMAL SCHISTOSOMULA.

(1) Schistosomular proteins pre-synthesized by cercariae are released. Also proteins translated from stored mRNA.

(2) Proteins synthesized by cercariae, and newly-translated proteins.

(3) Proteins translated from stored, mRNA, and also transcribed and translated after transformation.

(4) Hsp's recognise and remove any denatured proteins in the surface or cytoplasm. Also establish secretory, surface and internal proteins in their native conformations.

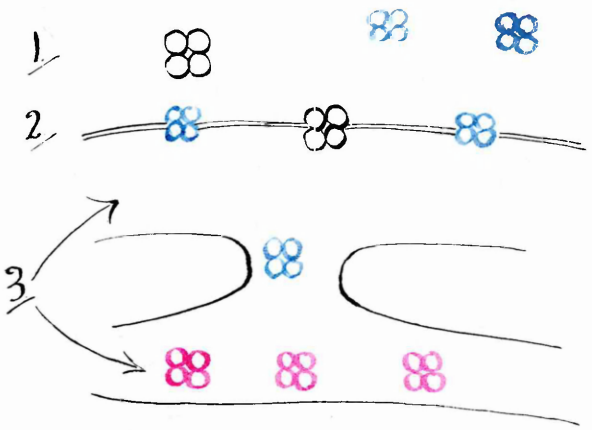
B. U.V.-IRRADIATED SCHISTOSOMULA.

Absence of heat-shock proteins acting on molecules produced in abnormal conformation before or after transformation means that the parasite antigens assume modified, non-native conformations.

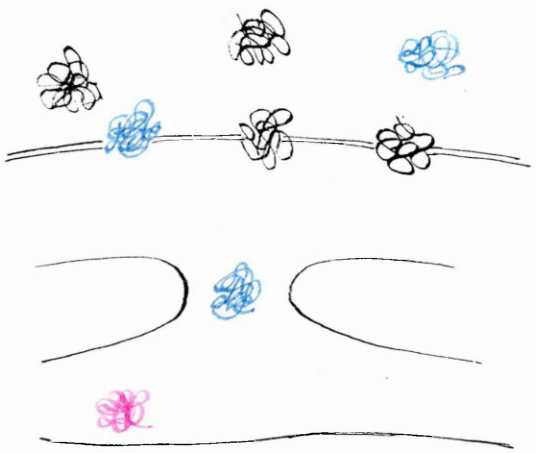
C. ACTINOMYCIN D-TREATED SCHISTOSOMULA.

Surface and secretory proteins are still in normal, native conformations, being derived mainly from material synthesized before drug treatment. However, as the stores of pre-existing mRNA and proteins are depleted, Actinomycin D-induced disruption of DNA means that the subtegumental cells cannot correctly transcribe and translate new proteins, including hsp's.

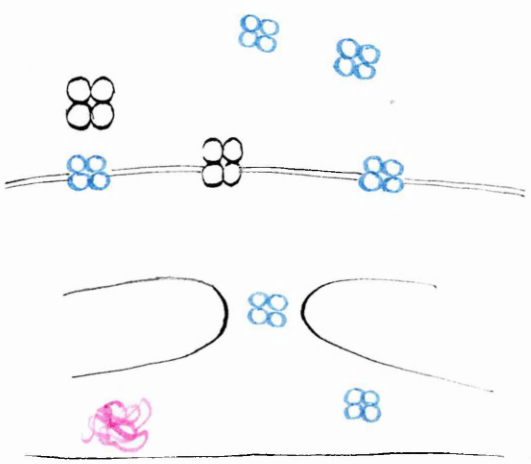
6-15 HOURS AFTER TRANSFORMATION.



A NORMAL SCHISTOSOMULA.



B U.V-IRRADIATED SCHISTOSOMULA.



C ACTINOMYCIN D-TREATED SCHISTOSOMULA.

3. 15-96 HOURS AFTER TRANSFORMATION.

A. NORMAL SCHISTOSOMULA.

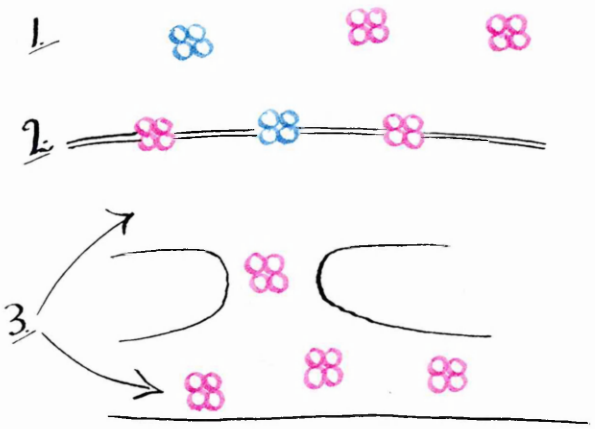
(1)-(3) Chiefly proteins both transcribed and translated after transformation.

(4) Role of hsp's in directing protein conformation as described for figures 1 and 2.

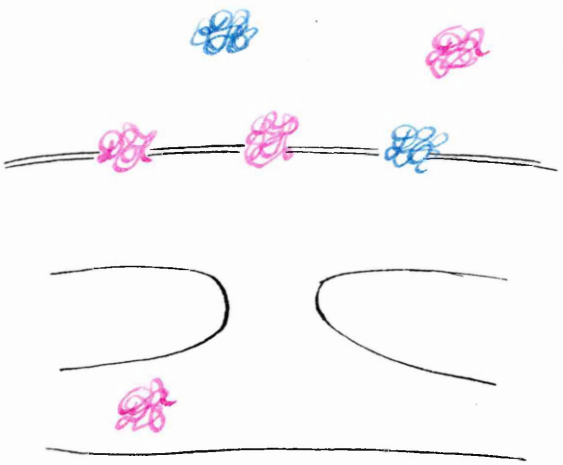
B. U.V.-IRRADIATED and C. ACTINOMYCIN D-TREATED SCHISTOSOMULA.

Internal, surface and secretory antigens adopt aberrant conformations in both attenuated forms.

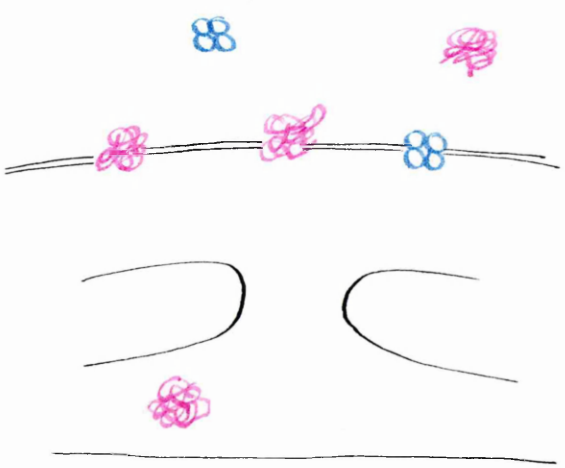
15-96 HOURS AFTER TRANSFORMATION.



A NORMAL SCHISTOSOMULA.



B U.V.-IRRADIATED SCHISTOSOMULA.



C ACTINOMYCIN D-TREATED SCHISTOSOMULA.

CHAPTER FOUR.

EFFECTS OF U.V.-IRRADIATION AND ACTINOMYCIN D TREATMENT ON OTHER METABOLIC PATHWAYS - NUCLEIC ACID SYNTHESIS, PHOSPHOLIPID SYNTHESIS, AND PROTEIN GLYCOSYLATION.

4. Effects of U.V.-irradiation and Actinomycin D treatment on other metabolic pathways - nucleic acid synthesis, phospholipid synthesis, and protein glycosylation.

4.1 Effects of U.V.-irradiation and Actinomycin D treatment on nucleic acid synthesis by schistosomula.

4.1.1. RNA synthesis.

Figures 4.1 and 4.2, and table 4.1, demonstrate that incorporation of ^{3}H-uridine and ^{3}H-adenosine into nucleic acid is severely inhibited in U.V.-irradiated and Actinomycin D-treated schistosomula, whether transformed mechanically or by skin penetration.

Both these nucleosides enter adult schistosomes by a combination of active transport and passive diffusion (Levy and Read, 1975). The mechanisms of uptake by the larval schistosomula may be similar (as yet, no investigation of nucleoside uptake has been performed for this stage). Conversion to the corresponding nucleotides occurs, in adult worms, and presumably in the schistosomula of our experiments, by salvage pathways:

Uridine + ATP⟶ uridine-5'-phosphate + ADP
(uridine cytidine kinase).

Adenosine + ATP⟶ adenosine-5'-phosphate + ADP.
(adenosine kinase).

Mitosis and DNA synthesis do not occur in schistosomula until day 4 of culture at the earliest (Clegg and Smithers, 1972; Salafsky *et al*, 1988; section 4.1.2.). Hence, the radiolabelled, TCA-precipitable nucleic acid in our experiments with ^{3}H-uridine and ^{3}H-adenosine should consist principally of RNA synthesized from these nucleosides.

Figure 4.1. Incorporation of ^{3}H-uridine into TCA-precipitable nucleic acid by normal, U.V.-irradiated and Actinomycin D-treated schistosomula from 24 to 96 hours after mechanical transformation.

——●——:Normal schistosomula

•-•-●-•-•:U.V.-irradiated schistosomula (400 μW min cm^{-2})

---○---:Actinomycin D-treated schistosomula

Each point represents the mean of two determinations.

Deviation of duplicates from mean = ±10.1%

PROTOCOL

Cercariae

↓

Divide in 3

↓

N I A

↓

U.V.-irradiate

(400 μW min cm^{-2})

↓

Transform mechanically

in Elac. Resuspend to 10 ml

Elac/10% hiFCS. Add Actinomycin D

to A.

Culture at 37°C/5% CO_2 for 20 hours.

↓

Wash 4 times in Elac.

Resuspend each 6 ml Elac/10% hiFCS.

Divide each into 6 1ml samples

To each sample add 50 μCi (1.85MBq) ^{3}H-uridine at 24h

↓

After 24, 48 and 72 hours of labelling,

remove 2 samples from each treatment group

to ice. Wash 3 times in ice-cold Elac;

freeze for TCA-precipitation.

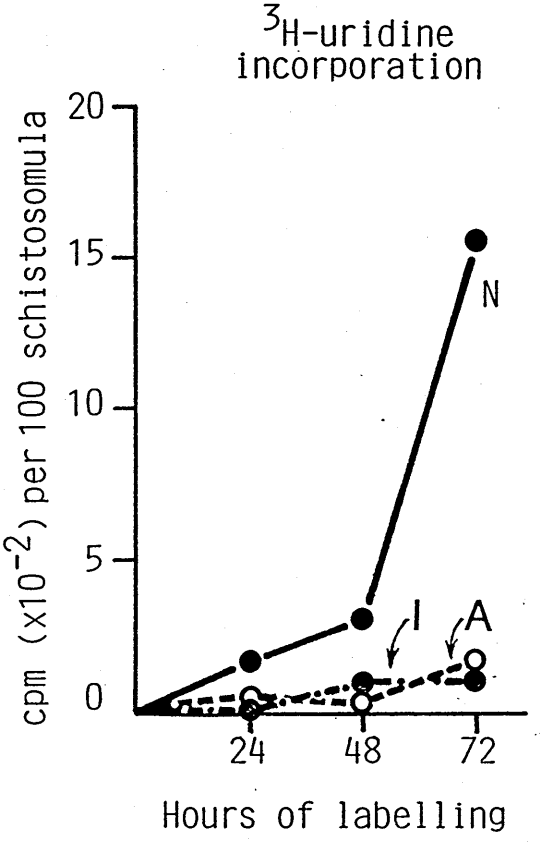

^{3}H-uridine
incorporation
cpm (x10^{-2}) per 100 schistosomula
20
15
10
5
0
N
I
A
24
48
72
Hours of labelling

Figure 4.2. Incorporation of ^{35}S-methionine, ^{3}H-uridine and ^{3}H-adenosine into TCA-precipitable protein or nucleic acid by normal, U.V.-irradiated and Actinomycin D-treated schistosomula from 24 to 72 hours after skin transformation.

M = ^{35}S-methionine incorporation into protein.

A = ^{3}H-adenosine incorporation into nucleic acid.

U = ^{3}H-uridine incorporation into nucleic acid.

N = normal schistosomula

I = U.V.-irradiated schistosomula (400 µW min cm^{-2})

A = Actinomycin D-treated schistosomula.

Each bar represents the mean of two determinations.

Deviation of duplicates from mean = ±7.6%.

Protocol

Cercariae

↓

Divide in 3

↓

N I A

U.V.-irradiate

(400 µW min cm^{-2})

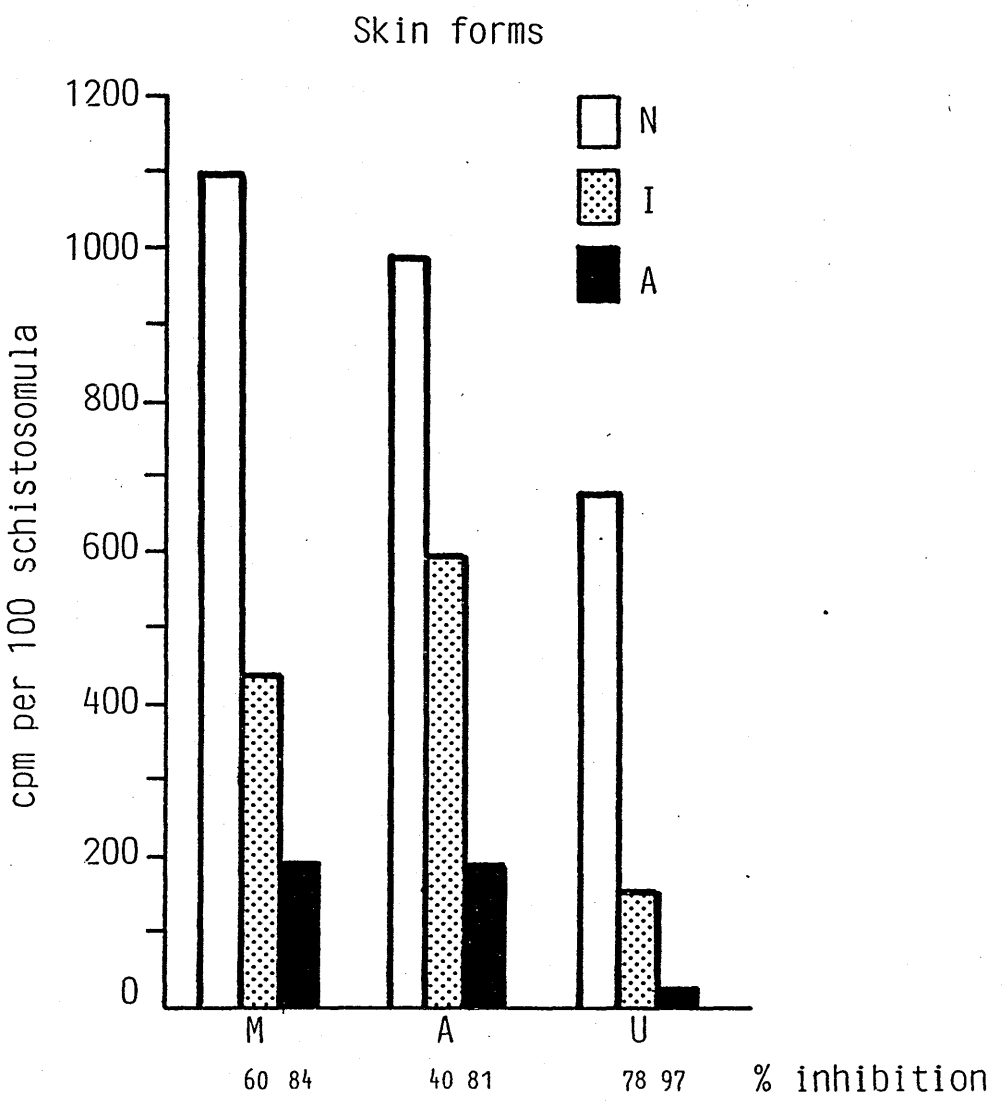
↓

Allow to penetrate excised mouse skins for 3 hours; collect into Elac.

↓

Wash; resuspend to 10 ml Elac/10% hiFCS, Add Actinomycin D to A. Culture at 37°C/ 5% CO_2 for 20 hours.

Continued on next page.


Skin forms
1200
1000
800
600
400
200
0
cpm per 100 schistosomula
N
I
A
M
A
U
60 84
40 81
78 97
% inhibition

Protocol for figure 4.2., continued.

Wash 4 times in Elac. Resuspend each to 6ml Elac/10% hiFCS. Divide each into 6 1 ml samples.

↓

To 2 samples, add 50 µCi (1.85MBq) ^{35}S-methionine
" " " ^{3}H-adenosine } at 24h
" " " ^{3}H-uridine

Culture at 37°C/5% CO_2 for 48 hours.

↓

Remove all samples to ice; wash 3 times in ice-cold Elac; freeze for TCA-precipitation.

Table 4.1. Inhibition of ^{3}H-uridine incorporation into TCA-precipitable RNA at each time-point in figure 4.1.

HOURS OF LABELLING	PERCENTAGE OF INHIBITION OF INCORPORATION BY	
	U.V.-IRRADIATED SCHISTOSOMULA	ACTINOMYCIN D-TREATED SCHISTOSOMULA
24	94.2	71.4
48	75.0	86.7
72	93.5	90.3

Two interesting points may be made about figures 4.1 and 4.2. Firstly, it was consistently observed that, in U.V.-irradiated schistosomula, incorporation of ^{3}H-adenosine into nucleic acid was less inhibited than ^{3}H-uridine incorporation (see figure 4.2). However, it is difficult to draw any conclusions about the nature of irradiation-induced inhibition of nucleic acid synthesis from this observation. By using radiolabelled nucleosides, rather than the purine and pyrimidine bases alone, we are limited to examining nucleic acid synthesis via salvage pathways. However, it is possible that schistosomes are capable of de novo synthesis of both purines and pyrimidines (Barrett, 1981; Chappell, 1979), although this has not been definitely established. If the de novo and salvage pathways for purines and pyrimidines are inhibited to different extents in the attenuated schistosomula, then our results, based on synthesis via the salvage pathway, may not give a representative picture of the real extent of inhibition of RNA synthesis.

From figure 4.1, it seems that, for normal schistosomula, ^{3}H-uridine incorporation into RNA rises very sharply between 48 and 72 hours of labelling. Since these schistosomula were cultured for 20 hours, then washed thoroughly before radiolabelling began (see accompanying protocol), they were approximately 68 and 92 hours old at the last 2 time points on the graph. The first indications of growth in cultured schistosomula appear at approximately 4 days (96 hours) of development (Clegg and Smithers, 1972). We might suggest that the striking enhancement of RNA synthesis just before day 4 could be a preliminary to the generally increased synthetic activity, and production of new proteins, which are presumably required for growth and maturation.

Although only a small percentage of the TCA-precipitable RNA in our experiments is likely to represent messenger RNA, as opposed to ribosomal RNA, and introns which must be spliced out from hnRNA to

produce the final message, the overall level of inhibition of RNA synthesis in irradiated and Actinomycin D-treated schistosomula is as might be predicted from the severe reduction in protein synthesis observed in both attenuated forms (chapter 3).

4.1.2. DNA synthesis.

Figures 4.3 and 4.4 show that uptake of ^{3}H-thymidine into TCA-precipitable nucleic acid by normal schistosomula is extremely low - only 150 to 180 cpm are incorporated by 1000 schistosomula during a labelling period of 52 or 72 hours. In skin forms, it does seem that this low uptake is further reduced after U.V.-irradiation or Actinomycin D treatment, although for mechanically-transformed schistosomula, uptake does not appear to be inhibited. An increase in ^{3}H-thymidine incorporation into nucleic acid is frequently used as an assay for repair of radiation lesions in DNA (Friedberg, 1985). There is no such evidence for extensive DNA repair in these experiments.

Results obtained by Salafsky et al (1988) confirm that incorporation of ^{3}H-thymidine into nucleic acid by recently transformed schistosomula is very low. This lack of measurable DNA synthesis is in accordance with the demonstration by Clegg and Smithers (1972) that schistosomula in vitro delay mitotic activity until at least the fourth day of culture.

It is not clear whether the low level of ^{3}H-thymidine incorporation detectable in normal schistosomula corresponds to repair of damaged DNA, or to cell division in a very small proportion of cells. Stirewalt et al (1983) and Cousin et al (1986) observed euchromasia in a percentage of schistosomula immediately after skin penetration. These workers claimed that the euchromasia was indicative of DNA activation prior to mitosis, but Salafsky et al (1988) offered a different explanation, considering that the trauma of skin

Figure 4.3. Incorporation of ^{3}H-thymidine into TCA-precipitable nucleic acid by normal, U.V.-irradiated and Actinomycin D-treated schistosomula from 24 to 96 hours after mechanical transformation.

—●— :Normal schistosomula

·-·-●-·-· :U.V.-irradiated schistosomula (400 μW min cm^{-2})

---O--- :Actinomycin D-treated schistosomula.

Each point represents the mean of two determinations.

Deviation of duplicates from mean = ±12.3%

PROTOCOL:

Cercariae

↓

Divide in 3

N I A

U.V.-irradiate

(400 μW min cm^{-2})

↓

Transform mechanically in Elac.

Resuspend to 10 ml Elac/10%

hiFCS. Add Actinomycin D to A.

Culture at 37°C/5% CO_2 for 20 hours.

↓

Wash 4 times in Elac.

Resuspend each to 6 ml Elac/10% hiFCS.

Divide each into 6 1ml samples.

Add 50 μCi (1.85MBq) ^{3}H-thymidine to each at 24h.

↓

After 24, 48 and 72 hours of labelling,

remove 2 samples from each group to ice.

Wash 3 times in ice-cold Elac; freeze

for TCA-precipitation.

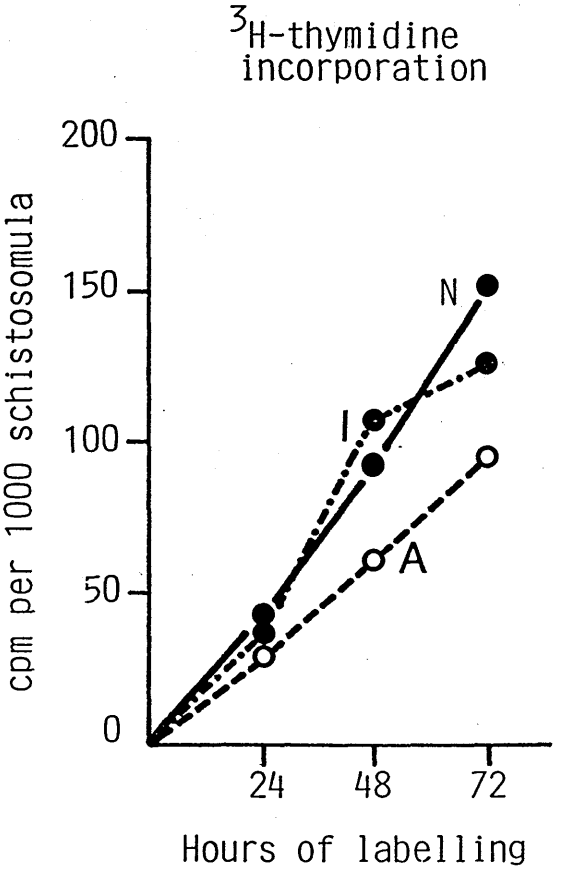

^{3}H-thymidine
incorporation
200
150
100
50
0
cpm per 1000 schistosomula
N
I
A
24
48
72
Hours of labelling

Figure 4.4. Incorporation of ^{3}H-thymidine into TCA-precipitable nucleic acid by normal, U.V.-irradiated and Actinomycin D-treated schistosomula from 24 to 72 hours after skin transformation.

N = normal schistosomula

I = U.V.-irradiated schistosomula (400 μW min cm^{-2}).

A = Actinomycin D-treated schistosomula.

Each point represents the mean of two determinations. Deviation of duplicates from mean = ±11.4%

PROTOCOL:

Cercariae

↓

Divide in 3

N I A

U.V.-irradiate

(400 μW min cm^{-2})

↓

Allow to penetrate excised mouse skins for 3 hours; collect into Elac.

↓

Wash; resuspend to 10 ml Elac/10% hiFCS.

Add Actinomycin D to A.

Culture at 37°C/5% CO_2 for 20 hours.

↓

Wash 4 times in Elac. Resuspend each to 2 ml Elac/10% hiFCS.

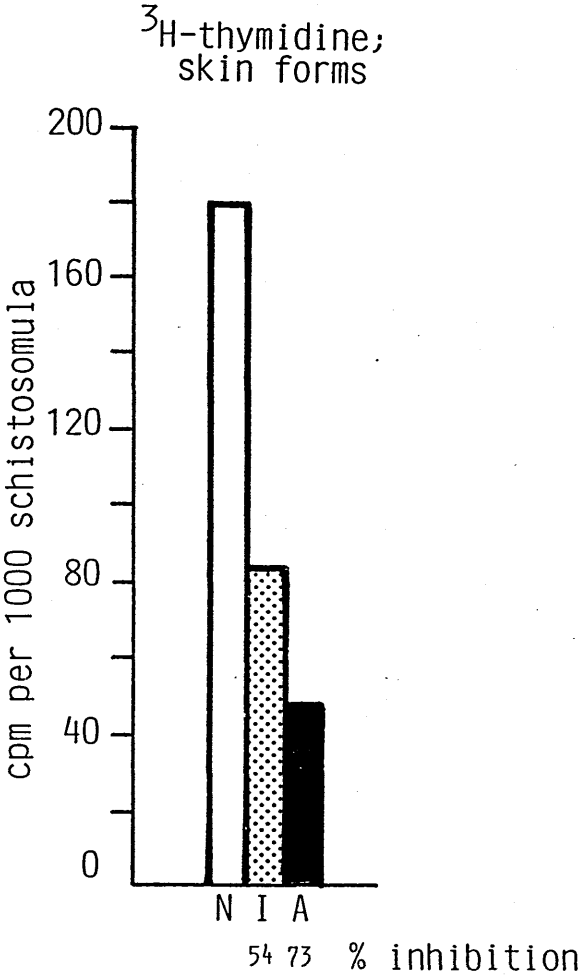
Divide each into 2 1ml samples

Add 50 μCi (1.85MBq) ^{3}H-thymidine to each at 24h.

Culture at 37°C/5% CO_2 for 48 hours.

↓

Remove all samples to ice; wash 3 times in ice-cold Elac; freeze for TCA-precipitation.


^{3}H-thymidine;
skin forms
200
160
120
80
40
0
cpm per 1000 schistosomula
N I A
54 73 % inhibition

penetration caused significant cell damage and death, with associated disruption of the DNA. Either interpretation might account for incorporation of a small amount of ^{3}H-thymidine into the nucleic acid, as observed in these experiments. It may also be noted that a small amount of thymidine is frequently incorporated into transfer RNA in established cellular systems (Lewin, 1983). This may also contribute to the overall uptake of ^{3}H-thymidine by normal schistosomula.

Similar explanations - low levels of cell division or of DNA repair - may be suggested for the incorporation of ^{3}H-thymidine into nucleic acid by U.V.-irradiated or Actinomycin D-treated schistosomula. Excision repair of damaged regions of DNA, containing radiation lesions, or intercalated Actinomycin D, might account for a proportion of the thymidine uptake. However, the low level of incorporation suggests that, if such repair processes do occur, they are not extensive.

4.2. Phospholipid synthesis by normal, U.V.-irradiated and Actinomycin D-treated schistosomula.

4.2.1. Metabolic labelling studies.

Figures 4.5 a) to d) show the incorporation of ^{32}P into phospholipids by normal, U.V.-irradiated, Actinomycin D-treated and Fenfluramine-treated schistosomula between 22 and 70 hours after transformation.

Fenfluramine inhibits the activity of phosphatidate phosphohydrolase (Brindley and Bowley, 1975). This drug decreases the rate of synthesis of triacylglycerols and the zwitterionic phospholipids - phosphatidylcholine and phosphatidylethanolamine. At the same time, phosphatidate and other acidic phospholipids such as CDP-diacylglycerol, phosphatidylinositol and phosphatidylglycerol accumulate (Brindley and Bowley, 1975; Sturton and Brindley, 1977). A Fenfluramine-treated group was included in the experiment in order to compare the results of specific inhibition of lipid synthesis alone with the effects of more general metabolic inhibitors - U.V.-irradiation and Actinomycin D.

The chromatograms of figure 4.5 are analysed quantitatively in figures 4.6 and 4.7. Figure 4.6a) shows the results of measuring ^{32}P-incorporation into the main phospholipid classes, while 4.6b) describes the percentage reduction, or accumulation, of each phospholipid, in the attenuated, as compared to normal, forms. Figure 4.7 compares the distribution of ^{32}P among the individual phospholipid classes by normal, U.V.-irradiated, Actinomycin D-treated and Fenfluramine-treated schistosomula.

Normal schistosomula incorporate inorganic phosphate primarily into PC, and approximately a third as much into each of PE and PI. Less than 5% as much ^{32}P is assimilated into PA, PS or PG as into PC, but

Protocol for figures 4.5, 4.6, 4.7.

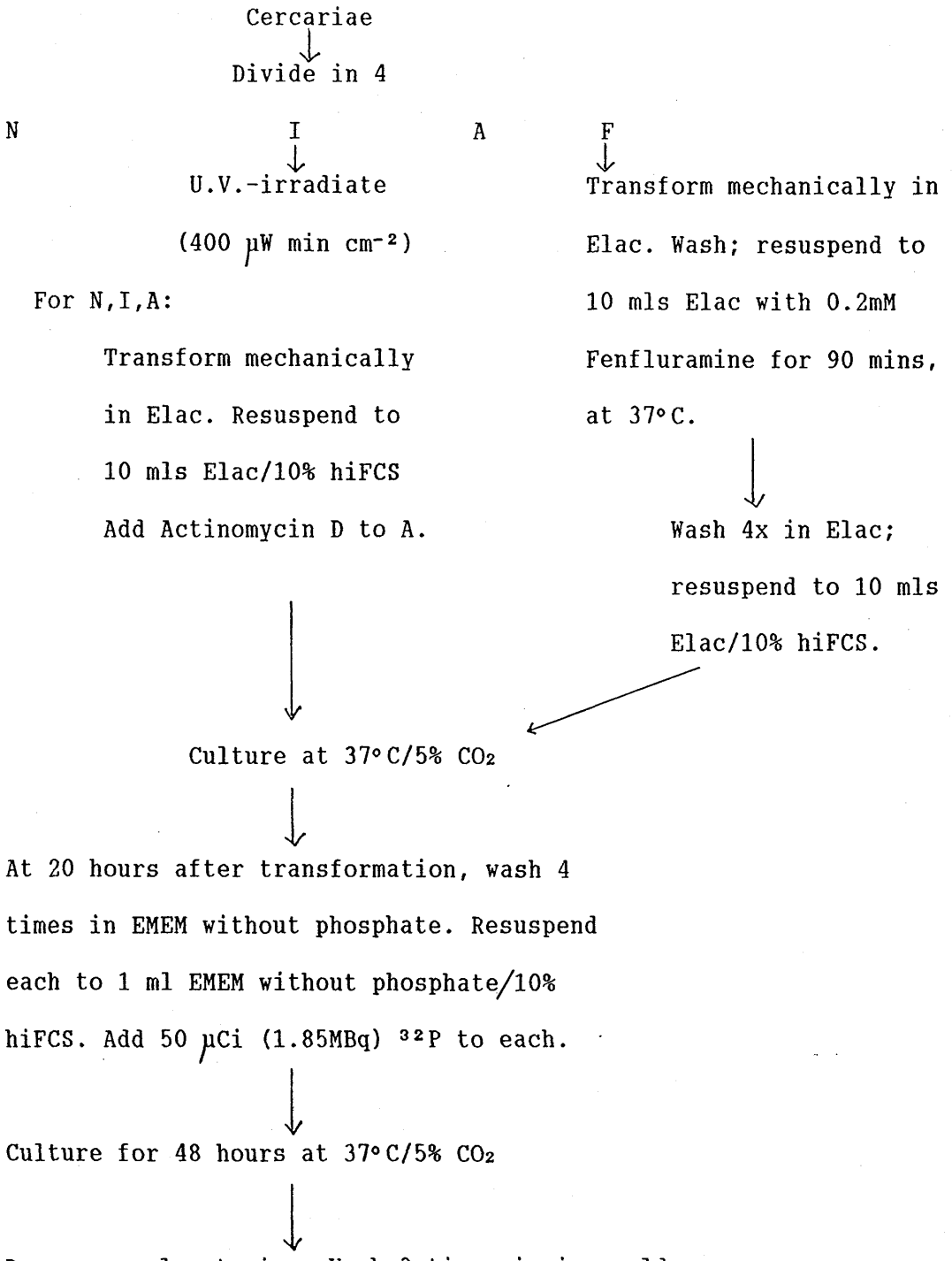


Figure 4.5 a) to d) Autoradiographs showing ^{32}P-incorporation into phospholipids by normal, U.V.-irradiated, Actinomycin D-treated and Fenfluramine-treated schistosomula between 22 and 70 hours after transformation.

a) Normal schistosomula

b) U.V.-irradiated schistosomula (400 μW min cm^{-2})

c) Actinomycin D-treated schistosomula

d) Fenfluramine-treated schistosomula.

1000 schistosomula on each chromatogram

ABBREVIATIONS:

PC = phosphatidylcholine

PE = phosphatidylethanolamine

PS = phosphatidylserine

PA = phosphatidic acid

PI = phosphatidylinositol

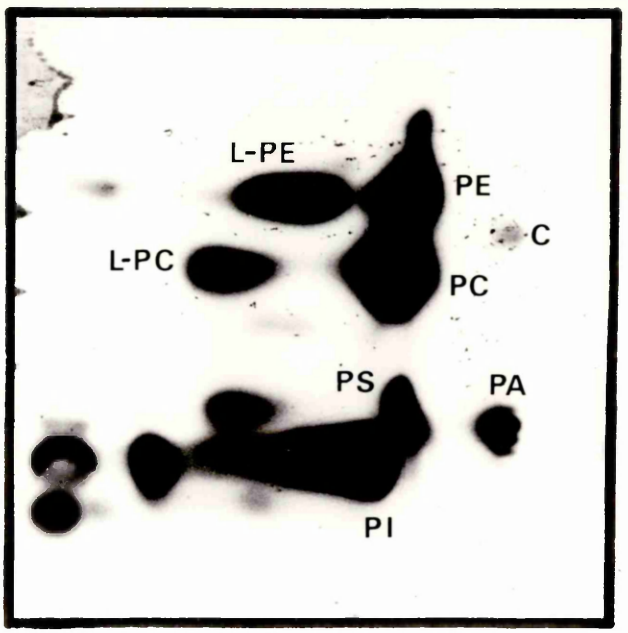
PG = phosphatidylglycerol

L-PC = lysophosphatidylcholine.

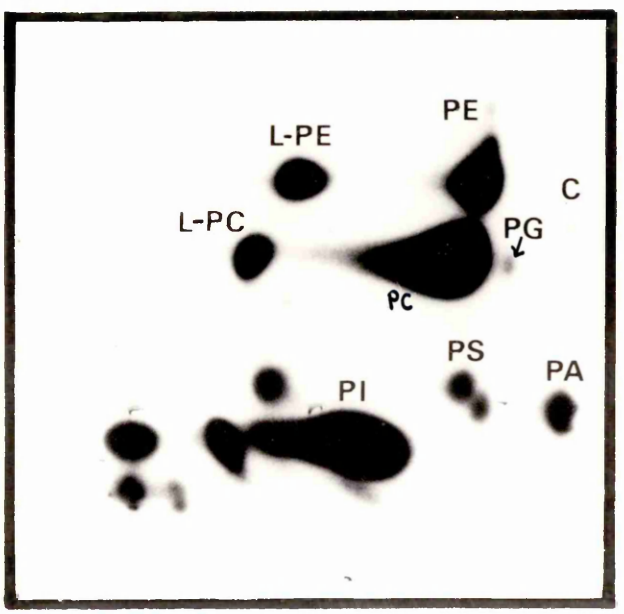
L-PE = lysophosphatidylethanolamine.

C = cardiolipin

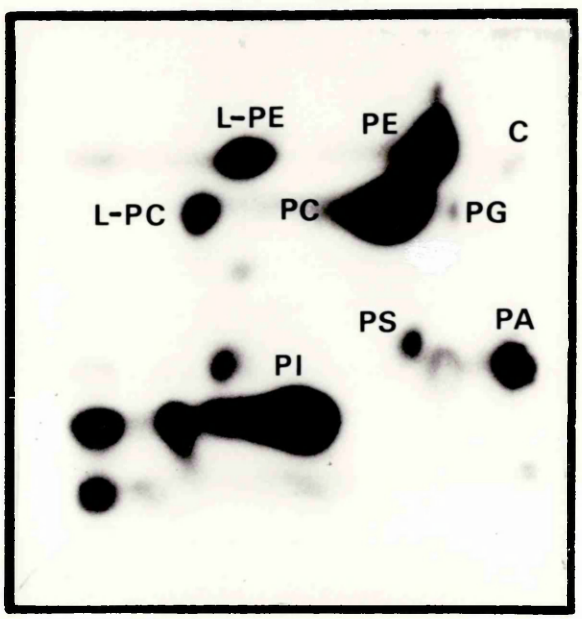
a Normal schistosomula



b Irradiated schistosomula



c Actinomycin D-treated schistosomula



d Fenfluramine-treated schistosomula

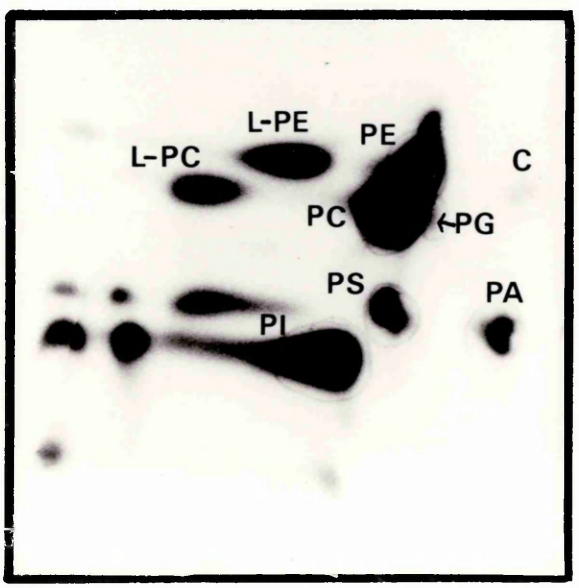


Figure 4.6a) Incorporation of ^{32}P into individual phospholipids by normal, U.V.-irradiated (400 μW min cm^{-2}), Actinomycin D-treated and Fenfluramine-treated schistosomula from 22 to 70 hours after transformation.

Figure 4.6b) Percentage decrease or increase in ^{32}P-incorporation into each class of phospholipid by the attenuated, as compared to normal, schistosomula.

Protocol and abbreviations for figure 4.6 are as for figure 4.5.
Each bar represents the mean of two determinations.
Deviation of duplicates from mean = ±7.8%.

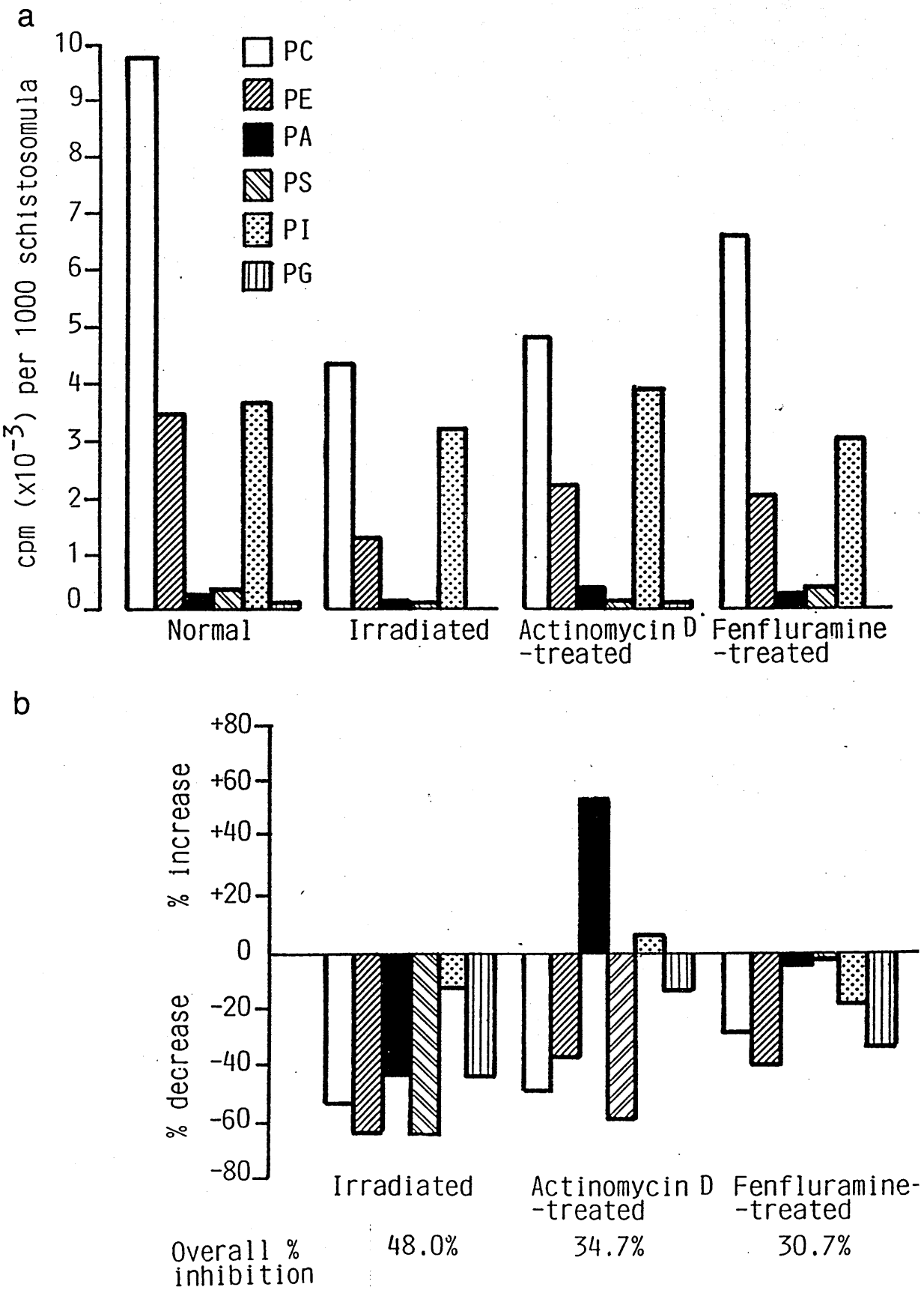
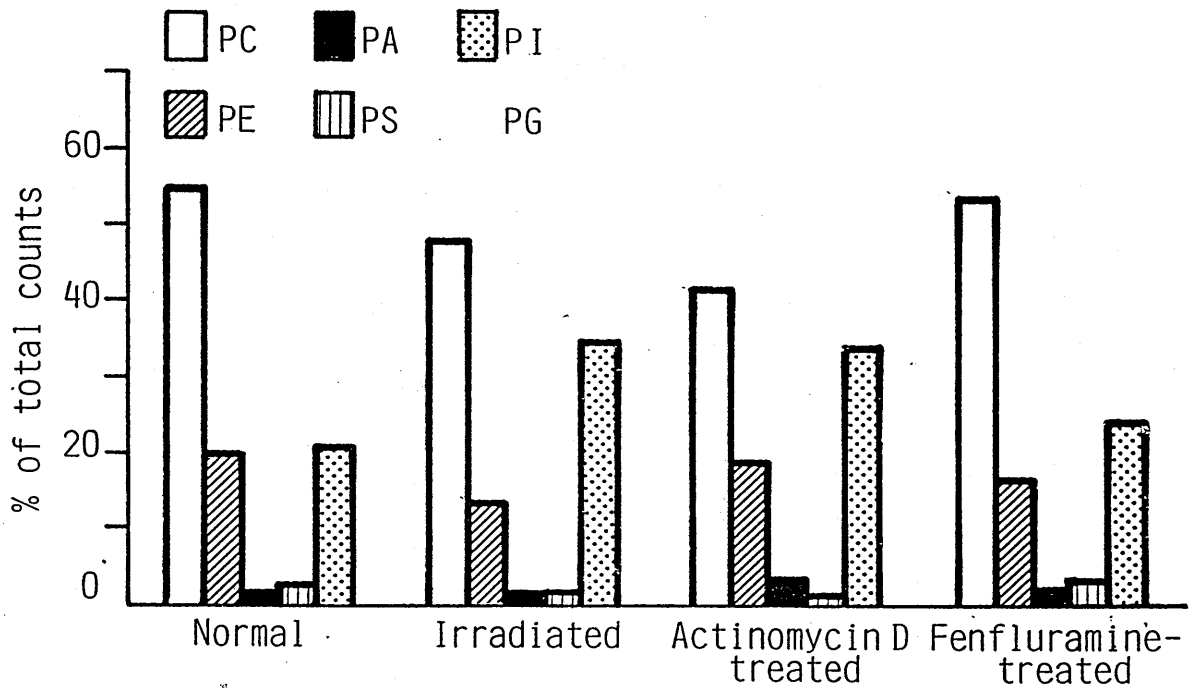

a
cpm (x10^{-3}) per 1000 schistosomula
10
9
8
7
6
5
4
3
2
1
0
PC
PE
PA
PS
PI
PG
Normal
Irradiated
Actinomycin D -treated
Fenfluramine -treated
b
+80
+60
+40
+20
0
-20
-40
-60
-80
% increase
% decrease
Irradiated
Actinomycin D -treated
Fenfluramine-treated
Overall % inhibition
48.0%
34.7%
30.7%

Figure 4.7 The distribution of ^{32}P among individual phospholipid classes by normal, U.V.-irradiated, Actinomycin D-treated and Fenfluramine-treated schistosomula.

For the 4 treatment groups, the radioactivity incorporated into each phospholipid class is expressed as a percentage of the total ^{32}P-incorporation into the 6 phospholipid classes of interest.

Protocol and abbreviations are as for figure 4.5.


PC
PA
PI
PE
PS
PG
60
40
20
0
% of total counts
Normal
Irradiated
Actinomycin D treated
Fenfluramine-treated

the radioactive spots are still readily detectable on the chromatogram.

U.V.-irradiated schistosomula show the most severe overall inhibition of phospholipid synthesis - 48%. PE and PS suffer the greatest decrease - almost 70% each - compared to a reduction of 54% for PC, and approximately 45% for PA and PG. Incorporation of ^{32}P into PI, in contrast, is only some 14% inhibited. This continued production of PI, while synthesis of other phospholipid classes is reduced, means that PI forms an abnormally high proportion of the newly synthesized phospholipids in U.V.-irradiated schistosomula - 35%, as opposed to 21% for normal parasites (figure 4.7). Although synthesis of the other phospholipid classes is reduced compared to normal forms, their relative contributions to overall phospholipid synthesis are almost the same. Thus, approximately 50% of the total phosphate incorporated into these 6 phospholipid classes is channelled into PC synthesis, some 15% into PE, and less than 5% into PA, PS and PG, by both normal and U.V.-irradiated schistosomula.

Actinomycin D-treated parasites show slightly less total inhibition of phospholipid synthesis than U.V.-irradiated schistosomula - 35%. Inhibition of ^{32}P incorporation into PS (60%), PC (50%), PE (38%), and PG (17%) is not as pronounced as in the irradiated larvae. PA shows a striking accumulation of ^{32}P - 53% more than normal schistosomula - while PI incorporation is also slightly increased (5%). However, PA constitutes such a small fraction of the total phospholipid production that the relative contributions of PC, PE, PA, PS and PG synthesis are again much the same as in normal parasites. Figure 4.7 shows that, despite the apparent accumulation of PA, the distribution of ^{32}P among the different phospholipid classes is very similar for normal, U.V.-irradiated and Actinomycin D-treated schistosomula. Only PI differs prominently from normal schistosomula. Just as for the U.V.-irradiated forms, this phospholipid shows increased incorporation of ^{32}P in Actinomycin D-treated parasites - 34% as opposed to 21% of

the incorporated phosphate.

Fenfluramine appears to induce the least total inhibition of phospholipid synthesis. Incorporation into PC, PE and PG is not as severely reduced as in irradiated schistosomula (30%, 42% and 36% decreases respectively). Synthesis of PA (6% decrease) and PS (2% decrease) is scarcely inhibited at all. PI, however, shows the greatest decrease of all three treatments - 20%. The relative proportions of the newly-synthesized phospholipid classes are virtually the same as for normal schistosomula, PE being reduced, and PI increased, by only a few per cent.

Thus, it seems that specific inhibition of phospholipid synthesis by Fenfluramine does not have the same effect on lipid composition as the general metabolic inhibition induced by U.V.-irradiation or Actinomycin D. These observations on the response of schistosomular phospholipid synthesis to Fenfluramine treatment might seem to contradict, to some extent, the established effects of this drug as reported in the literature (Brindley and Bowley, 1975; Sturton and Brindley, 1977). As mentioned above, Fenfluramine normally causes a marked accumulation of PA, PG and PI, with a reduction in PC and PE synthesis. In fact, when phospholipid synthesis by schistosomula is examined at earlier times after Fenfluramine treatment (1 to 3 hours) these phospholipids are found to accumulate (Dr. Janet Jones, personal communication). During the longer time course of the experiment described here (from 22 to 70 hours after transformation), some feedback regulatory system may aim to restore the proportions of the different phospholipids to approximately the same as in normal forms (see figure 4.7). In contrast, the profiles for U.V.-irradiated and Actinomycin D-treated schistosomula in figure 4.7 are rather different from normal, due chiefly to the accumulation of PI and PA.

Two important caveats should be applied to these results. Firstly, they describe total schistosomular phospholipids: those restricted to

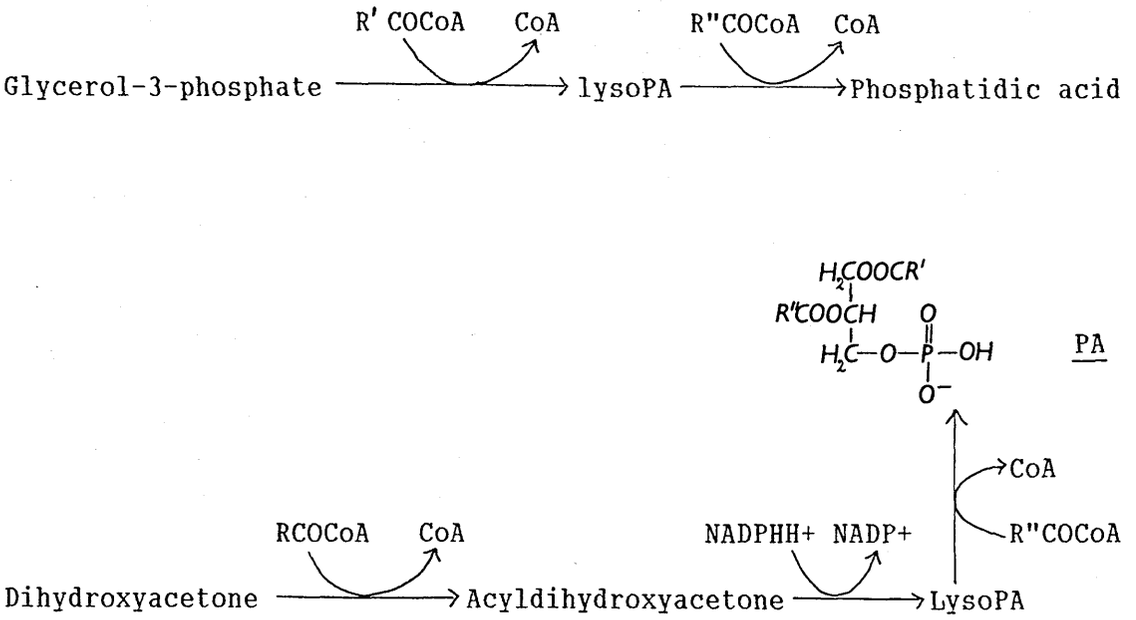
the surface were not identified. Secondly, these values for ^{32}P-incorporation into the various phospholipid classes reflect rates of turnover. Hence, they do not necessarily represent the proportions of each phospholipid class in the schistosomular membranes. For instance, very little ^{32}P is incorporated into PS. This might be due to the fact that PS turns over at an average rate, but constitutes only a small fraction of the total phospholipid. Alternatively, there could be substantial amounts of PS present, undergoing turnover at very low rates. A third possibility could be that PS turnover is very rapid, for instance, if it serves as a precursor for another phospholipid class, so that, at any one time, very little ^{32}P is present in PS.

4.2.2. Discussion: Lipid metabolism by schistosomula: normal, U.V.-irradiated, Actinomycin D- or Fenfluramine-treated.

4.2.2.1. Pathways of phospholipid synthesis by schistosomula.

An outline of the pathways of phospholipid synthesis should help explain the distribution of ^{32}P among the different phospholipid classes in normal and attenuated schistosomula. The following pathways apply in the first place to mammalian phospholipid synthesis, but have generally been demonstrated in helminths, too (Barrett, 1981). Vial *et al* (1985) demonstrated synthesis of phospholipids by both schistosomula and adult schistosomes, though the enzymic pathways were not worked out in detail.

i) Phosphatidic acid (PA). PA may be formed from glycerol-3-phosphate or dihydroxyacetone phosphate and 2 moles of fatty acyl CoA:



Alternatively, 1,2-diacylglycerol (DAG) can be converted to phosphatidic acid by diacyglycerol kinase:

1,2-Diacylglycerol + ATP $\longrightarrow$ Phosphatidic acid + ADP.

ii) Phosphatidylethanolamine. (PE)

Initially, ethanolamine is phosphorylated by ethanolamine kinase:

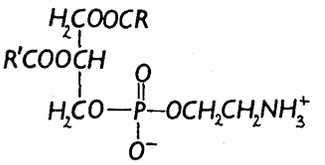
Ethanolamine + ATP $\xrightarrow{Mg^{2+}}$ Phosphoethanolamine + ADP

A cytidyl transferase then catalyses reaction of phosphoethanolamine with CTP to yield an activated CDP-derivative:

Phosphoethanolamine + CTP $\longrightarrow$ CDP-ethanolamine + PP_i.

CDP-ethanolamine is then transferred to diacylglycerol by ethanolamine transferase:

CDP-ethanolamine + 1,2-DAG ⟶ Phosphatidylethanolamine + CMP.



PE

iii) Phosphatidylcholine (PC).

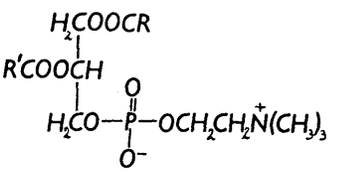
De novo synthesis of PC occurs by a similar route to that for PE synthesis:

Choline + ATP ⟶ phosphocholine + ADP (choline kinase)

Phosphocholine + CTP ⟶ CDP-choline + PP_i.

(choline-phosphate cytidyl transferase)

CDP-choline + DAG ⟶ phosphatidylcholine + CMP (choline transferase).



PC

An alternative pathway for PC synthesis is N-methylation of PE, in stages, by successive transfer of 3 methyl groups from S-adenosylmethionine:

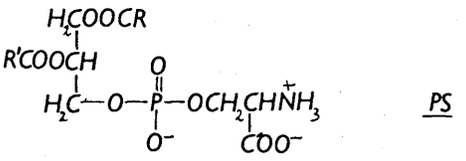
PE + 3 S-adenosylmethionine ⟶ PC + 3 S-adenosylhomocysteine.

Parra et al (1986) used S-adenosylhomocysteine as a methyltransferase inhibitor to show that this pathway occurs in schistosomula.

iv) Phosphatidylserine (PS).

In mammals, PS is synthesized by base exchange:

phosphatidylethanolamine + serine→phosphatidylserine + ethanolamine



PS

However, in H. diminuta (Webb and Mettrick, 1973), PS synthesis appears to be via PA, a pathway characteristic of bacteria:

PA + CTP ⟶ CDP-diacylglycerol + PP_i.

CDP-diacylglycerol + serine ⟶ PS + CMP.

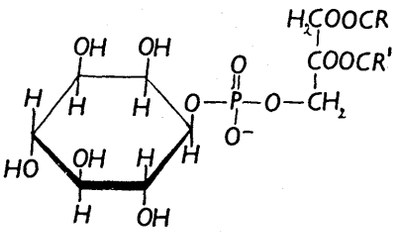
It would seem that schistosomula may also rely chiefly on this second pathway, since, from figures 4.6 and 4.7, Fenfluramine treatment inhibits PE synthesis, but has no effect on PS production. If an exchange reaction occurred, a similar decrease in PS synthesis would be predicted. In Actinomycin D-treated schistosomula, ^{32}P is incorporated into PA to approximately the same extent as it decreases in PS. The accumulation of newly-synthesized PA might therefore be due to inhibited synthesis of this last enzyme which converts PA to PS. This enzyme may have a considerably shorter half-life, or be produced in much smaller amounts, than those involved in synthesis of PI or PG from PA, since both of these phospholipids continue to be produced in Actinomycin D-treated parasites.

v) Phosphatidylinositol (PI) and phosphatidylglycerol (PG)

These phospholipids are derived from activated 1,2-diacylglycerol

by transferases:

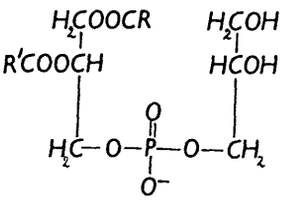
CDP-DAG + myoinositol ⟶ phosphatidylinositol + CMP.



PI

CDP-DAG + glycerol-3-phosphate ⟶ phosphatidylglycerol-phosphate + CMP.

Phosphatidylglycerol-phosphate ⟶ phosphatidylglycerol + P_i (phosphatase).



PG

Abbreviations. P_i = inorganic phosphate

PP_i = diphosphate

R, R', R", etc. = fatty acyl chains.

CoA = Coenzyme A.

DAG = 1,2-diacylglycerol.

CDP, CTP = cytidine diphosphate, cytidine triphosphate.

CDP-choline) activated cytidine derivatives of

CDP-DAG) choline; 1,2-DAG; ethanolamine, etc.

CDP-ethanolamine, etc.)

4.2.2.2. Fatty acid and neutral lipid metabolism in schistosomula.

Meyer et al (1970) demonstrated that schistosomes are unable to synthesize de novo sterols or fatty acids, and hence are dependent on dietary sources to synthesize their complex lipids. They retain the ability to elongate stearic acid and oleic acid chains by acetylation, but cannot desaturate them. This restricted ability to modify their

fatty acyl chains will mean that schistosomula are especially susceptible to external influences, such as temperature, pH and ionic strength, which affect membrane lipid organisation (section 4.2.3.).

Rumjanek and McLaren (1981) investigated the exchange of lipids between schistosomula and the serum in which they were cultured. Foetal calf serum induced a net loss of lipids from schistosomula, in particular, of mono- and diglycerides. Culture with human serum also resulted in loss of mono- and diglycerides, but this was accompanied by a substantial uptake of cholesterol and triglycerides into the schistosomulum surface. This exchange process appeared to be mediated by a lipid receptor, identified on SDS-PAGE as a doublet at Mr approximately 46 000. This receptor was normally buried in the schistosomulum double bilayer, but was immediately made available on the outer surface upon contact with human serum (Rumjanek et al, 1985). Gamma-irradiation of cercariae at 20 krad did not affect this lipid exchange process (Rumjanek and McLaren, 1981). As described in section 4.2.2.1., routes for the formation of PA from tri- and diacylglycerides could mean that the neutral lipids acquired from human serum in this way may be diverted to phospholipid synthesis.

Vial et al (1985) used radioactive precursors to examine the synthesis of neutral and phospholipids by schistosomula, as opposed to acquisition of these molecules from serum. Newly-transformed schistosomula incorporated 80% more glycerol and 150% more oleate into neutral lipids - DAG and TAG - than into phospholipids. In fact, triacylglyceride synthesis by recently-transformed schistosomula was higher than at any another stage.

4.2.2.3. Lipid catabolism by schistosomula.

Both Meyer et al (1970) and Vial et al (1985) were unable to detect breakdown of radioactive fatty acid precursors, and concluded

that fatty acid degradation by β-oxidation does not occur in schistosomes. Nor has any direct evidence for schistosome phospholipases been obtained. However, from the abnormal accumulation of PI in U.V.-irradiated and Actinomycin D-treated schistosomula (see figure 4.7), it might be inferred that, in non-inhibited schistosomula, a specific phospholipase might be involved in PI turnover.

The general absence of degradative enzymes suggests that lipid turnover by schistosomula may be largely accomplished by release or exchange from the surface membrane.

Section 4.2.4. will attempt to combine this information on lipid metabolism in normal schistosomula with a model for changes occurring in membrane lipids during transformation of cercariae. It is then possible to suggest how the organisation of membrane lipids and proteins might be altered in U.V.-irradiated and Actinomycin D-treated schistosomula.

4.2.3. Phospholipid synthesis during the transformation of cercariae to schistosomula.

In order to assess how U.V.-irradiation or Actinomycin D treatment might affect antigen expression during schistosomular development, it is of interest to examine the changes in organisation of membrane lipids during transformation of normal parasites.

Young and Podesta (1984) and McDiarmid and Podesta (1982) found that the major phospholipid component of adult Schistosoma mansoni was phosphatidylcholine. This observation agrees with the extensive synthesis of PC by schistosomula demonstrated in these experiments. These authors proposed that the schistosome double bilayer exposes principally PC on its extracytoplasmic face, while PE and PS are located principally in the inner bilayer. PG and PI are present in relatively small amounts - 8 to 9% each. A similar asymmetric

distribution exists in the single bilayer of rat and human erythrocytes (Houslay and Stanley, 1982), where PC partitions mainly to the extracytoplasmic side, while the aminophosphatides, PE and PS, are found mainly in the cytoplasmic monolayer.

However, the composition and arrangement of phospholipids in cercarial membranes are quite different from those of schistosomula. For cercariae, PE is the most abundant phospholipid (21%), with PS a close second (17%). PC and PG are present in significant, approximately equal amounts (16%), while PI constitutes 15% of the total (Young and Podesta, 1984).

Research in our laboratory supports the idea that cercariae synthesise different phospholipids from developing schistosomula and mature schistosomes. (Anne Davidson, unpublished results). It seems that free-living cercariae in water incorporate ^{32}P primarily into PG and PE. After transformation, PC becomes the phospholipid predominantly synthesized. Thus, it would appear that, upon transformation, the parasite immediately begins to replace the acidic phospholipids PI, PG, PS (one negative charge each), and PE, with an outer bilayer rich in the neutrally-charged PC and weakly hydrophilic neutral lipids derived in part by uptake from host serum (see 4.2.2.2. above). Radiolabelling cercariae in water with ^{32}P for 2 hours, then extracting the surface with digitonin, shows that the acidic PG is the major newly-synthesized phospholipid at the cercarial surface. Thus, free-living cercariae appear to synthesize and transport PG to their surfaces at a high rate. For schistosomula labelled with ^{32}P immediately after transformation, scarcely any radioactive components are detectable in the digitonin-extracted surface (Sharron MacFarlane, unpublished results). The labelled phospholipids are retained in the schistosomular body where they were synthesized, presumably in the subtegumental cells. It therefore seems that the phospholipid components of the double bilayer produced within 3 hours of

transformation (Hockley and McLaren, 1973) must be pre-synthesized and stored by developing cercariae. The newly-synthesized phospholipids may replace the vacuoles already released from the subtegumental cells. A similar situation applies to schistosomular proteins (see chapter 3).

Assuming that PS, PI, PG and PE are present in the cercarial surface in the same relative proportions as found in the whole body by Young and Podesta (1984), it would seem that the cercarial membrane is composed predominantly of negatively charged phospholipids. In an environment of low ionic strength, such as fresh water, these negatively charged molecules will tend to increase the area each occupies in the membrane, in order to decrease lateral repulsion between adjacent molecules and make the surface charge more diffuse. Consequently, this phospholipid composition may favour a lateral expansion of the bilayer, with creation of highly fluid lipid domains. An increase in ionic strength and cation concentration, as occurs upon transfer of cercariae to GMEM or mammalian skin, will reduce the bilayer surface charge, and may promote a more rigid, compact organisation of phospholipids. This phenomenon might help to explain the immediate, pronounced decrease observed in permeability to methionine upon transfer of cercariae to medium or salt solution (discussed in section 3.10.1; figure 3.26). Between 30 and 60 minutes after transformation, much of the original cercarial trilaminate membrane is discarded (Hockley and McLaren, 1973), and the neutral lipid- and PC-rich schistosomular double bilayer established, with its own permeability and fluidity characteristics (Foley et al, 1988; section 3.10.1; figure 3.26). These suggested alterations in organisation of the parasite membrane during transformation are time-tabled in figure 3.26.

The predominance of negatively charged phospholipids in cercariae may also play a role in osmoregulation. The predominance of PG in the surface of free-living cercariae in water is especially interesting,

for, in E. coli, the osmolarity of the medium appears to regulate PG synthesis. Kennedy (1982) demonstrated that bacteria grown in medium of low osmolarity produced 16 times as much PG as those grown in 0.4M NaCl. In the bacterial system, sn -1-phosphoglycerol derived from PG combines with membrane-derived oligosaccharides in the periplasmic space to produce highly anionic compounds which maintain a high osmotic pressure in the periplasmic compartment. The author postulated an osmotic sensor in the bacterial outer membrane which detects any alterations in turgor pressure, and relays the information to enzymes regulating the expression of genetic messages for production of PG.

It is tempting to extrapolate this system to transforming cercariae. Alterations in membrane lipid organisation, or in the conformation of surface proteins upon transfer of the parasites from water to medium of high osmotic strength, could repress messages encoding enzymes for the synthesis of negatively charged phospholipids, and enhance activation of those leading to PC and TAG production. As yet, it is unclear whether cercariae synthesise glycophospholipids that might play an osmoregulatory role like the bacterial oligosaccharides multiply-substituted with sn -1-phosphoglycerides. However, the highly polar, ^{32}P-labelled material which does not move from the origin on TLC's analysed for cercarial phospholipids (Anne Davidson, unpublished results), or the heavily-labelled ^{32}P-containing compounds that migrate to the bottom of SDS-gels (see figure 4.9) might well contain glycophospholipids.

A second role proposed for acidic phospholipids, which might be relevant to the transformation process, is active participation in insertion of proteins in the membrane, and protein secretion (Nesmeyanova, 1982; Leonard et al, 1981). From their observations in bacterial systems, these workers postulated a general model for insertion and secretory processes in procaryotes. The signal peptide of a protein destined for the membrane or for secretion interacts with

acidic phospholipids to modulate a non-bilayer configuration of phospholipids. A hydrophilic lipid channel consisting of an inverted micelle in the bilayer core (hexagonal phase lipid) is formed. The major part of the protein is translocated through this channel, pulled by moving phospholipids transferring to the outer bilayer, and with the signal peptide anchored in them. Figure 4.8 illustrates this process.

This model predicts that cercariae may have a particularly high requirement for acidic phospholipids such as PG, for they must insert proteins, not only into their own trilaminate surfaces, but also must be prepared to insert proteins into the new heptalaminate membrane produced during the first few hours after transformation. Integral membrane proteins seem to be inserted only after fusion of the multilaminate vacuoles with the schistosomular surface is achieved, for freeze-fracture electron microscopy indicates that these membranous vacuoles, prior to fusion with the surface, are devoid of intramembranous particles (apparently representing proteins). The intact surface membrane is rich in intramembranous particles, however (McLaren, 1980). It is also possible that the negatively charged phospholipids such as PG may be involved in the rapid release of cercarial proteins upon transformation (fig. 3.20; section 3.9.1; figure 4.8(2).), although this loss could be due to shedding of whole membrane fragments in association with the glycocalyx, rather than secretion of individual proteins.

In summary, this discussion envisages that channels of hexagonal phase lipid (formed by PG), interspersed in fluid domains of weakly-associated, negatively charged phospholipids, may be an important feature of the surface membrane of free-living cercariae. The organisation of cercarial membrane lipids is immediately modified upon contact with GMEM or the mammalian environment. Replacement of the cercarial trilaminate membrane with the neutral lipid- and PC-rich schistosomular bilayer completely restructures the parasite surface.

Figure 4.8(1) Insertion of integral membrane proteins, coupled with translocation of acidic phospholipids (based on Nesmeyanova, 1982).

1. Nascent peptide, containing N-distal charged site, begins to emerge from a ribosome.
2. Nascent peptide binds to a region rich in negatively-charged phospholipids.
3. Signal peptide inserts into the membrane interior.
4,5. Acidic phospholipids, losing their charge on interaction with signal peptide, start transbilayer movement, inducing the formation of a hydrophilic channel (hexagonal configuration), and pull the hydrophilic part of the protein across the channel.
6. Polypeptide elongation is completed, the signal sequence is removed by peptidase, and acidic phospholipids translocated to the opposite side of the membrane.

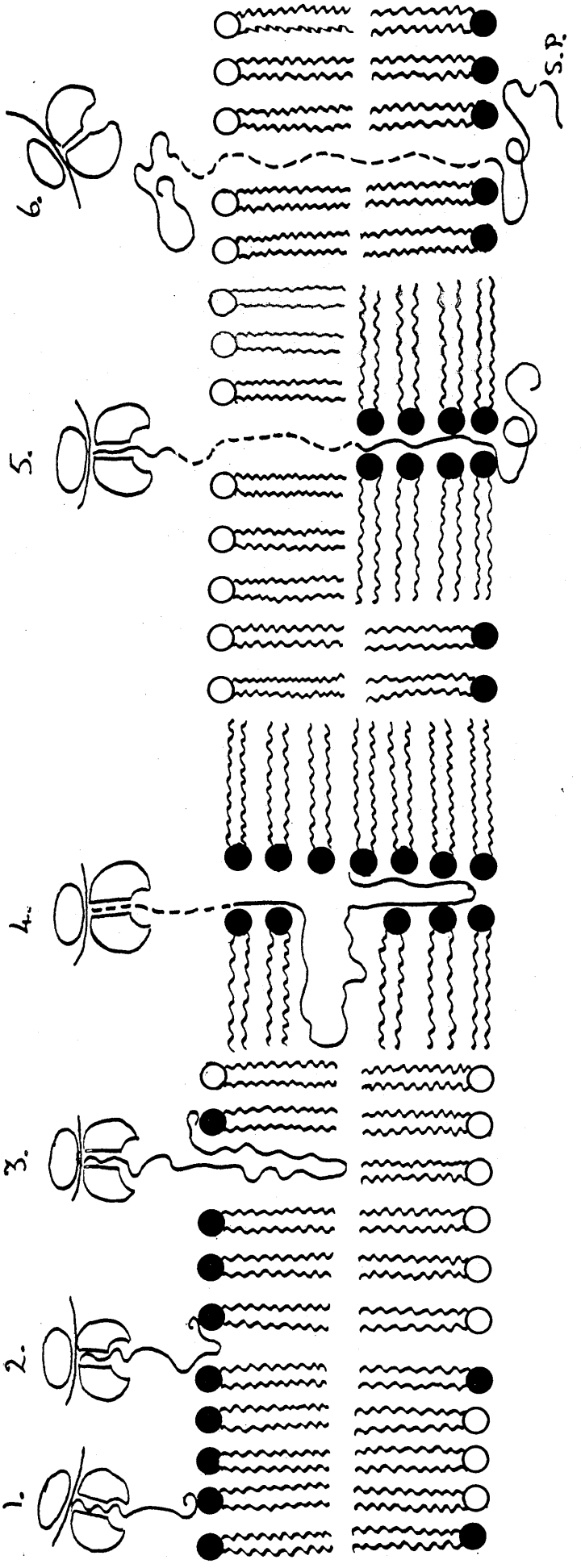

S.P.
6.
5.
4.
3.
2.
1.

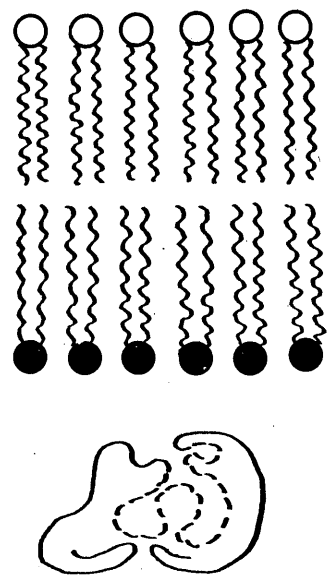
Figure 4.8(2) Protein secretion.

Steps 1-6 as in 4.8 (1).

Step 7: Secretory protein is released.

STEPS 1-6 AS ABOVE.

7.



4.2.4. Consequences of inhibited phospholipid synthesis for antigenicity of U.V.-irradiated and Actinomycin D-treated schistosomula.

It is possible to propose, speculatively, a number of mechanisms by which the inhibition of phospholipid synthesis in U.V.-irradiated and Actinomycin D-treated schistosomula might help to generate the enhanced immunogenicity of the attenuated parasites.

As discussed in the preceding section, formation of the parasite's new surface, at least during the first few hours of transformation, seems to rely principally on lipids synthesized and stored by developing cercariae. Thus, U.V.-irradiated schistosomula construct a morphologically normal double bilayer, despite the inhibition of phospholipid synthesis (see electron micrographs, chapter 6). However, as these stored lipids are utilized, then undergo turnover, by release into the surrounding medium, the parasite is predicted to become increasingly dependent on newly synthesized lipids, which attenuated schistosomula produce in greatly reduced amounts. Thus, irradiated and Actinomycin D-treated schistosomula will express an abnormally low proportion of *de novo* synthesized lipids at their surfaces. In compensation, the attenuated schistosomula are likely to acquire more lipids from host serum, or from interstitial spaces in the skin. This suggestion assumes that, as noted by Rumjanek *et al* (1983) for gamma-irradiated schistosomula, U.V.-irradiation and Actinomycin D treatment will not inhibit host lipid exchange. Since the principal lipids incorporated by schistosomula in this way are triacylglycerols and diacylglycerols, which the attenuated schistosomula will be largely unable to process further, there may be an increase in the proportion of neutral lipids, as opposed to phospholipids, in the parasite surface. Neutral lipids are weak amphiphiles, their biophysical properties dominated by their hydrophobic alkyl groups. Hence, there

may well be an increase in the hydrophobicity of the surface membrane of irradiated and Actinomycin D-treated schistosomula.

While the overall abundance of phospholipids at the parasite surface is expected to be reduced in U.V.-irradiated or Actinomycin D-treated forms, the negatively charged PI may form an abnormally high proportion of those present (see figure 4.7), assuming that total phospholipid synthesis provides a fair picture of surface phospholipid composition.

Thus, diverse factors may combine to make the lipid composition of attenuated forms quite different from that of normal schistosomula.

It is well established that the conformation of integral membrane proteins is largely modulated by the lipids of the bilayer. Integral membrane proteins are solvated by lipids in such a way as to maintain the native, functional structure of the inserted protein, while minimising leakage of material at the lipid - protein boundary. Thus, the activity of a number of enzymes and transport proteins embedded in the membrane is highly sensitive to changes in the properties of the bilayer, which affect the conformation and lateral mobility of the proteins (Houslay and Stanley, 1982). The enzyme β-OH butyrate dehydrogenase, for instance, which is integrated into the mitochondrial inner membrane, requires to associate with the headgroup of PC before its active site can adopt the correct conformation to bind and utilize NAD^+. Similarly, optimal functioning of rhodopsin, glycophorin and Na^+/K^+-ATPase demands segregation of acidic phospholipids into their immediate vicinity. Yeast mitochondrial cytochrome oxidase selects either cardiolipin or phosphatidic acid into its annular domain, while the function of Ca^{2+}-ATPase has a specific requirement for PE.

Thus, many proteins have a very precise requirement for a lipid environment which maintains and complements their native structure. In some instances, it seems that bilayer lipids, by sealing integral proteins into the bilayer, constrain their conformational flexibility.

Thus, certain enzymes are activated upon detergent solubilization, which releases them from such physical constraints (Houslay and Stanley, 1982).

Such evidence for close cooperation between membrane lipids and conformation or function of associated proteins strongly suggests that the modified composition of membrane lipids in U.V.-irradiated and Actinomycin D-treated schistosomula might affect the conformation of parasite antigens. Antigens secreted with membrane fragments, antigens at the parasite surface, or associated with internal membranes, may all assume non-native conformations in an altered lipid environment. It is suggested that these three types of antigens interact with the host immune system at different points in the migration and development of attenuated schistosomula (discussed in chapter 10). These denatured antigens may expose novel determinants, normally hidden in the hydrophobic interior of the native molecules, for interaction with the host immune system. We would suggest that exposure of new epitopes in this way may cause especially effective stimulation of helper T-cells, thus inducing high levels of protective immunity. Processing and presentation of such modified antigens from attenuated parasites are discussed in detail in chapter 10.

Parra et al (1986) suggested that PC, produced by N-methylation of PE (see section 4.2.2.1), might play a special role in masking schistosomular antigens from the host immune system. Inhibition of N-methylation enhanced binding of lethal antibody and complement to the parasite surface. The authors proposed that this phenomenon might be due to the fact that methylation of membrane phospholipids enhances membrane fluidity, as described by Hirata and Axelrod (1978), allowing structural displacement of antigens, so that they evade immune recognition. The non-methylated membrane, by contrast, is more rigid, and its antigens are therefore more susceptible to recognition and attack. Thus, the reduced amounts of newly-synthesized PC in the

irradiated and Actinomycin D-treated membranes might expose antigens, in abnormally rigid domains, to the host immune system.

Although the data presented here do not allow us to pinpoint precisely the effects of altered lipid composition on presentation of particular proteins, these various lines of evidence do suggest that inhibition of phospholipid synthesis in irradiated and Actinomycin D-treated schistosomula will result in modified presentation of antigens to the host immune system.

The legends accompanying figure 3.26 discuss the changes in lipid organisation which are postulated to occur during the development of normal, U.V.- irradiated and Actinomycin D-treated schistosomula.

4.2.5. Protein phosphorylation by normal and attenuated schistosomula.

Figure 4.9 shows the pattern of protein phosphorylation by the normal, U.V.-irradiated, Actinomycin D-treated and Fenfluramine-treated schistosomula whose phospholipid synthesis is illustrated in figures 4.5 to 4.7.

The major phosphorylated bands which can be identified for normal parasites have molecular weights 21 000, 39 000, 43 000, 50 000, 58 000, 65 000. There is also intense labelling of high molecular weight proteins, but it is difficult to distinguish distinct bands. Heavily phosphorylated material is also present at the foot of the gel. This may comprise phosphopeptides derived by proteolysis, glycophospholipids, or small phosphorylated proteins such as ubiquitin (Mr 7 000-8 000).

As might be predicted, protein phosphorylation in U.V.-irradiated schistosomula is generally inhibited. Surprisingly, however, no such inhibition is evident in the Actinomycin D-treated forms. Indeed, a band at Mr 92 000 seems to be especially heavily phosphorylated in the

Figure 4.9 Protein phosphorylation by normal, U.V.-irradiated (400 μW min cm^{-2}) and Actinomycin D-treated schistosomula during the period from 22 to 70 hours after transformation.

N = normal schistosomula

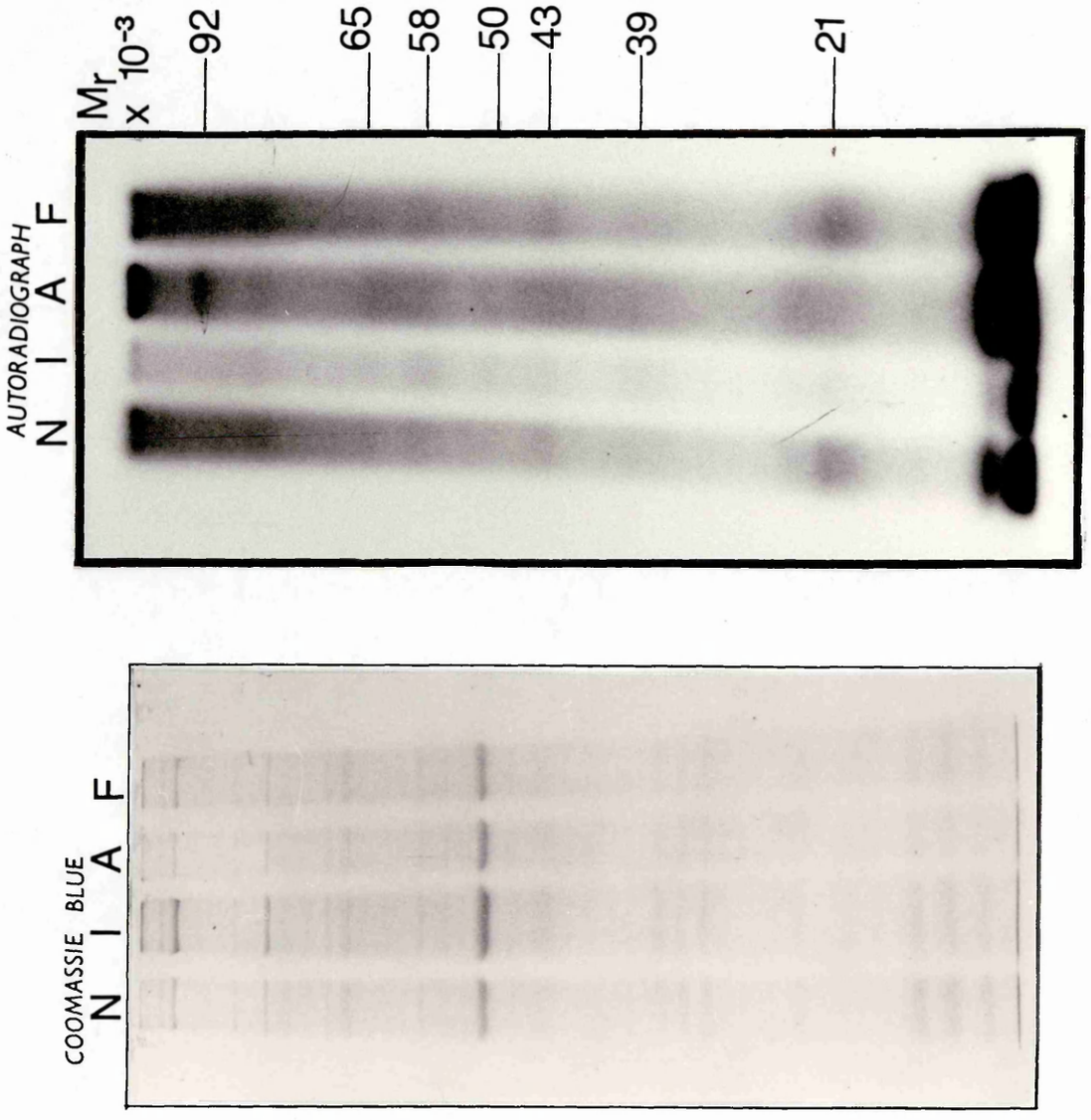
I = U.V.-irradiated schistosomula (400 μW min cm^{-2})

A = Actinomycin D-treated schistosomula.

F = Fenfluramine-treated schistosomula.

500 schistosomula per well. 3% stacking gel/10% resolving gel.

This gel accompanies figures 4.5 to 4.7, and the same protocol applies.


AUTORADIOGRAPH
N I A F
Mr x 10⁻³
92
65
58
50
43
39
21
COOMASSIE BLUE
N I A F

Actinomycin D track. Possibly, the Actinomycin D-treated schistosomula, whose protein synthesis is not seriously inhibited until some 15 to 20 hours of drug treatment, retain sufficient protein kinases to perform phosphorylation at later times. Regulation by protein phosphorylation of the activity of specific enzymes, involved for instance in repair of nucleic acid damage, might even be part of the parasite's response to noxious drugs such as Actinomycin D.

4.3 Protein glycosylation by normal, U.V.-irradiated and Actinomycin D-treated schistosomula.

4.3.1. Metabolic labelling studies.

Figure 4.10 shows the incorporation of ^{3}H-mannose into glycoproteins by normal, U.V.-irradiated and Actinomycin D-treated schistosomula. Figure 4.11 outlines the main pathways of protein glycosylation normally occurring in eucaryotic cells. The pathway in schistosomes is likely to be similar, although no definitive studies have yet been performed. However, Rumjanek (1980) and Simpson *et al* (1981) identified a number of glycosyltransferases in schistosomes.

Overall incorporation of ^{3}H-mannose is low, even in normal parasites, but it is still clear that protein glycosylation is reduced in attenuated schistosomula. Since protein synthesis is inhibited at the same time, these low levels of glycosylation could possibly be sufficient for the small quantity of new proteins produced. However, considering that the 70-80% inhibition of protein synthesis noted in chapter 3 will also apply to synthesis of the enzymes involved in the complex glycosylation pathway (figure 4.11), also that a high proportion of those enzymes which are present are likely to be in non-native conformations, and hence inactive (as described in chapter 3), it is most improbable that the detectable glycosylation in U.V.-irradiated and Actinomycin D-treated forms proceeds normally and to completion. Even supposing that the detectable glycosylation in attenuated schistosomula is efficient, there will still be a decrease in the amount of newly-synthesized carbohydrate expressed by these schistosomula.

Table 4.2 lists approximate molecular weights of the principal glycosylated proteins detected in normal schistosomula, together with references to molecules of similar molecular weights, reported in the

Figure 4.10 Incorporation of ^{3}H-mannose into glycoproteins by normal, U.V.-irradiated (400 μW min cm^{-2}) and Actinomycin D-treated schistosomula from 24 to 48 hours after transformation.

—O— = normal schistosomula

·····O····· = U.V.-irradiated schistosomula (400 μW min cm^{-2})

—●— = Actinomycin D-treated schistosomula.

Each point represents the mean of duplicate samples.

Deviation of duplicates from mean = ±9.8%

PROTOCOL:

Cercariae

↓

Divide in three

N I A

U.V.-irradiate

(400 μW min cm^{-2})

↓

Transform mechanically in Elac.

↓

Resuspend to 10 ml Elac/10% hiFCS.

Add Actinomycin D to A.

Culture for 20 hours at 37°C/5% CO_2

↓

Wash 4 times in EMEM containing pyruvate in place of glucose.

↓

From 24h:

Culture in 1ml EMEM (w/o glucose, + pyruvate)/ 10% hiFCS + 100 μCi (3.7MBq) ^{3}H-mannose for 24 hours at 37°C/5% CO_2.

Continued on next page.

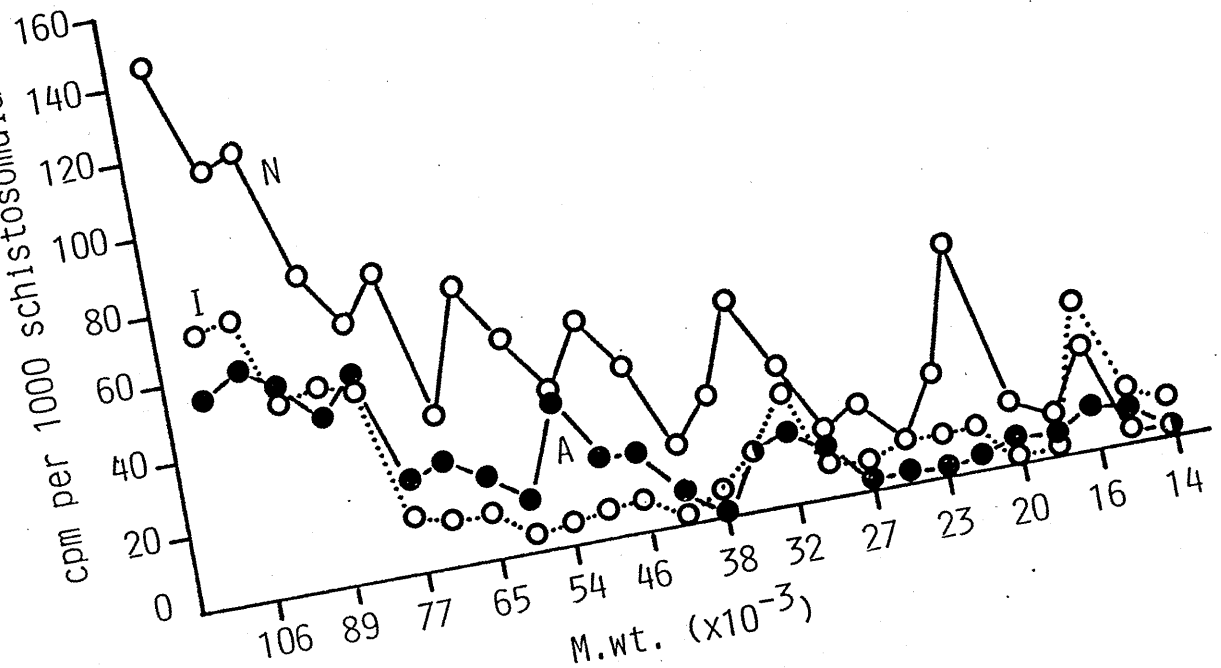

160
140
120
100
80
60
40
20
0
cpm per 1000 schistosomula
I
N
A
106 89 77 65 54 46 38 32 27 23 20 16 14
M.wt. (x10⁻³)

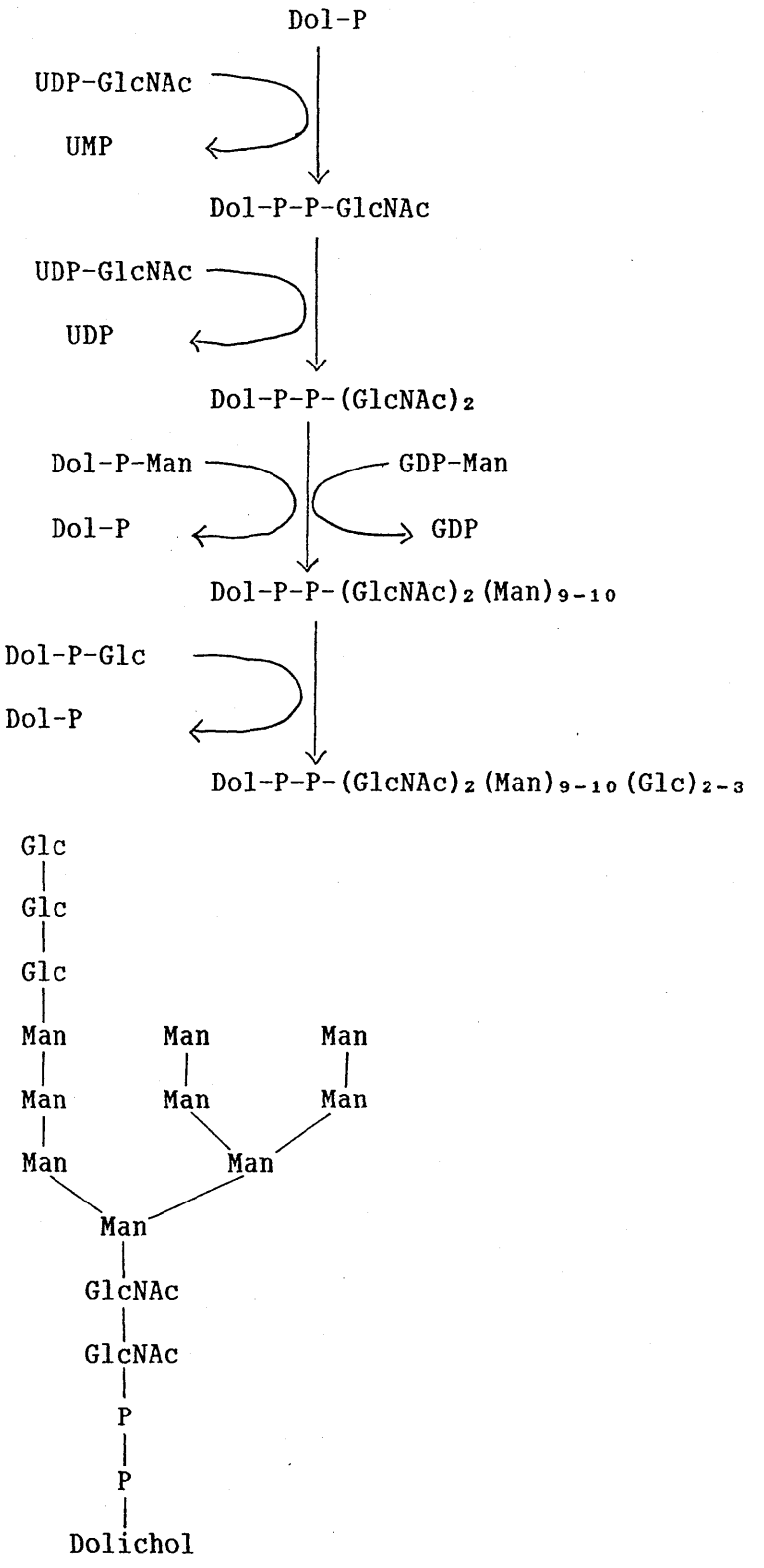
Figure 4.10 Cont.

Remove samples to ice; wash 3 times in ice-cold GMEM; freeze for SDS-PAGE.

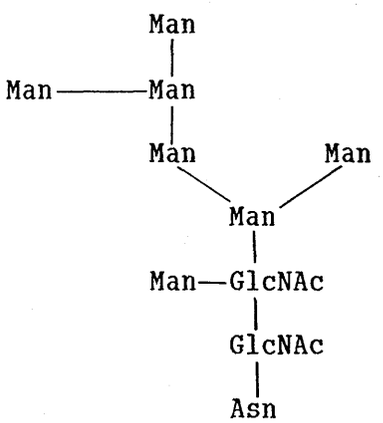
↓

Determine incorporation of radioactivity into different molecular weight species, as described in section 2.5.1.(5).

Figure 4.11 Synthesis and structure of high-mannose-type glycoproteins.



This pathway occurs in the rough endoplasmic reticulum (R.E.R.). The resultant oligosaccharide is transferred to an asparagine residue on a protein inserted in the R.E.R. membrane. It is then trimmed and elaborated by enzymes in the smooth endoplasmic reticulum and Golgi apparatus to produce the mature, high-mannose-type glycoprotein:



Abbreviations: P = phosphate

Dol-P = dolichol-phosphate

GlcNAc = N-acetylglucosamine

Man = mannose

Glc = glucose

Asn = asparagine

UDP = uridine diphosphate

UMP = uridine monophosphate

GDP = guanosine diphosphate

UDP-GlcNAc, etc. = activated nucleotide derivative of N-acetylglucosamine, etc.

Table 4.2. Proteins glycosylated by normal schistosomula between 24 and 48 hours after transformation, and glycoproteins of corresponding molecular weight, reported in the literature (see figure 4.10).

Table 4.2.

Mr of GLYCOPROTEIN (FROM FIGURE 4.10)	SCHISTOSOMULAR GLYCOPROTEIN OF CORRESPONDING MOLECULAR WEIGHT REPORTED IN THE LITERATURE	SUGGESTED IDENTITY OF GLYCOPROTEIN.
>100 000	Heavily glycosylated material above Mr 150 000 (Yi *et al*, 1986b). Mr 105 000 glycoprotein: Samuelson and Caulfield (1982).	Carbohydrate epitopes are: (1) shared with cercarial glycocalyx; (2) may induce both protective and blocking antibody responses in the concomitant immunity model.
77 000	Glycosylation of schistosome hsp's not reported.	Member of hsp 70 heat-shock protein family? (see chapter 3)
65 000	Mr 66 000 species: Taylor *et al* (1981); Taylor and Wells (1984); Simpson *et al* (1983b).	Alkaline phosphatase?
51 000	Mr 53 000 species: Samuelson and Caulfield (1982); Smith and Clegg (1985)	?
36 000	Series of related glycoproteins, Mr 32 000-38 000, having similar surface carbohydrates: Dissous *et al* (1981, 1982, 1985)	Carbohydrate epitopes shared with >100 000 species (above). These glycoproteins may also have a role in osmoregulation in cercariae.
21 000	Mr 22 000 and 20 000 molecules identified by Samuelson and Caulfield (1982)	Carbohydrate epitopes on these antigens are shared with the cercarial glycocalyx.
16 000	Mr 16 000 species identified by Samuelson and Caulfield (1982)	

literature. Only the peaks identified in figure 4.10 are included in this table. Since the radioactivity incorporated by normal schistosomula falls to base levels at very few points on the gel, mainly below Mr 27 000, it might be suggested that, in fact, the majority of schistosomular proteins above Mr 30 000 contain a proportion of bound oligosaccharides; the peaks represent those that are epecially heavily glycosylated.

Apart from glycoproteins, other carbohydrate-containing compounds reported to be synthesized by normal schistosomula include glycosaminoglycans (Stein and Lumsden, 1973) and glycolipids (Samuelson and Caulfield, 1982; Weiss and Strand, 1985). Judging by the inhibition of glycoprotein synthesis seen here, it is probable that synthesis of these compounds is also reduced in irradiated and Actinomycin D-treated schistosomula.

4.3.2. Effect of inhibition of glycosylation on antigen expression by U.V.-irradiated and Actinomycin D-treated schistosomula.

Ferguson and Homans (1988) pointed out that glycoconjugates synthesized by parasites often contain unusual carbohydrate structures which are very foreign to the mammalian host, and therefore activate the host's immune system very powerfully. This does seem to be the case for normal schistosome infections, where some 70% of the antibody response in chronically-infected mice appears to be directed against carbohydrate (periodate-sensitive) epitopes (Omer-ali et al, 1986, 1988). Schistosomes seem to conserve certain antigenic carbohydrate structures throughout their life-cycle. Thus, polyclonal antiserum raised against any one stage of development - eggs, miracidia, sporocysts, cercariae or adult worms - apparently contains antibodies which crossreact with some of the carbohydrate epitopes expressed by any other stage (Bayne et al, 1987; Dunn and Yoshino, 1988; Omer-Ali

et al, 1986, 1988; Yi et al, 1986a, b). It seems, however, that while some of these highly antigenic carbohydrate epitopes induce an immune response which helps eliminate challenge larvae in the concomitant immunity model, others protect the developing challenge schistosomula from host immune attack (Smithers et al, 1987; Butterworth, 1987a,b; Omer-Ali et al, 1986). By attracting a mask of blocking antibodies (section 1.6.3.2.; chapter 7), such carbohydrate moieties seem to shield those antigens whose recognition by the immune system would be detrimental for schistosomular survival. Omer-Ali et al (1986) found that the antibodies produced by mice rendered highly resistant to infection by multiple vaccinations with gamma-irradiated schistosomula reacted predominantly with periodate-insensitive, presumably polypeptide, antigens. They proposed that this recognition of non-carbohydrate epitopes was largely responsible for the efficacy of the irradiated vaccine (section 1.6.3.2.; chapter 7). This hypothesis coincides neatly with the observations presented here. Inhibited glycosylation in attenuated larvae may expose antigenic determinants in the polypeptide core of glycoproteins. In this way, protein epitopes which are masked by associated carbohydrate in normal schistosomula might be made available to stimulate the high levels of protective immunity characteristic of the irradiated vaccine.

Observations in a number of other biological systems support the idea that the absence of oligosaccharides may enhance the immunogenicity of glycoproteins. Feizi and Childs (1987) discuss the interaction with the immune system of the oncodevelopmental oligosaccharides which appear on tumour cells. These proteins are never processed to the normal, mature form. Thus, cryptic epitopes are exposed, not detectable on normal cells, where they are masked by the additional glycosylations of the complete glycosylation pathway. These abnormal, partially processed antigens frequently stimulate a powerful immune response. There are clear analogies between these cellular

systems and the enhanced immunogenicity of U.V.-irradiated or Actinomycin D-treated schistosomula, which, as demonstrated here, are also unable to express mature, fully-processed carbohydrate antigens.

Alexander and Elder (1985) used endoglycosidase F (endo F) treatment of leukaemia and influenza virus glycoproteins to show that the reactivities of most antibodies to these glycoprotein antigens were influenced by the associated carbohydrate moieties. From a panel of monoclonal antibodies raised against an antigen from leukaemia virus, "gp70", the majority showed an improved reactivity to the polypeptide component of the antigen after carbohydrate removal by endo F. Most of a series of monoclonal antibodies raised against synthetic peptide sequences of influenza virus haemagglutinin also improved in reactivity after carbohydrate removal from the antigen. The immune response to these glycoproteins was not simply a function of the immunogenicity of certain domains over others, but "a direct measure of carbohydrate influences on the host's perception of foreign antigen". Thus, in the influenza system, the presence of carbohydrate prevented interaction of antibodies with polypeptide epitopes, even when the polypeptide regions concerned were quite distant from any overlying carbohydrate. Presumably, in such cases, the carbohydrate moieties operated by some distal effect to alter protein conformation. Moreover, polyclonal antisera raised against intact glycoproteins lost all reactivity upon antigen deglycosylation. Thus, not only can carbohydrate moieties prevent the interaction of antibodies with protein antigenic determinants, but the carbohydrate either becomes the major immunogenic target of the glycosylated protein or directs the immune response to areas under the influence of attached carbohydrate. On this evidence, the authors proposed that the carbohydrate moieties of glycoproteins have a dual role - they mask certain polypeptide sites, and at the same time act as decoys, diverting the immune response towards themselves.

This theory can readily be applied to available information on the

rodent and human antibody responses to S. mansoni infection. In normal infections, carbohydrate antigens do indeed generate the larger proportion of the antibody response - the "decoy effect". It therefore seems feasible to suggest that inhibition of glycosylation in U.V.-irradiated or Actinomycin D-treated forms could expose previously masked polypeptide epitopes which are potent immunogens.

As described above, the work of Alexander and Elder (1985) emphasizes that carbohydrate moieties can affect the conformation of their associated proteins, hence altered glycosylation may lead to exposure of new polypeptide epitopes, normally hidden by protein folding. In particular, oligosaccharides enhance the hydrophilicity of associated molecules. In general terms, therefore, carbohydrate depletion may, increase the hydrophobicity of the antigens expressed by schistosomula.

Aberrant glycosylation by U.V.-irradiated and Actinomycin D-treated schistosomula may further aggravate the persistence of denatured antigens discussed in chapter 3, since incorporation of a carbohydrate residue - mannose-6-phosphate - seems to play an important role in directing denatured material to lysosomes for degradation (Rothman and Lenard, 1984; Kornfield, 1986). Inhibition of glycosylation may prevent attachment of this message to molecules that would normally be scheduled for breakdown. Moreover, synthesis of the unusual carbohydrate structure O-linked N-acetylglucosamine is remarkably pronounced in normal schistosomes (Nyame et al, 1987). It has been proposed that proteins containing this novel sugar moiety may assist in the proper assembly of multimeric protein complexes, such as membrane pores (Hart et al, 1988). This role in assembly of proteins is similar to that of heat-shock proteins, as discussed in chapter 3. U.V.- or Actinomycin D-induced inhibition of this form of glycosylation could therefore prevent normal protein associations. Antigenic determinants which would usually be masked by such associations might

be exposed in this way.

Taken together with the alterations in carbohydrate structure caused directly by the oxidising effects of irradiation (section 1.10), these observations on glycoprotein synthesis and conformation suggest that the glycoprotein antigens made available for interaction with the host immune system by irradiated or Actinomycin D-treated schistosomula will be highly modified compared with those expressed in their native forms by normal parasites. These aberrant glycoproteins are predicted to be processed by antigen presenting cells, and presented to host helper T-cells, by different routes from normal parasite antigens, in such a way as to stimulate a potent protective immune response (see chapter 10). In particular, the carbohydrate moieties associated with proteins appear to be important in determining sites of protease action (Feizi and Childs, 1987; Olden et al, 1982; Kornfield, 1986). Thus, it is possible to speculate that the proteases of antigen presenting cells may process material derived from attenuated schistosomula by different routes from those applied to normal parasite antigens.

Table 4.3 summarises some of the suggested ways in which immunogenicity may be enhanced as a result of metabolic inhibition in schistosomula.

Table 4.3. Summary of some possible mechanisms by which U.V.-irradiation- or Actinomycin D-induced metabolic inhibition may enhance the immunogenicity of schistosomular antigens.

Table 4.3.

Modified conformation of parasite antigens (secreted, surface-associated, and internal) due to:	1. Direct irradiation damage to proteins, carbohydrates and lipids (section 1.10). 2. Changes in temperature and osmotic environment, also mechanical stress and protease action, during transformation, modify protein antigens, and promote protein denaturation. 3. Interrupted or mutated genetic messages will produce aberrant proteins, carbohydrates and lipids. 4. Inhibition of glycosylation means that novel protein and carbohydrate determinants will be exposed on glycoprotein antigens. 5. Lack of heat-shock proteins prevents other proteins adopting their native conformations and forming normal protein-protein associations. Inhibition of O-linked N-acetylglucosamine incorporation may have a similar effect. 6. Altered lipid composition modifies the conformation of associated proteins.
Aberrant and denatured proteins persist because of:	1. Lack of heat-shock proteins to recognise, and renature or remove, non-native antigens. 2. Absence of the carbohydrate signals for protein degradation. 3. Enzymes for degradation of aberrant molecules are predicted to be in non-native conformations, hence inactive.