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
TPCK: L-1-tosylamide-2-phenylethylchloromethyl ketone } inhibitors
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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CHAPTER FIVE

EFFECTS OF GAMMA-IRRADIATION ON PROTEIN SYNTHESIS BY SCHISTOSOMULA.

5. Effects of gamma-irradiation on protein synthesis by schistosomula.

A series of experiments examining protein synthesis by gamma-irradiated parasites indicated that gamma-irradiation did inhibit schistosomular protein synthesis to a considerable extent, though less severely than U.V.- irradiation. Moreover, inhibition was evident only for a limited fraction of the 96 hour culture period examined.

5.1. Results of metabolic labelling studies.

Figure 5.1 shows the results of examining protein synthesis by normal, U.V.-irradiated and 20 krad gamma-irradiated parasites during the initial 3-hour transformation period. For the first hour, incorporation of ^{35}S-methionine into protein appeared to be inhibited by some 40% in both U.V.- and gamma-irradiated forms. At this early time after transformation, protein synthesis even in normal schistosomula is almost completely restricted to the proteins at Mr approximately 70 000-78 000 and 47 000 (see chapter 3). Hence, the inhibition observed refers to only a few molecules out of the whole range of schistosomular proteins. By 3 hours after transformation, however, when synthesis of a broader range of proteins can be compared for the normal and irradiated larvae, the gamma-irradiated schistosomula showed less inhibition than U.V.-irradiated ones - 66% as opposed to 82%.

A second experiment examined both protein and RNA synthesis by normal, U.V.-irradiated, gamma-irradiated and Actinomycin D-treated schistosomula during 72 hours of culture after transformation (figures 5.2a, 5.2b). The results in this instance confirm the suggestion of figure 5.1 that gamma irradiation does not inhibit protein synthesis as extensively as either U.V.- or Actinomycin D-treatment. Although

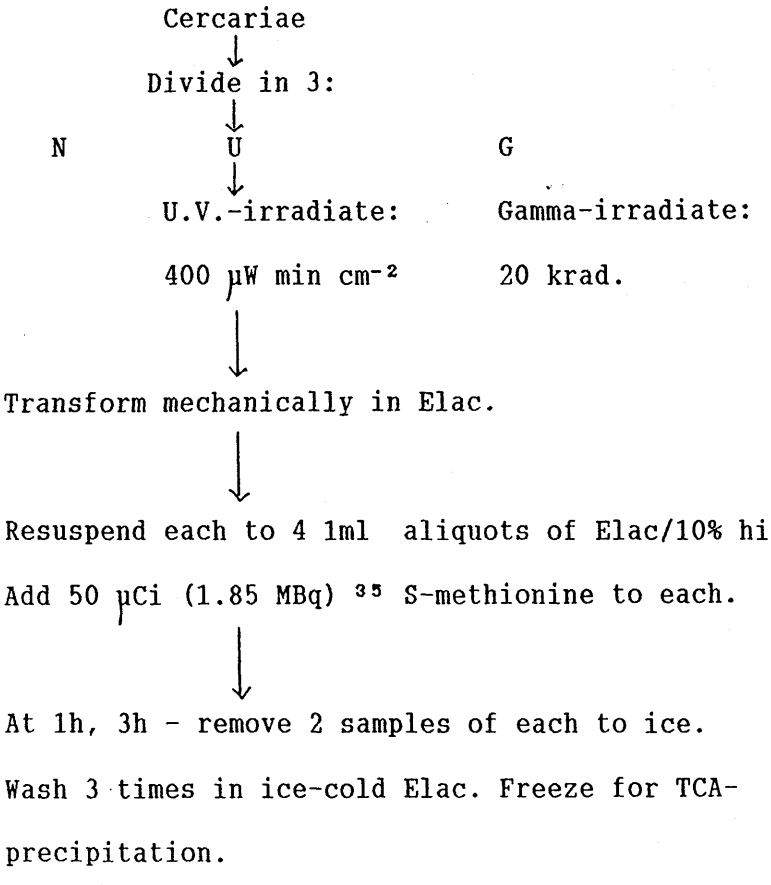
Figure 5.1 Effects of U.V.- and gamma-irradiation on protein synthesis by schistosomula during the initial 3-hour transformation period.

N = normal schistosomula.

U = U.V.-irradiated schistosomula (400 μW min cm^{-2})

G = gamma-irradiated schistosomula (20 krad; 0.45 krad/min).

PROTOCOL:



Each bar represents mean of paired samples. Deviation of duplicates from mean was ± 9.5%.

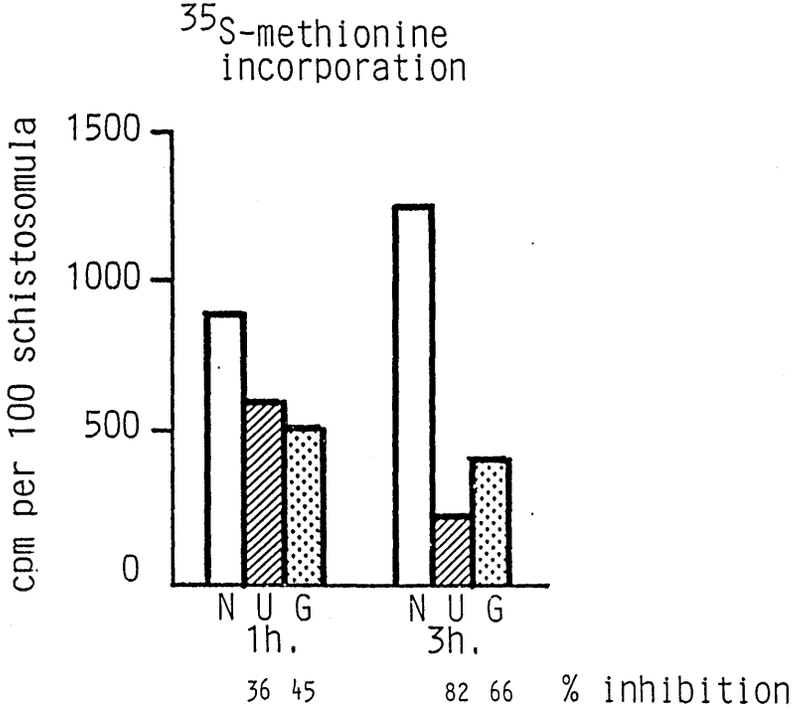

35S-methionine
incorporation
cpm per 100 schistosomula
1500
1000
500
0
N U G
1h.
N U G
3h.
36 45
82 66
% inhibition

Figure 5.2 a) Incorporation of ^{35}S-methionine into TCA- precipitable protein by normal, U.V.-irradiated and gamma-irradiated schistosomula during the 5-hour period following mechanical transformation. Also, incorporation of ^{35}S-methionine and ^{3}H-uridine into TCA-precipitable protein or nucleic acid by normal, U.V.- irradiated, gamma-irradiated (20 krad at 0.45 krad/min) and Actinomycin D-treated schistosomula during the culture period between 20 and 72 hours after transformation. Each bar represents the mean of paired samples. Deviation of duplicates from mean was ± 11.3%

(Abbreviations - see figure 5.2b)).

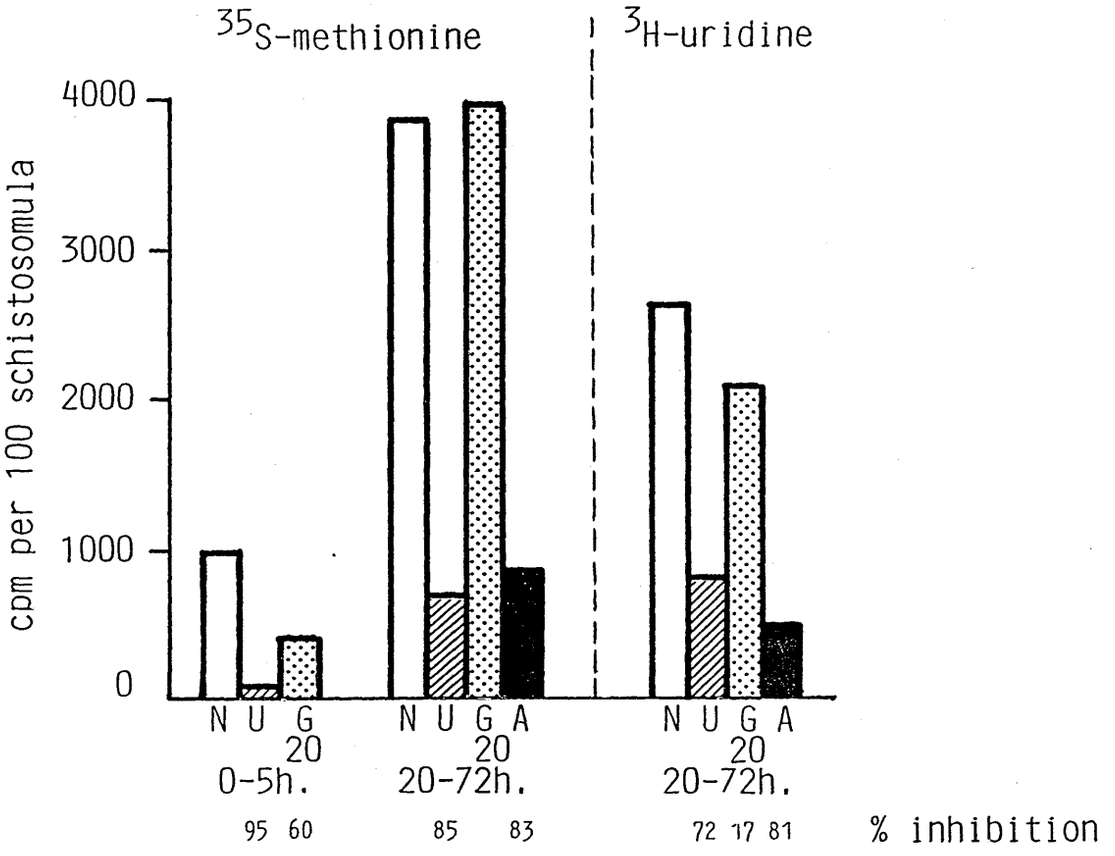

35S-methionine
3H-uridine
4000
3000
2000
1000
0
cpm per 100 schistosomula
N U G
20
0-5h.
95 60
N U G A
20
20-72h.
85 83
N U G A
20
20-72h.
72 17 81
% inhibition

Figure 5.2 b) Protein synthesis and secretion by normal, U.V.-irradiated and gamma-irradiated (20 krad at 0.45 krad/min) schistosomula during the 5-hour period following transformation. Also, protein synthesis by normal, U.V.-irradiated, gamma-irradiated (20 krad at 0.45 krad/min) gamma-irradiated and Actinomycin D-treated schistosomula during the culture period from 20 to 72 hours after transformation.

N = normal schistosomula.

U = U.V.-irradiated schistosomula (400 μW min cm^{-2})

G20 = Gamma-irradiated schistosomula (20 krad; 0.45 krad/min)

A = Actinomycin D-treated schistosomula.

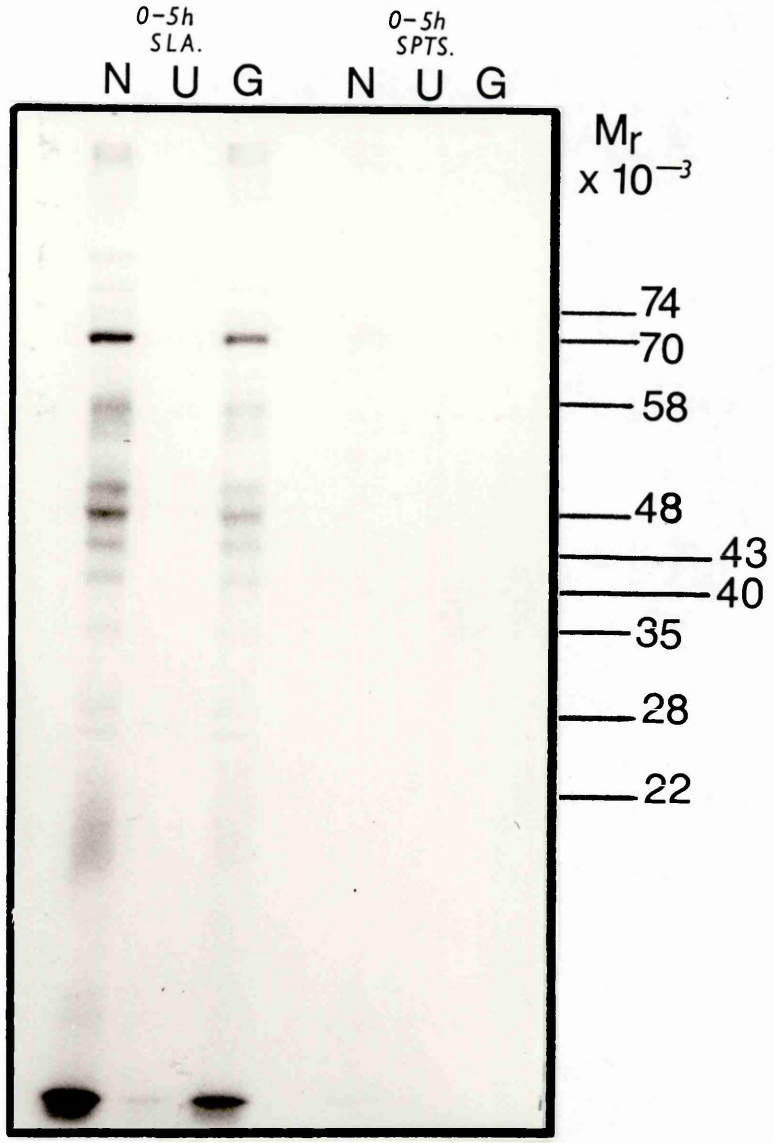
1000 schistosomula per well in 5.2 b.)

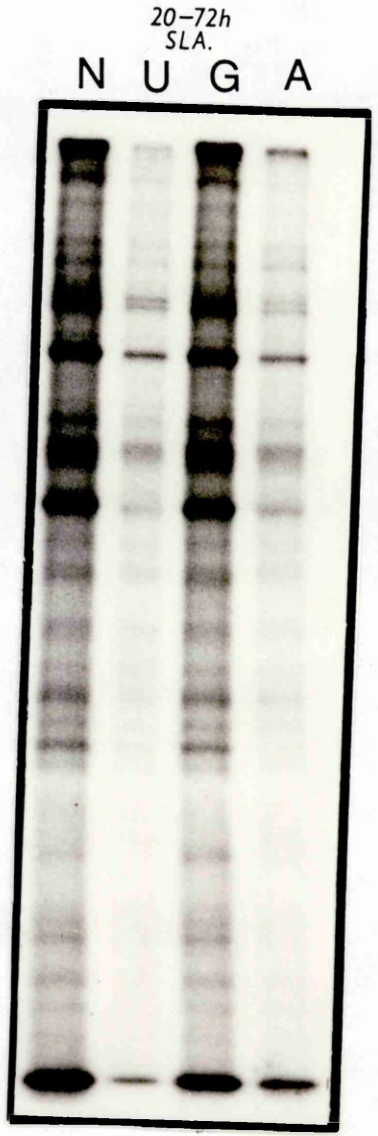
3% stacking gel/10% resolving gel.

SLA = SCHISTOSOMULA

SPTS = SUPERNATANTS.

(Protocol follows figures).


0–5h
SLA.
0–5h
SPTS.
N U G N U G
Mr
x 10–3
74
70
58
48
43
40
35
28
22


20–72h
SLA.
N U G A

PROTOCOL for figures 5.2 a) and 5.2 b)

Cercariae

↓

Divide in 4

N | U | G | A

U: ↓ U.V.-irradiate: 400 µW min cm^{-2}

G: ↓ Gamma-irradiate: 20 krad (0.45 krad/min)

Transform mechanically in Elac.

↓

Resuspend N, U, G to 6 ml Elac.

Resuspend A to 3 ml Elac/10% hiFCS.

Add Actinomycin D to A.

To 3 ml N, U, G: add hiFCS to 10%(v/v).

3 ml A is in Elac/10% hiFCS.

Culture for 20 hours at 37°C/5% CO_2

↓

Wash in Elac.

Resuspend each to 5 1ml samples of Elac/10% hiFCS

Add 50 µCi (1.85 MBq) ^{35}S-methionine to 3 samples

Add 50 µCi (1.85 MBq) ^{3}H-uridine to 2 samples

Culture at 37°C/5% CO_2 until 72h after transformation.

↓

Remove to ice. Wash 3 times in ice-cold Elac. Freeze.

CONTINUED ON NEXT PAGE.

To 3 1ml N, U, G samples in Elac, add 50 µCi (1.85MBq) ^{35}S-methionine.

↓

Culture at 37°C/5% CO_2 for 5 hours

↓

Remove to ice. Centrifuge

↓

Schistosomular pellet

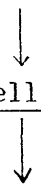
Wash 3 times in ice-cold Elac;freeze.

2 samples → TCA.

1 sample → SDS-PAGE.

Supernatants (secretions): add protease inhibitors. Remove salt; freeze-dry

FIGURE 5.2, CONTINUED.



20-72h labelled samples:

^{35}S label: 2 samples ⟶ TCA-precipitation

1 sample ⟶ SDS-PAGE

^{3}H label: 2 samples ⟶ TCA-precipitation.

inhibition was evident during the first 5 hours after transformation, the total of protein synthesis between 20 and 72 hours after transformation was the same in normal and gamma-irradiated forms.

Figure 5.2 b) shows the molecular weights of the proteins synthesized by each group of schistosomula during the two time-intervals (0-5 hours and 20-72 hours). On initial examination, it does not seem that protein synthesis in gamma-irradiated schistosomula is inhibited by as much as 60% during the first 5 hours. This may be partly due to a technical artifact - blackening of X-ray film is not directly proportional to the quantity of radioactivity to which it is exposed (Laskey and Mills, 1975). Comparing individual bands, however, it can be seen that each protein in the gamma-irradiated track is fainter than its counterpart in the normal one. In particular, all the bands below Mr 28 000 are much less intense than in the normal schistosomula. This suggests that there may have been preferential inhibition of synthesis of lower molecular weight proteins. Moreover, in the 0 to 5 hour secretions, a distinct labelled protein at Mr 66 000 could be seen in the material from normal schistosomula, but none from the gamma-irradiated parasites. While gamma-irradiated forms thus showed a partial inhibition of protein synthesis at 5 hours after transformation, virtually no labelling was detectable for the U.V.-irradiated schistosomula.

The range of proteins synthesized between 20 and 72 hours appeared to be the same in normal and gamma-irradiated parasites, as regards both molecular weights and total quantity. U.V.-irradiated and Actinomycin D-treated schistosomula, on the other hand, showed considerable reduction in protein synthesis during the 20 to 72 hour labelling period (approximately 85%). No proteins were inhibited preferentially over others in these experimental groups.

The incorporation of ^{3}H-uridine into TCA-precipitable nucleic acid from 20 to 72 hours after transformation agreed with these

observations on protein synthesis, for only a 17% reduction was observed in gamma-irradiated schistosomula, compared to 72% and 81% inhibition in U.V.-irradiated and Actinomycin D-treated forms.

These results suggested that protein synthesis by gamma-irradiated forms recovered after an initial inhibition period. Figures 5.3 a) and 5.3 b) show the result of an experiment which attempted to pinpoint the time at which this recovery began. Two doses of gamma irradiation - 20 krad and 50 krad - were used in this instance, to test whether a higher radiation dose might prolong the metabolic inhibition. Severe inhibition of protein synthesis in U.V.-irradiated forms was sustained at each of the four time-points measured over the 72-hour culture period, although it did decrease slightly, from 90% inhibition at 3 hours to 73% at 72 hours. 20 krad gamma-irradiated forms showed around 50% inhibition until 48 hours after transformation, but seemed to recover almost completely by 72 hours, when only 14% inhibition was evident. Surprisingly, protein synthesis by the 50 krad gamma-irradiated schistosomula seemed to recover more rapidly than the 20 krad forms - although inhibition was very pronounced at 3 hours and 24 hours (84% and 69%, respectively), it seemed to recover by 48 hours.

Figure 5.3 b) illustrates the histogram of 5.3 a) and allows us to identify the molecular weights of the proteins synthesized at each time point. Inhibition of protein synthesis in each group appears to be general throughout the whole range of molecular weights, except that, even at 3 hours and 24 hours, when their overall inhibition is severe, the 50 krad gamma-irradiated schistosomula maintain synthesis of a 34 000 molecular weight protein that is not detectable at all in the U.V.-irradiated or 20 krad gamma-irradiated parasites. Although the identity of this particular protein is unclear, it is interesting to speculate that it might play a role in the enhanced recovery of protein synthesis by the 50 krad gamma-irradiated schistosomula. It should be noted that, in this case, schistosomula were cultured in GMEM w/o

Figures 5.3 a) and 5.3 b)

Incorporation of ^{35}S-methionine into protein by normal, U.V.-irradiated and gamma-irradiated schistosomula at successive time-points over a 72-hour culture period.

N = normal schistosomula

U = U.V.-irradiated schistosomula (400 μW min cm^{-2})

G20 = 20 krad (0.3 krad/min) gamma-irradiated schistosomula.

G50 = 50 krad (0.3 krad/min) gamma-irradiated schistosomula.

1000 schistosomula per well in 5.3 b). 3% stacking gel/10% resolving gel.

PROTOCOL:

Cercariae

↓

Divide in 4

N — U — G20 — G50

U → U.V.-irradiate (400 μW min cm^{-2})

G20, G50 → Gamma-irradiate [20 krad/50 krad (0.3krad/min)]

↓

Transform mechanically in GMEM w/o methionine. Resuspend each to 12 1ml samples of GMEM w/o methionine/10% hiFCS. Add 20 μCi (740kBq) ^{35}S-methionine to each.

↓

At 3h, 24h, 72h - take 3 samples from each treatment group.

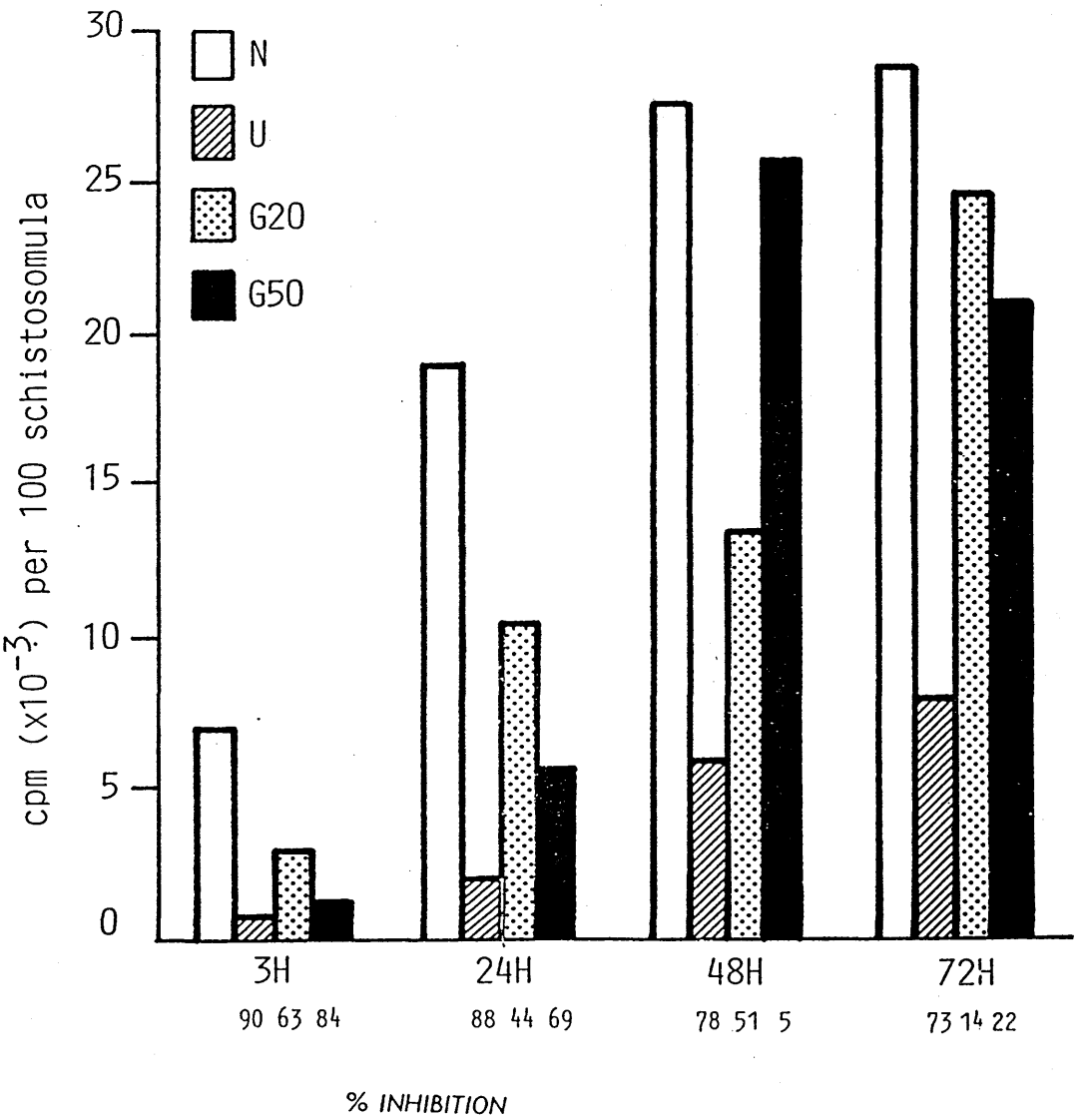
Wash 3 times in ice-cold GMEM.

Freeze. 2 samples ⟶ TCA-precipitation.

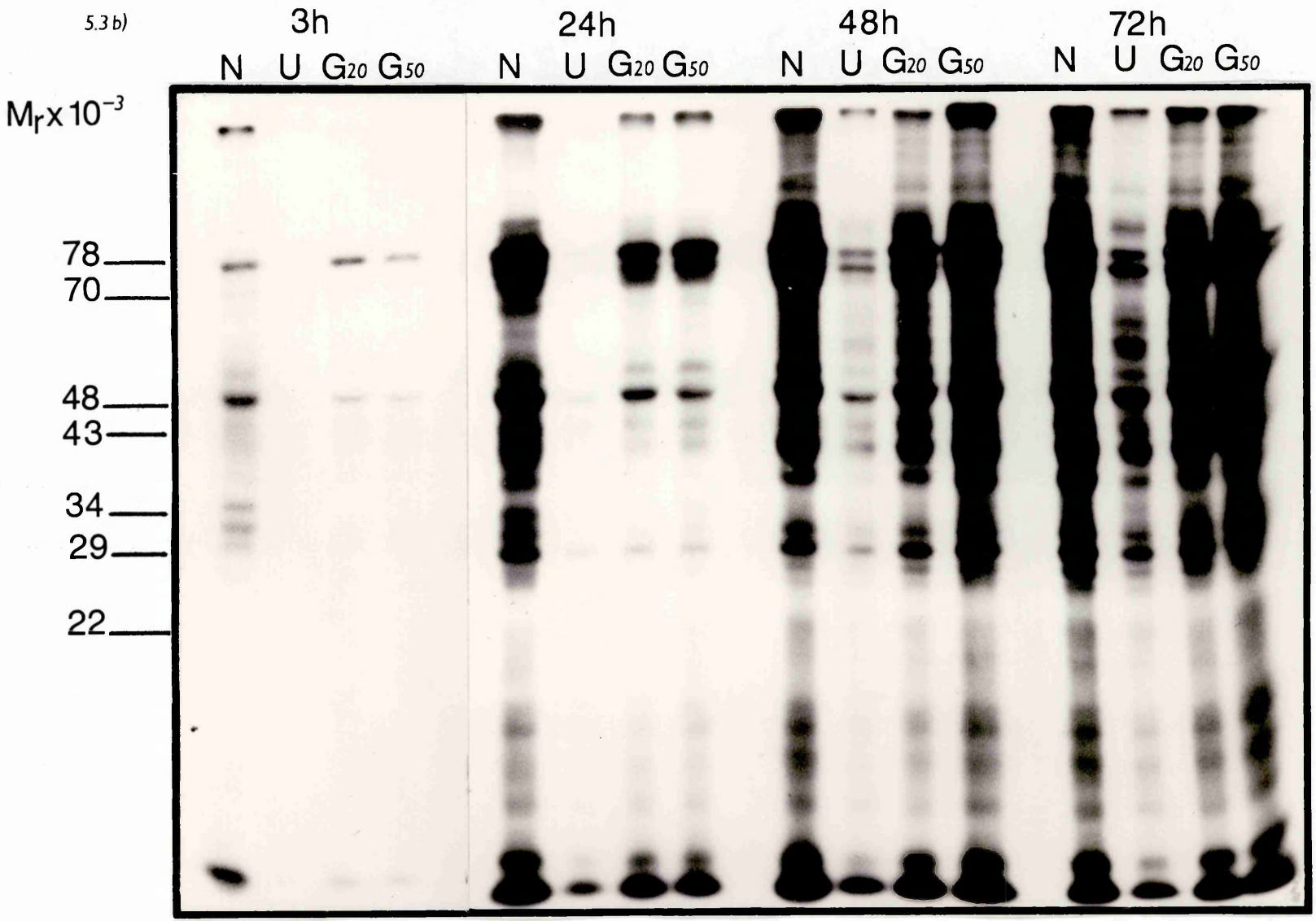
1 sample ⟶ SDS-PAGE

In figure 5.3 a), each bar represents the mean of two determinations. Deviation of duplicates from mean was ± 12.1%

5.3a) ^{35}S-methionine incorporation



5.3 b)


3h
24h
48h
72h
N U G20 G50
N U G20 G50
N U G20 G50
N U G20 G50
Mr x 10-3
78
70
48
43
34
29
22

methionine, rather than in Elac as for the previous two experiments. However, as will be described in chapter 8, the incubation medium did not appear to influence the extent or nature of inhibition of protein synthesis.

It is interesting to note, in passing, the differences in proteins synthesized by normal schistosomula in the experiments 5.2 and 5.3 of this series. Table 5.1 summarizes some of the differences in protein synthesis detected in these figures.

Table 5.1 Proteins synthesized by normal schistosomula in figures 5.2 and 5.3.

	SYNTHESIS DETECTED AT:			
	5 HOURS		72 HOURS	
Figure	5.2	5.3	5.2	5.3
Mr x 10^{-3}				
78	x	s	x	s
74	f	x	f	s
70	s	f	s	s
58	s	x	x	no discrete bands
47-48	✓	s	✓	s
43	✓	✓	✓	✓
28-40	40,35,28	38, 36(f) 34, 32, 29(s)	49,35,32,30,28	strong,diffuse
22-24	diffuse	x	diffuse	22

✓ = present

x = not detectable

s = strong band

f = faint band

In conclusion, it does appear that gamma-irradiation at 20 or 50 krad inhibits protein synthesis by schistosomula. However, the inhibition is not as severe as generally observed after U.V.-irradiation at 400 μW min cm^{-2}. Moreover, protein synthesis by gamma-irradiated larvae is restored to normal levels after some 24 to 48 hours of culture.

5.2 Discussion

Two alternative explanations might account for the different effects of gamma- and U.V.-irradiation on protein synthesis by schistosomula. Firstly, the lesions inflicted on parasite nucleic acid by gamma-irradiation might be more readily repaired than U.V.-irradiation damage. Alternatively, it may not be necessary to postulate repair processes; U.V.-irradiation may attack nucleic acid targets which are not as susceptible to gamma-radiation. Section 1.10 describes the various nucleic acid lesions which are inflicted by U.V.- and gamma-rays.

If repair of nucleic acid damage does occur in gamma-irradiated parasites, then restoration of normal synthetic processes is likely to be self-accelerating, as the initial, limited repair of nucleic acid lesions permits synthesis of more repair enzymes. The sudden, very marked increase in protein synthesis by gamma-irradiated schistosomula seen in figure 5.3 might be explained in this way.

However, if such repair processes do occur, they are evidently not wholly efficient, since, despite the restoration of protein synthesis to normal levels by 48 hours, no gamma-irradiated parasites survive to maturity. Clearly, some lethal lesions must persist. Moreover, no nucleic acid repair was detectable by uptake of ^{3}H-thymidine in U.V.-irradiated parasites (chapter 4).

An alternative explanation for the response of schistosomula to gamma-irradiation could be that gamma-irradiation does inactivate the nucleic acid responsible for growth and maturation of the juvenile schistosomula. However, the DNA responsible for protein synthesis during the period from approximately 24 hours until growth begins (at day 4 in culture, at the earliest - Clegg and Smithers, 1972) appears to be impervious to gamma-radiation damage. Despite this recovery of protein synthesis, the gamma-irradiated schistosomula die. We would suggest that death occurs because the class of nucleic acid responsible for growth and cell division is highly susceptible to gamma-radiation damage.

Using ionizing radiation of different penetration depths, it has been found that the most highly radiation-sensitive zone in interphase cells comprises a region about 0.2 μm thick, near the inner, nuclear surface (Munro, 1970; Datta et al, 1976). The damaging effects of radiation here, near the nuclear membrane, are more pronounced than those enforced by an equivalent amount of radiation energy deposited in the nucleoplasm. According to the model of Hancock and Boulikas (1982) for the spatial ordering of chromatin in interphase nuclei, the radiosensitive region just inside the nuclear envelope consists mainly of heterochromatin, organised into a condensed and inactive form by scaffolding proteins. Radiation damage persists in this DNA fraction, presumably because its highly compact organisation prevents access of protective compounds and repair enzymes (von Sonntag, 1987; Suciu, 1986). Transcriptionally active chromatin (euchromatin), on the other hand, is largely located in the nucleoplasm (Hancock and Boulikas, 1982), the region considerably less susceptible to radiation damage (Munro, 1970). Euchromatin is decondensed and partially released from the topological constraints of nucleosome organisation in order to accomplish replication and transcription (Hancock and Boulikas, 1982; Lewin, 1983). This unfolded DNA could therefore be more accessible to

repair enzymes. A third class of nucleic acid which is exposed to radiation damage is hnRNA, or messenger RNA, destined for, or undergoing, translation in the cytoplasm. Thus, three different classes of nucleic acid may be regarded as targets for gamma-radiation damage - cytoplasmic RNA, transcriptionally active DNA in the nucleoplasm, and, the most susceptible, inactive heterochromatin at the nuclear membrane.

As outlined in chapter 3, the time-course of Actinomycin D effectiveness indicates that, at early times after transformation, until about 15 to 20 hours, protein synthesis by schistosomula depends on pre-existing mRNA, synthesized by developing cercariae. Since our gamma-irradiation experiments pointed to a failure in protein synthesis until about 24 hours, we might suggest that gamma irradiation inhibits translation of these pre-formed RNA messages in the cytoplasm, either by direct disruption of the mRNA itself, or indirectly, for instance, by damaging the enzymes of translation.

Our examination of protein synthesis by Actinomycin D-treated schistosomula (chapter 3) indicated that de novo transcription of DNA into RNA is not essential for schistosomular protein synthesis until some 10 to 20 hours after transformation. From this time until growth and cell division begin, at some 4 days in culture after transformation (Clegg, 1965; Clegg and Smithers, 1972), both transcription and translation of genetic messages are required. We would suggest that the DNA involved at this stage may already be active and unfolded in the nucleoplasm of cercarial cells at the time of irradiation. Hence, according to the model described above, this class of nucleic acid is predicted to be relatively insusceptible to gamma-irradiation damage, also accessible to protective agents and repair enzymes. This explanation might account for the striking, almost complete, recovery of protein synthesis in gamma-irradiated schistosomula.

Inhibition of protein synthesis by U.V.-irradiated schistosomula is generally sustained throughout this time, presumably because 254 nm

U.V. radiation is so effectively absorbed by nucleic acid, regardless of its precise location in the nucleus. However, restoration (partial or complete) of protein synthesis in U.V.-irradiated larvae at this time was occasionally observed (experiments described in chapter 8). The accessibility of unfolded, active euchromatin to radioprotective compounds and repair enzymes might again help explain this observation.

We would postulate that, from about 96 hours onwards, as growth and cell division begin in culture, synthesis of new proteins and mitosis will require activation and decondensation of regions of the formerly inactive chromatin at the nuclear membrane. According to our model, since this class of DNA is apparently especially susceptible to radiation damage, growth and development of gamma-irradiated schistosomula are prevented. Thus, despite the apparent recovery of protein synthesis between 24 to 48 and 96 hours in culture, no gamma-irradiated larvae survive to adulthood in experimental hosts.

This model is described in more detail, in the context of the variable response to U.V.-irradiation, in chapter 8, and is illustrated in figure 8.20.

The pattern of protein synthesis in gamma-irradiated schistosomula suggests that there may be at least two distinct periods when the antigens expressed by such attenuated forms are different from, and more immunogenic than, the normal ones.

During the initial 24 hours (approximately), when protein synthesis depending on pre-existing RNA transcripts is inhibited, the gamma-irradiated schistosomula would be present in the skin (Mastin et al, 1983). At this time, the gamma-irradiated parasites are predicted to express antigens in modified conformations. Briefly, aberrant and denatured antigens may arise as a result of the direct effects of irradiation on structure of proteins, carbohydrates and lipids, and indirectly in consequence of the inhibited synthesis of all these macromolecules, just as described for U.V.-irradiated schistosomula in

chapters 3 and 4. Reduced synthesis of these molecules, in particular, of the heat-shock proteins, and carbohydrate signals responsible for recognition and removal of molecules in non-native conformations, is predicted to result in persistence of these modified structures. Since only a small proportion of gamma-irradiated schistosomula die during this early period after transformation (Mastin et al, 1983), it is suggested that these altered antigens may be made available chiefly in the form of secreted material and released membrane fragments for uptake by antigen presenting cells and presentation to the host immune system in the context of the major histocompatability complex.

When synthesis of proteins (especially hsps) recovers, however, at least partial clearance of the aberrant antigens is likely to occur. As normal synthetic activity proceeds, the gamma-irradiated schistosomula are likely to present an antigenic profile much more similar to normal parasites.

The experiments presented here followed protein synthesis by gamma-irradiated schistosomula only up to 96 hours after transformation. However, it is postulated that, when the developing normal schistosomula activate new regions of heterochromatin, as required for growth and cell division after day 4, the gamma-irradiated schistosomula cannot follow suit. We have suggested that the nucleic acid normally activated at this time is likely to be in the form of condensed, inactive heterochromatin restricted to the highly radiosensitive location of the nuclear membrane at the time of irradiation. Thus, in gamma-irradiated forms, inhibition of normal synthetic activity may again occur at this new stage in development, entailing, as before, disruption of the normal organisation and conformation of parasite antigens. As the lethally-damaged schistosomula die and disintegrate, the altered antigens of both the surface and the body interior should become available for processing and presentation by antigen presenting cells. This second stage in

modified antigen expression by attenuated schistosomula is predicted to occur chiefly in the lungs, where the gamma-irradiated forms begin to arrive from day 4 onwards (in the mouse model; Mastin et al, 1983).

Thus, susceptibility to radiation damage, entailing expression of antigens in modified, immunogenic conformations, is predicted to occur at discrete stages in schistosomular development. It is postulated that these "immunogenic stages" are associated quite specifically with different sites in the migration of irradiated larvae - firstly, in the skin, and secondly in the lung. Evidence supporting this model, whereby gamma-irradiated schistosomula induce immunity at distinct stages in development, and in consequence, at different sites in their migratory pathway - the skin and lung - will be discussed in chapters 9 and 10.

CHAPTER SIX

CHARACTERISATION OF ANTIGENS EXPRESSED BY NORMAL, IRRADIATED, AND ACTINOMYCIN D-TREATED PARASITES.

6. CHARACTERISATION OF ANTIGENS EXPRESSED BY NORMAL, IRRADIATED AND ACTINOMYCIN D - TREATED PARASITES.

6.1 Binding of antisera reactive with the cercarial glycocalyx.

It was considered that irradiation might alter expression of antigens in the cercarial glycocalyx, either directly as a result of oxygen radical damage, or indirectly in consequence of metabolic inhibition. Three antisera were used to investigate this question. Anti-coat 1 and anti-coat 2 were raised against different fractions of the cercarial glycocalyx; anti-snail haemolymph against snail material as described in Materials and Methods (chapter 2). The binding of these antisera to cercariae, and to schistosomula within four hours of skin or mechanical transformation, was examined.

Indirect immunofluorescence indicated that anti-coat 1 antibodies bound to approximately the same extent to normal cercariae and schistosomula up to 4 hours after mechanical transformation (fig. 6.1). For both normal and U.V.-irradiated schistosomula, binding to skin forms was greater than to mechanical ones. By 4 hours after skin penetration, reactivity was approximately 2-3 times as high as for mechanical schistosomula of the same age. Binding to irradiated schistosomula, whether skin or mechanical, was always greater than to normal ones, although binding to normal and irradiated cercariae was the same.

Immunoblotting results did not appear to correspond with the immunofluorescence data. Figure 6.2 shows that anti-coat 1 binds with the same intensity, and identifies the same proteins, in normal and irradiated cercariae and mechanically-transformed schistosomula. Immunofluorescence, in contrast, had indicated that anti-coat 1 bound more strongly to irradiated than normal, mechanical schistosomula. The molecular weights of the major proteins identified by Western blotting

in this instance were 100 000, 70 000, 47 000, 30 000, 23 000, 17 000, 14 000.

The contrast between immunoblotting and immunofluorescence data was even more pronounced for skin-transformed schistosomula (figure 6.3). In this case, binding to cercariae was intense, but decreased within an hour of skin penetration, and was not detectable at all by 5 hours after skin transformation. Immunofluorescence, however, showed a great increase in anti-coat 1 binding to the parasite surface during the four hours following skin transformation. The chief antigens detected in cercariae in this blot were found at Mr 74 000, 49 000, 35 000, 30 000, 28 000, 26 000, 20 000, 16 000.

In both these immunoblots, a small amount of antibody adsorption to the molecular weight standards is noticeable, in particular to serum albumin (Mr 67 000). The hydrophobic nature of serum albumin may encourage such non-specific binding. Anti-coat 1 binding to trypsin inhibitor at Mr 20 100 (figure 6.2), a glycosylated protein, might possibly be due to cross-reactivity with parasite oligosaccharide sequences.

Immunoblotting was also performed with antiserum raised against the second fraction of the cercarial glycocalyx - anti-coat 2. (see figure 6.4). Compared to anti-coat 1, this antiserum recognised a very limited range of proteins, at Mr 100 000, 70 000 and, for mechanically transformed schistosomula, at Mr 23 000. Low molecular weight antigens which migrated with the dye front were also very strongly recognised by anti-coat 2. This material may consist of glycopeptides, or glycolipids, which Weiss and Strand (1985) and Samuelson and Caulfield (1982) have identified in cercariae.

Like anti-coat 1, this antiserum reacted relatively strongly with antigens from cercariae and mechanical schistosomula, but only weakly with skin forms. Interestingly, skin schistosomula produced from cercariae irradiated at the higher dose (3 minutes), seem to retain

slightly more coat 2 antigens than normal or 1.5 minute-irradiated parasites. Similarly, figure 6.3 suggests that irradiated skin schistosomula may continue to express coat 1 proteins at a low level at 3 hours, when these epitopes are completely lost from normal skin forms.

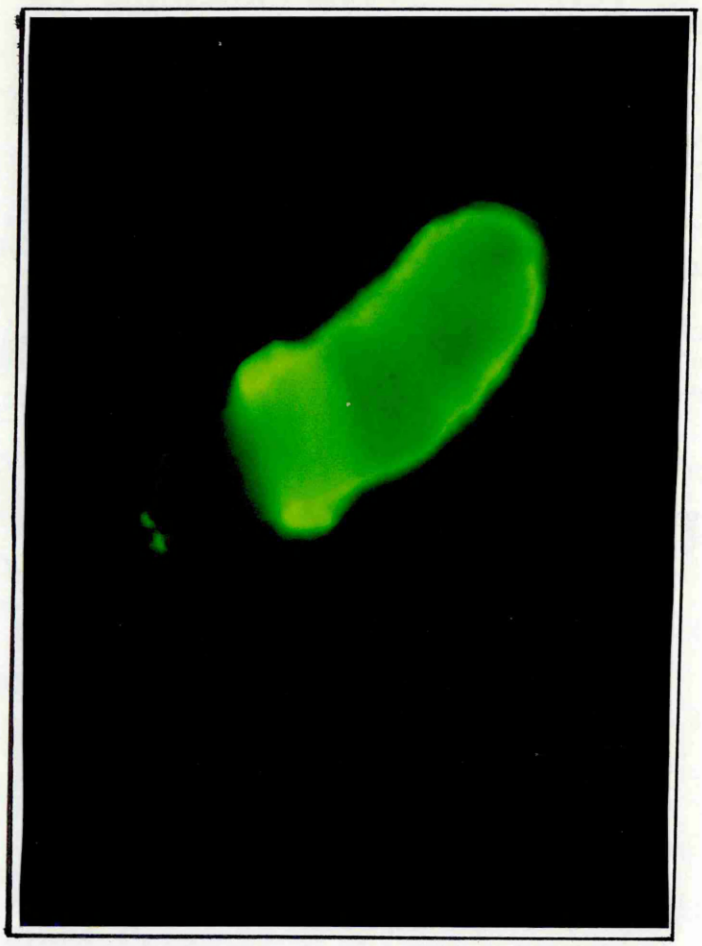
The presence of snail-like epitopes on normal and irradiated cercariae and schistosomula was investigated using antiserum raised against snail haemolymph proteins (figures 6.5, 6.6).

As tested by indirect immunofluorescence (figure 6.5), irradiated and normal cercariae bound anti-haemolymph antiserum approximately equally. After mechanical transformation, binding of this antiserum decreased to equal levels in both normal and irradiated schistosomula. The pattern for skin forms was very different, however. At 2 hours, normal skin schistosomula bound slightly less anti-haemolymph antiserum than cercariae, while irradiated 2-hour skin schistosomula also bound significantly more. Irradiated 2-hour skin schistosomula bound significantly more anti-haemolymph antibody than normal schistosomula of the same age. By 4 hours after skin transformation, binding to both normal and irradiated parasites had decreased to control levels.

Immunoblotting with cercariae and skin schistosomula supported the immunofluorescence results reasonably well in this case (see fig. 6.6). Thus, anti-haemolymph binding to both normal and irradiated forms was virtually undetectable at 4 hours, while binding to irradiated schistosomula at 2 hours, was higher than to normal ones. The major proteins identified by anti- haemolymph antiserum had molecular weights of 160 000, 100 000, 71 000, 67 000, 57 000, 47 000.

6.2 Exposure of new protein determinants by normal and irradiated cercariae and schistosomula.

6-Iodoacetamidofluorescein (6-IAF) binds covalently to SH groups



The cercarial glycocalyx has been labelled with infection serum and fluorescein — conjugated second antibody. In this picture, the fluorescent glycocalyx is being released during the cercarienhüllen reaction. (see p.25).

Dimensions: 200µm x 90µm (approx.).

Figure 6.1 Binding of anti-coat 1 antiserum to normal and U.V.-irradiated cercariae, and schistosomula. 2 hours and 4 hours after skin or mechanical transformation.

C = cercariae

S = schistosomula

Mech = mechanically-transformed schistosomula

Skin = skin-transformed schistosomula.

□ = Binding of anti-coat 1 antiserum.

▨ = Binding of normal rabbit serum.

U.V.-irradiation dose was 400 μW min cm^{-2}.

Indirect immunofluorescence was performed as described in section 2.6.3.1. Parasites were immobilized with carbachol. Each bar represents the mean of 20 to 25 samples.

An example of fluorescent labelling of the parasite surface is shown on p.408a)

STATISTICS: t-tests.

S, 2h, Mech: Normal vs Irradiated: $P<0.005$

S, 4h, Mech: Normal vs Irradiated: $P<0.005$

S, 2h, Skin: Normal vs Irradiated: $P<0.005$

S, 4h, Skin: Normal vs Irradiated: NOT SIGNIFICANT

S, 2h, Normal: Mech vs Skin: $P<0.005$

S, 4h, Normal: Mech vs Skin: $P<0.005$

S, 2h, Irradiated: Mech vs Skin: $P<0.005$

S, 4h, Irradiated: Mech vs Skin: $P<0.005$

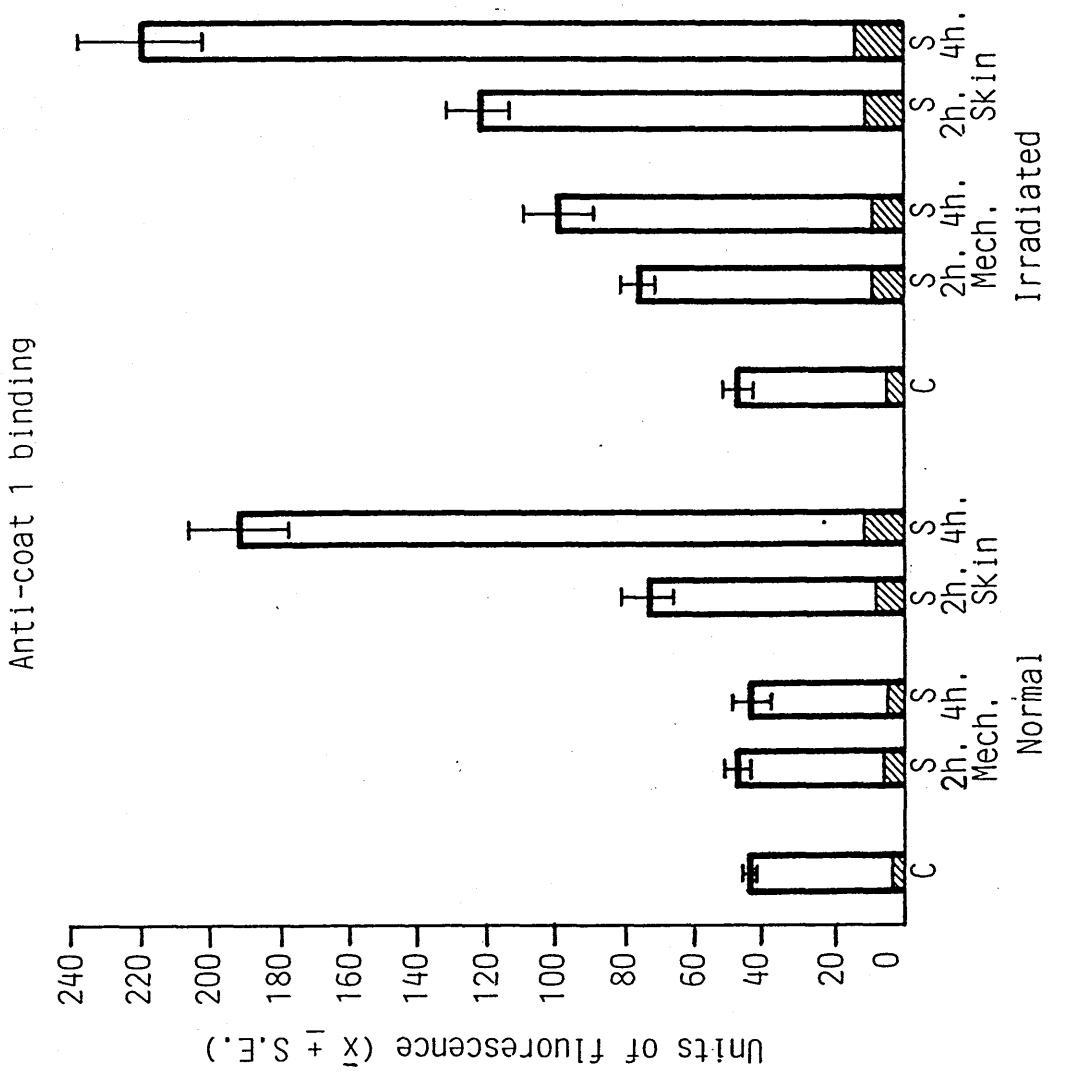

Anti-coat 1 binding
Units of fluorescence ($\bar{X} \pm$ S.E.)
240
220
200
180
160
140
120
100
80
60
40
20
0
C
S
2h. 4h. Mech.
S
S
2h. 4h. Skin
C
S
2h. 4h. Mech.
S
S
2h. 4h. Skin
Normal
Irradiated

Figure 6.2 Binding of anti-coat 1 antiserum to normal and irradiated cercariae, and schistosomula at 1, 3 and 5 hours after mechanical transformation.

C = cercariae

S = schistosomula

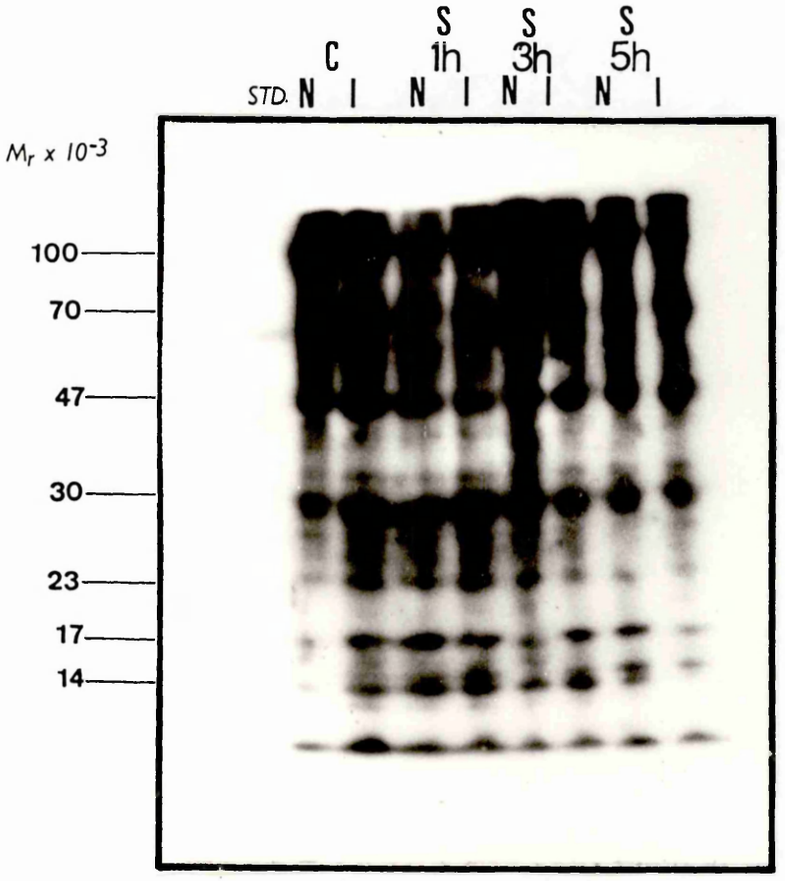
N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

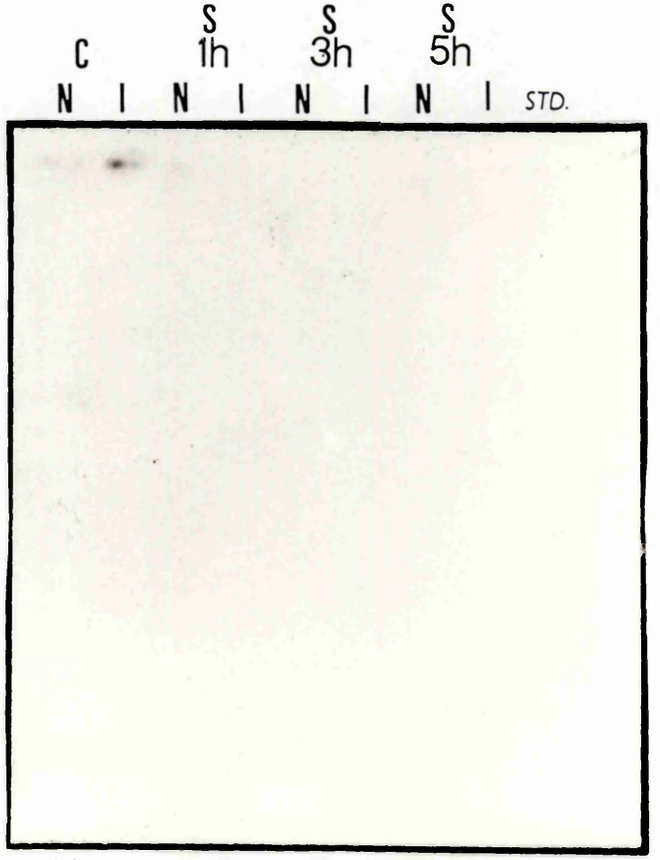
STD = protein standards

1000 parasites per well. 3% stacking gel/10% resolving gel.

Schistosomula were obtained by mechanical transformation of normal and irradiated cercariae, and were cultured in GMEM.



ANTI-COAT 1



N.R.S.

Figure 6.3 Binding of anti-coat 1 antiserum to normal and irradiated cercariae, and schistosomula at 1, 3 and 5 hours after skin transformation.

C = cercariae

S = schistosomula

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

STD = protein standards

500 parasites per well. 3% stacking gel/10% resolving gel.

Schistosomula were collected into GMEM after penetration of excised mouse skin.

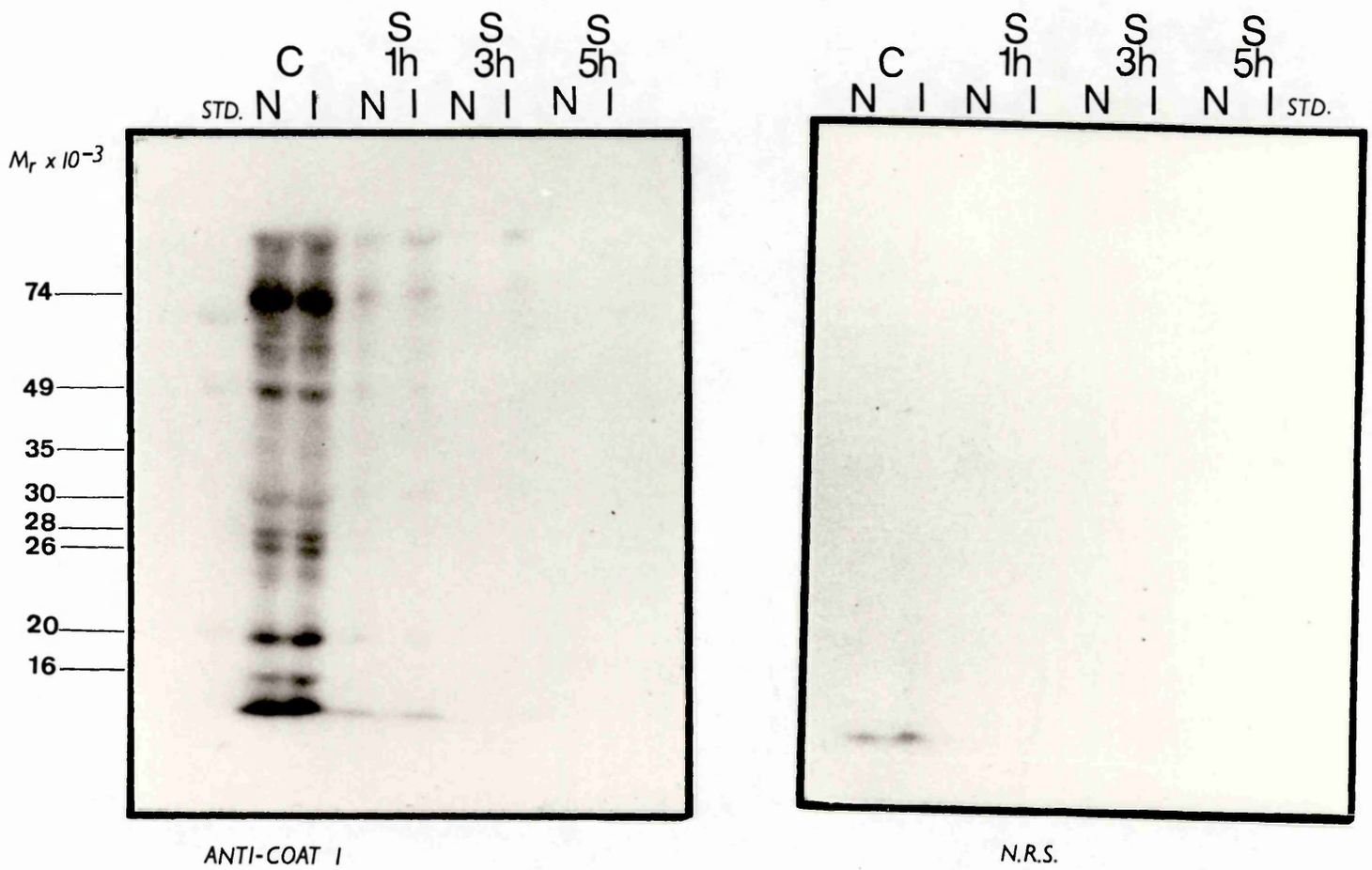

C
S 1h
S 3h
S 5h
STD.
N I N I N I N I
$M_r \times 10^{-3}$
74
49
35
30
28
26
20
16
ANTI-COAT I
C
S 1h
S 3h
S 5h
N I N I N I N I STD.
N.R.S.

Figure 6.4 Binding of anti-coat 2 antiserum to normal and irradiated cercariae, and schistosomula 3 hours after skin or mechanical transformation.

C = cercariae

M = schistosomula 3 hours after mechanical transformation

SK = schistosomula 3 hours after skin transformation

1.5 = U.V.-irradiated for 1.5 minutes (400 μW min cm^{-2})

3 = U.V.-irradiated for 3 minutes (800 μW min cm^{-2})

STD = protein standards

500 parasites per well

3% stacking gel/10% resolving gel.

Schistosomula were either transformed mechanically in GMEM or collected into GMEM after penetration of excised mouse skin.

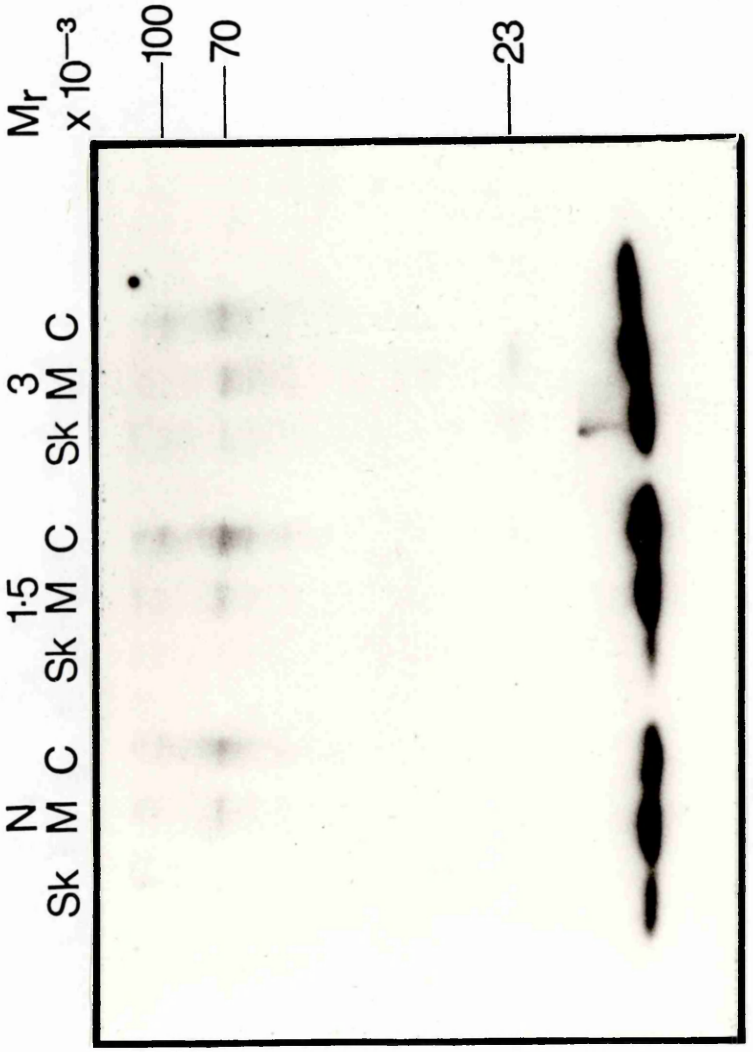

Mr
x 10⁻³
100
70
23
N
Sk M C
1·5
Sk M C
3
Sk M C

Figure 6.5 Binding of anti-haemolymph antiserum to normal and irradiated cercariae and schistosomula at 2 hours and 4 hours after skin or mechanical transformation.

C = cercariae

S = schistosomula

Mech = mechanically-transformed schistosomula

Skin = skin-transformed schistosomula.

U.V.-irradiation dose was 400 μW min cm^{-2}

□ = anti-haemolymph binding

▨ = normal rabbit serum binding

Cercariae were transformed mechanically in GMEM, or collected into GMEM after skin penetration.

Immunofluorescence was performed as described in section 2.6.3.1. Parasites were immobilized with carbachol. Each bar represents the mean of 20 to 25 readings.

STATISTICS: t-tests.

C: Normal vs Irradiated: $0.005<P<0.01$

S, 2h, skin: Normal vs Irradiated: $P<0.005$.

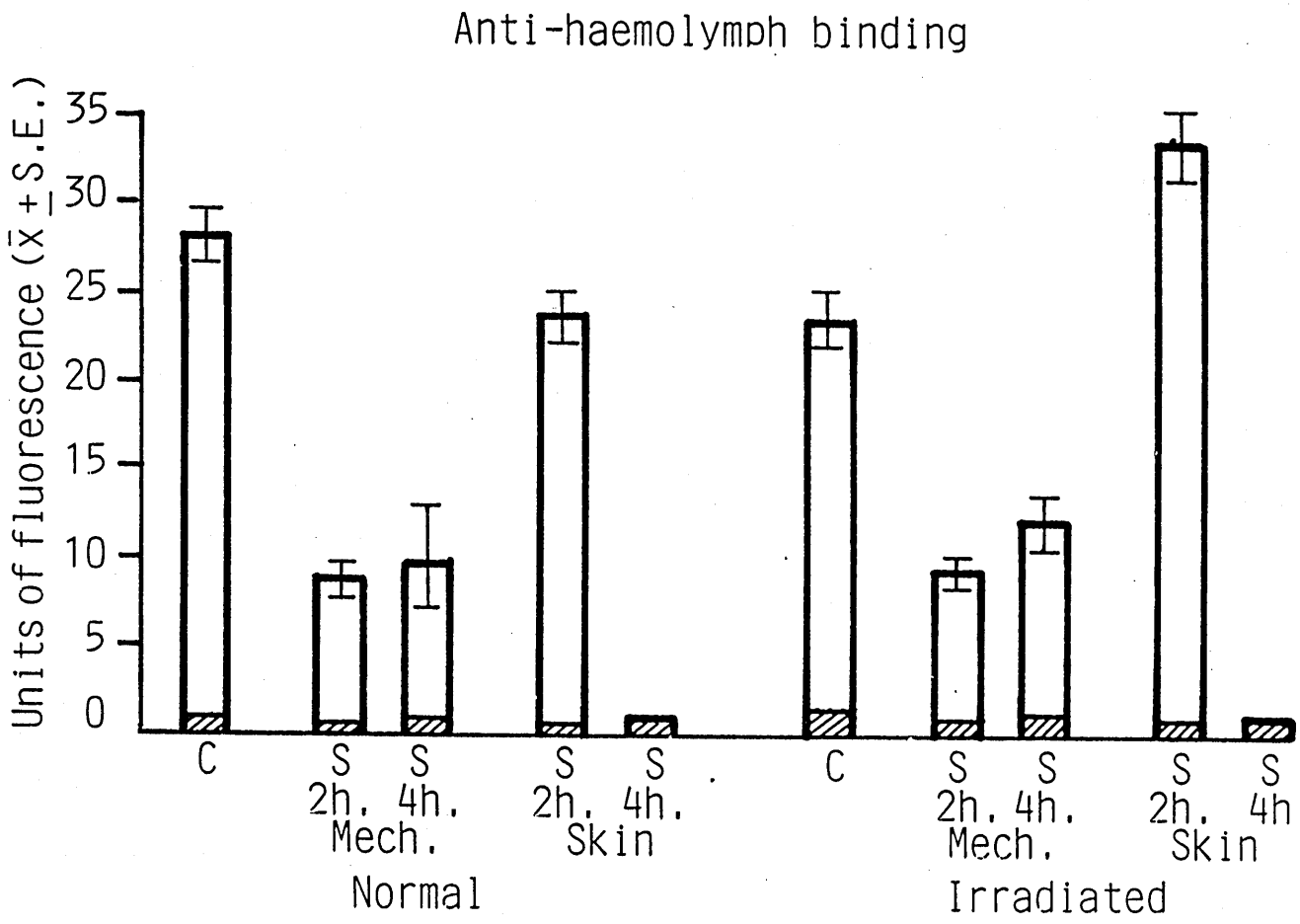

Anti-haemolymph binding
Units of fluorescence ($\bar{x} \pm$ S.E.)
35
30
25
20
15
10
5
0
C
S 2h.
S 4h.
Mech.
S 2h.
S 4h.
Skin
Normal
C
S 2h.
S 4h.
Mech.
S 2h.
S 4h.
Skin
Irradiated

Figure 6.6 Binding of anti-haemolymph antiserum to normal and irradiated cercariae, and schistosomula at 2 hours and 4 hours after skin transformation.

C = cercariae

S = schistosomula

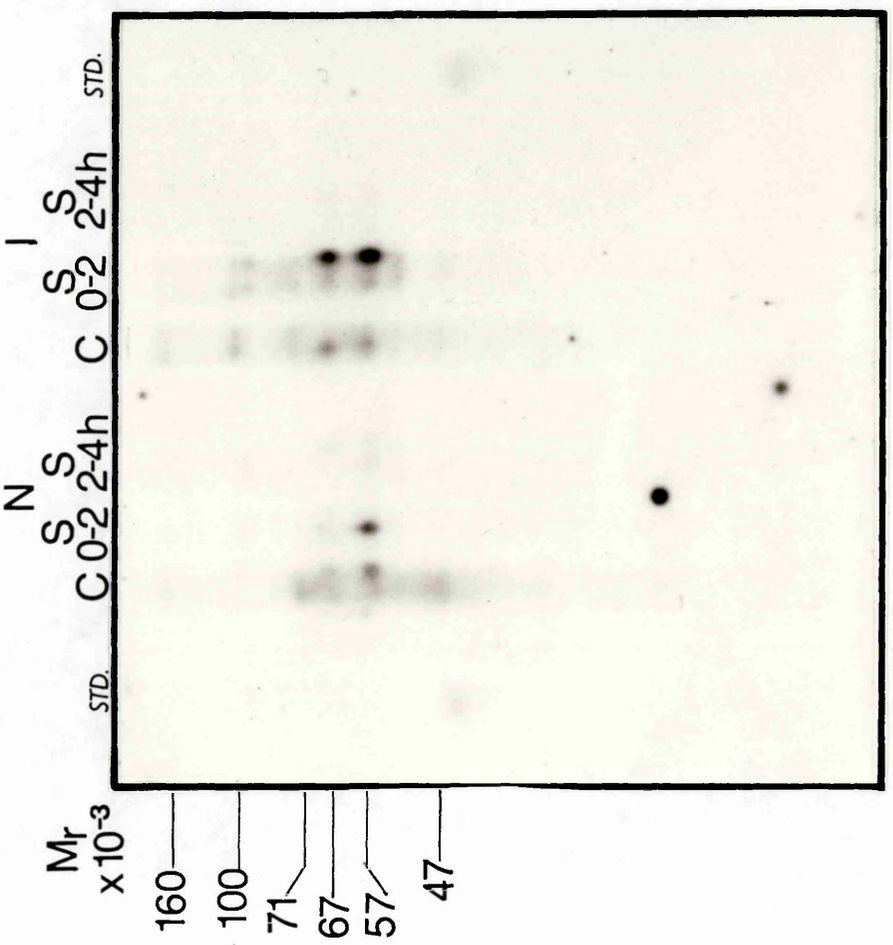
N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

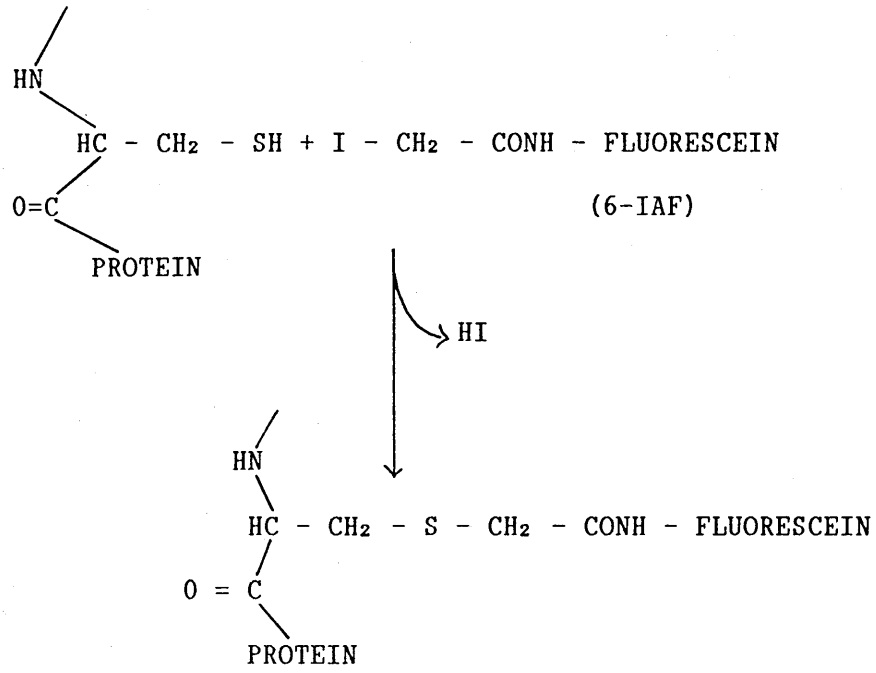
500 parasites per well.

3% stacking gel/10% resolving gel.

Schistosomula were collected into GMEM after penetration of excised mouse skin. At 2 hours, schistosomula were removed. The skin was then replaced, and a second batch of schistosomula collected after a further 2 hours.


STD.
C
S 0-2
S 2-4h
N
I
C
S 0-2
S 2-4h
STD.
Mr x10-3
160
100
71
67
57
47

exposed on proteins, by the following mechanism:



Gamma- or U. V.-irradiation apparently increased the number of SH groups available for 6-IAF binding at the surface of formaldehyde-fixed cercariae or schistosomula (fig. 6.7). Looking at cercariae first of all, both gamma- and U. V.-irradiation seem to enhance 6-IAF binding 5- to 8- fold over normal parasites. Higher doses of irradiation of either type seem to expose more binding sites for 6-IAF (i.e., SH groups). At 3 hours after transformation, 6-IAF binding was somewhat higher than for the cercarial stage, significantly so for normal and 20 krad gamma-irradiated forms. Binding to irradiated parasites was still much greater than to normal ones, however. Only normal and U.V.-irradiated schistosomula were examined at 24 hours after transformation, when binding was not significantly different from at 3 hours. 24-hour old, Actinomycin D-treated schistosomula did not show any enhancement of 6-IAF binding.

The increased 6-IAF binding to irradiated forms may be related to increased damage to the parasite surface, as indicated by uptake of the

Figure 6.7 Binding of 6-IAF to normal, irradiated and Actinomycin D-treated cercariae, and schistosomula at 3 hours and 24 hours after mechanical transformation.

N = normal

1'U = 1 minute U.V.-irradiated (400 μW min cm^{-2})

8'U = 8 minutes U.V.-irradiated (3.2 mW min cm^{-2})

G20 = 20 krad gamma irradiated

G50 = 50 krad gamma irradiated

A = Actinomycin D-treated

CERCS(C) = cercariae

SLA(S) = schistosomula.

Cercariae were transformed mechanically in GMEM, and cultured in GMEM/10% hiFCS. Parasites were formaldehyde-fixed, and 6-IAF binding measured as described in section 2.6.3.3.

Each bar represents the mean of 20 to 25 readings.

STATISTICS: t-tests.

C,N vs 1'U, 8'U, G20, G50: $P<0.005$

S, 3h, N vs 1'U: NOT SIGNIFICANT

8'U: $0.025<P<0.01$

G20, G50: $P<0.005$

S, 24h, N vs 1'U, $0.005<P<0.01$

8'U: $P<0.005$

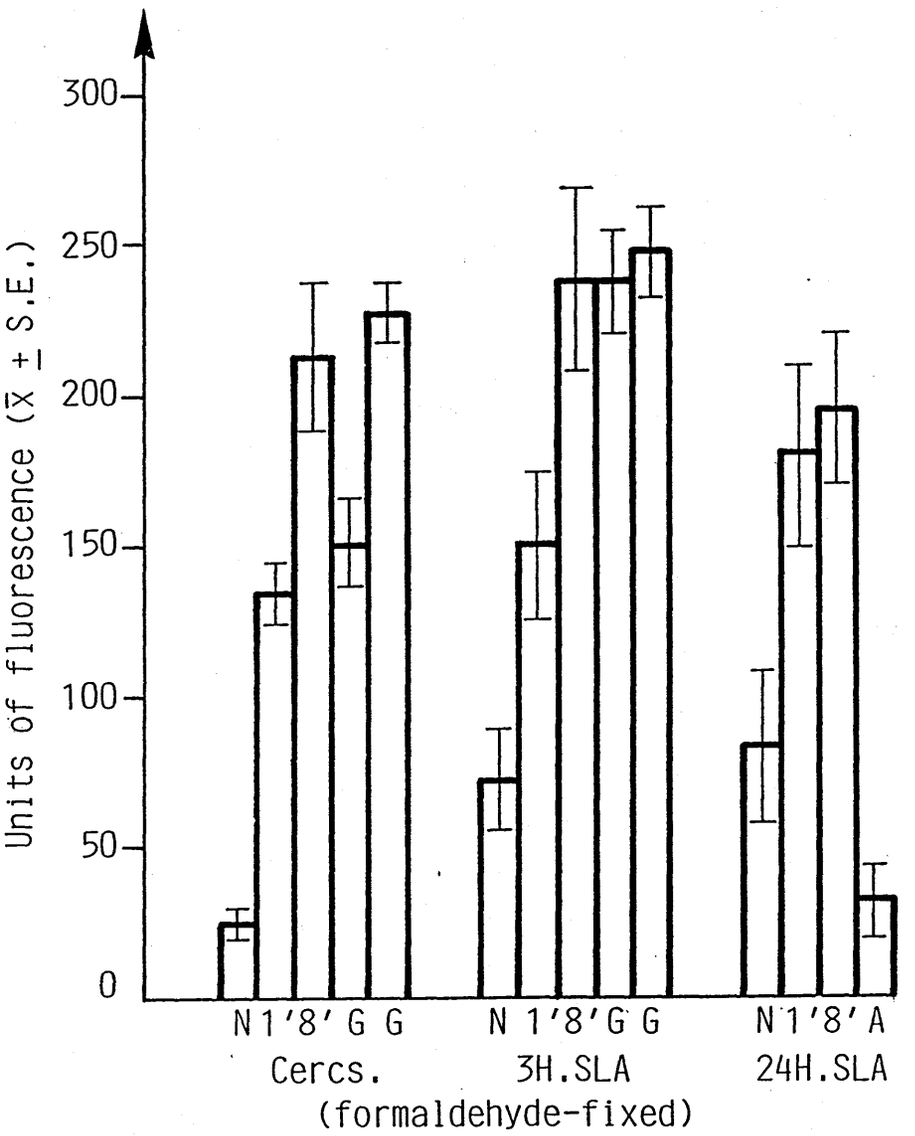

Units of fluorescence ($\bar{X} \pm$ S.E.)
300
250
200
150
100
50
0
N 1'8' G G
N 1'8' G G
N 1'8' A
Cercs.
3H.SLA
24H.SLA
(formaldehyde-fixed)

Figure 6.8 Hoechst (H33258) uptake by normal and irradiated cercariae, and schistosomula 3 hours after mechanical transformation.

N = normal

1′ = 400 μW min cm^{-2}

4′ = 1.6 mW min cm^{-2}

8′ = 3.2 mW min cm^{-2}

16′ = 6.4 mW min cm^{-2}

} dose of U.V.-irradiation.

Hoechst uptake was measured for cercariae in aquarium water. Schistosomula were obtained by mechanical transformation in GMEM, and Hoechst uptake in GMEM measured as described in section 2.6.3.5.

Each bar represents the mean of 20 to 25 determinations.

STATISTICS: t-tests.

CN vs 1′: NOT SIGNIFICANT

4′, 8′, 16′: $P<0.005$

C1′ vs C4′: $0.025<P<0.01$.

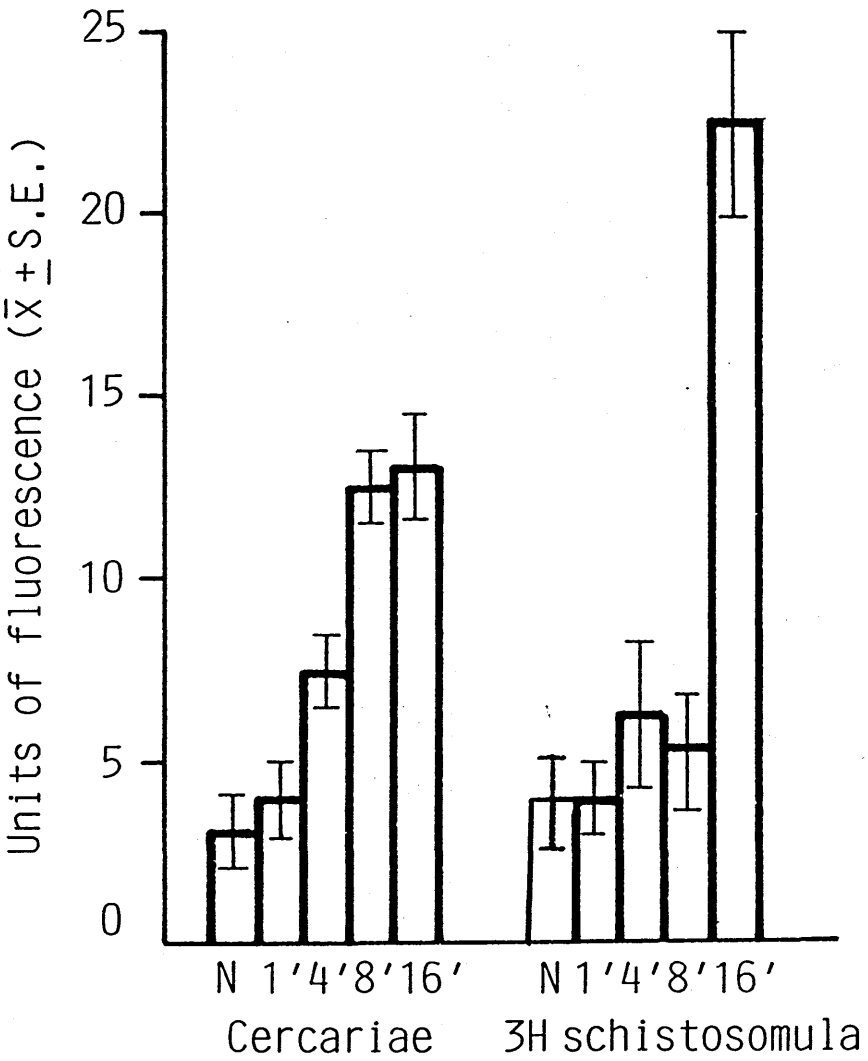
C4′ vs C8′: $P<0.005$

S, 3h, N vs 1′, 4′, 8′: NOT SIGNIFICANT

16′: $P<0.005$

C = cercariae

S = schistosomula


25
20
15
10
5
0
Units of fluorescence ($\bar{X} \pm$ S.E.)
N 1'4'8'16'
N 1'4'8'16'
Cercariae
3H schistosomula

normally excluded dye Hoechst H33258. Figure 6.8 shows that higher doses of irradiation were accompanied by increased internalization of Hoechst by cercariae or 3-hour schistosomula. Despite the surface damage indicated by Hoechst uptake, less than 5% of the irradiated parasites at these times were dead when assessed by light microscopy as described in chapter 2.

6.3 Monoclonal antibodies, and antisera, showing differences in binding to normal and irradiated cercariae or schistosomula.

A number of other reagents showed preferential binding to normal or irradiated cercariae or schistosomula.

The monoclonal antibody A3, obtained from S. R. Smithers, showed different binding patterns to normal and irradiated parasites. Figure 6.9 demonstrates that U.V.-irradiated cercariae showed a striking increase in capacity for A3 binding. After transformation, binding to the irradiated surface decreased, but remained higher than for normal parasites until 2 hours after transformation. From 2-3 hours, however, binding to irradiated schistosomula declined markedly, while that to normal parasites continued to increase.

For the monoclonal M7B3A, on the other hand, no difference could be detected in binding to normal and irradiated cercariae or schistosomula (Fig 6.10). This monoclonal, produced and characterised by Bickle et al (1986) recognises a 16 000 Mr antigen on the surface of newly transformed schistosomula. This antigen was also identified by serum from mice vaccinated with gamma-irradiated cercariae, but not from animals with a chronic infection. The importance of this Mr 16 000 antigen in immunity is indicated by the fact that M7B3A will transfer passive resistance (28-70% protection) to cercarial infection in mice.

Infected human serum recognised irradiated cercariae preferentially over normal ones, as assessed by immunofluorescence. Binding to 24-hour

Figure 6.9 Binding of monoclonal A3 to normal and irradiated cercariae and schistosomula during 3 hours after mechanical transformation.

N = normal cercariae

I = U.V.-irradiated (400 μW min cm^{-2}) cercariae

—○— = normal schistosomula

---●--- = U.V.-irradiated (400 μW min cm^{-2}) schistosomula

Cercariae were transformed mechanically, and incubated in GMEM at 37°C.

Immunofluorescence was performed as described in section 2.6.3.1. Parasites were immobilized by carbachol treatment. Each point represents the mean of 20 to 25 readings.

STATISTICS: t-tests.

CERCARIAE: N vs I: $P<0.005$.

SCHISTOSOMULA: 30' N vs I: $P<0.005$

1h: N vs I: $0.025<P<0.05$

2h: N vs I: NOT SIGNIFICANT

3h: N vs I: $P<0.005$.

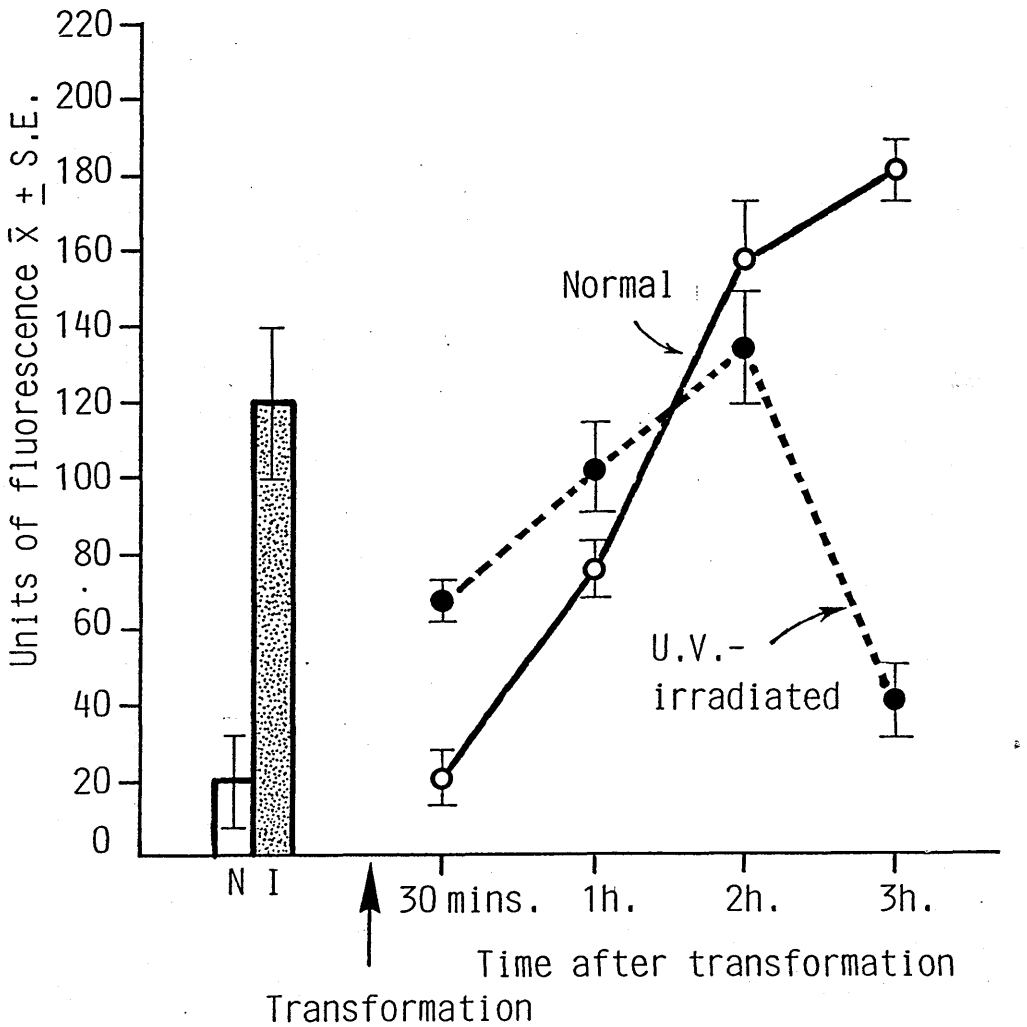

Units of fluorescence $\bar{X} \pm$ S.E.
220
200
180
160
140
120
100
80
60
40
20
0
Normal
U.V.-
irradiated
N I
30 mins.
1h.
2h.
3h.
Time after transformation
Transformation

Figure 6.10 Binding of monoclonal M7B3A (Bickle et al, 1986) to normal and irradiated cercariae, and schistosomula during 3 hours after mechanical transformation.

N = normal cercariae —o—

I = U.V.-irradiated cercariae (400 μW min cm^{-2}) ---●---

Cercariae were transformed mechanically, and incubated in GMEM at 37°C. Immunofluorescence was performed as described in section 2.6.3.1.

Parasites were immobilized by carbachol treatment. Each point represents the mean of 20 to 25 readings.

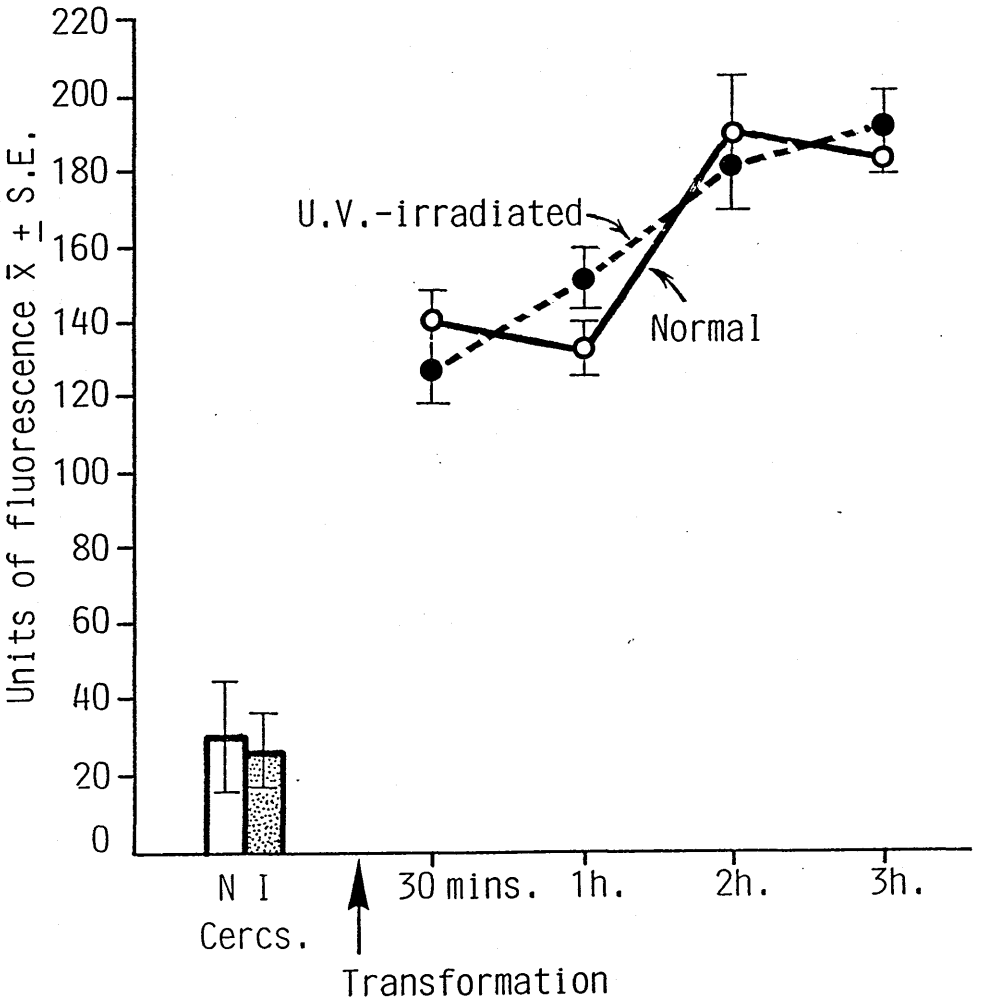

220
200
180
160
140
120
100
80
60
40
20
0
Units of fluorescence $\bar{X} \pm$ S.E.
U.V.-irradiated
Normal
N I
Cercs.
30 mins.
1h.
2h.
3h.
Transformation

Figure 6.11 Binding of infected human serum (I.H.S.) to normal and irradiated cercariae, and normal, irradiated and Actinomycin D-treated schistosomula at 24 and 48 hours after mechanical transformation.

N = normal
I = U.V.-irradiated (400 μW min cm^{-2})
A = Actinomycin D-treated

C = cercariae
S = schistosomula

Binding of normal human serum is subtracted in each case.

Cercariae were transformed mechanically in Elac, and cultured in Elac/10% hiFCS at 37°C/5% CO_2. Immunofluorescence was performed as described in section 2.6.3.1. Parasites were immobilized by carbachol treatment. Each bar represents the mean of 20 to 25 readings.

STATISTICS: t-tests.

CN vs CI: $P<0.005$.

S, 24h: N vs I: $P<0.005$
N vs A: $0.025<P<0.05$

S, 48h: N vs I,A: NOT SIGNIFICANT

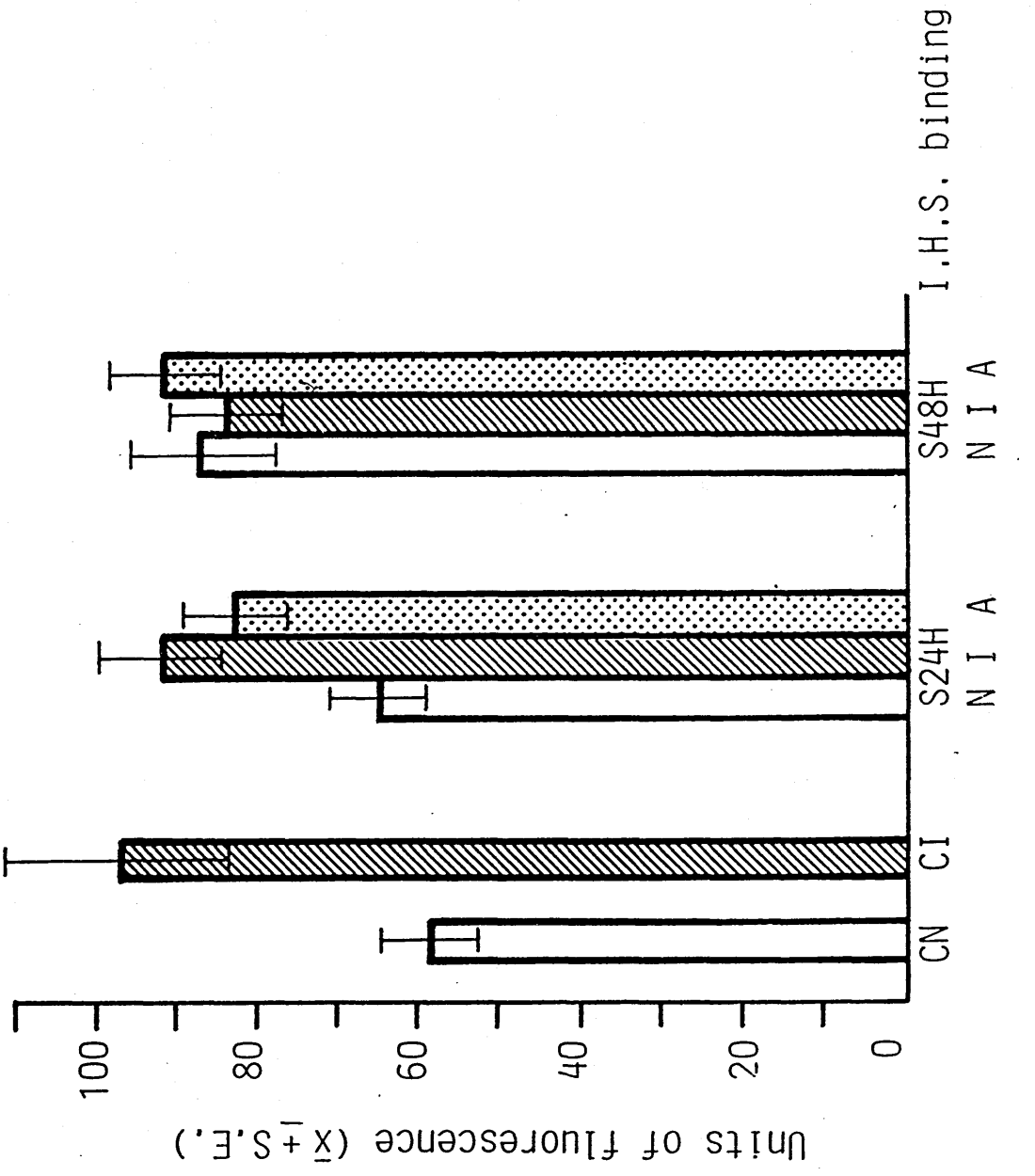

I.H.S. binding
S48H
S24H
NIA
NIA
CN
CI
0
20
40
60
80
100
Units of fluorescence (X̄ ± S.E.)

Figure 6.12 Binding of antiserum raised against cercarial membrane antigens (anti-CMAG antiserum) to normal and irradiated cercariae and normal, U.V.-irradiated and Actinomycin D-treated schistosomula at 24 hours and 72 hours after mechanical transformation.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

A = Actinomycin D-treated

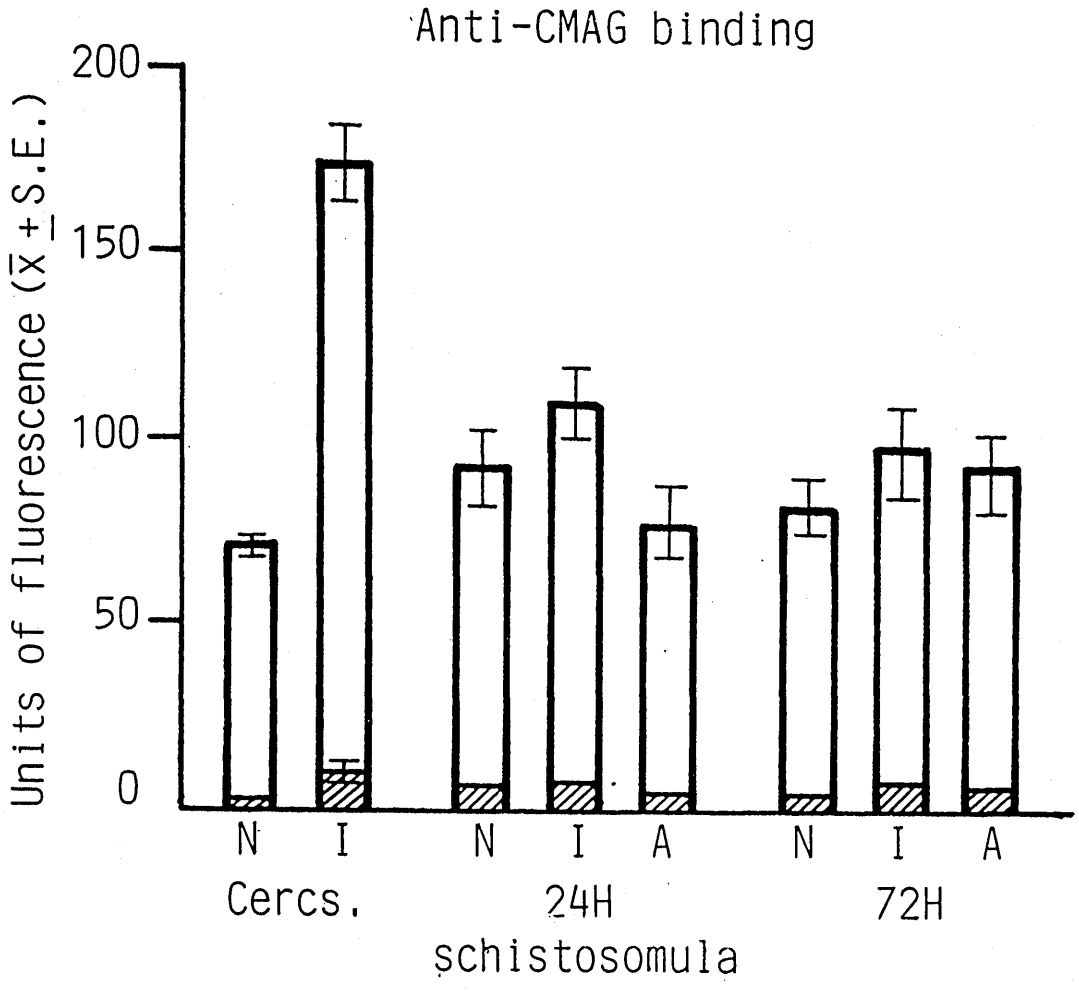
□ = anti-CMAG binding

▨ = normal rabbit serum binding

Cercariae were transformed mechanically in Elac, and cultured in Elac/10% hiFCS at 37°C/5% CO_2. Immunofluorescence was performed as described in section 2.6.3.1. Parasites were immobilized by carbachol treatment. Each bar represents the mean of 20 to 25 readings.

STATISTICS: t-test.

CN vs CI: $P<0.005$.


Anti-CMAG binding
Units of fluorescence ($\bar{x} \pm$ S.E.)
200
150
100
50
0
N
I
N
I
A
N
I
A
Cercs.
24H
72H
schistosomula

mechanically-transformed schistosomula was also slightly higher for irradiated and Actinomycin D-treated forms than for normal ones. By 48 hours after transformation, however, this serum bound equally to normal, irradiated and Actinomycin D-treated schistosomula (fig. 6.11).

Finally, antiserum raised against "cercarial membrane antigens" released during the first 90 minutes of transformation (anti-CMAG) showed twice as much binding to irradiated as to normal cercariae. However, the patterns of binding to normal, irradiated and Actinomycin D-treated, mechanically-transformed schistosomula at 24 hours and 72 hours were indistinguishable from one another. The level of binding at these times was also approximately the same as to normal cercariae (fig. 6.12).

6.4 Surface iodination of normal, irradiated and Actinomycin D-treated schistosomula at 3, 20 and 72 hours after transformation.

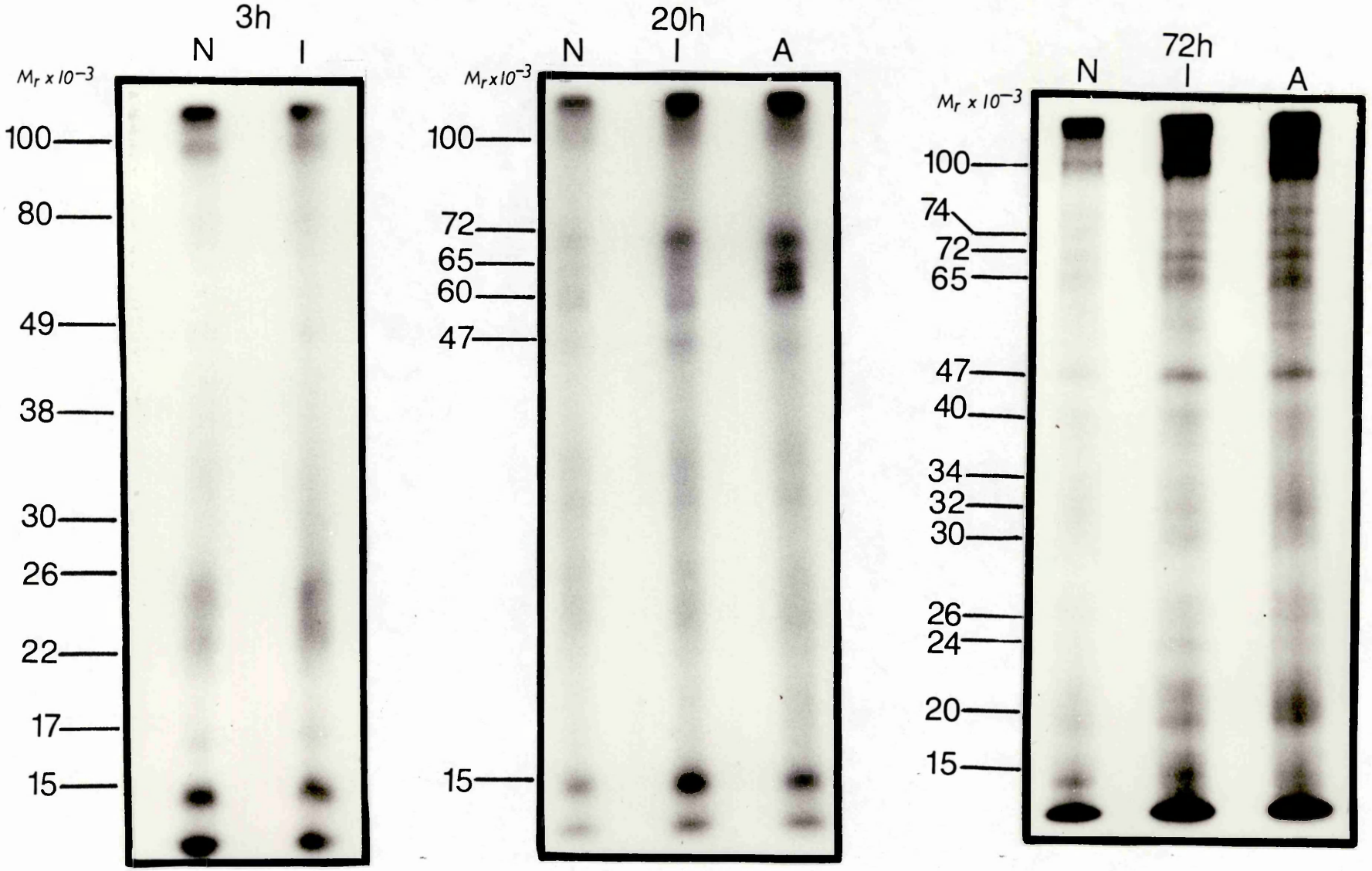
While immunofluorescence and immunoblotting with different antisera and monoclonal antibodies give information on the expression of particular epitopes by normal and irradiated parasites, a more general picture of the antigens exposed at the surface of normal and attenuated schistosomula should be obtained from surface iodination, and SDS-PAGE of the labelled proteins, without any selection of antigens by specific antibody.

Figure 6.13 a) to c) shows the antigens exposed for reaction with Iodogen by normal, irradiated and Actinomycin D-treated schistosomula from 3 to 72 hours of culture after mechanical transformation. No differences can be distinguished between normal and attenuated parasites, but new antigens seem to be exposed at the surface as the parasite develops. Species at Mr 100 000 and 150 000 are labelled at all 3 time points. Proteins at Mr 82 000 and 17 000 are prominent on the surface of 3-hour schistosomula, but are not detected at 20 hours.

Figure 6.13 Surface iodination of normal, irradiated and Actinomycin D-treated schistosomula at 3, 20 and 72 hours after transformation.

U.V.-irradiation dose was 400 μW min cm^{-2}

Schistosomula were cultured in Elac/10% hiFCS at 37°C/5% CO_2. Iodogen iodination was performed as described in section 2.6.1.
1000 schistosomula per well.
3% stacking gels/10% resolving gels.


3h
N
I
$M_r \times 10^{-3}$
100
80
49
38
30
26
22
17
15
20h
N
I
A
$M_r \times 10^{-3}$
100
72
65
60
47
15
72h
N
I
A
$M_r \times 10^{-3}$
100
74
72
65
47
40
34
32
30
26
24
20
15

A heavily-labelled, diffuse band between Mr 22 000 and 26 000 at 3 hours is less prominent in 20-hour forms, while in 72-hour schistosomula, it is replaced by discrete, faintly-labelled proteins at Mr 22 000, 24 000, 26 000. Similarly, the diffusely labelled group of proteins between Mr 30 and 38 000 in normal schistosomula is replaced by discrete, faint bands at Mr 30 000, 32 000, 34 000, 40 000 in 72-hour forms. Proteins at Mr 60 000, 65 000, 72 000 are very pronounced in 20-hour schistosomula, but fainter by 72 hours. Bands at Mr 20 000 and 40 000 appear to be unique to the 72-hour schistosomulum surface, while a band at Mr 47 000 becomes increasingly heavily labelled as the schistosomulum develops.

6.5 Interaction of surface antigens of normal, irradiated and Actinomycin D-treated schistosomula with mouse peritoneal macrophages.

Normal, U.V.-irradiated and Actinomycin D-treated, 48-hour old schistosomula were surface-iodinated, and cultured with mouse peritoneal macrophages for 5 hours. Release of iodinated proteins into the culture medium, and uptake of these surface antigens by the macrophages were then examined.

Figure 6.14 shows that the major antigens exposed for iodination by 48-hour schistosomula, whether normal, U.V.-irradiated or Actinomycin D-treated, had Mr 150 000, 100 000, 72 000, 47 000, 38 000, 32 000, 31 000, 30 000, 20 000, 18 000 and 14 000. Some very low molecular weight labelled material can also be seen at and below the dye-front. Some proportion of all these labelled antigens seem to be released into culture, as shown by the overall decrease in intensity of labelling of the surface antigens after a five-hour incubation. Looking at the released antigens, antigens at Mr 150 000, 100 000, 72 000, 47 000, 40 000, 30 000, 14 000 and at or below the dye-front

Figure 6.14 Release of antigens from ^{125}I-surface labelled, 48-hour normal, U.V.-irradiated and Actinomycin D-treated schistosomula during a 5-hour incubation, and uptake of these iodinated antigens by mouse peritoneal macrophages during a 5-hour incubation with the parasites.

Protocol. Mechanically-transformed parasites were cultured in Elac/10% hiFCS at 37°C/5% CO_2. At 48 hours, surface-iodination was performed.

48h N ↓ (normal) I ↓ (U.V.-irradiated, 400 µW min cm^{-2}) A ↓ Actinomycin D-treated (^{125}I-labelled)

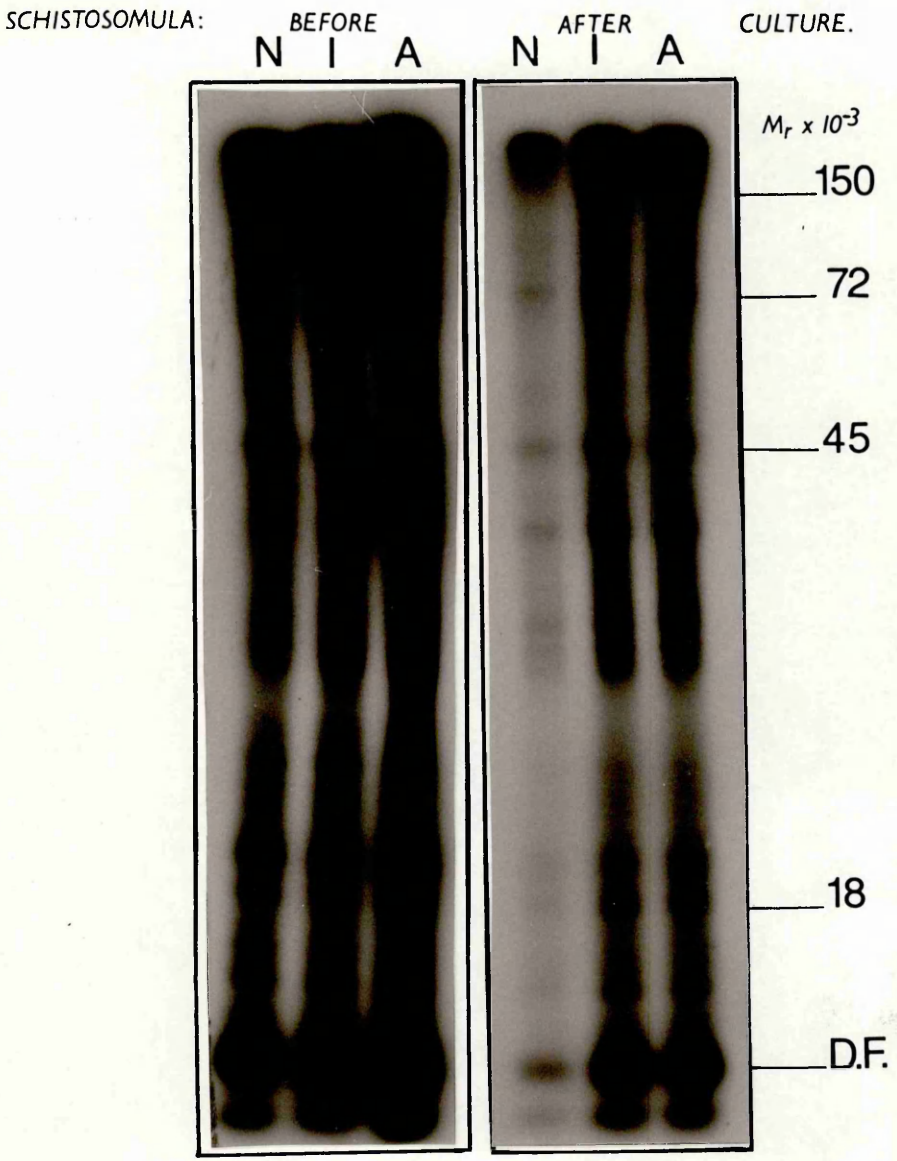
Divide each group in 3.

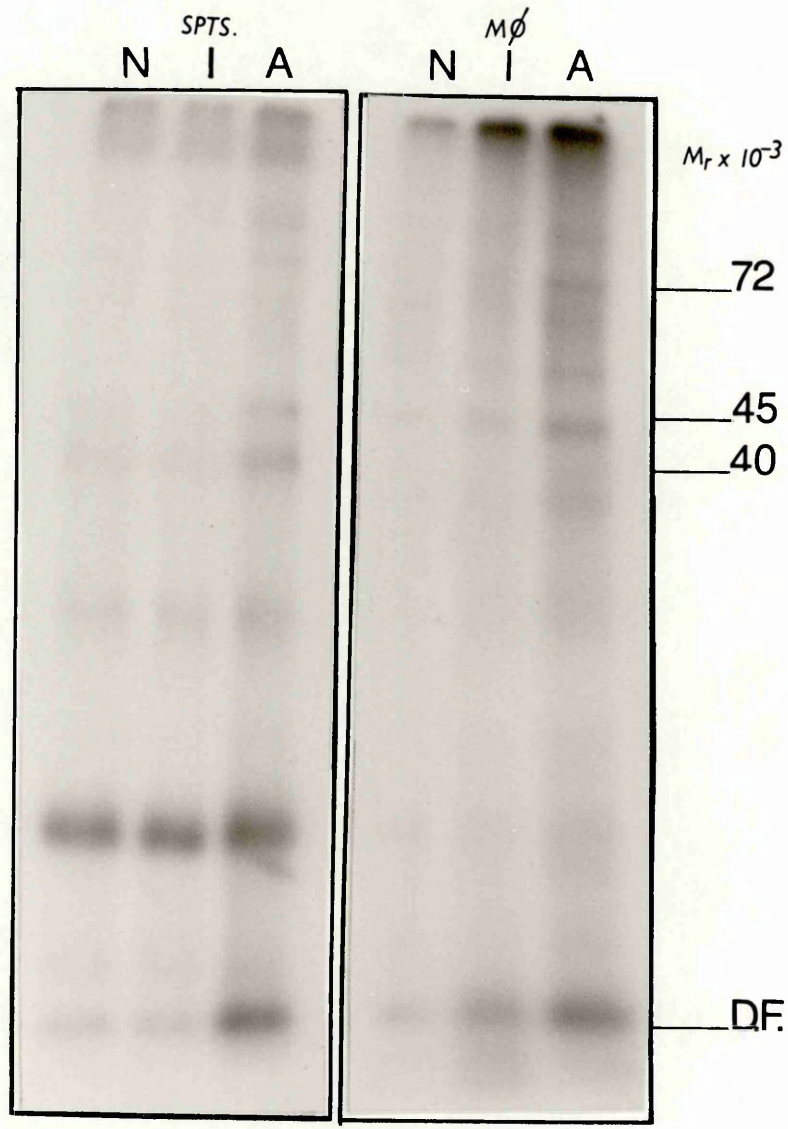
1.	2.	3.
Freeze immediately for SDS-PAGE	Culture in Elac for 5h at 37°C. Separate parasites from supernatant, containing released material. Add protease inhibitors, remove salt and freeze-dry for SDS-PAGE.	Culture with mouse peritoneal macrophages in Elac for 5 hours. Then separate parasites and cells. Wash and freeze both for SDS-PAGE.

ABBREVIATIONS.

N = normal

I = U.V.-irradiated (400 µW min cm^{-2}) (Cont. on next page)


SCHISTOSOMULA:
BEFORE
AFTER
CULTURE.
N I A
N I A
$M_r \times 10^{-3}$
150
72
45
18
D.F.


SPTS.
MØ
N I A
N I A
$M_r \times 10^{-3}$
72
45
40
D.F.

PROTOCOL FOR FIGURE 6.14, CONTINUED.

A = Actinomycin D-treated.

SPTS = ^{125}I-labelled proteins released by schistosomula during a 5-hour incubation without macrophages.

Mϕ = ^{125}I-labelled proteins associated with mouse peritoneal macrophages after a 5-hour incubation with surface-iodinated schistosomula.

"Before culture" } ^{125}I-labelled surface proteins on
"After culture" } schistosomula before and after 5 hours of culture with mouse peritoneal macrophages.

D.F. = Dye-front.

500 schistosomula per well.

3% stacking gel/10% resolving gel.

can be identified. However, by far the most prominent secreted protein has molecular weight 20 000. The antigens associated with well-washed macrophages have Mr 100 000, 72 000, 47 000 and 38 000. The bands at 150 000, 30 000, 20 000 Mr in the macrophage tracks are very faint compared with the corresponding bands in the supernatants. Very low molecular weight material at and below the dye-front is also evident in the macrophage samples. Since the Mr 150 000, 30 000, 20 000 proteins are heavily labelled in the supernatants but only faintly so in the macrophage tracks, it would seem that the labelled proteins identified as associated with the cells are not simply adhering nonspecifically to the cell surfaces. The absence of the Mr 150 000, 30 000 and 20 000 secreted proteins from the macrophages may be due either to their not being taken up by the antigen presenting cells, or, at the opposite extreme, they may be phagocytosed and digested very rapidly, within the 5-hour incubation period. Pulse-chase experiments, examining cellular processing of the parasite antigens at much earlier times, might help distinguish between these possibilities. The presence of very low molecular weight material in the macrophage tracks is especially interesting, for it may represent peptides derived from antigen processing.

No differences appeared in the surface-labelled proteins of 48-hour old normal, irradiated and Actinomycin D-treated parasites, the schistosomular secretions, or the antigens taken up by macrophages cultured with the normal or attenuated forms.

6.6 Lectin binding to normal, irradiated and Actinomycin D-treated parasites.

Figure 6.15 illustrates the ability of various lectins to bind to the surface of normal, U.V.-irradiated and Actinomycin D-treated schistosomula, at 3 hours and 48 hours after mechanical transformation.

The lectins tested have the following specificities (in order of affinity):

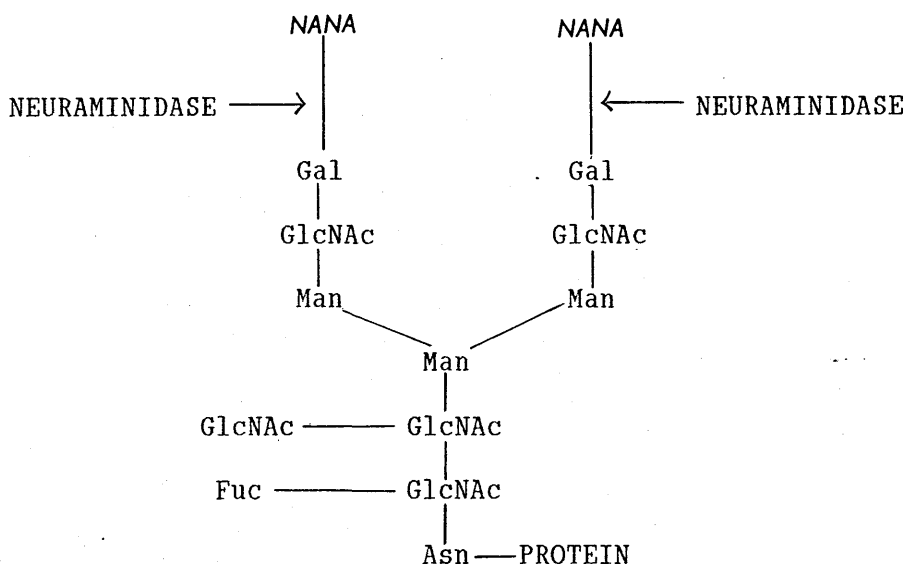
Concanavalin A: (Con A) — (1) Methyl α-D-mannopyranoside; (2) D(+)-mannose; (3) D(+)-glucose; (4) N-acetyl-D-glucosamine.

Wheat Germ Agglutinin: (WGA) — (1) N,N',N"- acetylchitotriose; (2) N-acetyl-D-glucosamine

Peanut Agglutinin: (PNA) — D(+)-galactose.

Specificity of lectin binding was assessed using an appropriate inhibitory sugar at high concentration (0.2M).

The presence of sialic acid (N-acetyl neuraminic acid) was tested for, using neuraminidase. By removing terminal sialic acid residues, this enzyme uncovers formerly hidden sugars, for instance, galactose on lactosomine-type oligosaccharides:



NANA = N-acetylneuraminic acid (sialic acid)

Gal = galactose

GlcNAc = N-acetylglucosamine

Man = mannose

Fuc = fucose

Asn = asparagine.

The resulting increased availability of galactose should be detectable by enhanced PNA binding.

Figure 6.15 demonstrates that U.V.-irradiation or Actinomycin D treatment had no quantitative effect on Con A, WGA or PNA binding to the schistosomular surface. Nor was total binding significantly different at 3 hours and 48 hours. Neuraminidase treatment had no effect on PNA binding, suggesting that no sialic acid was present. 0.2M N-acetyl-D-glucosamine only partially inhibited WGA binding. However, subsequent results indicated that N-acetyl-D-glucosamine may not be the ideal inhibitor for this lectin.

Experiments with 24-hour old, mechanically-transformed schistosomula gave some more information on the nature of the sugars exposed at the surface, but again revealed no differences between normal, irradiated and Actinomycin D-treated forms.

Figure 6.16 shows that similar amounts of Con A bound to normal, irradiated and Actinomycin D-treated parasites. Con A apparently bound to both mannose and N-acetylglucosamine residues on the schistosomular surface, since binding was almost equally inhibited by 0.2M methyl-α-D-mannopyranoside (80.5%) and N-acetyl-D-glucosamine (75%). However, steric hindrance, rather than specific blockade of lectin binding to a particular sugar moiety, is probably partly responsible for the reduction in binding effected by each sugar, since their total inhibition adds up to well over 100%.

WGA also showed no difference in binding to normal,

U.V.-irradiated and Actinomycin D-treated schistosomula (fig. 6.17). 0.2M N-acetyl-D-glucosamine blocked binding by 35-50%, but 0.2M N,N',N" - acetylchitotrose inhibited binding almost by 100%. This observation is in accordance with the report of Goldstein et al (1975) that N-acetyl-D-glucosamine is 4000 times less inhibitory for WGA than N,N',N" - acetylchitotriose. Ideally, however, a control should have been included to test the ability of N,N',N"-acetylchitotriose to block, non- specifically, binding by an irrelevant lectin such as Con A.

PNA also showed no difference in ability to bind to normal, U.V.-irradiated or Actinomycin D-treated schistosomula 24 hours after mechanical transformation. As in the previous experiments, neuraminidase treatment did not suggest that sialic acid was present (see fig. 6.18).

Simpson et al (1983a) found that fucose binding protein from L. tetragonolobus, but not UEA, another fucose-specific lectin, bound to schistosomula 3 hours after mechanical transformation. In our hands, binding of both lectins to 24-hour, formaldehyde-fixed schistosomula was low, but FBP-binding could be specifically inhibited by 44-50% in the presence of 0.2M fucose (see figure 6.19). The low level of binding as compared with the results of Simpson et al (1983a) may be due to loss of FBP-binding sites during the fixation procedure, or to the greater age of the parasites in our experiment, for Simpson et al (1983a) observed a pronounced decrease in FBP binding as the parasite developed. As for the other lectins, there was no difference in FBP binding to normal, irradiated or Actinomycin D-treated schistosomula (fig. 6.19).

Figure 6.15 Binding of FITC-conjugated Con A, WGA and PNA to normal, irradiated and Actinomycin D-treated schistosomula at 3 hours and 48 hours after mechanical transformation.

N = normal schistosomula.

I = U.V.-irradiated schistosomula (400 μW min cm^{-2})

A = Actinomycin D-treated schistosomula.

C: □ = Con A binding

▒ = Con A binding in presence of 0.2M methyl-α-D-manno-pyranoside

W: □ = WGA binding

▒ = WGA binding in presence of 0.2M N-acetyl-D-glucosamine

P: □ = PNA binding

▒ = PNA binding in presence of 0.2M D(+)-galactose.

N/P: □ = PNA binding after neuraminidase treatment.

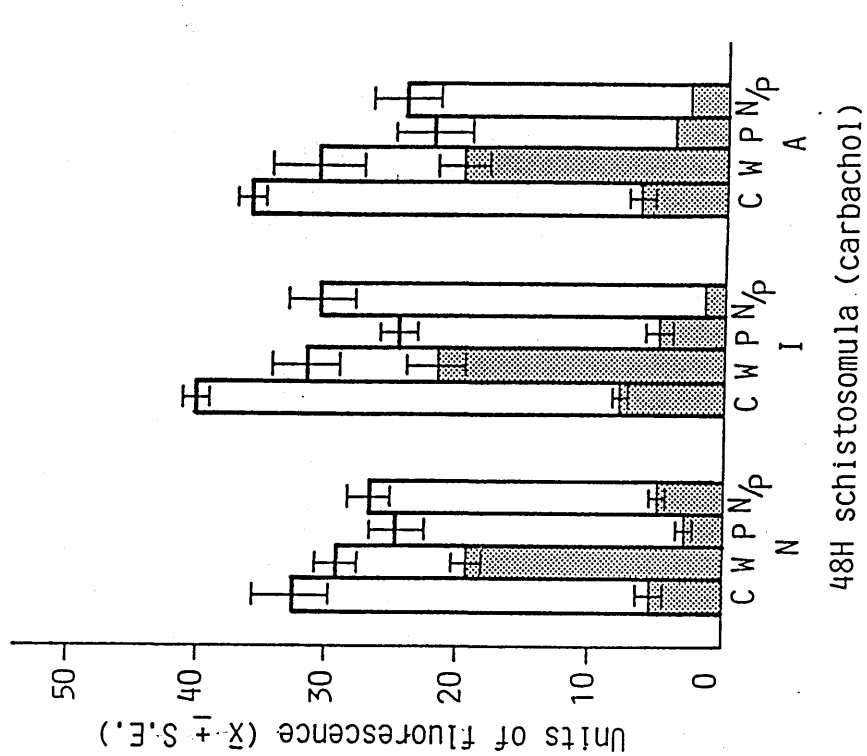
▒ = PNA binding in presence of 0.2M D(+)-galactose, after neuraminidase treatment.

Cercariae were transformed mechanically in Elac, and cultured in Elac/10% hiFCS at 37°C/5% CO_2.

Parasites were immobilized by carbachol treatment.

Fluorescent lectin binding was performed as described in section 2.6.3.2.

Each bar represents the mean of 20 to 25 readings.


Units of fluorescence (X̄ + S.E.)
0
10
20
30
40
50
C W P N/P
N
C W P N/P
I
C W P N/P
A
48H schistosomula (carbachol)

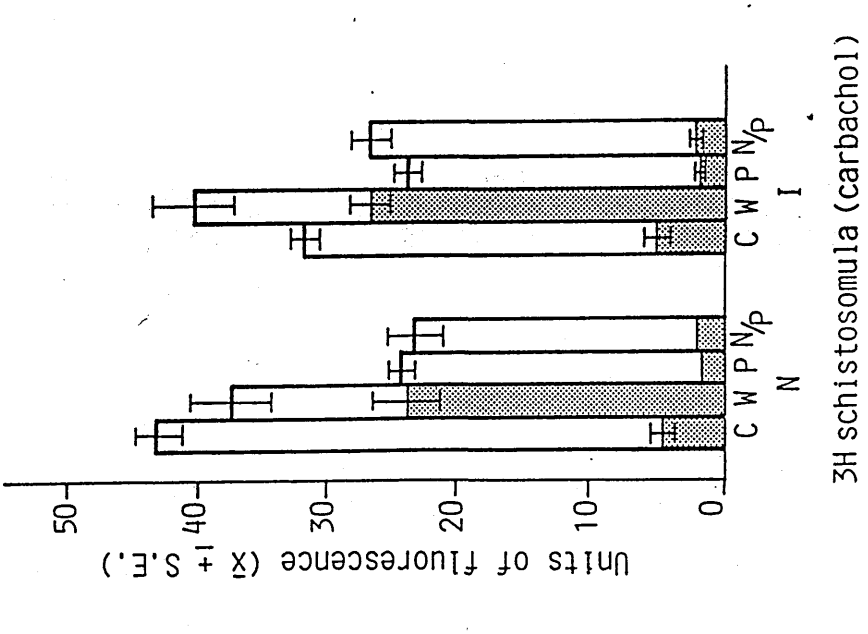

Units of fluorescence (X̄ + S.E.)
0
10
20
30
40
50
C W P N/P
N
C W P N/P
I
3H schistosomula (carbachol)

Figure 6.16 Binding of FITC-Con A to normal, irradiated and Actinomycin D-treated schistosomula, formaldehyde-fixed 24 hours after mechanical transformation.

N = normal schistosomula.

I = U.V.-irradiated schistosomula (400 μW min cm^{-2})

A = Actinomycin D-treated schistosomula.

C  = Con A binding

C + G  = Con A binding in presence of 0.2M methyl-α-D-mannopyranoside

C + N  = Con A binding in presence of 0.2M N-acetyl-D-glucosamine.

Cercariae were transformed mechanically in Elac, and cultured in Elac/10% hiFCS for 24 hours at 37°C/5%CO_2. Formaldehyde-fixation and fluorescent lectin binding were performed as described in section 2.6.3.2.

Each bar represents the mean of 20 to 25 readings.

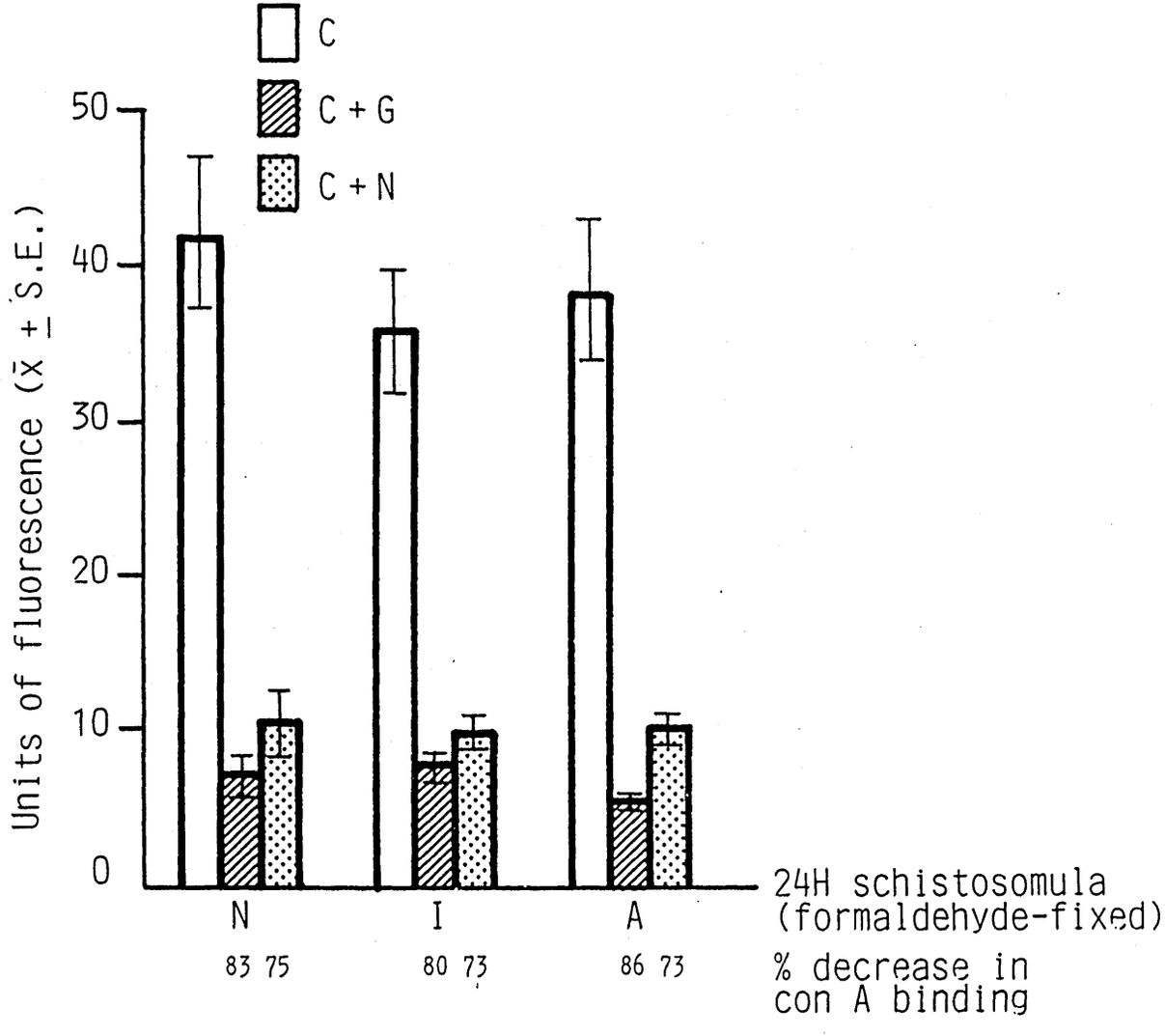

C
C + G
C + N
Units of fluorescence ($\bar{x}$ ± S.E.)
50
40
30
20
10
0
N
I
A
24H schistosomula
(formaldehyde-fixed)
83 75
80 73
86 73
% decrease in
con A binding

Figure 6.17 Binding of FITC-WGA to normal, irradiated and Actinomycin D-treated schistosomula, formaldehyde-fixed 24 hours after mechanical transformation.

N = normal schistosomula.

I = U.V.-irradiated schistosomula (400 μW min cm^{-2})

A = Actinomycin D-treated schistosomula.

W  : WGA binding.

W + N  : WGA binding in presence of 0.2M N-acetyl-D-glucosamine.

W + Ch  : WGA binding in presence of 0.2M NNN"-acetylchitotriose.

Cercariae were transformed mechanically in Elac, and cultured in Elac/10% hiFCS at 37°C/5% CO_2.

Formaldehyde-fixation and lectin binding were performed as described in section 2.6.3.2.

Each bar represents the mean of 20 to 25 readings.

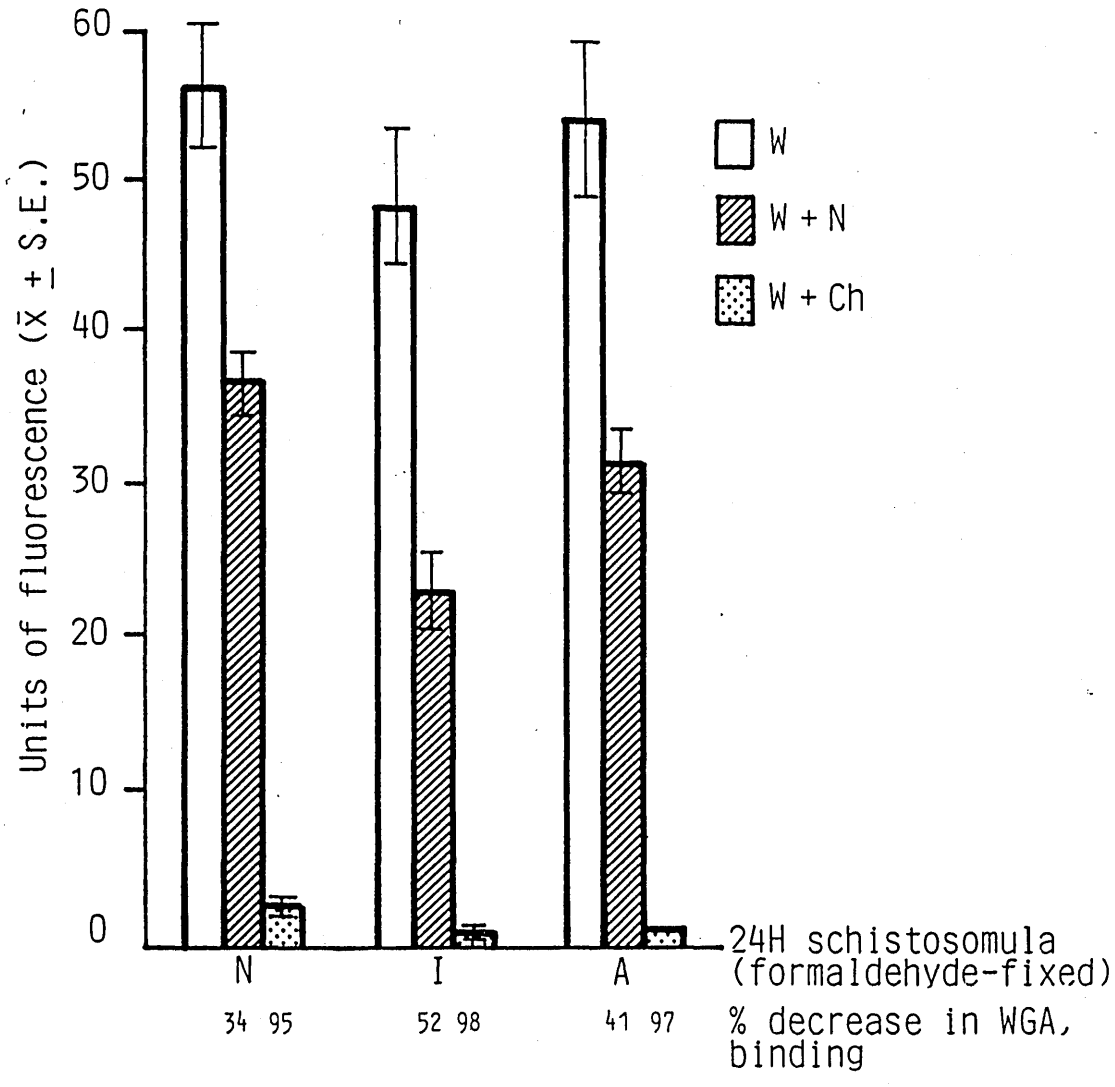

60
50
40
30
20
10
0
Units of fluorescence ($\bar{X} \pm$ S.E.)
W
W + N
W + Ch
N
I
A
24H schistosomula (formaldehyde-fixed)
34 95
52 98
41 97
% decrease in WGA, binding

Figure 6.18 Binding of FITC-PNA to normal, U.V.-irradiated and Actinomycin D-treated schistosomula, formaldehyde-fixed 24 hours after mechanical transformation.

P  = PNA binding.

P + G  = PNA binding in presence of 0.2M D-(+)-galactose

N/P  = PNA binding after neuraminidase treatment.

N/P + G  = PNA binding in presence of 0.2M D-(+)-galactose after neuraminidase treatment.

Schistosomula were transformed mechanically in Elac, and cultured in Elac/10% hiFCS at 37°C/5% CO_2.

Formaldehyde-fixation, neuraminidase treatment and fluorescent lectin binding were performed as described in section 2.6.3.2.

Each bar represents the mean of 20 to 25 readings.

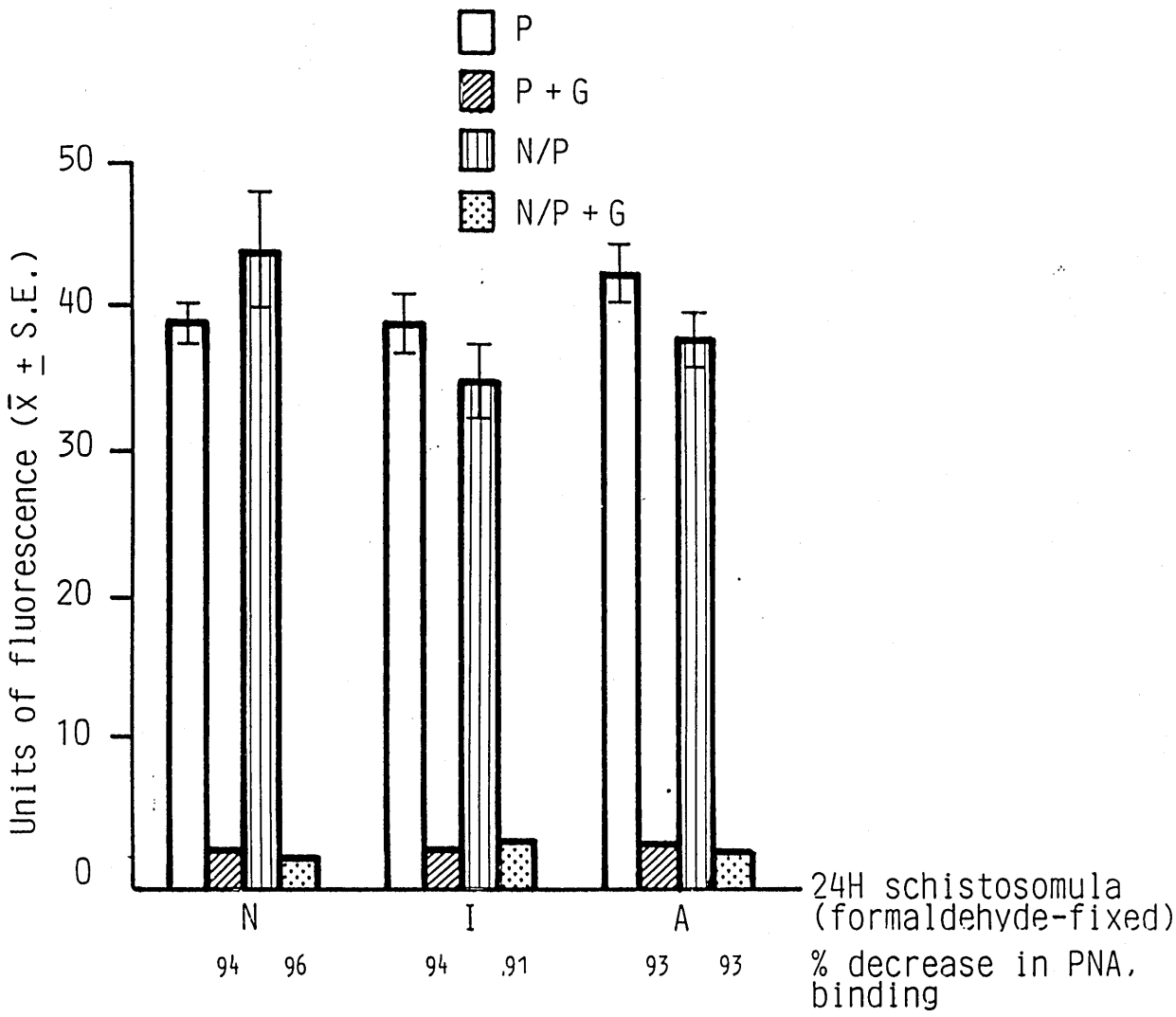

P
P + G
N/P
N/P + G
Units of fluorescence ($\bar{x} \pm$ S.E.)
50
40
30
20
10
0
N
I
A
24H schistosomula (formaldehyde-fixed)
94 96
94 91
93 93
% decrease in PNA. binding

Figure 6.19 Binding of FITC-conjugated UEA and FBP to normal, U.V.-irradiated and Actinomycin D-treated schistosomula, formaldehyde-fixed 24 hours after mechanical transformation.

U = UEA binding

U + f = UEA binding in presence of 0.2M L(+)-fucose.

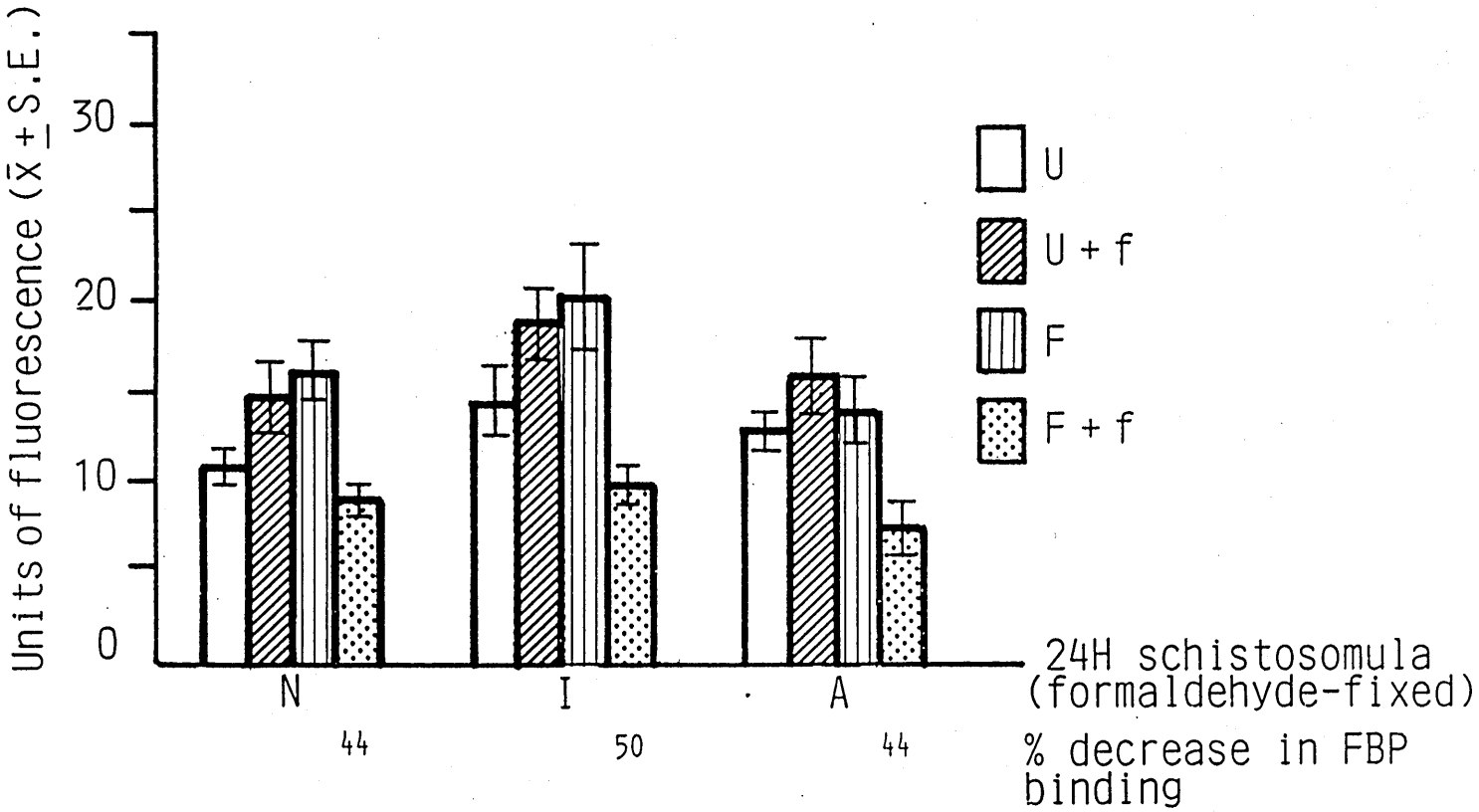
F = FBP binding

F + f = FBP binding in presence of 0.2M L(+)-fucose.

Cercariae were transformed mechanically in Elac, and cultured in Elac/10% hiFCS.

Formaldehyde-fixation and fluorescent lectin binding were performed as described in section 2.6.3.2.

Each bar represents the mean of 20 to 25 readings.


Units of fluorescence ($\bar{X} \pm$ S.E.)
30
20
10
0
U
U + f
F
F + f
N
I
A
24H schistosomula
(formaldehyde-fixed)
44
50
44
% decrease in FBP
binding

6.7 Electron microscopy of the surface of normal and irradiated schistosomula during 80 hours in culture.

The surfaces of normal and irradiated schistosomula were examined by electron microscopy at 30, 50 and 80 hours of culture following skin penetration.

(1) 30-hour normal and irradiated schistosomula

Figures 6.20 a) to d) show the surface of normal schistosomula cultured in Elac/10% human serum for 30 hours after skin transformation. The highly folded and pitted nature of the surface, as described by Hockley and McLaren (1973) and McLaren (1980) is evident (a, b). The trilaminate basal membrane underlying the granular tegumental cytoplasm can be seen (a, c), and the circular and longitudinal muscle layers beneath. As described by Hockley and McLaren (1973), the outer surface shows the heptalaminate structure typical of bloodstream helminths (McLaren and Hockley, 1977). The tegumental bodies, consisting of tightly-packed membranous whorls, contribute to and renew this heptalaminate surface (c, d).

The surface of irradiated schistosomula after 30 hours in culture appears identical to the normal one (Figure 6.21a, b, c). Even at the highest magnification, there seems to be no defect in formation of the heptalaminate membrane (figure c). The tegumental cytoplasm is intact, and rich in multilaminate vesicles, as in normal schistosomula (figures a, b). However, the interior of the irradiated parasite body does show some evidence of disorganisation compared to the normal 30-hour form. Internal disruption of the irradiated parasite body would be predicted from observations at the light microscope level, for by 30 hours after transformation, the irradiated schistosomula begin to lose translucency, becoming increasingly granular and shrunken with time in

culture.

(2) 50-hour normal and irradiated schistosomula.

At 50 hours, the surface is convoluted and clearly heptalaminate in both normal and irradiated parasites (figures 6.22, 6.23).

(3) 80-hour normal and irradiated schistosomula.

Even at 80 hours, when some disintegration of the parasite membrane might be expected in the irradiated parasites, since, at the level of the light microscope, they are granular and shrunken, electron microscopy shows no disruption of the surface; the heptalaminate membrane is still intact (figures 6.24, 6.25).

In many of these specimens, a thin layer of diffuse granular material can be seen on the outer surface of the parasite. Hockley and McLaren (1973) also observed this diffuse material adhering to the surface of skin schistosomula maintained in Elac/human plasma for 24 or 48 hours and concluded that this diffuse "coat" was artifactual, being material absorbed from the culture medium.

Although no differences were observed in the surfaces of normal and irradiated parasites at these time points, it would have been valuable to examine the morphology of the parasite surface during the 5 hours immediately following transformation, when the immunofluorescence results of figs., 6.1, 6.6, 6.9, 6.11, 6.12 suggest that irradiation disrupts the organisation of the glycocalyx and underlying membrane (see discussion in section 6.9.1).

6.8 Acquisition of blood-group antigens by normal and irradiated schistosomula.

Acquisition of host blood-group substances is considered to be a

Figures 6.20-6.25 Samples were processed for electron microscopy as described in section 2.6.5.

Figure 6.20 a)-d). The surface of normal schistosomula, cultured in Elac/10% A^+ human serum for 30 hours after skin penetration

a) MAGNIFICATION x 6638

S = spines

B.M. = basal membrane (trilaminate)

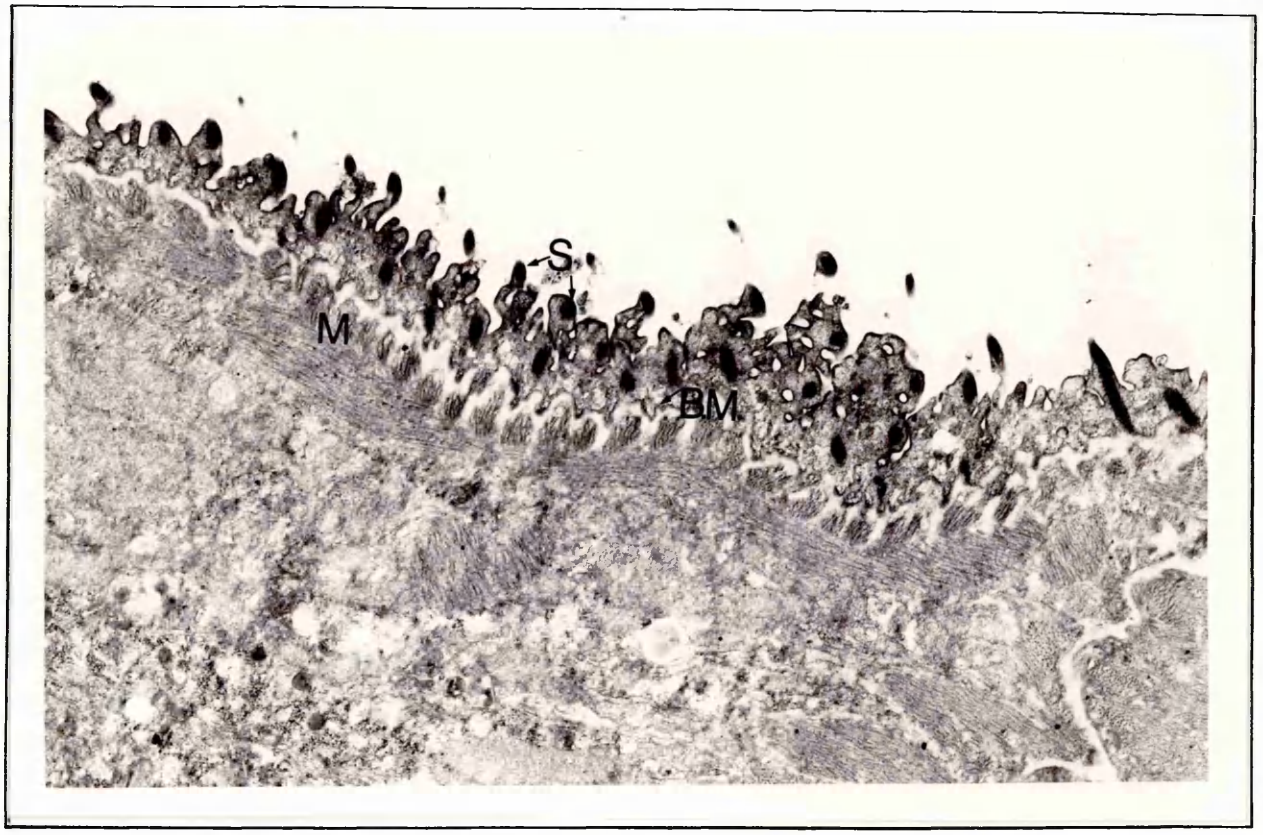
M = muscle layers.

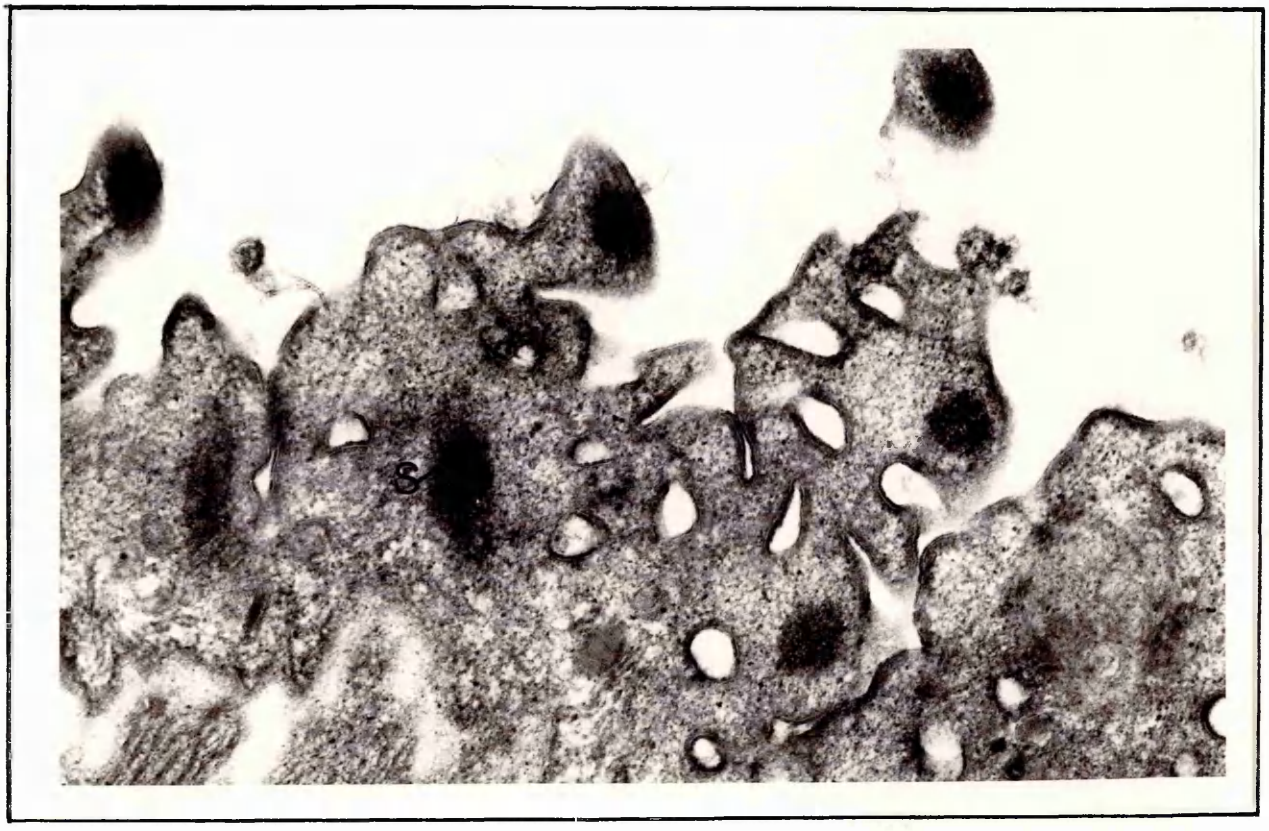
b) MAGNIFICATION x 31500

The pitted and convoluted nature of the surface is clear.

S = spine

6.20a





6.20b

c) MAGNIFICATION x 40500

S = spine

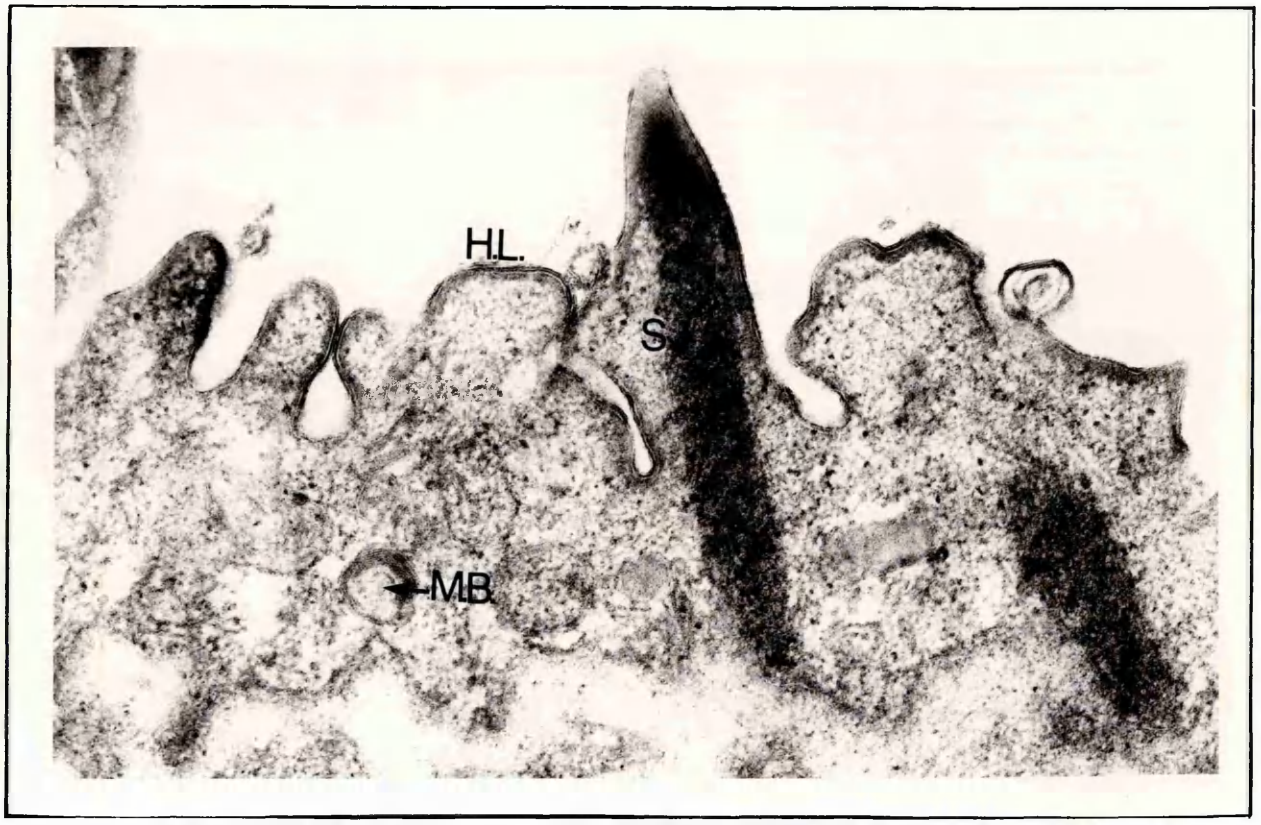
H.L. = heptalaminate membrane

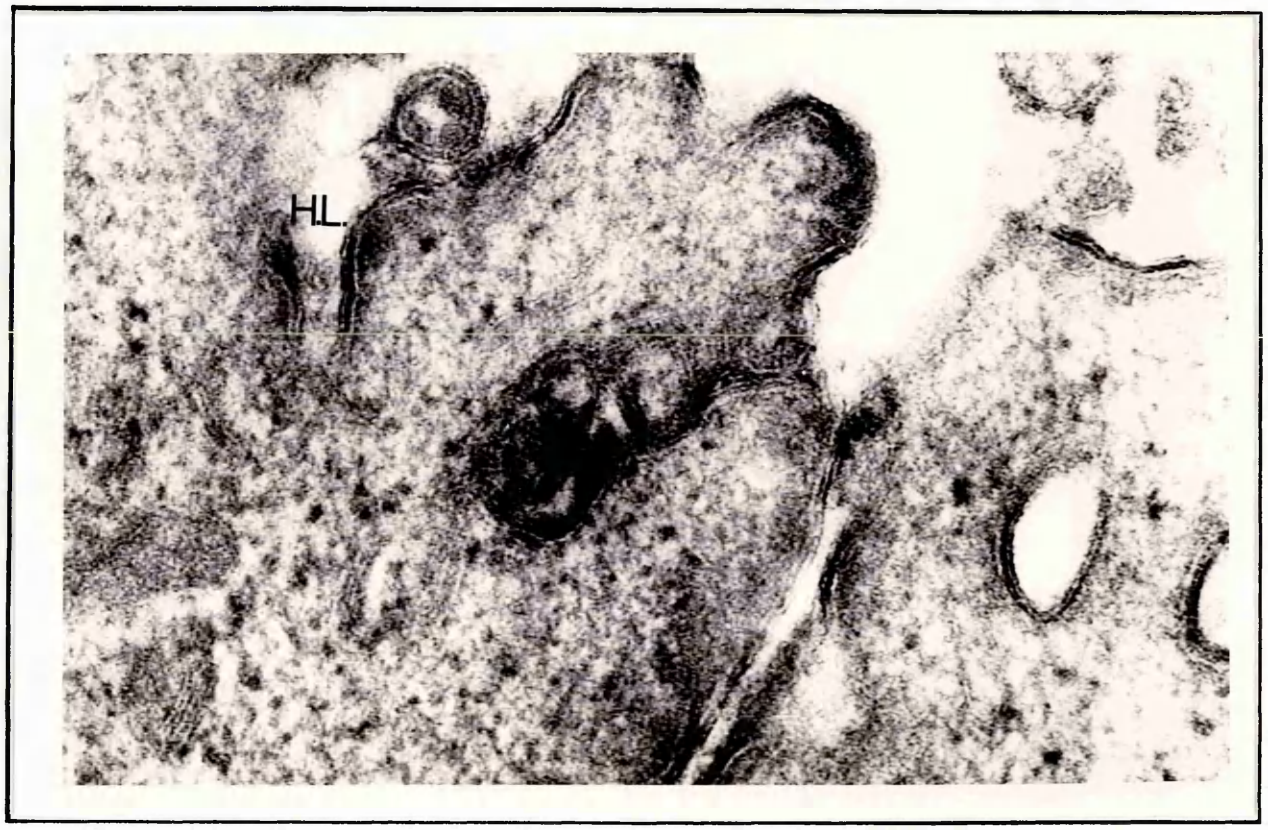
M.B. = membranous bodies

d) MAGNIFICATION x 110250

The heptalaminate nature of the outer surface (H.L.) is especially clear at this very high magnification.

6.20c





6.20d

Figure 6.21 a) to c)

The surface of U.V.-irradiated schistosomula (800 μW min cm^{-2}), cultured in Elac/10% A^+ human serum for 30 hours following skin penetration.

a) MAGNIFICATION x 6638.

S = spine

B.M. = basal membrane

M = muscle layers

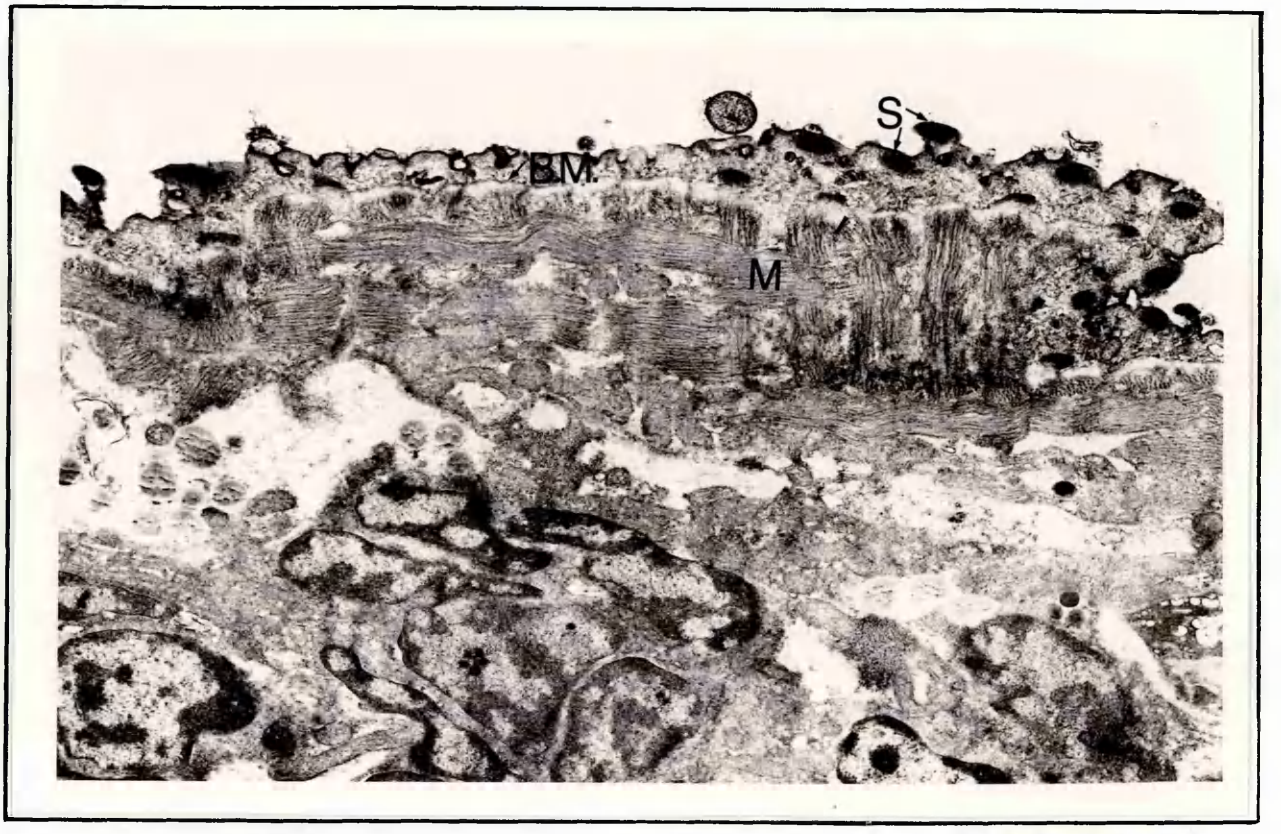
b) MAGNIFICATION x 48375

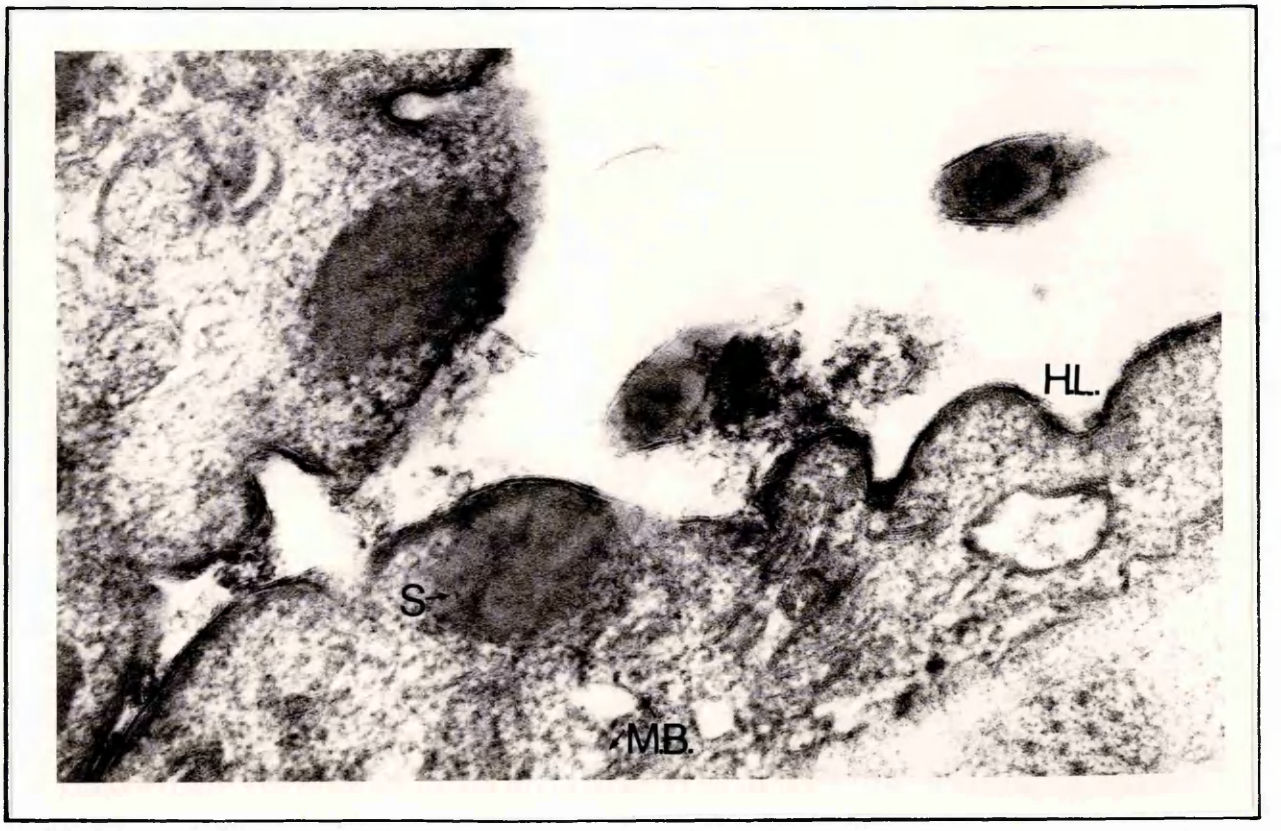
S = spine

H.L. = heptalaminate membrane

M.B. = membranous bodies

6.21a





6.21b

c) MAGNIFICATION x 110250

The heptalaminate membrane (H.L.) is very clear at this high magnification.

M.B. = membranous body.

6.21c

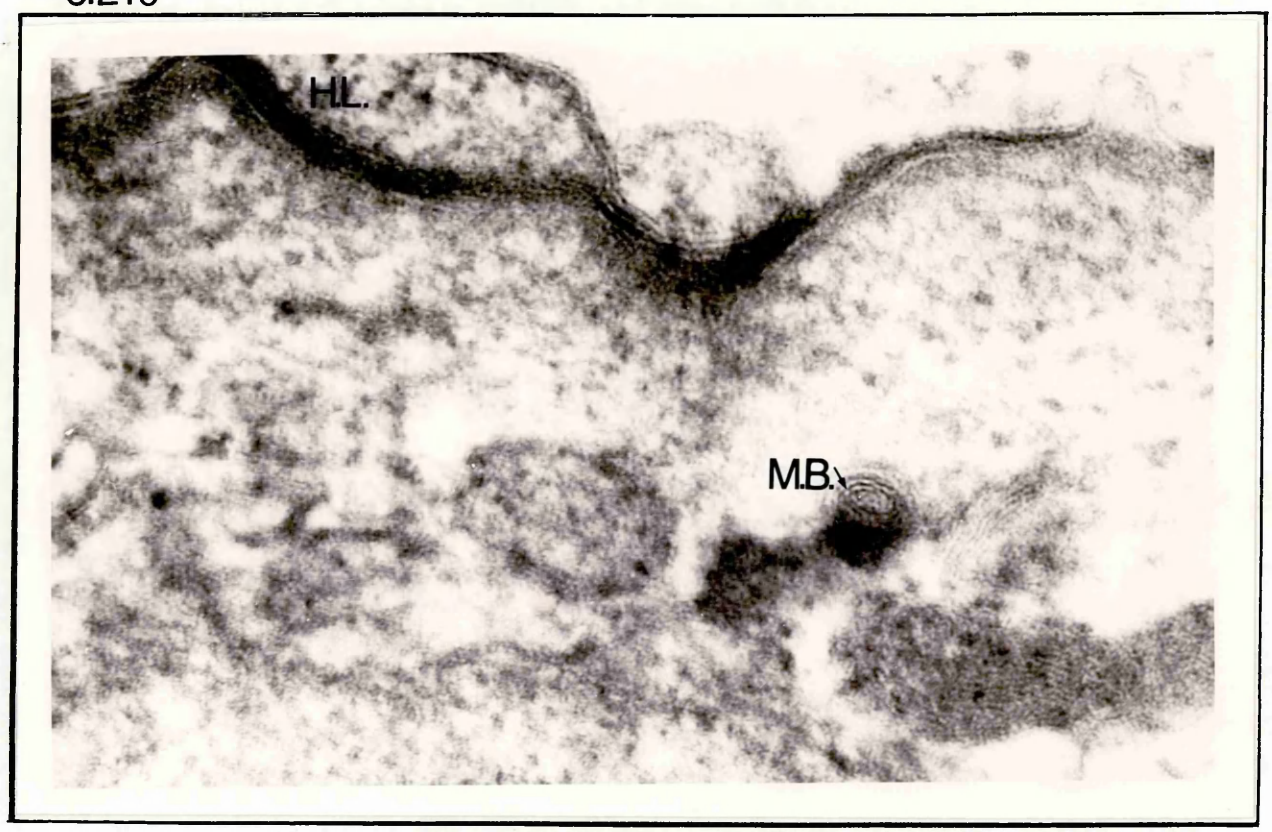


Figure 6.22 a), b)

Surface of normal schistosomula, cultured in Elac/10% A^+ human serum for 50 hours after skin penetration.

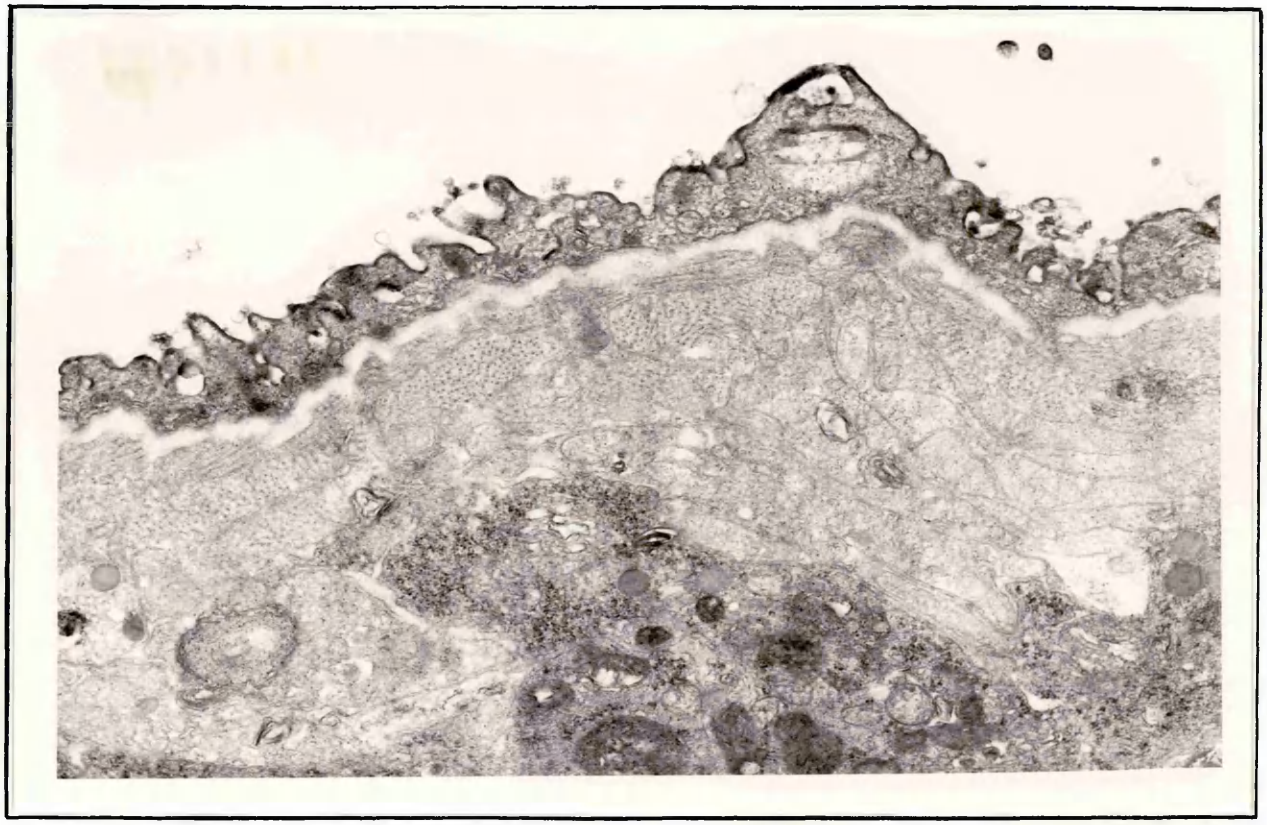
a) MAGNIFICATION x 11025

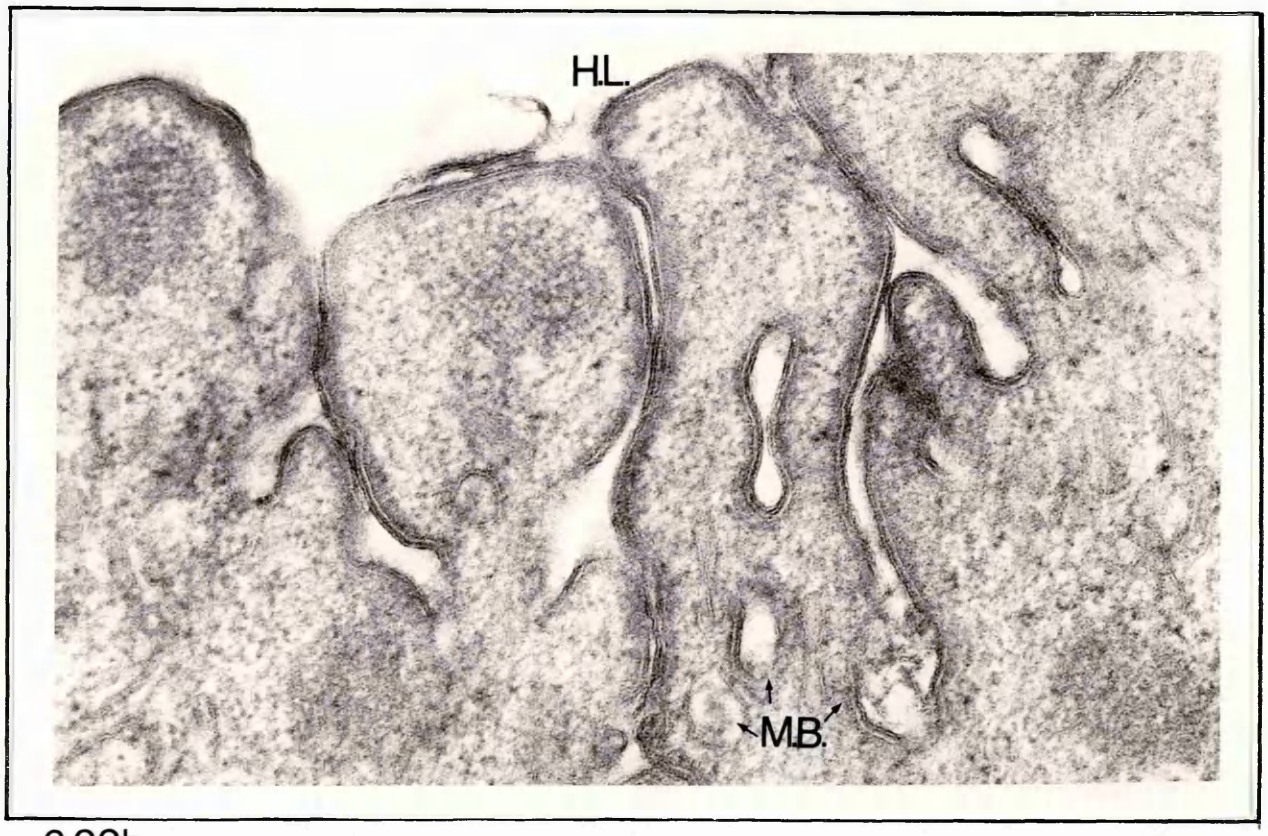
b) MAGNIFICATION x 66375

Shows the highly convoluted surface and heptalaminate membrane (H.L.).

M.B. = membranous bodies.

6.22a





6.22b

Figure 6.23 a), b). Surface of U.V.-irradiated schistosomula (800 μW min cm^{-2}), cultured in Elac/10% A^+ human serum for 50 hours after skin penetration.

a) MAGNIFICATION x 40500

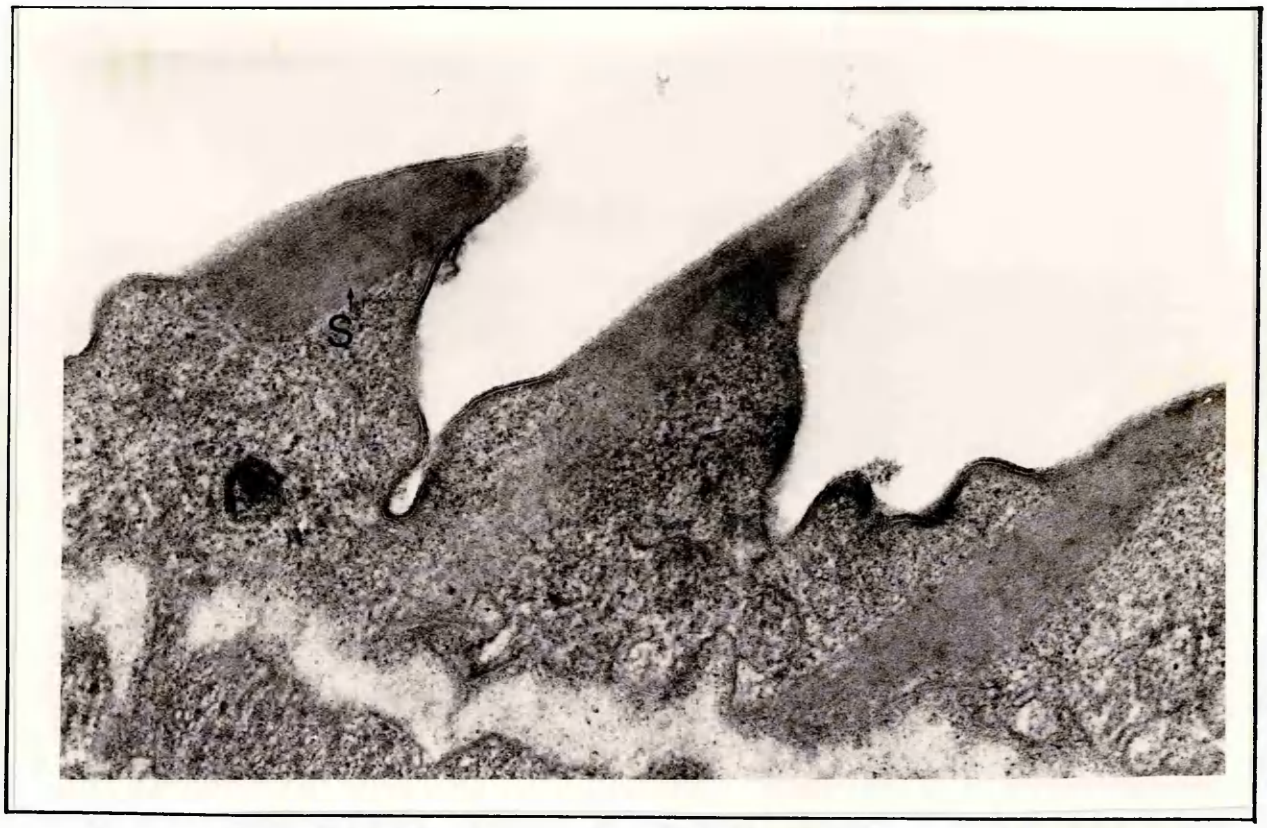
S = spine

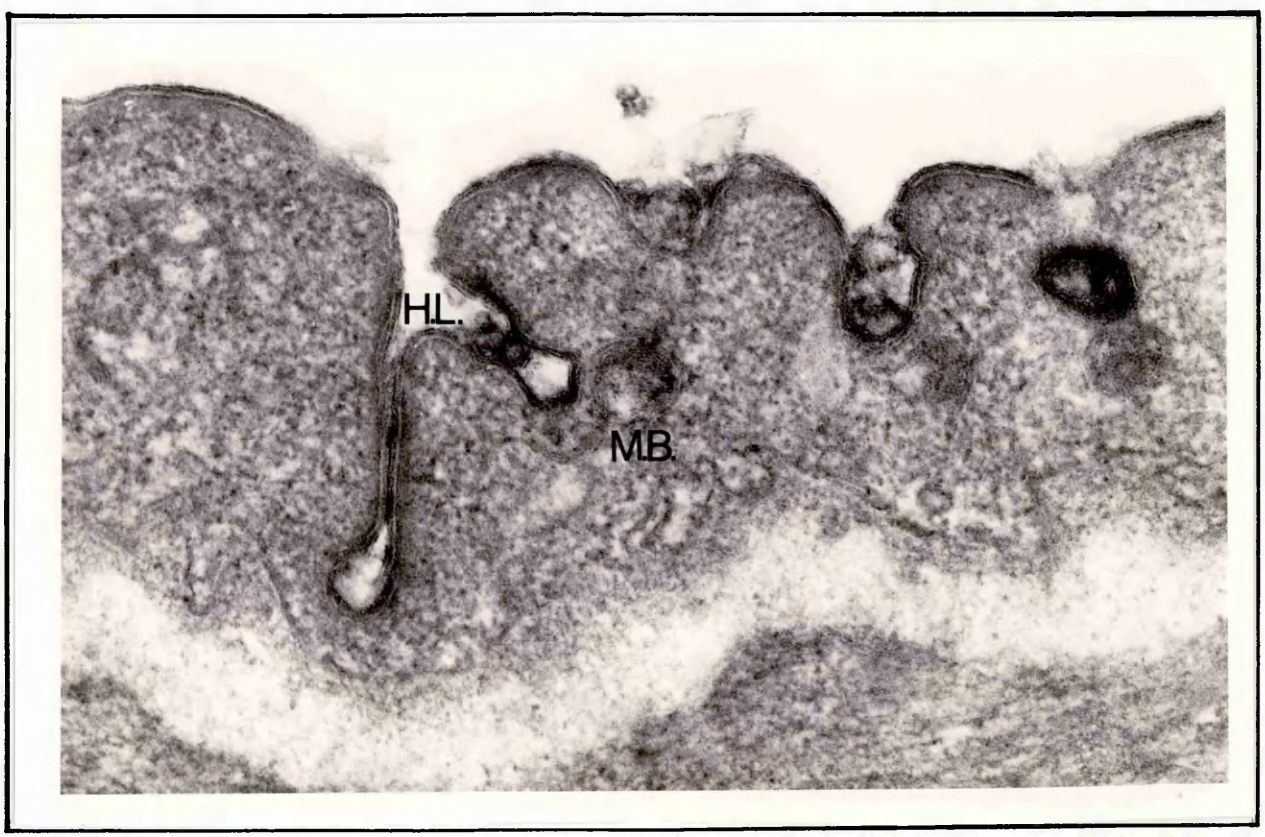
b) MAGNIFICATION x 60750

H.L. = heptalaminate membrane

M.B. = membranous bodies.

6.23a





6.23b

Figure 6.24

Surface of normal schistosomulum, cultured in Elac/10% A^+ human serum for 80 hours after skin penetration.

MAGNIFICATION x 66375

H.L. = heptalaminate membrane

M.B. = membranous body

6.24

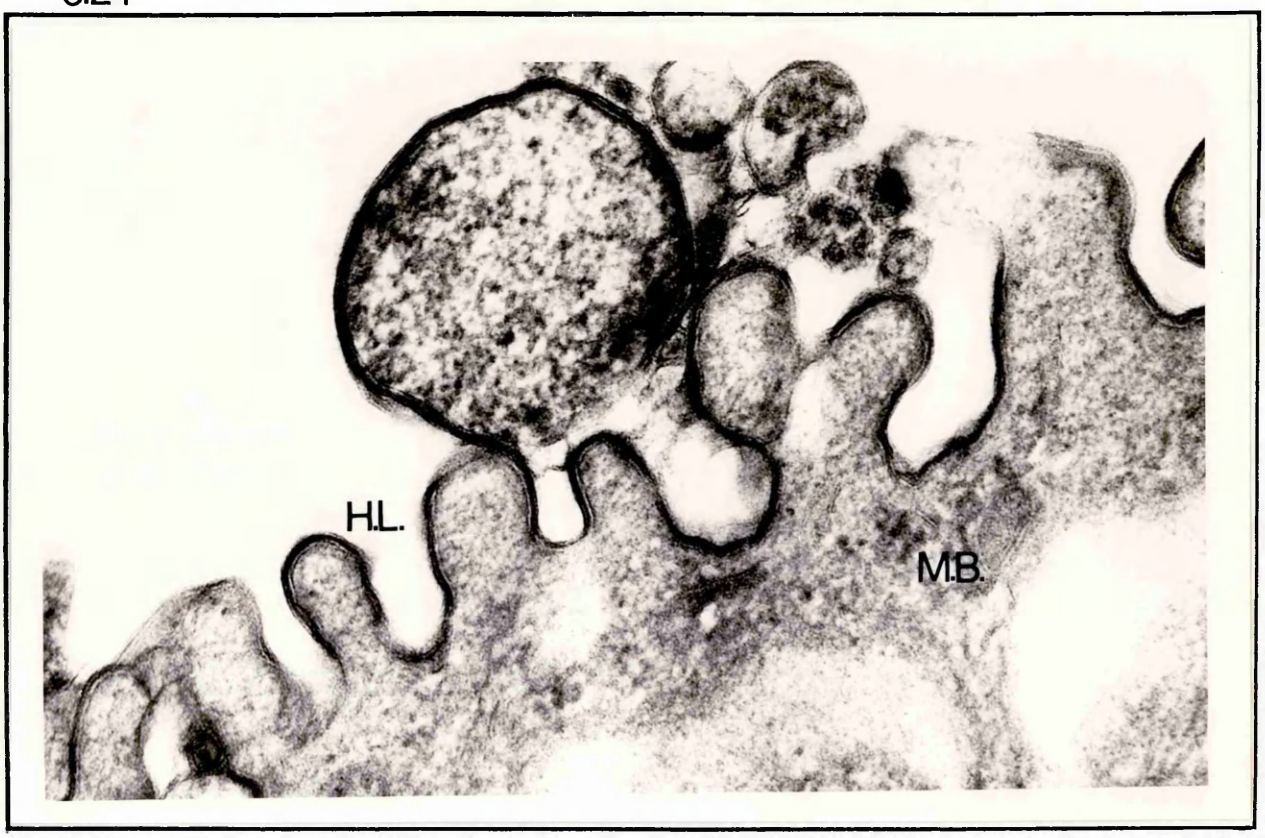


Figure 6.25 Surface of U.V.-irradiated schistosomulum (800 μW min cm^{-2}) cultured in Elac/10% A^+ human serum for 80 hours after skin penetration.

MAGNIFICATION x 84375

H.L. = heptalaminate membrane

M.B. = membranous body.

6.25

stratagem for masking of parasite antigens by developing schistosomula (Smithers et al, 1969; Clegg et al, 1971; Clegg, 1974; McLaren, 1984; section 1.5.7). If this uptake were inhibited in irradiated schistosomula, the host immune system could be exposed to parasite antigens normally disguised by host material. Since Imohiosen et al (1978) had shown that schistosomula, cultured in vitro with mouse red blood cells for as short a time as 24 hours, showed a measurable, though low, reaction with anti-mouse red blood cell serum, it seemed justifiable to investigate uptake of human blood group A substance by normal and U.V.-irradiated schistosomula after 72 hours of culture with A+ human plasma and A+ human red blood cells.

Figure 6.26 shows the results of this experiment. Uptake of blood group A antigens was not inhibited in U.V.-irradiated schistosomula.

6.9 Discussion: Antigens expressed by normal, U.V.-irradiated and Actinomycin D-treated schistosomula.

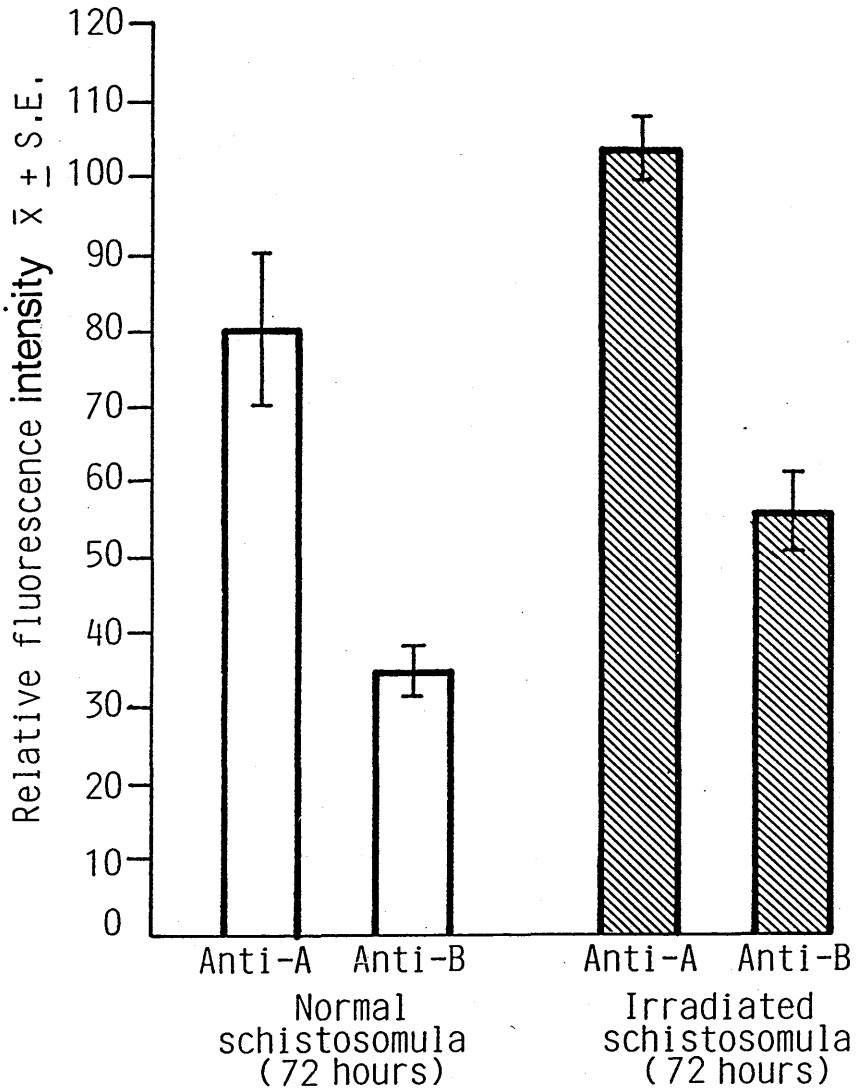
6.9.1 Effects of irradiation on glycocalyx structure and immunogenicity.

Infected human serum, anti-CMAG antiserum and monoclonal antibody A3 all showed increased binding to U.V.-irradiated cercariae, while anti-coat 1 and anti-haemolymph antiserum showed higher binding to the U.V.-irradiated schistosomular surface during the 5-hour period after transformation. A3 showed an initial increase in binding to the surface of newly-transformed schistosomula, but by 3 hours after transformation, this declined to well below normal levels. These observations suggest that irradiation may severely disrupt the organisation of the cercarial glycocalyx and surface membrane during transformation. This disruption may be reinforced and exaggerated by the metabolic effects of the irradiation. Such alterations in surface

Figure 6.26 Binding of anti-blood group substance A and anti-blood group substance B monoclonals to normal and U.V.-irradiated schistosomula cultured for 72 hours in Elac in the presence of 50% A^+ human plasma/1% A^+ human red blood cells.

Cercariae were transformed mechanically, and cultured in Elac supplemented as described above, at 37°C/5% CO_2 for 72 hours.
Irradiation dose was 400 μW min cm^{-2}
Indirect immunofluorescence was performed as described in section 2.6.3.1.
Parasites were immobilized by carbachol treatment.
Each bar represents the mean of 20-25 readings.

Binding of anti-blood group substance A and anti-blood group substance B monoclonals to normal and irradiated schistosomula cultured for 72 hours in the presence of human A^+red blood cells



antigenicity may contribute to the enhanced immunogenicity of the irradiated parasite.

6.9.1.1. The cercarial glycocalyx: its structure and loss during transformation.

The scanning electron micrographs of Samuelson and Caulfield (1985) depicted the cercarial glycocalyx as a dense mesh of 15-30 nm. fibrils. Subsequent morphological and biochemical studies (Caulfield et al, 1987) led these authors to describe its structure as "particulate"; particles of minimum size 15-20nm appeared to be held together by noncovalent forces to form larger polymers. During transformation, the cercarial trilaminate membrane and associated glycocalyx are assembled into 3-5μm microvilli which are then shed from the parasite surface.

Caulfield et al (1987) described release of the glycocalyx as a process of depolymerisation, aggregates of glycocalyx material being broken up into smaller particles, either physically, by mechanical stress, or in response to the increase ionic strength of the transformation medium.

However, it seems that transformation, at least by mechanical means, does not completely clear the glycocalyx. Thus, when Samuelson and Caulfield (1982) mechanically transformed cercariae whose surfaces had been radiolabelled by galactose oxidase and NaB^3H_4, more than 30% of the tritium label was retained by schistosomula several hours after mechanical transformation. This residual glycocalyx took the form of a 0.1-0.5 μm fibrillar network on the parasite surface. In mechanical forms, these glycocalyx epitopes appear to persist for several days. This observation may explain the results shown in figure 6.12, where antiserum raised against cercarial membrane antigens binds to normal cercariae to the same extent as to 72-hour schistosomula

cultured in Elac/10% hiFCS after mechanical transformation. In vivo, however, these antigens characteristic of cercariae disappear as the schistosomula develop into lung forms. Thus, Imohiosen et al (1978), using immunofluorescence with infected mouse serum, observed that, when 3-hour, mechanically-transformed schistosomula were injected intravenously in mice, and collected a day later from mouse lung, there was a striking reduction in availability of parasite antigens. In contrast, when the schistosomula developed for the same period in Elac/40% hiFCS in vitro, their surface antigens remained unchanged, as detected by antibody binding. This latter observation is consistent with the results presented here.

It seems that even a brief exposure to in vivo conditions may induce loss or concealment of parasite antigens, for Dean (1977) found that schistosomula transformed by penetration of excised mouse skin prior to culture in defined medium showed decreased binding of infection serum compared to cercariae or mechanical forms. This surface change also occurred in the presence of metabolic inhibitors. Thus, it did not appear to depend on active physiological processes. Figure 6.6 presented here supports these observations on antigen loss from skin forms, for the "snail-like" epitopes identified by anti-haemolymph antiserum were not detectable on 4-hour skin forms, but remained present at a constant level on the surface of mechanical schistosomula of the same age. Irradiated schistosomula show a similar loss of snail epitopes at this time, despite the metabolic inhibition in these forms. Reduced antibody binding to the schistosomulum surface correlates with development of resistance to the cytotoxic effects of antibody by the parasite maturing in the host (McLaren, 1980). This phenomenon has been attributed to both the masking effects of acquired host antigen, and development and rapid turnover of the schistosomular heptalaminate membrane (Dean, 1977; McLaren et al, 1975; McLaren, 1980; see section 1.5.7).

As regards the composition of the glycocalyx, the biochemical analysis of Samuelson and Caulfield (1987) indicated that more than 80% of the cercarial glycocalyx was carbohydrate in nature, the remainder consisting of protein. The principal sugar moieties identified were fucose, galactose, mannose, N-acetylgalactosamine and N-acetyl-glucosamine. Gel filtration analysis gave a molecular weight of approximately 5 000 000 for the glycocalyx.

The altered pattern of antibody binding to irradiated larvae - cercariae and newly-transformed schistosomula - might be attributed to the fact that irradiation induces degradative changes in carbohydrate polymers such as the glycocalyx. Such disruption may make antigenic determinants in the glycocalyx itself more accessible to antibody binding, or may expose epitopes which are normally hidden in the underlying membrane (see figure 6.27).

As described in section 1.10.3.5, radicals produced by irradiation can induce scission of glycosidic bonds, and loss of fragments from carbohydrate polymers. Disruption of the cell wall of M. radiodurans irradiated in aqueous suspension has been attributed to the action of the ·OH radical in this way (Mitchel, 1981). A small number of breaks or crosslinks can then markedly alter the physical properties of a carbohydrate polymer. Irradiated cellulose, for instance, shows a decrease in tensile strength, elongation and elasticity (Phillips, 1970; von Sonntag, 1987). Partial depolymerisation of irradiated hyaluronic acid, heparin and amylose have been observed (Phillips, 1970).

Reactions 63 to 68 in section 1.10.3.5 describe how radiolysis of oligosaccharides may result in fragmentation of carbohydrate polymers, and release of sugar moieties.

Disaggregation of the tightly coherent fibrils of the glycocalyx could make its antigenic sites more available for antibody binding in irradiated parasites, both before transformation and during its

Figure 6.27 Availability of epitopes for antibody binding in the normal and irradiated glycocalyx.

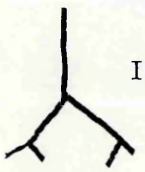
1. The normal glycocalyx.

The compact, highly cross-linked organisation of the carbohydrate polymers prevents access of many antibodies to the antigenic sites.

△ = Epitope accessible to antibody in normal glycocalyx.

▲ = Epitope not accessible to antibody in normal glycocalyx.

× In this region, antibody binding is sterically prevented.



IgG molecule.

2. The irradiated glycocalyx.

Radiation-induced fragmentation and degradation of the carbohydrate polymers exposes more antigenic sites for antibody binding.

△ = Epitope accessible to antibody in normal and irradiated glycocalyx.

▲ = epitope not accessible to antibody in normal glycocalyx, but available in the irradiated glycocalyx.

▲ = site not accessible to antibody in normal or irradiated glycocalyx.

(2.)

(1.)

removal. Figure 6.27 illustrates how the irradiated glycocalyx structure might become more accessible to antibody binding, as indicated by the immunofluorescence studies of figures 6.1, 6.6, 6.11, 6.12.

A slightly different explanation might be proposed for the altered binding by monoclonal A3 to irradiated parasites. A3 apparently binds specifically to the surface of normal schistosomula, at 3 hours after mechanical transformation, but not to normal cercariae (see figure 6.9). The enhanced binding of this monoclonal to the surface of irradiated cercariae, and irradiated schistosomula up to one hour after transformation, suggests that irradiation - induced disruption of the glycocalyx may expose antigens in the underlying membrane, or even the tegument, which are exposed by normal schistosomula only as the glycocalyx is released. The decline in A3 binding to the irradiated surface coincides approximately with the time of completion of the heptalaminate membrane (Hockley and McLaren, 1973). Completion of the double bilayer and maximal exposure of A3 antigens occur at approximately the same time in normal schistosomula (3 hours). It is therefore surprising that irradiated parasites, whose formation of the double bilayer is apparently unimpaired (figures 6.20-6.25), display A3 antigen only transiently. A possible explanation might be that irradiation reveals the antigen sites by inducing a change in protein conformation, as a consequence of deglycosylation or some other radiolesion. However, such aberrant protein structures, especially when abnormally glycosylated or non-glycosylated, are especially vulnerable to protease digestion (Goldberg and St. John, 1976; Rademacher et al, 1988; Olden et al, 1982). Thus, irradiation might prematurely reveal antigenic epitopes, but proteolysis removes them just when normal development of the schistosomular membrane would expose the same antigenic sites.

6.9.1.2. Effects of altered glycocalyx structure on parasite immunogenicity.

The importance of the glycocalyx in stimulating gamma-irradiated vaccine immunity is indicated by the work of Sher and Benno (1982) and Bickle et al (1979), who both observed that better protection was induced in mice by exposure to irradiated cercariae than schistosomula.

As described in chapter 4, alterations in the carbohydrate moieties of glycoproteins may enhance their immunogenicity by exposing new antigenic epitopes. The irradiation-induced disruption of the cercarial glycocalyx may have such an effect. Howans et al (1987) have described how oligosaccharides exist in solution with regions of well-defined 3-dimensional structure. Their overall conformation is modulated by certain key residues, so that even limited radiation damage, if it affects these important targets, may markedly alter the structure assumed by the carbohydrate polymers of the glycocalyx.

Alexander and Elder (1985) and Feizi and Childs (1987) describe how alterations in carbohydrate moieties can expose cryptic antigens that may be processed and presented to stimulate the host immune system especially effectively. The carbohydrate moieties of glycoproteins play a dominant role in determining the sites of action of proteases (Goldberg and St. John, 1976; Rademacher et al, 1988). Thus, we would suggest that irradiated schistosomula may release glycocalyx antigens whose structure is modified in such a way as to alter the pattern of processing by the proteolytic enzymes of antigen-presenting cells. As a result, novel determinants from antigens may be presented to helper T-lymphocytes, stimulating potent protective immunity (see Chapter 10).

6.9.2. Glycocalyx epitopes expressed by normal and irradiated cercariae and schistosomula.

6.9.2.1 Coat 1 and coat 2 antigens.

The molecular weights of the antigens identified here with anti-coat 1 and anti-coat 2 antisera correspond to some of those identified in cercariae or newly-transformed schistosomula in the literature. Most studies have concentrated on surface antigens, ^{125}I-labelled, then immunoprecipitated with infected rodent or human serum. Using infected mouse or rat serum, several workers have identified the major surface antigens of cercariae as a series of related proteins which migrate as a diffuse band ranging from Mr 32 000 to 38 000 (Dissous et al, 1985; Payares et al, 1985; Simpson and Smithers, 1985). Payares et al (1985) also precipitated a 140-150 000 Mr antigen from ^{125}I-surface-labelled cercariae. However, when surface iodination of cercariae is followed directly by SDS-PAGE, with no selection of antigenic proteins by antibody, proteins of a broad range of molecular weights are identified (Payares et al, 1985; Snary et al, 1980). The cercarial surface is thus more complex than the immunoprecipitation results would suggest.

Transformation appears to expose new antigens on the parasite surface. Proteins consistently recognised by infected or vaccinated rodent serum have molecular weights 92 000, 20 000, 17 000 and 15 000. The 32 000-38 000 Mr cercarial complex is replaced by discrete bands at Mr 32 000 and 38 000 on the schistosomular surface (Simpson et al, 1984, 1985; Dissous et al, 1981, 1985; Payares et al, 1985; Simpson and Smithers, 1985). Using ^{125}I-iodosulphinilic acid as iodination reagent, rather than lactoperoxidase or Iodogen, however, Taylor et al (1981) identified quite different proteins, at Mr 105 000, 68 000, 25 000 as the principal antigens of the newly-transformed schisto-

somular surface.

Dalton et al (1986, 1987) used monoclonal antibodies rather than whole infection serum to identify some other components of the cercarial glycocalyx. Polypeptides of Mr >800 000, 220 000, 180 000, 170 000, 15 000 apparently share at least one common epitope. After transformation, these molecules are shed from the surface, and are therefore no longer accessible to surface labelling.

Samuelson and Caulfield (1982) identified a large number of components on the surface of newly-transformed schistosomula, using galactose oxidase and NaB^3H_4. Molecules at Mr 220 000, 180 000, 170 000, 38 000-32 000, 18 000, 12 000 were observed, all of which were reactive with Con A. Other components at Mr > 800 0000, 65 000, 50 000 and 15 000 did not bind Con A, but did react with FBP.

Interpretation of these results, and our own, on surface-labelling of schistosomula at early times after mechanical transformation, is complicated by the persistence of glycocalyx material on the schistosomulum surface for several hours after transformation, as indicated by E. M. studies (Samuelson and Caulfield, 1985; McLaren, 1980). This makes it difficult to be sure whether some of the proteins identified are integral to the developing heptalaminate membrane or belong to the residual glycocalyx. The molecular weights of a number of proteins reported on the schistosomular surface correspond with ones identified in the cercarial glycocalyx. Thus, in the studies summarised above, Mr 38 000 and 32 000 antigens have been observed in both stages. The galactose oxidase labelling technique of Samuelson and Caulfield (1982) demonstrated many more molecules, at Mr > 800 000, 220 000, 170 000 and 150 000, on the surface of newly-transformed schistosomula. Using a monoclonal antibody, Dalton et al (1986, 1987) immunoprecipitated ^{125}I-labelled antigens of these same molecular weights from the cercarial glycocalyx (see above).

It is evident that Samuelson and Caulfield's galactose oxidase

labelling method detected a number of antigens on the newly-transformed schistosomulum surface which are not evident after ^{125}I-labelling. An explanation for these conflicting observations could be that, after transformation, the development of the heptalaminate surface, and loss of much of the glycocalyx may render tyrosine residues in the polypeptide regions of some antigens inaccessible to Iodogen, but the oligosaccharides are still exposed for reaction with galactose oxidase.

The approach employed here, immunoblotting of the proteins separated on SDS-gels, avoids such problems of accessibility of epitopes to labelling reagents. Since whole parasite material was used, antigens from the interior of the parasite body, which share epitopes with glycocalyx material, will also be identified, whereas the studies reported in the literature concentrate on surface antigens only. Since our model proposes that both surface and internal parasite antigens are presented to the host immune system as the attenuated schistosomula die and disintegrate, it was relevant to investigate the antigenicity of the whole spectrum of parasite proteins. The molecular weights of some antigens identified by our anti-coat 1 and anti-coat 2 antisera do coincide with those reported in studies of cercarial and schistosomular antigens in a number of publications. Together with the studies described above, we detected binding of antibodies from anti-coat 1 and anti-coat 2 antisera to bands at Mr 100 000, 30 000-38 000, 20 000, 17 000, 16 000, 14 000, in both cercariae and mechanically-transformed schistosomula.

It is interesting that such a limited number of antigens are identified by anti-coat 2 antiserum, in contrast to the broad range of proteins recognised by anti-coat 1. This observation suggests that a greater variety of antigens may be released immediately following mechanical trauma than during the subsequent incubation period (see chapter 2 for definition of coat 1 and coat 2 fractions; figures 6.2, 6.3, 6.4). A Mr 23 000 antigen from mechanically- (but not skin-)

transformed schistosomula is uniquely recognised by anti-coat 2 antibodies.

Table 6.1 draws together our results for molecular weights of glycocalyx antigens and some of those reported in the literature.

It is difficult to explain the discrepancy between immunofluorescence and immunoblotting results for anti-coat 1 antiserum. Reports in the literature state that antibody-binding to skin forms is less than to mechanical ones (Brink et al, 1977; Bickle et al, 1983). Immunoblotting with anti-coat 1 against mechanical and skin schistosomula supports this claim (figures 6.2, 6.3) but immunofluorescence gives the opposite result (figure 6.1). It seems possible that immunofluorescence may detect the highly cross-linked network of the glycocalyx, which is too large to enter the polyacrylamide gel. It might be speculated that the greater stresses of skin penetration, as opposed to mechanical transformation, result in more severe disaggregation of the particulate matrix forming the glycocalyx. In this way, more sites may be exposed for antibody binding to the surface, as detected by immunofluorescence (figure 6.1). However, no morphological evidence has been reported to support this proposition. Anti-coat 1 blotting of SDS-polyacrylamide gels might identify individual glycoproteins released from the cross-linked network during transformation, or else glycoproteins within the cercarial tegumental vesicles, destined to fuse with the membrane and release their contents for integration into the matrix of the glycocalyx. The depletion of these proteins from skin schistosomula, but not mechanical forms, might be due to a more rapid loss of these vesicles, or degradation of their contents in response to the stresses of transformation (temperature, osmotic and mechanical shocks). Again, however, no morphological evidence exists to confirm this speculation.

It is especially interesting that immunoblotting suggests that irradiated skin forms retain both coat 1 and coat 2 antigens for

Table 6.1 Cercarial and schistosomular proteins identified with anti-coat 1, anti-coat 2 and anti-haemolymph antisera, and schistosomular surface proteins identified by iodogen iodination. Our results are compared with surface antigens described in the literature.

Notes: c = cercariae

s = schistosomula.

In our experiments, anti-coat 1, anti-coat 2 and anti-haemolymph antisera were blotted against whole parasite proteins, i.e. both surface and internal.

Our iodogen iodinations, and the references from the literature, should detect surface proteins only.

Levels of immunity associated with the various proteins were determined as follows:

1) Monoclonal antibody specific for a particular protein or set of proteins transferred passively to mice or rats before cercarial challenge. Grzych et al (1982): one monoclonal recognises Mr > 200 000 and 38 000 antigens. Zodda and Phillips (1982): one monoclonal recognises Mr 20 000, 17 000 antigens.

2) Purified antigen injected, with adjuvant, in mice, before challenge.

Table 6.1.

Mr ($x10^{-3}$)	ANTI-COAT 1	ANTI-COAT 2	ANTI-HAEMOLYMPH	SURFACE IODINATION AT 3h	20h	72h	REFERENCE TO SURFACE PROTEIN OF THIS Mr	SUGGESTED FUNCTION	LEVEL OF IMMUNITY ASSOCIATED WITH ANTIGEN OF THIS Mr.
>200				s	s	s	s:Grzych et al (1982)		63%: Grzych et al, 1982 (1).
180							c: Dalton et al (1987) s: Samuelson, Caulfield (1982)		
170							c: Dalton et al (1987) s: Samuelson, Caulfield (1982)		
150			c,s	s	s	s	s: Dissous et al (1985)		
105	c,s	c,s	c,s				s: Taylor et al (1981)		
80-82				s(82)			s: Taylor et al (1981)		
70-75	c,s	c,s	c,s		s	s		heat-shock proteins?	
65-67	c,s		c,s(67)		s(65)	s(65)	s: Samuelson, Caulfield (1982) Taylor & Wells (1984)	Alkaline phosphatase?	
57-60			c,s(57)		s(60)	s(60)	s: Samuelson, Caulfield (1982)		25%: Smith & Clegg, 1985 (2)
47 44	c,s		c,s	s	s s	s s intense	s:44-46 :- Rumjanek et al (1983)	LDL receptor?	
43	c,s							Actin	
40						s	s: Dissous et al (1981)		
30-38	c,s:32,34, 38.			s diffuse	s diffuse	s (32,34)	c(diffuse): Dissous et al (1985)	Role in osmo-regulation.	38: 63% Grzych et al, 1982 (1) 32: 27-58% Bickle et al, 1986 (1)
22-26		c,s:(23)		s diffuse	s diffuse	s 22,24,26	s: Samuelson, Caulfield (1982)	Stimulate IgE production?	

(1), (2): see table legend.

(Continued on next page).

Table 6.1 continued.

Mr. ($x10^{-3}$)	ANTI-COAT 1	ANTI-COAT 2	ANTI-HAEMOLYMPH	SURFACE IODINATION AT 3h	20h	72h	REFERENCE TO SURFACE PROTEIN OF THIS Mr.	SUGGESTED FUNCTION	LEVEL OF IMMUNITY ASSOCIATED WITH ANTIGEN OF THIS Mr.
20/19	c,s					s	s:20 Samuelson, Caulfield (1982)		20: Zodda and 48% Phillips, 1982 (1)
18/17	c,s						s:17 Simpson et al (1983b)		17: Zodda and 48% Phillips, 1982 (1)
16/15	c,s			s	s	s	s:15 Simpson et al (1983b)	Recognised only by gamma-irrad. vaccine serum	16:28-70% Bickle et al, 1986 (1)
14	c,s								
DYE-FRONT	c,s	c,s		s	s	s		peptides/ glycolipids?	

(1), (2): see table legend.

slightly longer than their normal counterparts. On the basis of the above model, it might be suggested that the irradiated parasites either could utilize, or degrade, the contents of their cercarial tegumental vesicles less rapidly and efficiently than normal larvae.

6.9.2.2. Expression of snail-like epitopes by normal and irradiated cercariae and schistosomula.

Cross-reactivity between S. mansoni antigens and those of the intermediate host B. glabrata has been described by several authors (Kemp et al, 1974; Yoshino and Cheng, 1978; Stein and Basch, 1979; Kemp et al, 1982; Bayne et al, 1987; Grzych et al, 1987). Jackson and de Moor (1976) showed similar cross-reactivity between S. haematobium antigens and its intermediate host Bulinus africanus. Kemp et al (1974) and Jackson and de Moor (1976) used the cercarienhüllen reaction to demonstrate that antigens typical of their respective intermediate hosts are also present on the glycocalyx of S. mansoni and S. haematobium cercariae. Kemp et al (1974) suggested that snail-derived antigens might be carried with cercariae into the mammalian host, thus exposing the immune system to snail antigens. Dissous et al (1986) and Grzych et al (1987) have described the presence of a common epitope in S. mansoni, B. glabrata and four other molluscs. This epitope was identified as carbohydrate in nature, and monoclonal antibodies reactive with this carbohydrate moiety protected against infection with S. mansoni cercariae. Diehl et al (1987) found that antigens from B. alexandrina partially protected against subcutaneous infections with cercariae of S. mansoni, but were ineffective against infection by percutaneous invasion. However, snail antigens do not consistently induce immunity in experimental hosts. (Diehl et al, 1987).

It is tempting to interpret such cross-reactivity between the

antigens of parasite and intermediate host in terms of the molecular mimicry hypothesis (Capron et al, 1969; Damian, 1964, 1987). However, Bayne et al (1987) warned that cross-reactivity between invertebrate antigens, when detected by mammalian antisera, should not be automatically interpreted as genuine molecular mimicry. The unusual carbohydrate sequences and structures of invertebrate molecules are so foreign to the mammalian immune system (Ferguson and Homans, 1988) that glycoproteins from any one invertebrate will stimulate a powerful antibody response which cross-reacts broadly with other invertebrate material, regardless of genuine phylogenetic or "mimicry" relationships between the antigens concerned.

Bayne et al (1987) found that antiserum raised against snail plasma proteins recognised at least twenty-two antigens in a sporocyst tegumental extract. Surface iodination of sporocysts (Boswell et al, 1987) picked out approximately 12 bands, ranging in molecular weight from Mr $>$ 200000 to $<$ 12 000. Antigens of Mr $>$ 200000, 170 000, 116 000, 89 000, 70 000, 54 000, 27 000, 22 000 were immunoprecipitated by monoclonal antibodies. In tissue sections, these monoclonals also bound to vesicles in the tegument of developing cercariae. Some of these species may coincide approximately in molecular weight with antigens identified here, using anti-haemolymph antiserum, at Mr 160 000, 100 000, 71 000, 67 000, 57 000 in cercariae and schistosomula (figure 6.6).

Proteins at Mr 100 000, 70-75 000, 65-67 000, 47 000, are recognised by anti-coat 1 or anti-coat 2 as well as anti-haemolymph. Moreover, the antigens recognized by anti-haemolymph, anti-coat 1 and anti-coat 2 antibodies all disappear from skin schistosomula by 4 hours after transformation, but persist in mechanical forms. These coincidences in molecular weights and time course of antibody binding suggest that the 3 antisera may recognise some of the same glycocalyx components. Anti-haemolymph antiserum does not react with all the

antigens recognised by anti-coat 1. This observation implies that only some of the glycocalyx components may share carbohydrate epitopes with snail antigens. Since anti-coat 1, on the other hand, does detect (though sometimes faintly) all the bands recognised by anti-haemolymph, all the snail-like epitopes may be released with the first fraction of the glycocalyx (see chapter 2 for definition of coat 1.).

These techniques do not allow us to distinguish whether snail-like antigens are synthesized by the parasite itself, or represent snail material which adheres to the parasite surface. Microscopical evidence for a close association between the tegument of developing cercariae and the snail material forming the matrix of the brood chamber (Caulfield et al, 1988) could provide one possible route for incorporation of snail proteins into the glycocalyx. Alternatively, such antigens might adhere during migration of cercariae through the snail body.

6.9.3. Effects of irradiation on conformation and immunogenicity of protein antigens.

The 6-IAF binding experiments indicate that new SH groups, hence novel protein regions, may be presented at the surfaces of irradiated cercariae and schistosomula.

Novel protein regions may be exposed by irradiated cercariae initially either as a result of disruption of the glycocalyx, or as a direct consequence of the effects of irradiation on protein conformation. Later in development, exposure of new antigenic epitopes on proteins in non-native conformations will be accentuated by the inhibition of protein, lipid and glycoprotein synthesis, and persistence of aberrant forms induced by irradiation, as described in chapters 3 and 4.

Direct effects of irradiation on protein structures have been

extensively researched (reviewed by von Sonntag, 1987). As described in section 1.10.3.4., radicals produced by irradiation can induce ring cleavage in aromatic amino acids, destabilization of the amide bond, also deamination and decarboxylation of peptides. The peptide radicals formed in these reactions tend to dimerise, resulting in -C-C- cross-linking of proteins. Another source of radicals for protein cross-linking results from oxidation of SH groups by the following reactions:

$$RSH + \cdot OH \longrightarrow RS\cdot + H_2O$$

$$RS^- + \cdot OH \longrightarrow RS\cdot + OH^-$$

These sulphur radicals combine to produce disulphide links between peptides.

Evidence for cross-linking of proteins in response to irradiation has been obtained in a number of systems (von Sonntag, 1987). For instance, Cantafora et al (1987) found that, after irradiation of erythrocytes, many cytoskeletal proteins, spectrin in particular, formerly detectable in the resolving gel on SDS-PAGE, formed high molecular weight aggregates at the top of the gel. These new bonds appeared to be covalent, since they were not disrupted by SDS treatment. Nor were they reducible by dithiothreitol, hence apparently were not disulphide-linked.

The tertiary structure of proteins may also be altered, by new hydrophobic and electrostatic interactions induced by irradiation. For instance, lactate dehydrogenase forms non-covalent aggregates upon irradiation (Buchanan and Armstrong, 1976). Unfolding of ribonuclease with increasing radiation damage has also been described (Schüssler et al, 1977).

Protein denaturation in these ways may reveal new antigenic regions, and hence partially explain the increased exposure of SH groups in irradiated parasites. However, it should also be noted that,

at high doses, the solvated electron produced in consequence of irradiation can directly induce formation of SH groups from -S-S- bridges, by the following reaction mechanism (von Sonntag, 1987).

$$e^-_{aq} + R\text{-}S\text{-}S\text{-}R \longrightarrow RSSR^{\cdot-}$$

$$RSSR^{\cdot-} + H^+ \rightleftharpoons R - S\overset{H}{\cdot}\cdot S - R \longrightarrow RS\cdot + RSH$$

Thus, Edwards et al (1984) describe how, at high radiation doses, oxidation of SH groups in S. cerevisiae is outbalanced by radiolysis of disulphides.

Chapter 10 will describe how antigens whose conformation is altered so that they expose novel epitopes in this way may induce the high levels of protective immunity characteristic of the irradiated cercarial vaccines.

Uptake of Hoechst H33258 indicates some surface disruption in live but irradiated cercariae and schistosomula. This membrane damage may also contribute to exposure of previously hidden SH groups.

No such evidence for exposure of new protein antigens was obtained for 24-hour old, Actinomycin D-treated schistosomula. Since inhibition of protein synthesis in these forms only becomes detectable after 10 to 15 hours of Actinomycin D treatment (chapter 3), inhibition may not have been sustained long enough for modified protein conformations to appear.

6.9.4. Surface antigens expressed during the later development of normal, irradiated and Actinomycin D-treated schistosomula.

6.9.4.1 Proteins identified by surface iodination during the culture period from 3 to 72 hours after mechanical transformation.

Surface iodination and SDS-PAGE, without any selection of antigens by specific antibody, revealed more of the proteins expressed at the

schistosomular surface during development.

Figure 6.13 shows that, although different surface proteins are exposed as the parasite develops over the 72-hour period following mechanical transformation, there are no differences in the molecular weights of the antigens expressed by normal, as compared to irradiated or Actinomycin D-treated schistosomula.

A number of authors have documented changes in surface antigens during the first few days of development. Snary et al (1980) used lactoperoxidase-catalysed iodination to examine the surface of cercariae, and schistosomula cultured for 3 and 24 hours after mechanical transformation. 3-hour schistosomula gave a pattern very similar to cercariae, with bands at Mr 80 000 and 25 000. In figure 6.13 presented here, we do see bands at approximately these molecular weights after surface Iodogen iodination, but also many others. On the 24-hour schistosomular surface, Snary et al (1980) identified the major antigens as a heavily-labelled series of proteins between Mr 67 000 and 80 000, also at approximately Mr 53 000, 40 000 and 20 000. These might correspond to the bands in figure 6.13 at Mr 82 000, 72 000, 65 000 and 22 000, although others at Mr 100-200 000, 60 000, 30-38 000, 22-26 000, 15 000 are also identified at 20 hours in this figure.

By immunoprecipitation with infected mouse serum, Simpson et al (1984) and Payares et al (1985) both observed, firstly, replacement of the diffuse band between Mr 32 000 and 38 000 in cercariae, by discrete bands at Mr 32 000 and 38 000 after transformation. The Mr 38 000 antigen was then gradually lost from the schistosomular surface over the subsequent 48 hours in culture. Dissous et al (1981) also followed expression of this antigen at Mr 38 000 using a monoclonal antibody. It could be radiolabelled on cercariae, 50% of the molecules originally expressed being shed during transformation into schistosomula. Thereafter, up to 24 hours, this antigen continued to be

shed, but more slowly. All three research groups found that, simultaneously with loss of the 38 000 Mr molecule, the Mr 32 000 surface antigen became more prominent. The iodogen-labelling pattern of fig. 6.13 corresponds reasonably well with these established immunoprecipitation results, in that the diffuse band seen between Mr 30 000 and 38 000 at 3 hours is replaced by discrete, though faint bands at Mr 30 000, 32 000, 34 000 at 72 hours. Simpson et al (1984) observed that a Mr 17 000 antigen on the surface of 3-hour schistosomula disappeared by 48 hours after transformation. Similarly, figure 6.13 and table 6.1 show that by 20 hours, a Mr 17 000 molecule on the 3-hour schistosomular surface has been lost.

Our inability to detect any difference in molecular weights of the antigens expressed at the surface of normal and irradiated schistosomula or of those identified in normal and irradiated larvae by antisera raised against the glycocalyx agrees with the conclusions of Simpson et al (1984) and Dalton et al (1986). These authors determined which antigens had been presented to the immune system by normal and gamma-irradiated schistosomula during their existence in the host by immunoprecipitating ^{125}I- or ^{35}S-labelled normal schistosomular proteins with antisera from mice exposed to normal or gamma-irradiated infections. All the antigens immunoprecipitated by vaccine sera were also precipitated by infection serum, with the exceptions of an antigen of Mr 15 000 on 3-hour schistosomula (Simpson et al, 1984) and a Mr 38 000 glycoprotein on schistosomula cultured for 5 days (Dalton et al, 1986). These antigens were uniquely recognized by gamma-irradiated vaccine sera. Since our results indicate that both normal and attenuated - U.V.-irradiated or Actinomycin D-treated - schistosomula continue to express antigens of the same molecular weights, such differences in the host response may arise from differences in the presentation of these antigens to the immune system.

However, figure 6.14 shows no differences in the interaction of

these surface proteins with mouse peritoneal macrophages. It would be valuable to extend this preliminary study on antigen presentation. For instance, it is intriguing to question whether absence from the macrophages of the 30 000 and 20 000 molecular weight surface antigens so prominently labelled in the schistosomular secretions is due to their rapid digestion by the macrophages prior to presentation of the resulting peptides at the cell surface.

This figure also supports the conclusions of chapter 3 that release of surface antigens is not inhibited by irradiation or Actinomycin D treatment.

Table 6.1 summarizes the molecular weights of the antigens identified on the surfaces of normal and attenuated schistosomula, together with proteins of similar weight, postulated identities and immunogenicity as reported in the literature.

6.9.4.2. Analysis of the developing schistosomular surface by immunofluorescence with antisera and lectins.

Antiserum raised against cercarial membrane antigens, human infection serum, and a variety of lectins, showed no differences in binding to normal, irradiated and Actinomycin D-treated schistosomula. Binding to all 3 forms from 24 to 72 hours after mechanical transformation remained at least as high as to normal cercariae.

This observation conflicts with well-established evidence that, as young schistosomula develop, either *in culture* *in vitro*, or *in vivo* as they migrate from the skin to the lungs, they change or mask their original surface antigens (reviewed in section 1.5.7). It seems, however, that schistosomula cultured *in vitro* do not show as pronounced a loss or disguise of surface antigenicity as those recovered from an animal host, or those obtained after even a brief exposure to *in vivo* conditions by penetration of excised mouse skin.

Thus, Brink et al (1977) demonstrated by indirect immunofluorescence that mechanically-transformed schistosomula bound chronic mouse serum more strongly than skin forms. Bickle et al (1983) also observed significant differences between mechanically- and skin-transformed schistosomula in surface binding of chronically-infected mouse serum. Imohiosen et al (1978) observed that mechanically-transformed schistosomula bound the same amount of immune mouse serum at successive time-points during a 24-hour period after transformation. However, if freshly transformed schistosomula were injected intravenously in mice and recovered from the lungs 24 hours later, binding of immune serum markedly decreased.

Indeed, when compared by various criteria of development, mechanical schistosomula seem to develop more slowly than their in vivo counterparts. Stirewalt et al (1983) observed that mechanically-transformed schistosomula retained features characteristic of cercariae - a positive cercarienhüllen reaction, glycocalyx material, heterochromatic nuclei - for longer than schistosomula prepared by penetration of dried rat skin. Brink et al (1977) found that fewer mechanical than skin-transformed schistosomula reached the closed-gut stage of development after 12 days in culture. They also demonstrated slight differences in adult worm recoveries between skin- and mechanically - transformed schistosomula injected into mice.

The results presented here support this evidence for the delayed development of mechanically-transformed schistosomula. Thus, the continued reactivity of anti-CMAG, infected human serum and anti-glycocalyx antisera with mechanical forms (figures 6.11, 6.12) might be attributed to the absence of in vivo factors which decrease surface antigenicity, either directly by incorporation into the parasite surface (eg. masking host antigens) or by triggering some developmental change by the schistosomulum itself. L. Q. Vieira and J. R. Kusel (manuscript in preparation) have found that schistosomula

cultured in the peritoneal cavity of a mouse for 5 days, or recovered from lungs, show no binding of anti-coat 1, anti-coat 2 or anti-haemolymph sera, while counterparts cultured *in vitro* for the same length of time continue to react with these antisera. Thus, loss of glycocalyx antigens seems to reflect the *in vivo* situation more truly.

Antisera and lectin binding studies with normal and attenuated parasites obtained after skin penetration or a period of *in vivo* development might thus give more information on the nature of the antigens presented to the host *in vivo*.

6.9.4.3. Electron microscopy of the surface of developing schistosomula.

Electron microscopy of schistosomula derived by skin penetration showed no significant differences between normal and irradiated forms after 30, 50 and 80 hours of culture in Elac/10% human serum. This observation is consistent with the model of Hockley and McLaren (1973) and Nagai *et al* (1977) that the heptalaminate membrane is derived from pre-synthesized membranous vacuoles, and does not require *de novo* protein or lipid synthesis immediately after transformation (see chapters 3 and 4).

Acquisition of host lipids also does not appear to depend on active metabolic processes, for, when cultured with A+ human red blood cells, irradiated schistosomula incorporated as much of the glycolipid blood group A antigen as normal ones. This result is consistent with the observation of Rumjanek *et al* (1983) that expression of the LDL receptor, which may mediate uptake of host lipids, can be detected in gamma-irradiated schistosomula. It also supports the model described in chapter 4, whereby reduced lipid synthesis by irradiated or Actinomycin D-treated parasites might be compensated for by assimilation of host

lipids into the membrane, resulting in a severely altered composition of the parasite surface.

6.9.4.4. Identification of surface carbohydrates by lectin binding to normal and attenuated schistosomula during 72 hours in culture.

A number of studies have attempted to analyse the surface membrane carbohydrates of normal schistosomula. Murrell et al (1978) detected Con A and WGA binding to 2-hour and 18-hour schistosomula. Con A binding was highly specific, being completely inhibited by α-methylmannoside, and slightly less so by N-acetylglucosamine. These authors concluded that WGA binding was partly non-specific, for N-acetylglucosamine exerted an incomplete (though significant) inhibition. Soybean Agglutinin, specific for N-acetylgalactosamine, and Ulex europaeus Agglutinin, specific for fucose, did not appear to bind to schistosomula. Simpson et al (1983a), working with newly-transformed schistosomula, also found that these last two lectins did not bind. However, Con A, WGA, Fucose Binding Protein from L. tetragonolobus, and PNA (galactose-specific) did bind to newly-transformed schistosomula. Like Murrell et al (1978), these workers found that N-acetylglucosamine did not completely prevent WGA binding. Both research groups found that neuraminidase treatment of schistosomula had no effect on lectin binding.

The results presented here on lectin binding to schistosomula coincide well with these published observations. Like Murrell et al (1978), we observed considerable Con A binding, which could be inhibited by both α-methylmannoside and N-acetylglucosamine. WGA binding was also observed, and could be only partially inhibited by N-acetylglucosamine, although N,N',N"- acetylchitotriose blocked binding completely, as predicted by Goldstein et al (1975). PNA

binding indicated that galactose residues were exposed, and FBP showed that fucose was also present, although 24-hour, formaldehyde-fixed schistosomula do not seem to expose as much fucose as the 3-hour, unfixed ones studied by Simpson et al (1983a). Our results with neuraminidase treatment also suggest that no sialic acid is present on schistosomula, at least up to 48 hours after mechanical transformation.

In conclusion, it seems that mechanically-transformed schistosomula expose on their surfaces mannose, galactose, N-acetylglucosamine and fucose. No differences in lectin binding were apparent when normal, U.V.-irradiated and Actinomycin D-treated schistosomula were compared. This observation might seem to contradict previous results on disruption of the glycocalyx and inhibition of glycosylation in attenuated forms. Indeed, a number of studies in the literature report marked effects of irradiation on the nature of the carbohydrates exposed at cell surfaces, as detected by lectin binding. Thus, Köteles et al (1976) showed that gamma irradiation of W138 fibroblasts resulted in a rapid, transient increase in [^{3}H]-Con A binding to the cells. Moullier et al (1986) observed that low levels of gamma irradiation (<1 Gy) of rat splenic lymphocytes led to removal of terminal sialic acid residues from oligosaccharides, exposing new sites for WGA binding. At higher doses of irradiation, more internal damage to glycoproteins occurred, detected as a striking decrease in WGA binding sites. Banchereau et al (1982) also demonstrated partial disialylation of lymphocyte membranes after high in vitro doses of gamma irradiation (10-50 Gy). Long-wave U.V.-irradiation, in combination with 8-methoxypsoralen, led to an increase in Dolichos biflorus Agglutinin and UEA binding to guinea pig epidermis, but staining with Con A, Soybean Agglutinin and WGA decreased in intensity (Danno et al, 1984). In view of the alterations in lectin binding observed after irradiation in these cellular systems, it was especially surprising that attenuated schistosomula showed the same level of

lectin binding as normal ones.

6.9.5. Conclusions: antigenicity of developing normal, U.V.-irradiated and Actinomycin D-treated schistosomula.

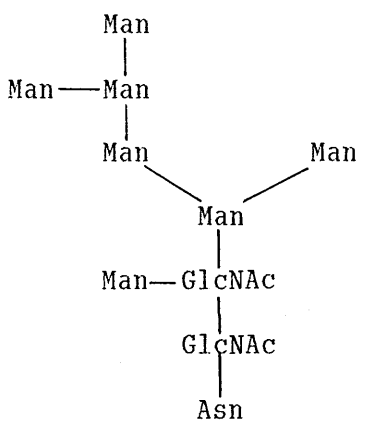
During the initial stages of transformation, normal and irradiated parasites appeared to present glycocalyx antigens differently (see section 6.9.1). In constrast, the studies on binding by anti-CMAG antiserum, infected human serum and various lectins, also electron microscopy and blood group antigen uptake revealed no differences in the nature of the antigens exposed at the surfaces of normal, irradiated and Actinomycin D-treated parasites during culture from 24-72 hours after mechanical transformation. These observations might appear to conflict with previous results, suggesting that inhibition of protein, phospholipid and glycoprotein synthesis in attenuated schistosomula results in expression of antigens in novel, highly immunogenic conformations (chapters 3, 4, 5). However, a major caveat in all the binding studies must be that mechanical forms do not always give a reliable picture of the *in vivo* situation (Wilson, 1987). It would be valuable to repeat these lectin- and antiserum-binding experiments on schistosomula recovered from experimental hosts, or by staining of schistosomula in sections cut from excised skin and lung sites.

Secondly, electron microscopy and labelling studies (Hockley and McLaren, 1973; Samuelson and Caulfield, 1982) have shown clearly that mechanically- transformed schistosomula retain more than 30% of their glycocalyx material for at least 3 hours after transformation. Since antiserum raised against cercarial membrane antigens (anti-CMAG) shows identical binding, as measured by immunofluorescence, to schistosomula up to 72 hours after mechanical transformation, it seems plausible to suggest that residual glycocalyx material may be retained on mechanical

schistosomula throughout this culture period. This covering of glycocalyx material could conceal the schistosomular antigens in the underlying membrane.

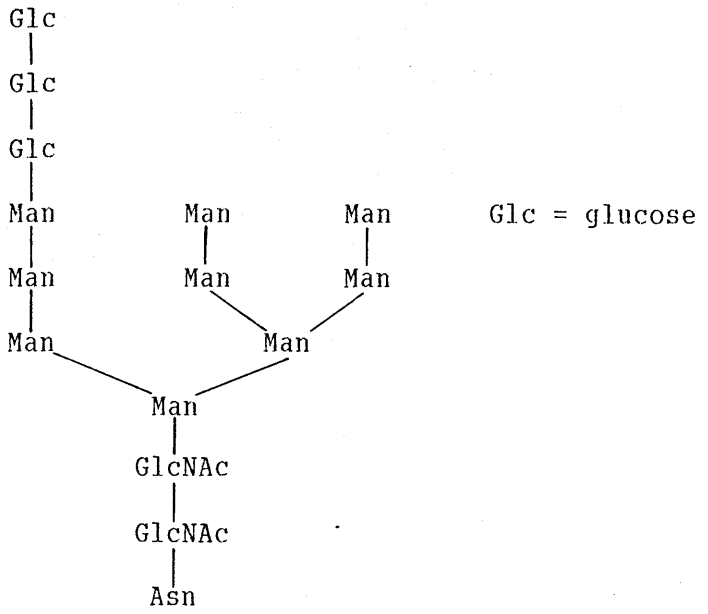
Thirdly, the antisera, being raised against preparations from normal parasites,may only react with normal epitopes; they might not indicate the presence of the new epitopes which, we propose, are also exposed on attenuated forms.

Finally, the fact that quantitative binding of various antisera and lectins to normal and attenuated schistosomula, is identical, does not necessarily indicate that the epitopes recognised by these reagents are the same in both cases. Thus, examining the structure of mature high mannose oligosaccharides, which do appear to be present in S. mansoni (Rumjanek, 1979; Rumjanek et al, 1981):



Man = mannose
GlcNAc = N-acetylglucosamine
Asn = asparagine

It can be seen that the absence of one or more mannose residues will be compensated by exposure of other mannose or N-acetylglucosamine moieties, so that binding of Con A, or antibodies specific for certain carbohydrate sequences may not decrease. Indeed, if processing of the precursor oligosaccharide:



is inhibited, more binding sites than normal could be exposed. The continued exposure of galactose, N-acetylglucosamine and fucose in irradiated or Actinomycin D-treated schistosomula might have a similar explanation, but at present, the composition of the parasite oligosaccharides containing these sugars is unclear. The absence of sialic acid implies that they are not present in the lactosamine-type oligosaccharides typical of mammalian cells.

In conclusion, it seems that irradiation may disrupt the cercarial glycocalyx and alter the conformation of protein antigens exposed at the parasite surface. Both these effects may be relevant to the enhanced immunogenicity of irradiated cercariae. No differences were observed in surface membrane carbohydrates of normal, U.V.-irradiated and Actinomycin D- treated schistosomula from 24 to 72 hours after mechanical transformation. This may be partly due, however, to artifacts arising from the use of mechanical forms, and to the limits to the information which lectins and antisera raised against normal antigens can provide about the surfaces of attenuated schistosomula.

CHAPTER SEVEN

EXPRESSION OF CARBOHYDRATE AND POLYPEPTIDE EPITOPES BY NORMAL, IRRADIATED, AND ACTINOMYCIN D-TREATED SCHISTOSOMULA. ROLES OF THE TWO CLASSES OF ANTIGEN IN STIMULATING PROTECTIVE IMMUNITY.

7. Expression of carbohydrate and polypeptide epitopes by normal, irradiated, and Actinomycin D-treated schistosomula. Roles of the two classes of antigen in stimulating protective immunity.

7.1 Introduction

Various lines of evidence suggest that antigens from S. mansoni eggs stimulate human and rodent hosts to produce high concentrations of antibodies which crossreact with carbohydrate epitopes on the schistosomulum surface. Some of these antibodies may play a role in resistance to reinfection (Smithers et al, 1987), but others inhibit binding to the schistosomulum surface by effector antibodies which are detrimental to parasite survival. Under these circumstances, invading schistosomula of a challenge infection are able to evade the host's immune effector mechanisms. Only in the absence of such blocking antibodies can antibody-dependent cell-mediated cytotoxicity mechanisms (ADCC) prevent establishment of challenge larvae. It has been proposed that such effector mechanisms may account, at least in part, for the resistance to infection induced by gamma-irradiated cercariae (Omer-ali et al, 1986, 1988). Since the radiation-attenuated larvae do not develop into egg-laying adults, the powerful antigenic stimulus afforded by egg polysaccharides does not dominate the host's antibody response.

In studies of the role of these carbohydrate determinants, periodate treatment of the schistosomulum surface has been used. This reagent apparently destroys the carbohydrate epitopes recognised by blocking antibodies, but not the polypeptide antigens involved in vaccine-induced resistance (Omer-ali et al, 1986; Smithers et al, 1987).

7.1.1. Evidence for this model in the literature.

a) In vitro studies.

Omer-ali et al (1986) used the radioimmunoassay technique to examine the effect of periodate treatment of glutaraldehyde-fixed schistosomula on binding by sera from mice that had been chronically-infected (CMS), or immunised three times with gamma-irradiated cercariae (VMS). Periodate treatment led to a 90% decrease in CMS binding, but slightly enhanced VMS binding, by some 10%. The resultant binding of both sera was approximately equal. The authors concluded that CMS reacted with a large number of periodate-sensitive, presumably carbohydrate, epitopes, not recognised by VMS. The interspecific cross-reactivity of CMS indicated that such carbohydrate structures were common to different schistosome species, also to different life-cycle stages - schistosomula, cercariae, miracidia and eggs. Western blotting and immunoprecipitation located these epitopes to antigens of Mr > 200 000, 38 000 and 17 000 on the schistosomular surface.

Yi et al (1986a, b) subsequently demonstrated that the CMS antibodies specific for these ubiquitous carbohydrate epitopes blocked in vitro parasite killing dependent on other, effector antibodies. Such inhibition was observed both when effector antibodies were derived from whole sera and when they were in the form of monoclonals.

These authors demonstrated that CMS depleted of anti-carbohydrate antibodies by absorption with formaldehyde-fixed cercariae showed enhanced activity in an antibody-dependent, complement-mediated killing assay directed against schistosomula. Similar absorption of VMS, however, had no effect on the lethal activity of the serum. Fractionation of CMS into IgG and IgM subclasses on protein A-sepharose indicated that different antibody isotypes were likely to be

responsible for the blocking and killing activities. The IgG fraction of CMS displayed enhanced lethal activity compared with unfractionated serum, but this was not true of VMS. Moreover, the IgM fraction of CMS was shown to block *in vitro* killing mediated by the IgG fractions of both CMS and VMS.

A panel of IgM monoclonals was also produced. These IgM antibodies recognized a carbohydrate determinant on the schistosomulum surface antigen at Mr > 200 000, and cross-reacted with the surfaces of cercariae, miracidia, and also schistosomula, of *S. haematobium, S. bovis* and *S. mansoni.* An IgG1 monoclonal, on the other hand, was specific for an antigen of Mr 20 000 on the surface of *S. mansoni* schistosomula only. This monoclonal exhibited high levels of cytotoxicity for schistosomula *in vitro,* but the IgM monoclonals blocked this lethal activity. These IgM monoclonals also reduced the complement-dependent schistosomulicidal activity of VMS. In radioimmunoassays, CMS was able to compete with a ^{125}I-labelled IgM monoclonal, but VMS showed no such competition.

It was concluded that IgM antibodies present in CMS, but not VMS, expressed blocking activities, directed against carbohydrate epitopes common to the egg, different stages of the life-cycle, and various *Schistosoma* species. Mice vaccinated with irradiated cercariae lacked high levels of anti-carbohydrate antibodies, and recognition of schistosomulum surface antigens was largely by species-specific polypeptide epitopes.

Working on the rat model, Grzych *et al* (1984) had previously suggested a similar dynamic balance between protective and regulatory (blocking) mechanisms, mediated by different antibody subclasses. Thus, a rat IgG2c monoclonal which did not exhibit any killing activity for schistosomula specifically inhibited the eosinophil-dependent cytotoxicity mediated by an IgG2a monoclonal. These monoclonals recognised different epitopes on the same antigen (Mr 38 000), whereas

Yi et al (1986b) demonstrated blocking and effector antibodies recognising different molecules.

It was suggested that the IgM antibodies of CMS expressing the cross-specific anti-carbohydrate activity observed in the experiments of Omer-ali et al (1986) were induced by the massive antigenic stimulation provided by schistosome eggs during a chronic infection. Further investigations supported this hypothesis, in that absorption of infection serum with schistosome eggs largely abolished its binding to schistosomula in radioimmunoassays. Binding was reduced to the same extent when the serum was absorbed with eggs that had been subjected to 100°C for 30 minutes, then treated with protease, confirming the carbohydrate nature of the antigens involved (Omer-ali et al, 1988).

Interestingly, both CMS and VMS seem to recognise many of the same antigens on immunoprecipitation, in particular, molecules at Mr > 200 000 and 38 000 (Omer-ali et al, 1988). The different efficacies of the two sera in killing schistosomula are due to the different antibody isotypes involved, and the nature of the particular epitopes recognised in each case. IgM antibodies in CMS react with carbohydrate moieties; in VMS, on the other hand, IgM is not the predominant isotype, and the antibodies are directed largely against polypeptide epitopes.

Smithers et al (1987) have proposed a second model for modulation of host protective responses by IgM anti-egg antibodies. They suggest inflammatory responses other than ADCC (eg. hypersensitivity types I, III, IV - see figure 1.5) may be the basis of protection. Suppression of these reactions reduces protective immunity. A role for antibodies directed against egg antigens has already been established in suppression of cell-mediated responses manifested as granulomas formed around eggs injected intravenously in mice (Pelley and Warren, 1978). Such antibodies may act in a similar way to inhibit cell-mediated responses directed against migrating schistosomula.

It should be borne in mind, however, that although some carbohydrate epitopes on the schistosomulum surface seem to induce this undesirable suppression of host immunity, passive transfer in mice of IgM monoclonals recognising oligosaccharide moieties indicates that other carbohydrate epitopes may be targets of protective immunity (Smithers et al, 1987).

Moreover, although carbohydrate epitopes common to eggs and schistosomula modulate antibody-dependent damage to schistosomula in vitro, other workers have been unable to demonstrate similar blocking of immune mechanisms in vivo, by passive transfer of monoclonals recognising such shared carbohydrate antigens (Bickle and Andrews, 1988). However, extensive field studies have suggested that IgM anti-egg antibodies may modulate immunity in man.

b) Field studies on human immunity.

Analysis of sera obtained from infected schoolchildren and adults in a Kenyan village where schistosomiasis is endemic led to similar conclusions as regards the role of blocking antibodies in preventing expression of human immunity. Schistosome infections were initially cleared by oxamniquine treatment; reinfection rates were monitored by faecal egg counts. Blood samples were taken before treatment, and at intervals afterwards. When various immune responses were assayed in the serum samples, a strong positive correlation was observed between pretreatment levels of anti-egg antibodies and susceptibility to subsequent reinfection. Following fractionation of individual infection sera by S. aureus protein A absorption, it was found that IgM fractions blocked immune cell-mediated killing of schistosomula dependent on IgG from the same sera (Khalife et al, 1986). These IgM-enriched fractions recognised both the schistosomular surface, and egg antigens. Further analysis of the sera indicated that intensities of

reinfection after treatment were positively correlated with pretreatment levels of IgM specific for the Mr 38 000 schistosomular surface antigen, also with IgM and IgG titres against the major egg polysaccharide K3 (Dunne et al, 1988).

The work of Dunne et al (1988) further extended Capron's original hypothesis that different antibody isotypes suppress, or contribute to, expression of immunity. The human infection sera used in this study showed antigen-directed restriction of immunoglobulin isotype responses to certain egg and adult worm antigens which shared common epitopes with the schistosomulum surface. IgM, IgG1, IgG2 and IgG3 responses to polysaccharide epitopes common to the egg and schistosomulum declined as the intensity of infection increased, or with longevity of the initial infection. At the same time, there was an increase in titres of IgG4 antibodies, restricted to non-polysaccharide (periodate insensitive) antigens shared by the schistosomulum and adult worm. This switch in dominant antibody isotypes was correlated, not only with duration and intensity of infection, but also with increasing age of the subjects. The authors drew comparisons with studies in healthy European children showing that levels of IgG4 rose only slowly with age, reaching adult levels at about 12 years.

These observations were combined to produce the following hypothesis on development of human immunity to schistosomiasis mansoni. During early infections of young children, it is proposed that the major immunogenic stimuli are antigens released from eggs. These antigens, including polysaccharides and heavily-glycosylated glycoproteins, elicit predominantly IgM responses, and certain inappropriate IgG isotypes. These antibodies cross-react with major glycoproteins on the schistosomulum surface. At the same time, potentially protective IgG responses are mounted against the same glycoproteins. These may be directed against the same epitopes as the blocking antibodies, or against different ones, in which case their

binding is blocked sterically. Since the balance of this early response is towards the blocking antibodies, the child is not resistant to challenge infection. Under the influence of persistent antigen stimulation, and as the child ages, there is a switch from predominantly blocking, anti-polysaccharide responses, to antibodies of one or more different isotypes, directed against periodate insensitive epitopes. Under these circumstances, the child expresses partial resistance to further infection.

One caveat to this interpretation of the serum analyses and reinfection data is that, in the first serum samples taken after oxamniquine treatment, at 5 weeks, the blocking anti-egg responses of children subsequently found to be resistant or susceptible had declined to the same level for both groups (Butterworth et al, 1985, 1987). Since blocking antibodies were reduced to the same baseline level in all children, it is difficult to explain, in terms of the above model, why new infections should establish preferentially in certain subjects. It should also be noted that similar field studies on S. haematobium in the Gambia were unable to demonstrate similar antagonism between blocking and effector antibodies in expression of immunity to this species (Hagan, 1987).

In view of these observations, it seemed worthwhile to compare the expression of periodate-sensitive and insensitive antigens by normal, irradiated and Actinomycin D-treated schistosomula, also the recognition of the two classes of antigen by experimental hosts exposed to normal and attenuated parasites.

7.2 Results

7.2.1. Effect of periodate treatment of normal, U.V.-irradiated and Actinomycin D-treated schistosomula on binding by sera from mice exposed to normal, U.V.-irradiated or Actinomycin D-treated larvae.

20-hour cultured, normal, U.V.-irradiated and Actinomycin D-treated schistosomula were formaldehyde-fixed, and divided into periodate-treated and untreated samples. Their ability to bind sera from mice bled ten weeks after p.c. exposure to normal or U.V.-irradiated larvae, or s.c. immunisation with Actinomycin D-treated schistosomula, was tested by indirect immunofluorescence (figures 7.1a)-c)). The U.V.-irradiated and Actinomycin D-treated parasites induced, respectively, 78% and 62% resistance to cercarial challenge at six weeks after immunisation (chapter 9).

Binding of CMS to normal schistosomula was high, but periodate treatment of the parasites reduced this binding to the same level as the two vaccine sera. Binding to normal schistosomula by UVMS was low, and even less for AMS, though both were significantly higher than unimmunised controls. Periodate treatment did not lower binding by UVMS, and slightly increased that by AMS. Periodate-treated schistosomula also showed an increase in binding by NMS.

Binding characteristics of the four types of sera to U.V.-irradiated and Actinomycin D-treated schistosomula before and after periodate treatment were strikingly similar to the pattern for normal schistosomula. UVMS and AMS did not, as might have been expected, show enhanced binding to U.V.-irradiated or Actinomycin D-treated schistosomula.

This set of experiments pointed to a marked similarity between the antibody responses of mice exposed to U.V.-irradiated and Actinomycin

Figure 7.1 a)-c)

Effect of periodate treatment of normal, U.V.-irradiated and Actinomycin D-treated schistosomula on binding by sera from NIH mice infected with normal, U.V.-irradiated or Actinomycin D-treated parasites.

SERA: NIH female mice were infected:

1. p.c. with 150 normal cercariae (CMS)
2. p.c. with 500 U.V.-irradiated (400 μW min cm^{-2}) cercariae (UVMS).
3. s.c. with 500 Actinomycin D-treated, 20-hour schistosomula (AMS).

NMS was obtained from a pool of uninfected NIH mice.

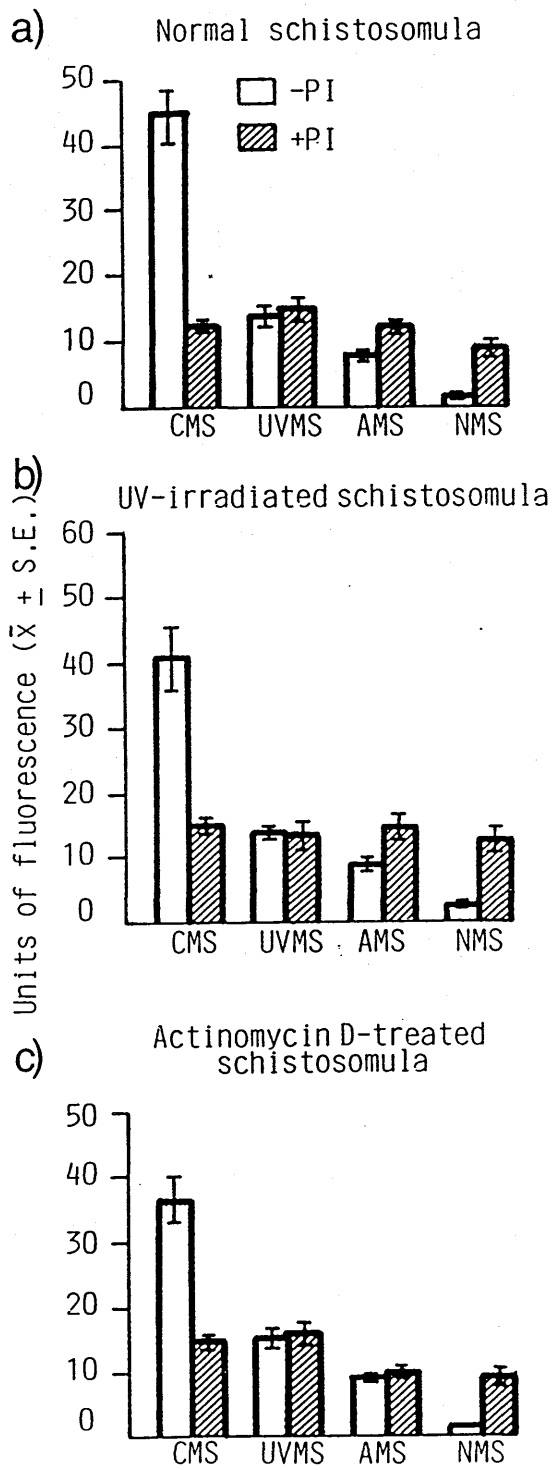
Mice were bled 11-12 weeks after infection or immunisation.
For CMS, UVMS and AMS, sera from three mice were pooled.

SCHISTOSOMULA: Normal, U.V.-irradiated (400 μW min cm^{-2}) and Actinomycin D-treated schistosomula were formaldehyde-fixed after 20 hours of culture in Elac/10% hiFCS.

Periodate treatment and indirect immunofluorescence were performed as described in 2.6.3.1.

- PI = no periodate treatment.
+ PI = periodate treatment.

STATISTICS: No significant difference in CMS binding to normal, U.V.-irradiated and Actinomycin D-treated schistosomula, non-periodate-treated, by Student's t-test.


a)
Normal schistosomula
-PI
+PI
50
40
30
20
10
0
CMS
UVMS
AMS
NMS
b)
UV-irradiated schistosomula
Units of fluorescence ($\bar{X}$ ± S.E.)
60
50
40
30
20
10
0
CMS
UVMS
AMS
NMS
c)
Actinomycin D-treated schistosomula
50
40
30
20
10
0
CMS
UVMS
AMS
NMS

D-treated schistosomula. Both showed low levels of antibody reactivity, unaffected by destruction of carbohydrate epitopes on their target antigens.

7.2.2. Effect of competing sugars on binding by infected and vaccine mouse sera to periodate-treated and untreated schistosomula.

Figure 7.2 a) compares the effects of periodate treatment and the presence of competing sugars on binding to 20-hour schistosomula by infected mouse sera and sera obtained from mice immunized with U.V.-irradiated, Actinomycin D-treated or gamma-irradiated schistosomula. Methyl-α-D-mannopyranoside, N-acetyl-D-glucosamine and D-(+)-galactose were chosen as competitors, because lectin binding studies (chapter 6) suggested that these sugars were the most abundant on the surface of 24-hour schistosomula.

In this instance, periodate treatment did not decrease the binding of CMS to normal schistosomula as markedly as in figure 7.1a). However, figure 7.2b shows a decrease in CMS reactivity with U.V.-irradiated schistosomula after periodate treatment, hence there seems to have been no defect in the chemical effectiveness of periodate. The discrepancy between figures 7.1 a) and 7.2 a) in CMS binding to periodate-treated normal schistosomula may therefore be due to the fact that a different serum pool was used in each experiment. Since sera from only 3 mice were used in each experiment, one individual with antibody specificities that deviate from average may significantly influence the binding patterns of the serum pool as a whole.

High concentrations of competing sugars reduced slightly but significantly the binding of CMS to periodate-treated schistosomula, but had no effect on its reactivity with untreated parasites.

As in figure 7.1, binding of UVMS to normal schistosomula was low, increasing slightly after periodate treatment. Competing sugars

Figure 7.2 Binding of infection and vaccine sera to

a) normal schistosomula.
b) U.V.-irradiated schistosomula (400 μW min cm^{-2}).

} in presence and absence of competing sugars.

SERA: CMS, UVMS, AMS - as for figure 7.1, but 3 different mice contributed to the serum pools.

GMS: NIH females were immunised p.c. with 500 gamma-irradiated cercariae, and bled 11-12 weeks later.

SCHISTOSOMULA: treated as for figure 7.1.

Periodate treatment, addition of competing sugars, and indirect immunofluorescence were performed as described in 2.6.3.1.

-PI: no periodate treatment. + PI: periodate-treated

-S: 1st antibody added with GMEM in place of any competing sugars.

+S: 1st antibody added simultaneously with α-methylmannoside, N-acetyl-glucosamine and D(+)-galactose, each at a final concentration of 0.2M.

STATISTICS t-tests Figure 7.2a) Binding to normal schistosomula.

CMS: -PI is greater than +PI: $0.025 < P < 0.05$

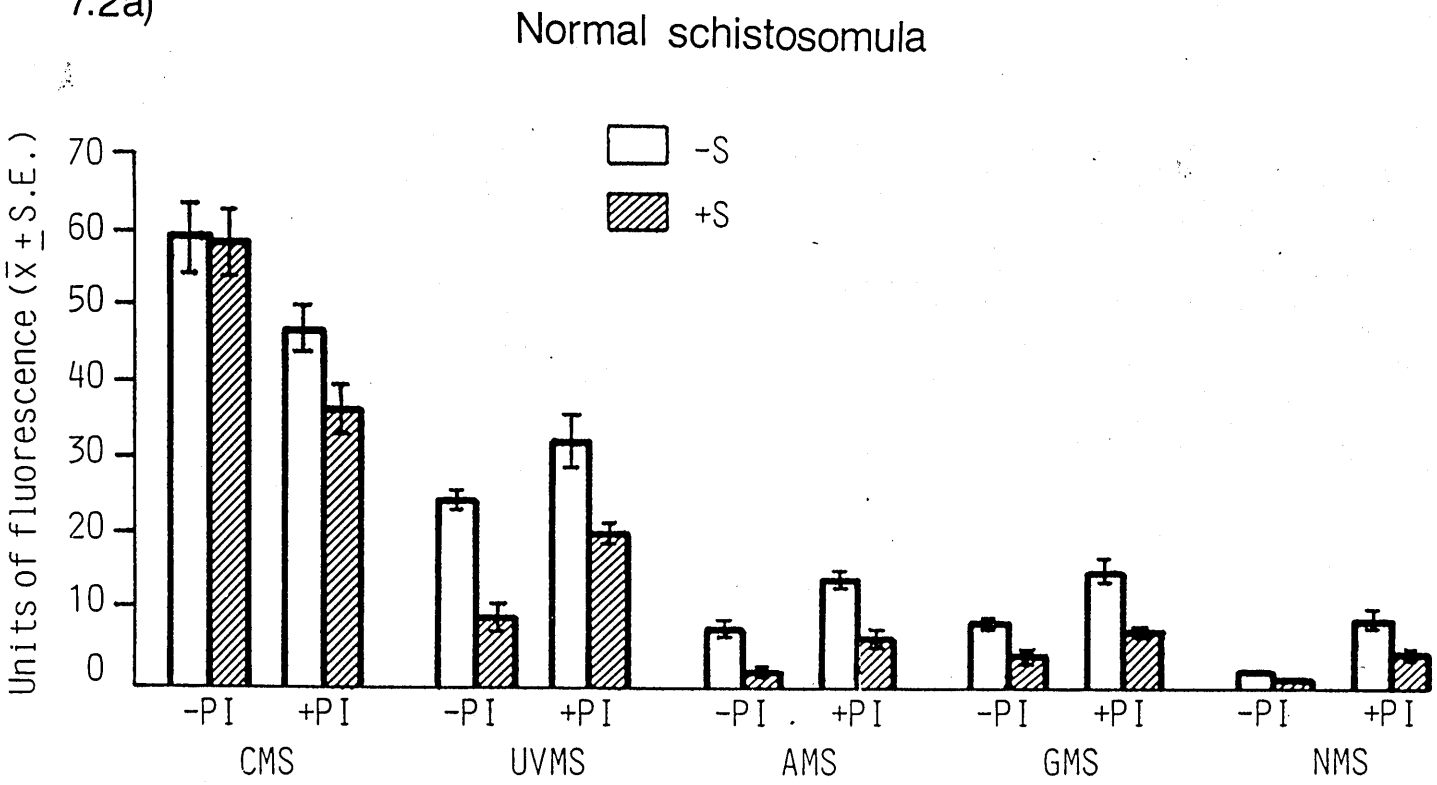
CMS(+PI): -S is greater than +S: $0.005 < P < 0.01$.

Figure 7.2b) Binding to U.V.-irradiated schistosomula.

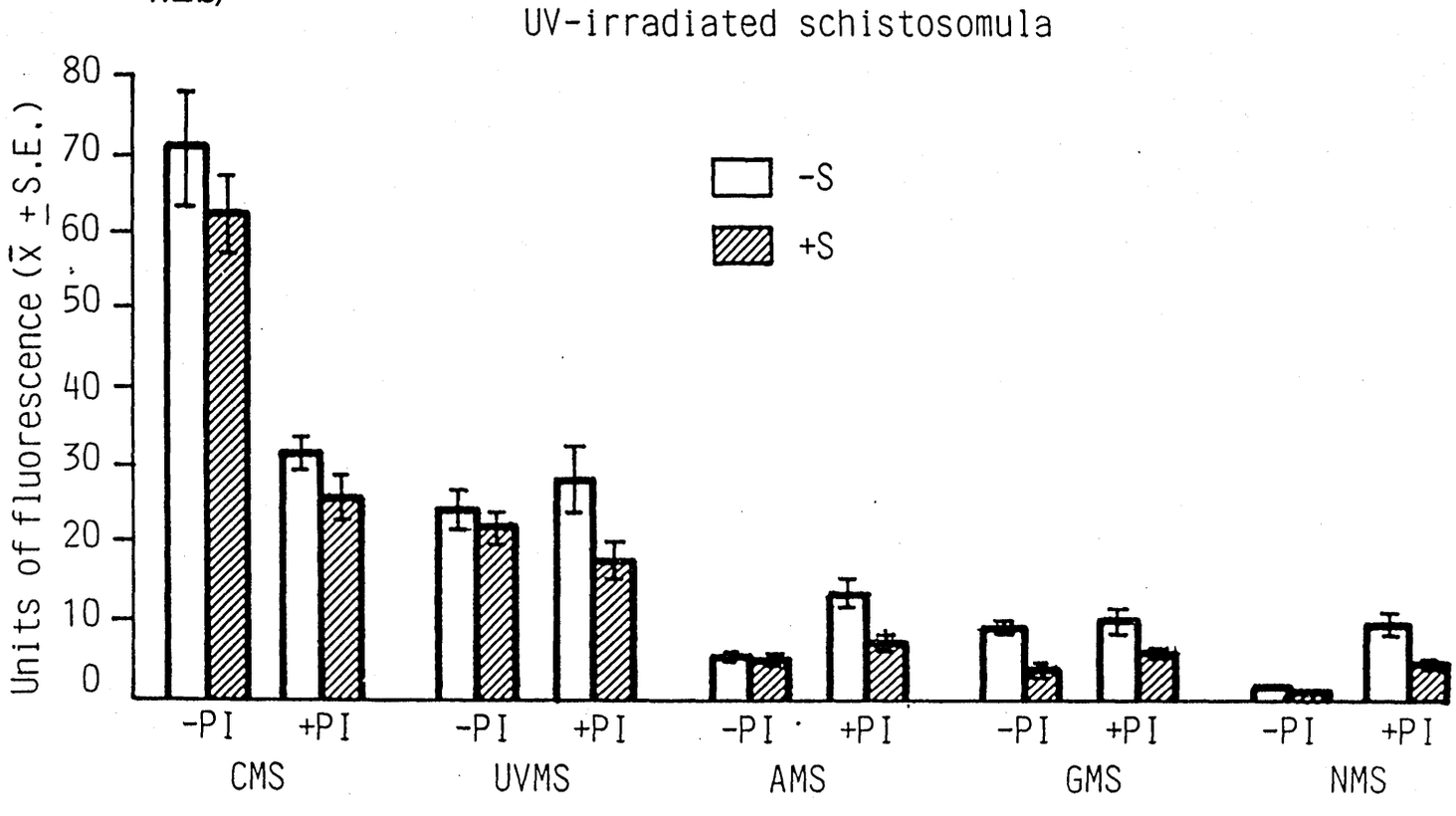
CMS(+PI): -S is not significantly different from +S.

UVMS(+PI): -S is significantly greater than +S: $0.01 < P < 0.025$

7.2a)


Normal schistosomula
-S
+S
Units of fluorescence ($\bar{X} \pm$ S.E.)
70
60
50
40
30
20
10
0
-PI
+PI
CMS
UVMS
AMS
GMS
NMS

7.2b)


UV-irradiated schistosomula
Units of fluorescence ($\bar{x}$ ± S.E.)
80
70
60
50
40
30
20
10
0
-S
+S
-PI
+PI
CMS
UVMS
AMS
GMS
NMS

reduced UVMS binding to normal schistosomula, both periodate-treated and untreated.

AMS and GMS were strikingly similar. Both showed even lower reactivity with normal schistosomula than UVMS, again slightly enhanced after periodate treatment. As for UVMS, competing sugars reduced this binding, whether the normal, target schistosomula were periodate-treated or not.

Competing sugars had no effect on UVMS or AMS binding to U.V.-irradiated schistosomula. This contrasted with the observations on normal schistosomula (figure 7.2a), where sugars markedly reduced binding by these sera. However, when the U.V.-irradiated schistosomula had been periodate-treated, competing sugars again reduced UVMS and AMS binding.

When U.V.-irradiated schistosomula, instead of normal ones, were the target of antibody binding, GMS showed different binding characteristics from UVMS and AMS in that competing sugars reduced reactivity, whether the parasites were periodate-treated or not.

Periodate-treatment and competing sugars had the same effect on NMS binding to U.V.-irradiated as to normal schistosomula.

7.2.3. Effect of periodate treatment of normal, U.V.-irradiated and Actinomycin D-treated schistosomula on binding by serum from mouse strains responsive and non-responsive to the Actinomycin D vaccine.

Subcutaneous injection of Actinomycin D-treated schistosomula induced immunity in NIH mice to subsequent cercarial challenge, but apparently had no effect on resistance in CBA mice (chapter 9). It therefore seemed worthwhile to examine the binding characteristics of sera from the two mouse strains. Sera were obtained after 11-12 weeks from mice infected subcutaneously with normal or Actinomycin D-treated

schistosomula, and from mice challenged percutaneously with normal cercariae five weeks after initial infection or immunisation. The specificity of the different types of sera for periodate-sensitive and insensitive epitopes on normal, U.V.-irradiated and Actinomycin D-treated, 20-hour old schistosomula was examined (figures 7.3a, 7.3b).

As in the preceding experiments (figure 7.1), chronic infection serum, from either mouse strain, showed higher binding to normal than to Actinomycin D-treated schistosomula. For both NIH and CBA mice, periodate treatment of schistosomula decreased binding of chronic serum, and slightly increased that by Actinomycin D vaccine serum. Secondary challenge of infected or immunised mice with normal cercariae led to a striking increase in the antibody response. Although binding of sera from Actinomycin D-vaccinated and challenged mice was not as high as for those rechallenged after a normal infection, it did reach much higher levels than for mice exposed to normal infection only, without prior immunisation. Periodate treatment of normal schistosomula markedly reduced binding of pre-infected or immunised, and challenged sera. However, the level of antibody binding remained two to three times higher than that of infected or immunised, but unchallenged, serum to periodate-treated parasites.

Levels of antibody binding did not appear to reflect the immune status of the two mouse strains. For each category of serum examined, the non-immune CBA strain showed higher antibody levels than the resistant NIH mice.

When the binding to U.V.-irradiated or Actinomycin D-treated schistosomula by sera from mice infected 11-12 weeks previously with normal or Actinomycin D-treated parasites was investigated, the patterns of antibody binding by the two mouse strains were indistinguishable from binding to normal schistosomula (figure 7.3b). Whether normal or attenuated schistosomula were the targets, and whether NIH or CBA sera were being tested, CMS showed higher levels of

Figure 7.3

7.3 a) Binding of NIH and CBA sera to normal, 20 hour-cultured schistosomula.

SERA: Obtained from NIH and CBA female mice.

N = Mice infected s.c. with 150 normal 20-hour schistosomula, and bled 11-12 weeks later.

A = Mice infected s.c. with 500 Actinomycin D-treated, 20-hour schistosomula, and bled 11-12 weeks later.

N/C = Mice infected s.c. with 150 normal, 20-hour schistosomula. Challenged after 6 weeks with 100 normal cercariae p.c. Bled after a further 5-6 weeks.

A/C = Mice infected s.c. with 500 Actinomycin D-treated, 20-hour schistosomula. Challenged after 6 weeks with 100 normal cercariae p.c. Bled after a further 5-6 weeks.

In each case, sera from three mice were pooled.

NMS: Obtained from a pool of uninfected NIH mice.

SCHISTOSOMULA: Normal schistosomula were formaldehyde-fixed after 20 hours of culture in Elac/10% hiFCS.

- PI: not periodate-treated.

+ PI: periodate-treated.

Periodate treatment and indirect immunofluorescence were performed as described in 2.6.3.1.

7.3 a) STATISTICS. t-tests

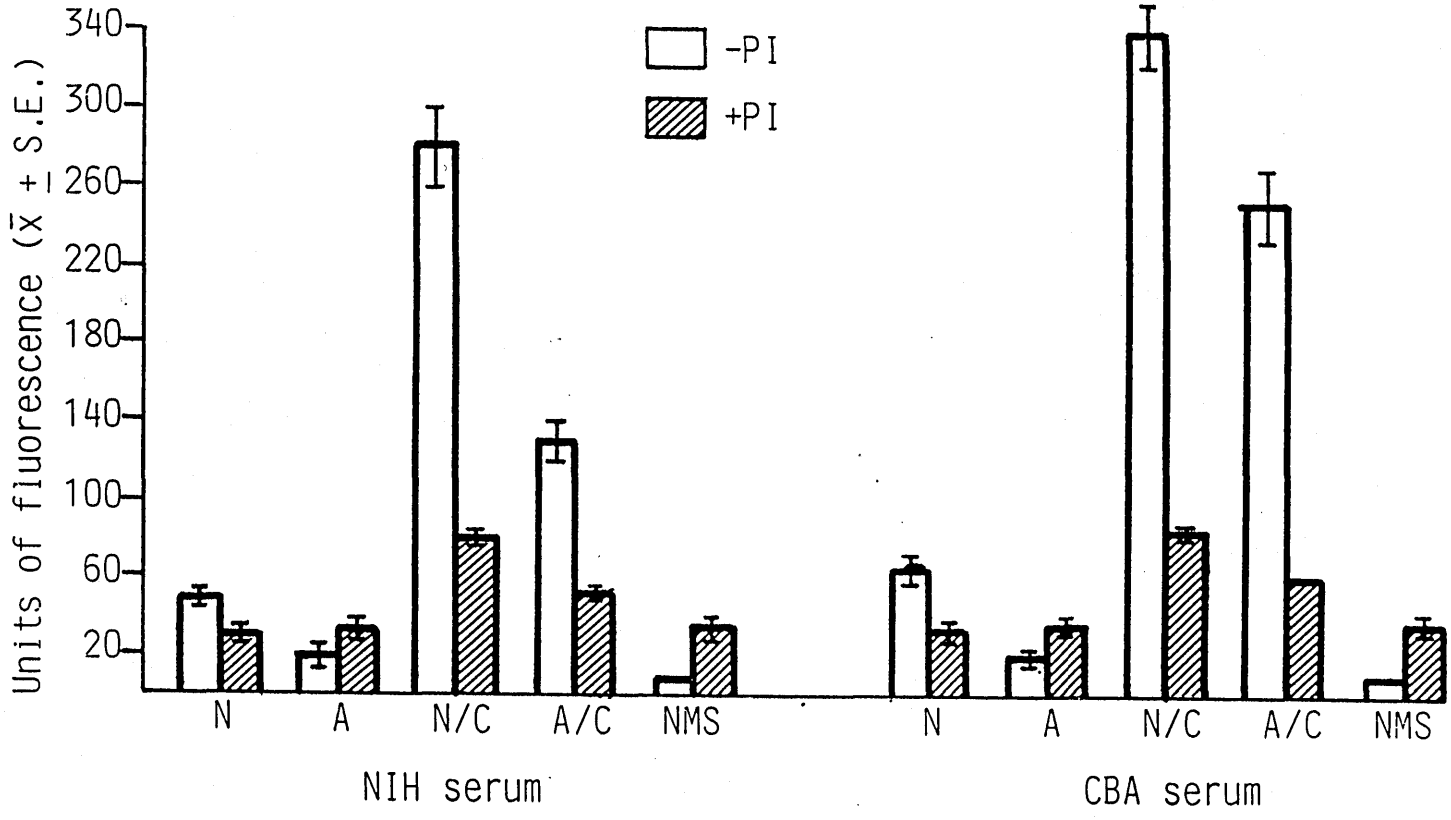
(-PI) N serum: CBA > NIH: $0.025<P<0.05$

(-PI) A serum: CBA and NIH not significantly different.

(-PI) N/C serum: CBA > NIH: $P<0.005$

(-PI) A/C serum: CBA > NIH: $P<0.005$.

7.3 a)


Units of fluorescence ($\bar{x} \pm$ S.E.)
340
300
260
220
180
140
100
60
20
-PI
+PI
N
A
N/C
A/C
NMS
NIH serum
CBA serum

7.3 b) Binding of NIH and CBA sera to normal, U.V.-irradiated (400 μW min cm^{-2}) and Actinomycin D-treated schistosomula.

SERA: N, A } as for 7.3 a)

SCHISTOSOMULA: Normal, U.V.-irradiated (400 μW min cm^{-2}) and Actinomycin D-treated schistosomula were formaldehyde-fixed after 20 hours of culture in Elac/10%hiFCS.

- PI: not periodate-treated
+ PI: periodate-treated.

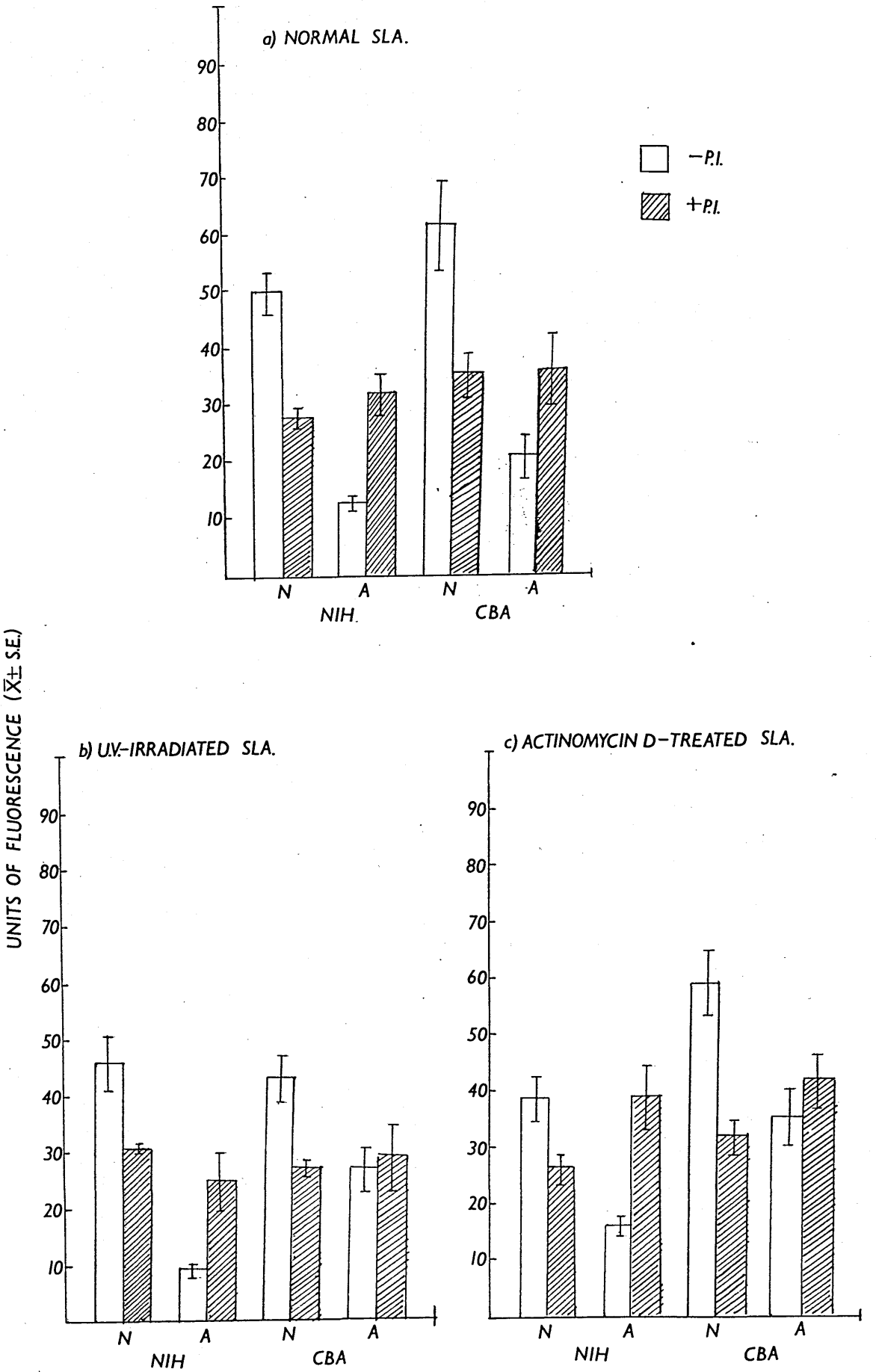
Periodate treatment and indirect immunofluorescence were performed as described in 2.6.3.1.

STATISTICS: t-tests.

Normal schistosomula.	N serum (-PI):	NIH not sig. different from CBA.
	A serum (-PI):	CBA > NIH: $0.025 < P < 0.05$.
U.V. schistosomula.	N serum (-PI):	NIH not sig. different from CBA.
	A serum (-PI):	CBA > NIH: $P < 0.005$
A. schistosomula	N serum (-PI):	CBA > NIH: $P < 0.005$
	A serum (-PI):	CBA > NIH: $P < 0.005$.

CBA, A serum: Act. D sla (-PI) > N sla (-PI): $0.01 < P < 0.025$.

7.3 b)


a) NORMAL SLA.
−P.I.
+P.I.
UNITS OF FLUORESCENCE (X̄± S.E.)
90
80
70
60
50
40
30
20
10
N
A
N
A
NIH.
CBA
b) U.V.-IRRADIATED SLA.
c) ACTINOMYCIN D-TREATED SLA.
NIH
CBA

binding than AMS, and periodate treatment always decreased CMS binding, while increasing that of AMS. CBA chronic or vaccine serum showed greater binding to normal, U.V.-irradiated and Actinomycin D-treated schistosomula than the corresponding NIH sera, except in the case of CMS against U.V.-irradiated schistosomula, when the two mouse strains showed equal binding. Serum from CBA mice vaccinated with Actinomycin D-treated parasites showed greater reactivity with Actinomycin D-treated parasites than with normal ones, but this was not true for NIH sera.

7.2.4. ELISA assays: periodate-sensitive and insensitive epitopes in whole schistosomular homogenates. Antibody isotypes induced by chronic infection or vaccination.

In order to complement the results, obtained by immunofluorescence, on antibodies directed against schistosomular surface antigens, chronic infection serum and sera from mice immunized with U.V.- or gamma-irradiated schistosomula were used in ELISA assays on homogenates of whole schistosomular proteins. The different antibody isotypes present in infection and vaccine sera were also compared (figures 7.4a)-e)).

As already demonstrated in the immunofluorescence assays, CMS showed much higher antibody levels than either gamma- or U.V.-irradiated vaccine sera. None of the sera examined showed levels of schistosome-specific IgG2a or IgG2b significantly above those of NMS (figures d,e). The dominant IgG isotypes in CMS were IgG1 and IgG3, and a considerable proportion of each seemed to be directed against periodate-sensitive, presumably carbohydrate, epitopes (figures b,c). Recipients of U.V.-irradiated or gamma-irradiated schistosomula showed very low levels of IgG1 and IgG3 antibodies, which increased slightly after challenge with normal cercariae. Periodate treatment of the

Figure 7.4 a)-e).

Effect of periodate treatment of 3-hour schistosomular homogenates on binding of different antibody isotypes, in infected or vaccine sera, measured by ELISA.

Sera were obtained from NIH female mice. Sera from 3 mice were pooled in each case.

ABBREVIATIONS FOR SERA

Ch: Mice were infected p.c. with 150 normal cercariae and bled 11-12 weeks later.

G: Mice immunised with 500 20 krad gamma-irradiated cercariae p.c., and bled after 11-12 weeks.

G/C: Mice immunised with 500 20 krad gamma-irradiated cercariae p.c.; challenged 6 weeks later with 150 normal cercariae p.c.; bled after a further 5-6 weeks.

U: Mice immunised with 500 U.V.-irradiated (400 μW min cm^{-2}) cercariae p.c.; bled after 11-12 weeks.

U/C: Mice immunised with 500 U.V.-irradiated (400 μW min cm^{-2}) cercariae p.c.; challenged 6 weeks later with 150 normal cercariae p.c.; bled after a further 5-6 weeks.

NMS: Obtained from a pool of uninfected NIH mice.

Homogenate of 3-hour schistosomula was prepared as described in 2.6.3.4. ELISA assays and periodate treatment were performed as described in 2.6.3.4.

a): IgM binding
b): IgG1 "
c): IgG3 "
d): IgG2a "
e): IgG2b "

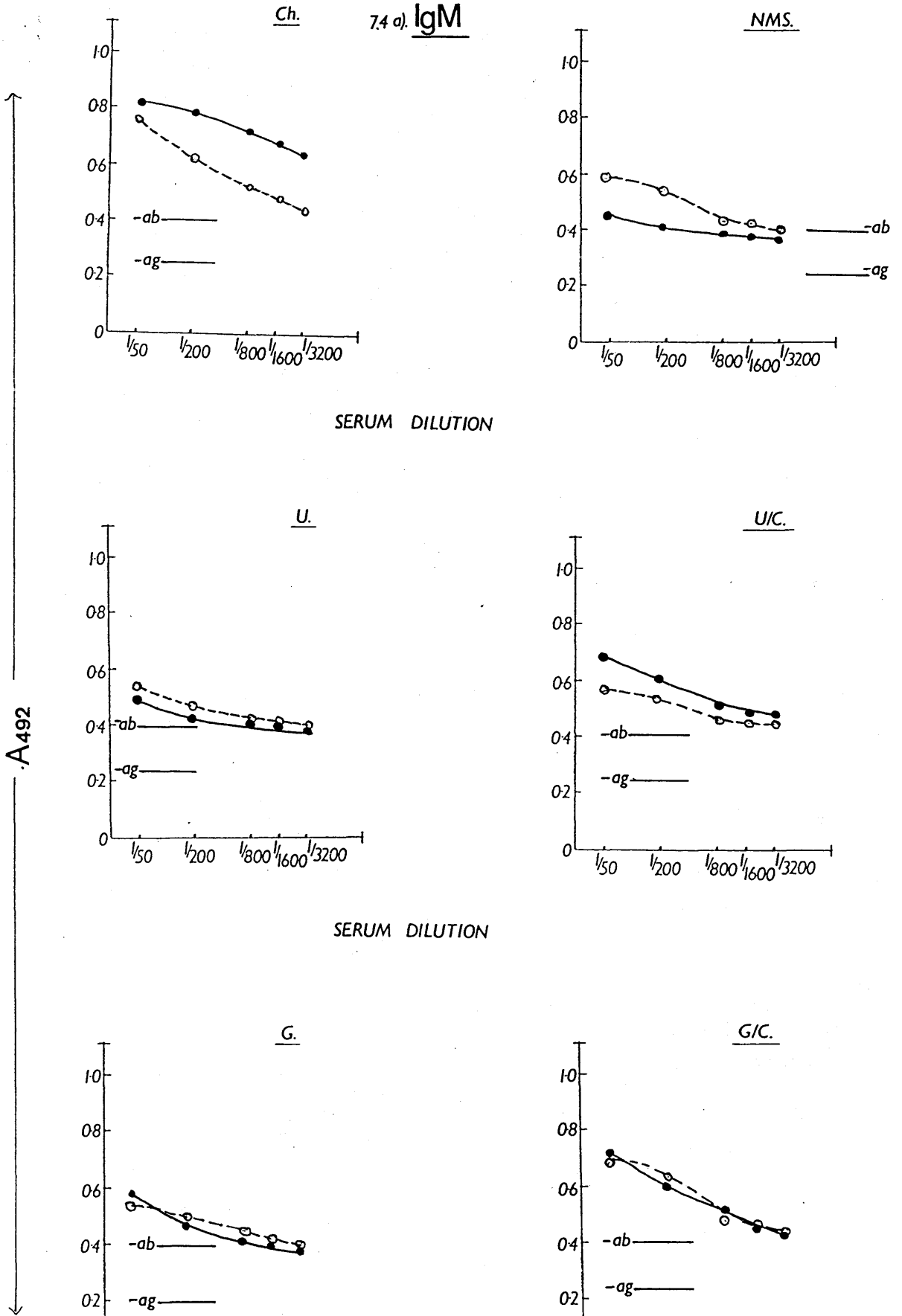
—•— = untreated antigen

----o---- = periodate-treated antigen

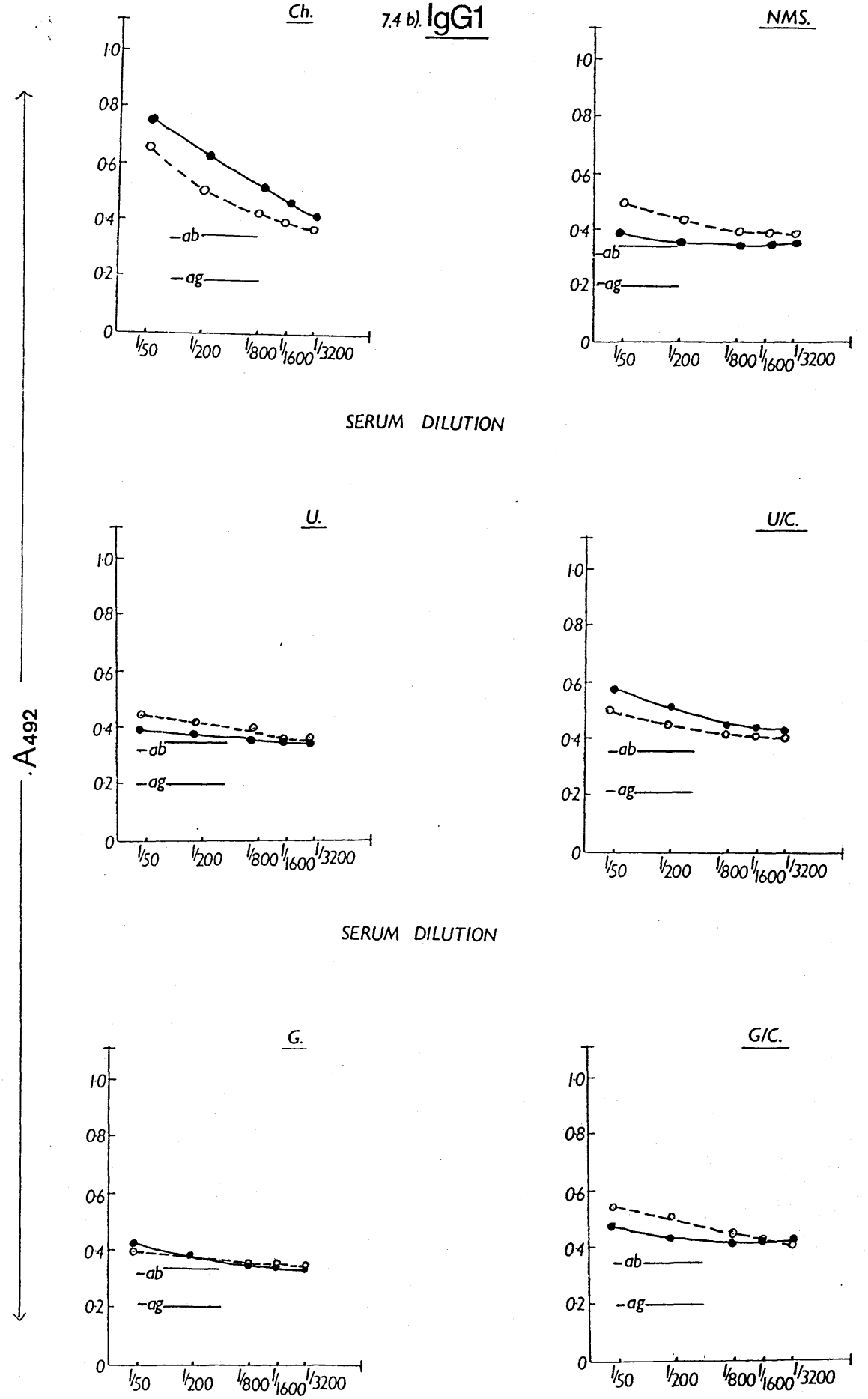
-ab: no Ist Ab in assay

-ag: no Ag in assay; Ch serum as Ist Ab

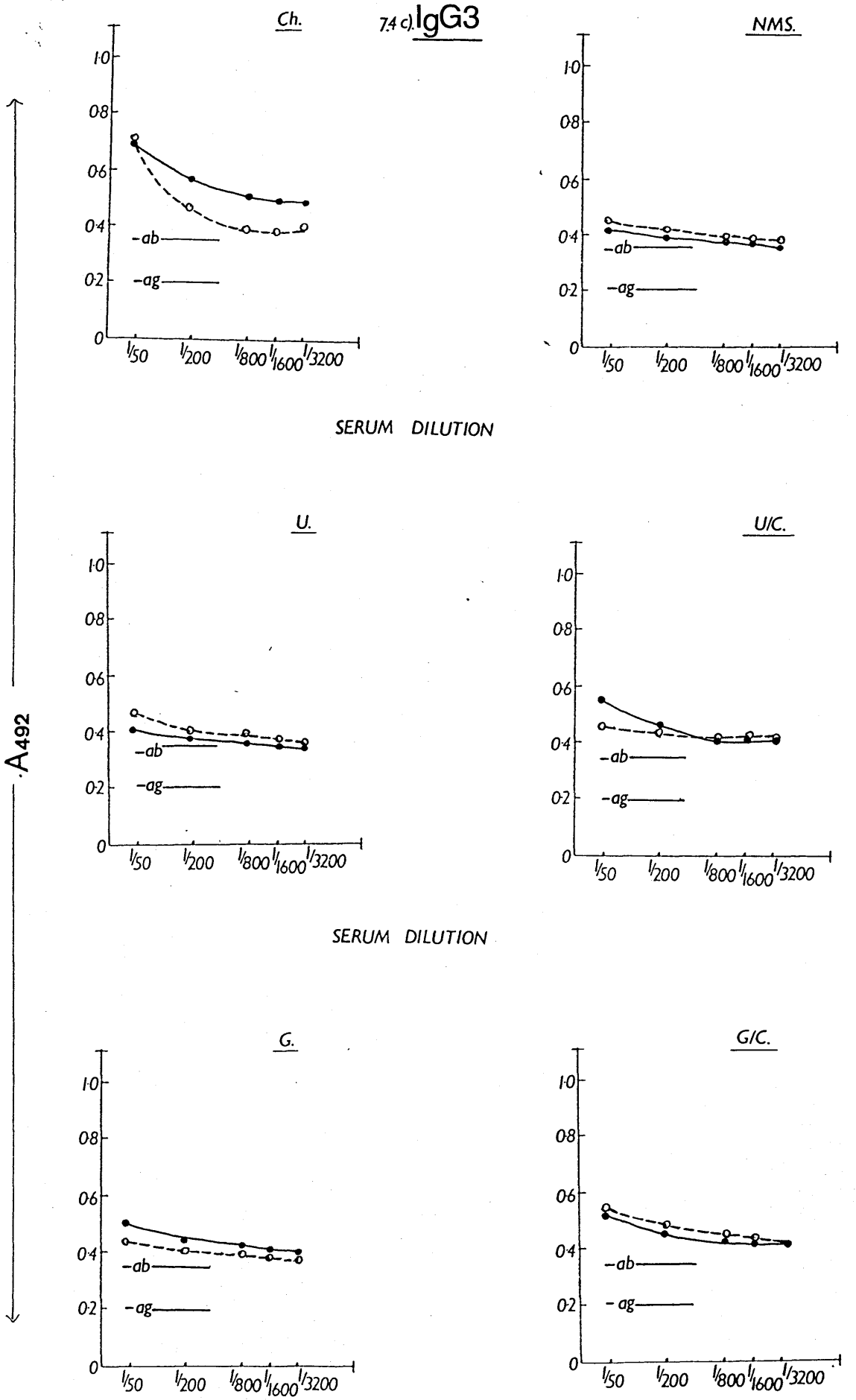
7.4 a). IgM


Ch.
NMS.
U.
U/C.
G.
G/C.
1·0
0·8
0·6
0·4
0·2
0
-ab
-ag
1/50
1/200
1/800
1/1600
1/3200
SERUM DILUTION
A492

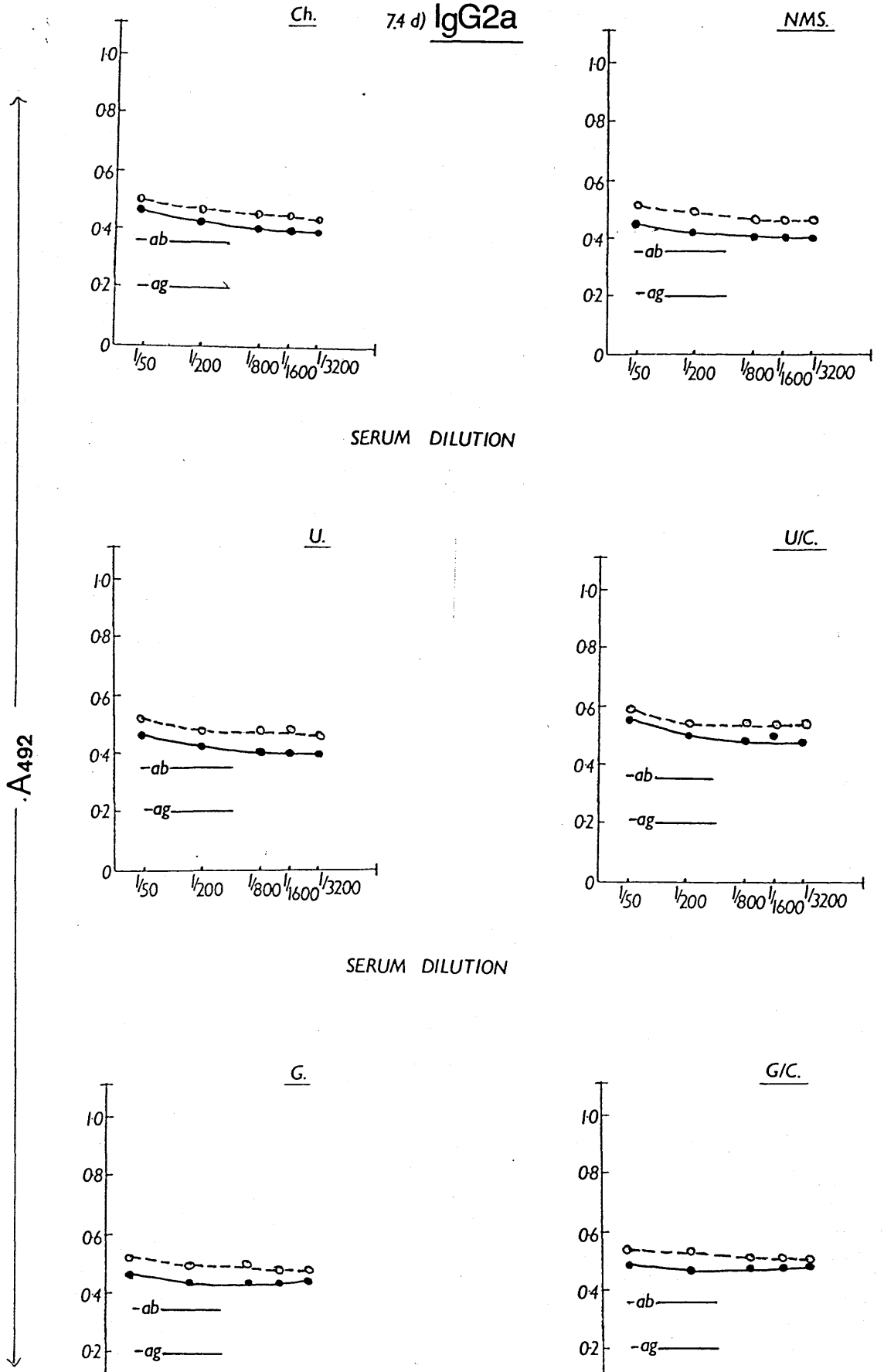
7.4 b). IgG1


Ch.
NMS.
U.
U/C.
G.
G/C.
1·0
0·8
0·6
0·4
0·2
0
-ab
-ag
1/50
1/200
1/800
1/1600
1/3200
SERUM DILUTION
SERUM DILUTION
SERUM DILUTION
A492

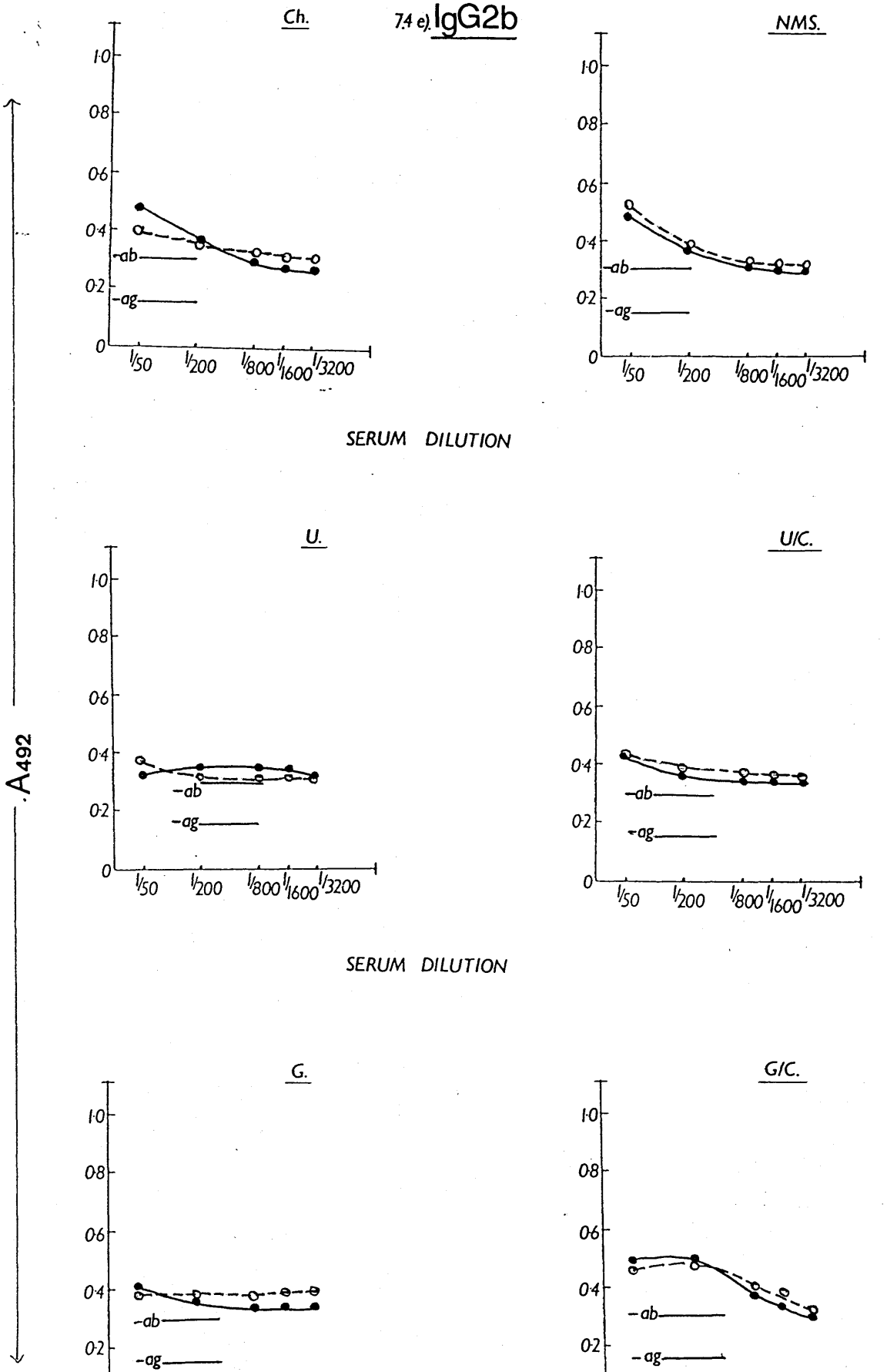
7.4 c). IgG3


Ch.
NMS.
U.
U/C.
G.
G/C.
1·0
0·8
0·6
0·4
0·2
0
-ab
-ag
1/50
1/200
1/800
1/1600
1/3200
SERUM DILUTION
A492

7.4 d) IgG2a


Ch.
NMS.
U.
U/C.
G.
G/C.
1·0
0·8
0·6
0·4
0·2
0
-ab
-ag
1/50
1/200
1/800
1/1600
1/3200
SERUM DILUTION
A492

7.4 e). IgG2b


Ch.
NMS.
U.
U/C.
G.
G/C.
1·0
0·8
0·6
0·4
0·2
0
-ab
-ag
1/50
1/200
1/800
1/1600
1/3200
SERUM DILUTION
A492

schistosomular antigens did not reduce binding of these isotypes in the two vaccine sera; indeed, there was even a slight increase. Levels of IgM antibodies directed against schistosomular antigens in CMS wre higher than any of the IgG isotypes, and a high proportion showed specificity for periodate-sensitive, carbohydrate epitopes. IgM levels in GMS and UVMS, though much lower than in chronically-infected mice, were also noticeably higher than the IgG isotypes, and increased quite markedly after challenge. Since periodate treatment did not reduce binding, these IgM's may not be directed against carbohydrate antigens.

7.3. Discussion

7.3.1. Antisera from mice immunised with irradiated or Actinomycin D-treated larvae show similar binding to periodate-treated and untreated schistosomula.

These observations impress upon us that UVMS, AMS and GMS show almost identical binding patterns to normal, irradiated and Actinomycin D-treated schistosomula (figs. 7.1 a)-c)). Periodate treatment of the schistosomula, or the addition of competing sugars to the assay also had very similar effects on binding by the three antisera. Thus, the three types of attenuation appear to be comparable, not only in their metabolic effects on parasites, but also as regards the humoral immune response which they evoke in the host. The model which attributes the immunogenicity of the gamma-irradiated vaccine, at least in part, to production of antibodies predominantly specific for capable of mediating ADCC (see sections 7.1.; 1.9.2.3.7) might polypeptide epitopes on the schistosomular surface and of isotypes therefore also apply to the biochemically similar U.V.-irradiated and Actinomycin D-treated schistosomula. In contrast, the polysaccharides derived from eggs during chronic infection stimulate massive production

of immunoglobulins of blocking isotypes which cross-react with carbohydrates on the larval surface, and prevent binding of those antibodies potentially detrimental to parasite survival. The observation of Omer-ali et al (1988) that, despite the differences in binding of CMS and VMS to the intact parasite surface, both sera identify the same antigens by immunoprecipitation, is especially interesting, for it supports the suggestion that the two types of sera could recognise different epitopes on the same antigens. CMS is likely to react with the outermost sugars of glycoproteins, since these will be exposed on the normal schistosomular surface. VMS, obtained from mice exposed to larvae with prematurely terminated or unfolded glycoproteins, exposing normally hidden protein determinants, as proposed in chapter 4, may respond chiefly to more interior polypeptide epitopes.

7.3.2. Irradiated and Actinomycin D-treated schistosomula induce only a low antibody response.

The low level of binding to the schistosomular surface by UVMS, GMS and AMS (figure 7.1) suggests that humoral responses might not be the major mechanism by which resistance to challenge infection is induced in these vaccine models. These assays used antibodies from mice at a comparatively long time after immunisation - 11-12 weeks. However, Omer-ali et al (1986) found that binding to schistosomula by sera obtained only 4 weeks after the last of 3 successive immunisations with gamma-irradiated larvae was almost one-tenth of CMS binding - an even smaller proportion than observed here. Nor does the antibody response reflect the degree of resistance to experimental challenge induced by Actinomycin D-treated schistosomula - CBA mice, which are non-resistant, actually show higher antibody binding than the resistant NIH strain (figures 7.3a, 7.3b). The fact that as many as three successive

vaccinations are required for passive transfer of serum in mice to confer 50% immunity (Mangold and Dean, 1986) also suggests that humoral responses may not be the primary mechanism of immunity in these attenuated vaccines. Nevertheless, the assays involving periodate treatment or incubation in the presence of competing sugars should give some indication of the carbohydrate or polypeptide nature of the epitopes exposed by the irradiated and Actinomycin D-treated parasites. After challenge of mice exposed to the Actinomycin D-treated vaccine, responses to both periodate-sensitive and insensitive antigens markedly increased, perhaps indicating that the initial exposure to attenuated schistosomula had primed the host immune system to both carbohydrate and polypeptide epitopes. Indeed, Correa-Oliveira et al (1984) suggested that the anamnestic antibody response to challenge in vaccinated mice is so rapid and produces such high levels of immunoglobulins that it might help to eliminate the challenge larvae which themselves stimulated the heightened response.

7.3.3. Irradiated and Actinomycin D-treated schistosomula do express carbohydrate epitopes.

The fact that CMS binding to U.V.-irradiated and Actinomycin D-treated schistosomula decreases when these parasites are subjected to periodate treatment (figures 7.1, 7.2) suggests that, despite inhibition of glycosylation and expression of proteins in unfolded conformations, some carbohydrate epitopes are still exposed on the surface of the attenuated parasites. This claim is further supported by the observation that competing sugars reduced binding of CMS, UVMS, AMS and GMS to U.V.-irradiated schistosomula that had been subjected to periodate (figure 7.2b). Moreover, UVMS, AMS and GMS binding to normal schistosomula also decreased in presence of competing sugars (figure 7.2a), implying that in all three cases, the attenuated schistosomula

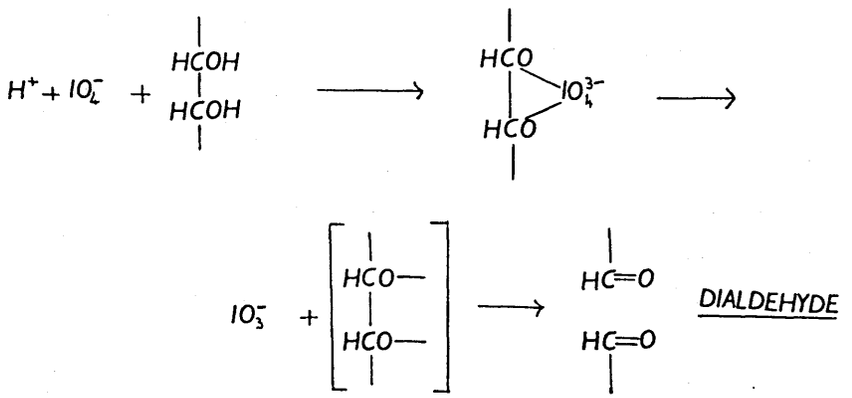
had primed their hosts to recognise carbohydrate epitopes.

Competing sugars further reduced binding of all sera to schistosomula already subjected to periodate treatment (figures 7.2a, 7.2b), implying that periodate does not completely destroy all carbohydrate epitopes. Indeed, when Omer-ali et al (1986) used trifluoromethanesulphonate (TFMS) treatment to destroy carbohydrate epitopes on normal schistosomula, some 40% of the binding capacity of gamma-irradiated vaccine serum was abolished, whereas periodate treatment had no effect on binding by this serum. These findings support our own conclusions, suggesting, firstly, that hosts vaccinated with irradiated larvae do produce some anti-carbohydrate antibodies, and, secondly, that periodate does not completely destroy carbohydrate antigens.

7.3.4. Models for binding of the different sera to normal, irradiated and Actinomycin D-treated schistosomula.

7.3.4.1. Mechanism of action of periodate.

Periodate oxidation of 1, 2-diol groups in sugars apparently takes place through the intermediate formation of an unstable ester, resulting in cleavage of a C-C bond and production of a dialdehyde:



(Pigman and Goepp, 1948).

As regards polysaccharides, periodate oxidises only some of the total carbohydrate residues. Thus, Lotan et al (1975) found that periodate treatment of soybean agglutinin resulted in disruption of, at most, five out of the nine mannose residues present in each of its subunits. Periodate only oxidises pyranose sugars in particular types of polysaccharide linkage (see figure 7.5). Non-reducing end-groups (i) and hexose residues linked through C_1 and C_6 (v) contain three contiguous free hydroxyl groups, which will both be attacked, with consumption of two moles of periodate. Pyranose residues linked through C_1 and C_4 (ii) or C_1 and C_2 (iii) possess two adjacent hydroxyl groups, hence reduce one mole of periodate for every unit, and are themselves cleaved through C_2 and C_3, and C_3 and C_4 respectively. In contrast, 1, 3-linked pyranose sugars resist oxidation, as do branch points in 1,2- or 1,4-linked linear polysaccharides ((vi): 1,4-linked unit). Residues involved in such linkages have no α-glycol groups, therefore are immune to periodate attack.

Lotan et al (1975) pointed out that, besides its action on carbohydrate, periodate can also oxidise SH groups in the polypeptide domain of glycoproteins. Thus, in interpretation of our results, it should be remembered that periodate may disrupt, not only carbohydrate, but also polypeptide, structures.

There is a notable similarity between the action of periodate and some of the effects of irradiation. The free radicals induced by irradiation also oxidise SH groups (von Sonntag, 1987; section 1.10) and may also form aldehydes from pyranose sugars, though in this case they are not derived by C-C bond cleavage:

Free radical produced by irradiation-induced abstraction of H from C-H bond.

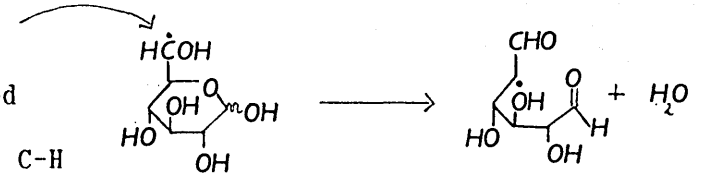
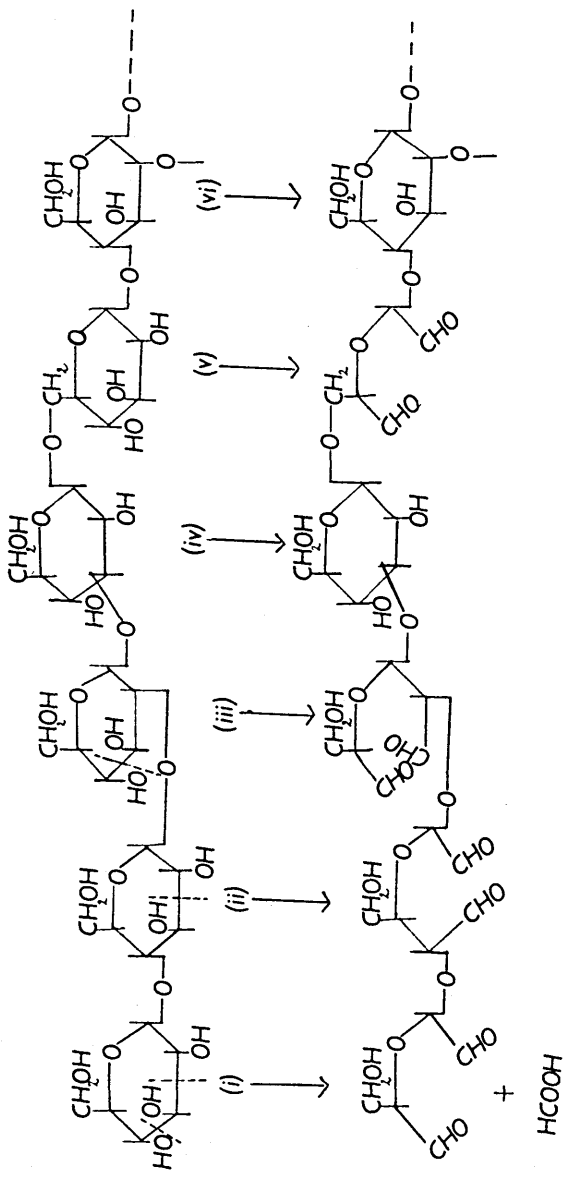


Figure 7.5. Action of periodate on different types of polysaccharide linkage.



Thus, the antigens exposed by periodate treatment may, to some extent, adopt similar conformations to those expressed by irradiated schistosomula.

7.3.4.2. Antigenic determinants exposed by normal and attenuated schistosomula in the presence and absence of periodate.

We would suggest that the nature of the antigens expressed by normal and attenuated schistosomula may explain the patterns of binding by infection and vaccine sera, in presence and absence of periodate, as follows (see figures 7.6a), b)).

a) Binding of CMS to normal schistosomula.

i) Untreated normal schistosomula.

The work of Omer-ali et al (1986) indicates that the predominant binding by CMS is to carbohydrate epitopes. Although antibodies against polypeptide moieties are also present, their contribution to the total binding capacity of the serum appears to be comparatively minor (fig. 7.6a), i)).

ii) Periodate-treated normal schistosomula.

Destruction of a proportion of carbohydrate groups by periodate means that fewer antigenic sites are available for anti-carbohydrate antibodies. However, polypeptide epitopes should remain largely intact. Since CMS contains a lower proportion of anti-polypeptide antibodies than anti-carbohydrate ones, total binding decreases considerably. (fig. 7.6 a), ii).

Figure 7.6 a), b) Models for binding of chronic infection serum and vaccine sera to normal and attenuated schistosomula, untreated or periodate-treated.

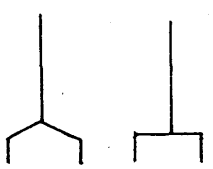

= polypeptide domain of glycoprotein embedded in the schistosomular surface membrane.


= polypeptide epitope exposed on normal glycoprotein.


= carbohydrate epitope exposed on normal glycoprotein.


= carbohydrate or polypeptide epitope modified by irradiation or Actinomycin D treatment.


= carbohydrate or polypeptide epitope modified by periodate treatment.


,etc. = Antibodies of different specificities.

7.6 a) Binding of chronic infection serum.

i) To untreated, normal schistosomula.

Antibodies to polypeptide epitopes are present, but binding is predominantly to carbohydrates.

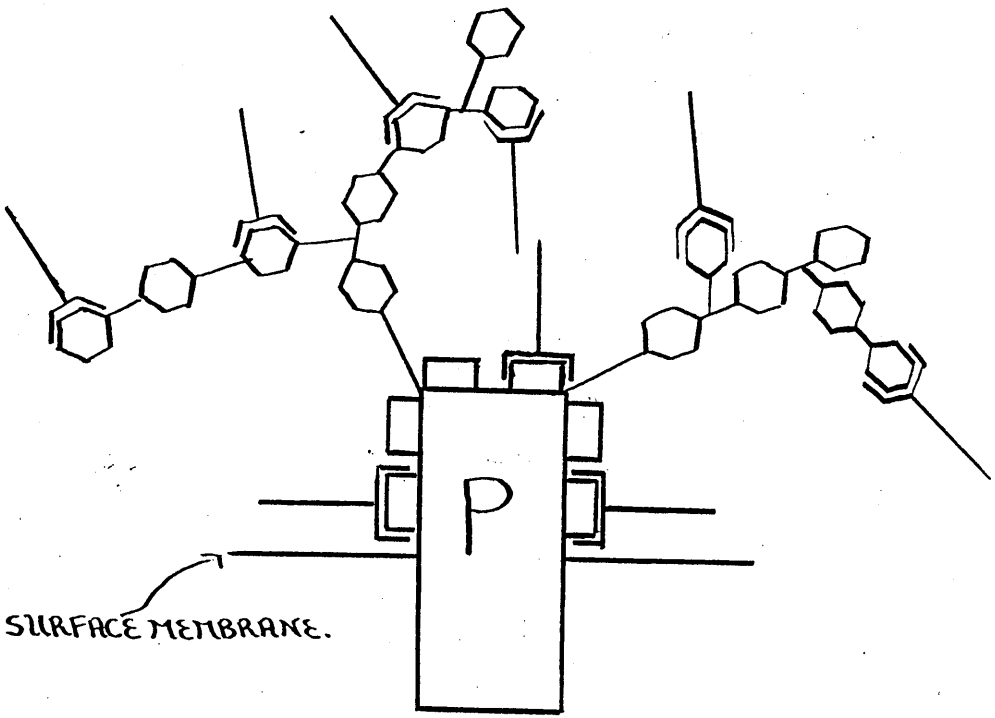
ii) To periodate-treated, normal schistosomula.

Periodate-oxidation of carbohydrate epitopes means that predominantly anti-peptide antibodies bind.

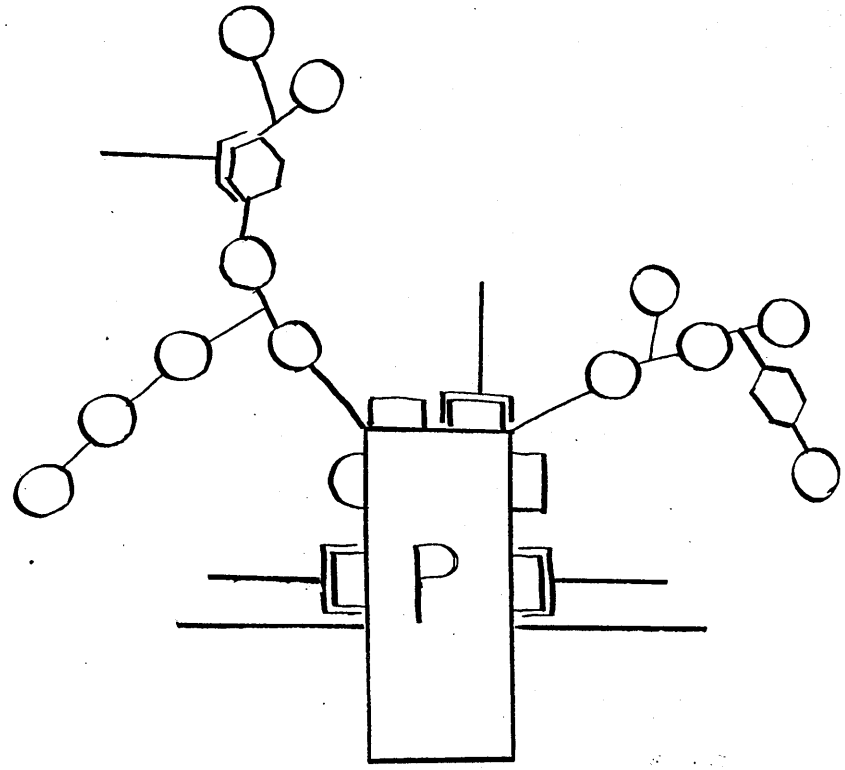
There is still some minimal binding to those carbohydrate structures unaltered by periodate treatment.

a) BINDING OF CHRONIC INFECTION SERUM.

i) TO UNTREATED, NORMAL SCHISTOSOMULA.



ii) TO PERIODATE-TREATED, NORMAL SCHISTOSOMULA.



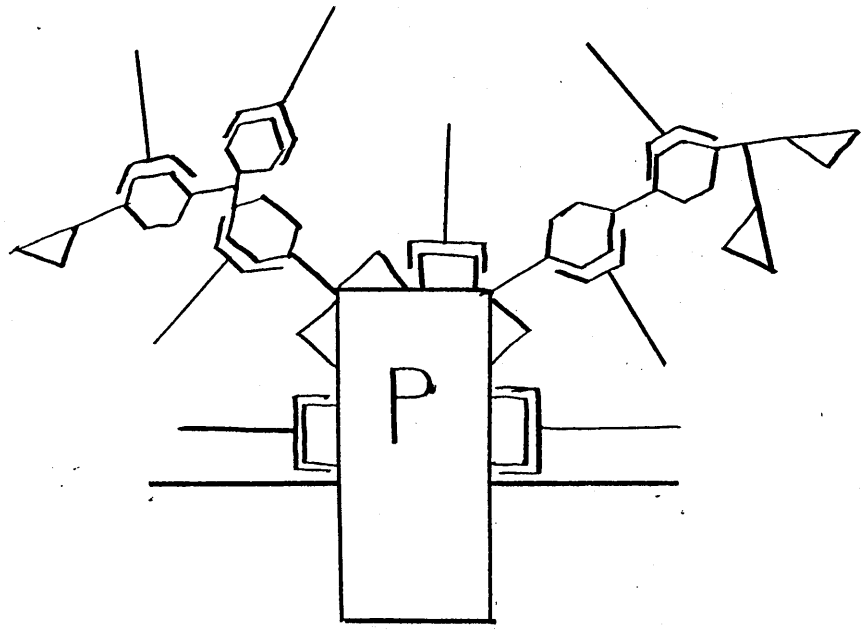
7.6 a) iii) To U.V.-irradiated or Actinomycin D-treated schistosomula.

Oligosaccharide structures are incomplete; many polypeptide and carbohydrate epitopes are modified, but antigens recognised by CMS are still available for binding.

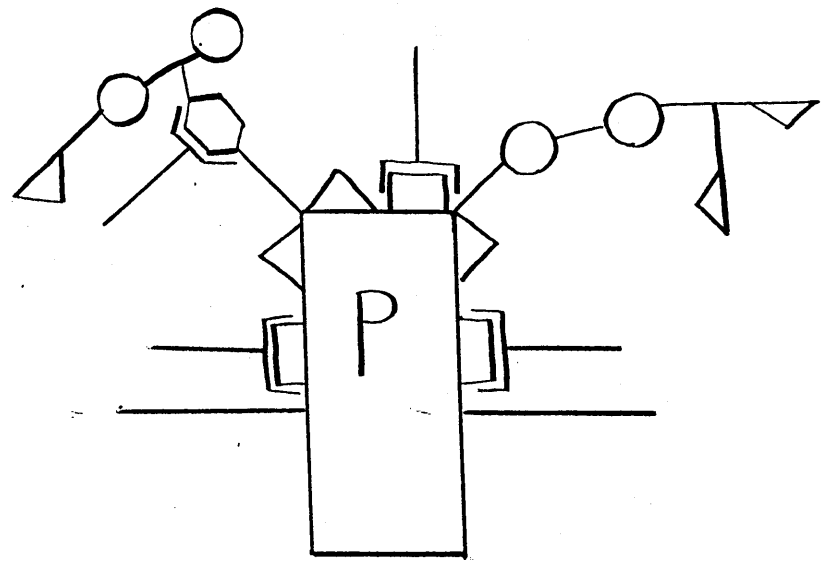
iv) To U.V.-irradiated or Actinomycin D-treated schistosomula, subjected to periodate treatment.

Periodate-oxidation of sugars means that predominantly anti-peptide antibodies bind.

iii). To U.V.-IRRADIATED OR ACTINOMYCIN D-TREATED SCHISTOSOMULA.



iv). To U.V.-IRRADIATED OR ACTINOMYCIN D-TREATED SCHISTOSOMULA, SUBJECTED TO PERIODATE TREATMENT.



7.6 b) Binding of UVMS, AMS, GMS

i) To normal schistosomula.

The vaccine sera are raised against incomplete and modified structures, as shown in a)iii) and b)iii). Hence, the antibodies should bind to the normal schistosomular antigens at internal carbohydrates in the oligosaccharide structures, and to polypeptide epitopes. Total binding is much lower than for chronic infection serum, because vaccine sera contain fewer anti-carbohydrate antibodies, only a proportion of which are directed against native carbohydrate structures as expressed by normal schistosomula.

ii) To periodate-treated, normal schistosomula.

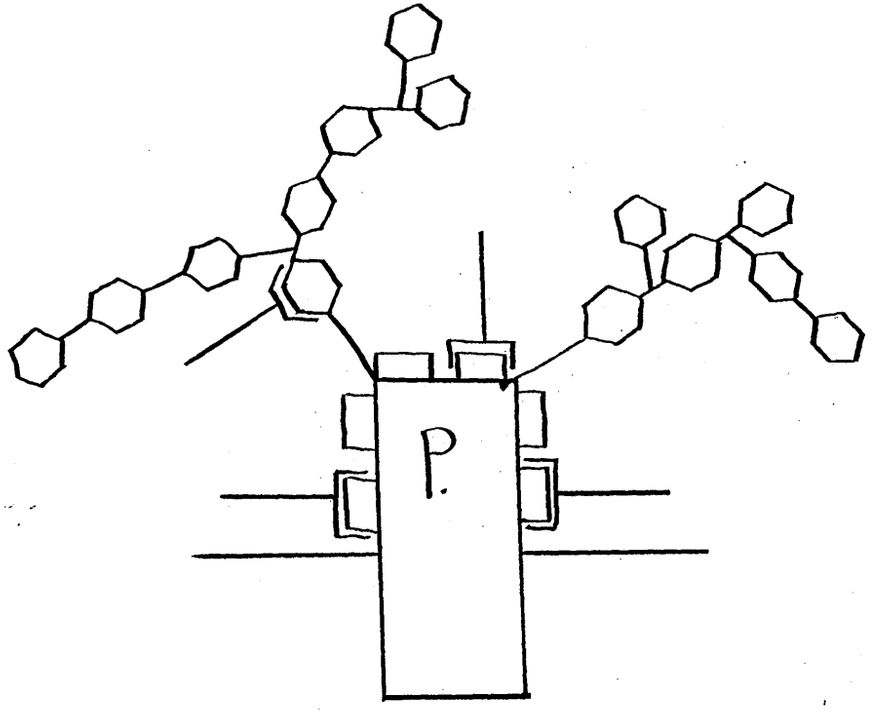
Periodate treatment has little effect on binding by vaccine antibodies, since they are chiefly directed against polypeptide epitopes.

The carbohydrate structures that remain unaffected by periodate treatment are sufficient to bind the comparatively few anti-carbohydrate antibodies.

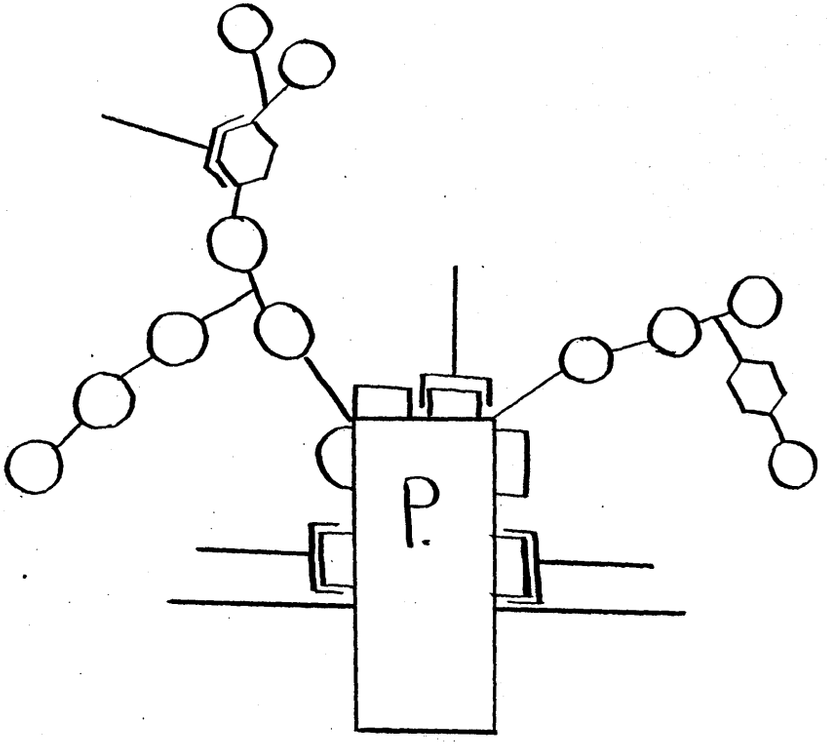
Total antibody binding is therefore not reduced by periodate treatment.

b) Binding of IIVMS., GMS., AMS.

i) To normal schistosomula.



ii). To periodate-treated, normal schistosomula.



7.6 b) iii) To irradiated or Actinomycin D-treated schistosomula.

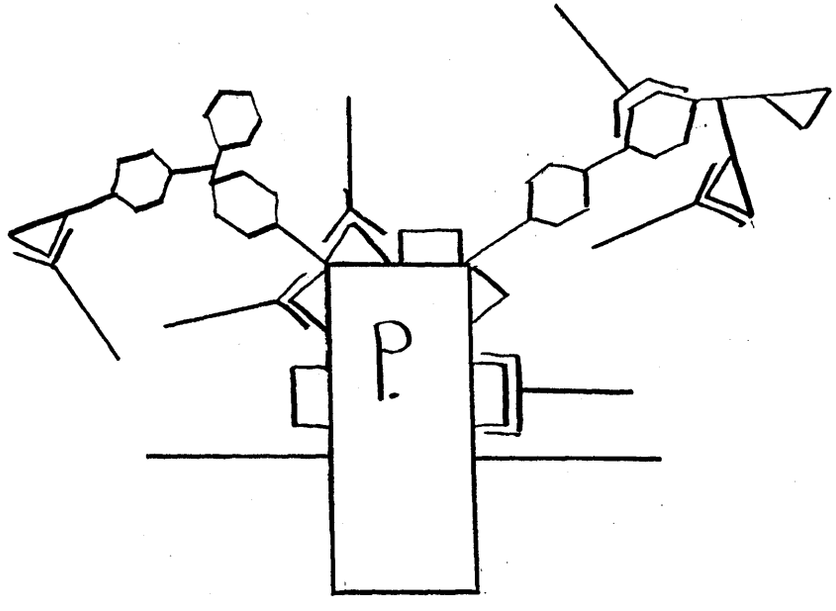
Vaccine sera contain antibodies directed against those antigens △ whose conformation is modified by irradiation or drug treatment.

iv) To irradiated or Actinomycin D-treated schistosomula subjected to periodate treatment.

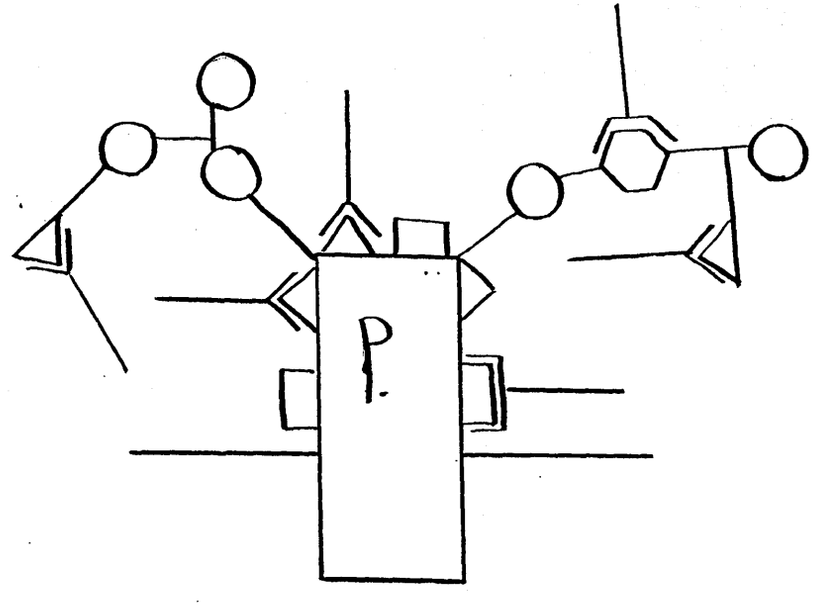
Periodate treatment has little effect because binding is directed predominantly against peptide epitopes.

The carbohydrate structures that remain unaffected by periodate treatment are sufficient to bind the comparatively few anti-carbohydrate antibodies.

iii). BINDING TO IRRADIATED OR ACTINOMYCIN D-TREATED SCHISTOSOMULA.



iv). TO IRRADIATED OR ACTINOMYCIN D-TREATED SCHISTOSOMULA SUBJECTED TO PERIODATE TREATMENT.



b) Binding of UVMS, AMS, GMS to normal schistosomula.

The various vaccine sera apparently contain predominantly anti-polypeptide antibodies, presumably because the carbohydrate moieties of the immunising, attenuated larvae are disrupted or incomplete (chapter 4). Although these protein antigens expressed by irradiated or Actinomycin D-treated schistosomula are predicted to be in partially-denatured conformations, the determinants recognised by the antibodies must share some epitopes with native parasite antigens in order to permit the observed binding to the normal schistosomular surface. Indeed, to induce effective protection against normal, challenge parasites, the attenuated schistosomula must induce an immune response which recognises native antigens. We propose that the modified antigens of irradiated and Actinomycin D-treated larvae are processed by antigen-presenting cells differently from normal ones. As a result, epitopes shared with the normal antigens may be presented to the host immune system in a novel manner which stimulates a powerful protective response against challenge. This model will be discussed fully in chapter 10.

The effects of competing sugars on binding to normal schistosomula by the three sera and the fact that periodate treatment of U.V.-irradiated or Actinomycin D-treated schistosomula reduces binding by CMS implies that some carbohydrate epitopes are expressed by attenuated parasites, and that these carbohydrate determinants do stimulate an antibody response. Since glycosylation is inhibited in attenuated schistosomula, it might be predicted that the carbohydrate structures recognised on normal schistosomula would be found chiefly in the internal core of oligosaccharide chains (fig. 7.6b), i)).

ii) Periodate-treated normal schistosomula.

Periodate treatment will destroy some carbohydrate epitopes, but the disruption it causes may expose formerly hidden epitopes on underlying polypeptides and in internal core regions of oligosaccharides. The slightly preferential binding of UVMS, AMS and GMS to periodate-treated schistosomula may be due to the fact that vaccinated hosts were originally exposed to such cryptic antigens. The similarity between periodate and irradiation in producing dialdehydes and oxidising SH groups might also help explain the increased binding of UVMS and GMS to periodate-treated schistosomula. (figure 7.6 b), ii)).

c) Binding of CMS to U.V.-irradiated and Actinomycin D-treated schistosomula.

i) Non-periodate-treated, U.V.-irradiated and Actinomycin D-treated schistosomula.

The antibodies involved in recognition in these instances should be those specific for the more internal sugars of oligosaccharides, and for polypeptide epitopes. Although we would predict that the antigens expressed by the attenuated parasites should be in partially modified conformations, whereas the antibodies in chronic mouse serum were originally directed against native molecules, there must be sufficient similarity in structure for CMS to recognise the antigens expressed by the attenuated parasites (figure 7.6 a), iii)).

ii) Periodate-treated, U.V.-irradiated and Actinomycin D-treated schistosomula.

Periodate treatment seems to destroy a high proportion of the epitopes recognised by CMS on U.V.-irradiated schistosomula. This suggests that many of the antigens recognised on the untreated forms may be carbohydrate in nature. After exposure to periodate, CMS presumably recognises chiefly the remaining polypeptide epitopes (figure 7.6 a), iv)).

d) Binding of UVMS, AMS and GMS to U.V.-irradiated and Actinomycin D-treated schistosomula.

i) Untreated, U.V.-irradiated and Actinomycin D-treated schistosomula.

These vaccine sera should bind to the incomplete and denatured carbohydrate and polypeptide structures on attenuated schistosomula with high affinity, because the antibodies involved were originally induced by antigens in such modified conformations. Binding to the attenuated schistosomula is no higher than to normal ones, despite the fact that the modified antigens would be predicted to accommodate these antibody binding sites better. We would suggest that all the antibodies in the vaccine sera may bind to both normal and attenuated schistosomula, but binding affinity is predicted to be higher for attenuated schistosomula. The effect of competing sugars seems to support this idea that vaccine sera bind with higher affinity to attenuated than to normal schistosomula - see e) below (figure 7.6 b), iii)).

ii) Periodate-treated, U.V.-irradiated and Actinomycin D-treated schistosomula.

Binding of all three vaccine sera increased slightly when the attenuated parasites were subjected to periodate. We would suggest that the conformation in which the U.V.-irradiated and Actinomycin D-treated schistosomula were formaldehyde-fixed may expose only a limited number of the total epitopes. Periodate treatment, although destroying some of the antigens, could expose others which the immune system would have recognised on the unfixed, attenuated schistosomula (figure 7.6 b) iv)).

e) The effects of competing sugars.

One interpretation of the effects of competing sugars on the binding of infection and vaccine sera to schistosomula is that competing sugars only intercept antigen/antibody interaction when the antibody does not have a high affinity for the epitope concerned. CMS would be predicted to have especially high affinity for normal schistosomula, and UVMS for U.V.-irradiated schistosomula. Hence, competing sugars would not interfere with these binding reactions. However, CMS apparently recognises antigens of attenuated schistosomula with much lower affinity. The same applies to the vaccine sera as regards normal parasite antigens. In each case, the carbohydrate conformations recall relatively faintly the structures against which the antibodies were originally raised. Thus, the antibodies may have an equally high affinity for exogenously added competing sugars. This explanation might also account for the fact that competing sugars always inhibit the interaction with periodate-treated antigens. Despite some similarities with the effects of irradiation, the disrupted carbohydrate structures produced by periodate are expected to be

different from those on both normal and attenuated parasites. Hence, such periodate-treated antigens may bind the antibodies in these assays with only low affinity. Confirmation of this idea would of course require detailed studies on affinity of antigen/antibody interactions.

f) Problems in interpretation caused by NMS binding.

Periodate increases the binding of NMS to normal, U.V.-irradiated and Actinomycin D-treated schistosomula. This may be due to exposure of new, cryptic antigens by periodate treatment, as described above. Serum from normal mice may contain antibodies capable of recognising certain carbohydrate antigens, perhaps in consequence of bacterial infection. Competing sugars inhibit the interaction of NMS with periodate-treated schistosomula, supporting the idea that recognition of carbohydrate epitopes may occur with low affinity. However, the binding by NMS could be at least partly non-specific. Since NMS binding to periodate-treated schistosomula is almost as high as for the infection or vaccine antisera, a major caveat to be borne in mind in interpreting these results is that a considerable proportion of their binding might also be non-specific.

7.3.4.3. Antibody isotypes in chronic and vaccine sera.

The ELISA results (figures 7.4 a)-e)), demonstrating a high level of anti-carbohydrate IgM antibodies, in CMS but not in the irradiated vaccine sera, are compatible with the model described in section 7.1, whereby vaccinated subjects express resistance to challenge because, unlike normal infected hosts, they do not develop high levels of antibodies of blocking isotypes, reactive with carbohydrate epitopes on the schistosomulum surface.

7.4. Conclusions

Overall, our results on the effects of periodate treatment of schistosomula on binding by UVMS and AMS lead to similar conclusions to those drawn by Omer-ali *et al* (1986) from studies on gamma-irradiated vaccine sera from mouse hosts, and applied to human immunity by Butterworth *et al* (1985, 1987). It does seem that attenuated vaccines induce a far smaller proportion of anti-carbohydrate antibodies than chronic infection. Hence, blocking antibodies should not inhibit development and expression of the immune response in vaccinated hosts.

However, it does seem that carbohydrates are expressed by both U.V.-irradiated and Actinomycin D-treated schistosomula, and that these attenuated larvae do stimulate an anti-carbohydrate antibody response. The vaccinated host seems to be primed to both carbohydrate and polypeptide epitopes.

U.V.-irradiated schistosomula do seem to expose altered carbohydrate epitopes as compared to normal schistosomula. Firstly, in figures 7.2 a) and b) periodate treatment has no effect on CMS binding to normal forms, but does inhibit CMS binding to U.V.-irradiated forms. Secondly, the fact that competing sugars do not interfere with binding of UVMS to U.V.-irradiated schistosomula, whereas interaction of UVMS with normal parasites is inhibited by the same competing sugars, suggests that the anti-carbohydrate antibodies elicited by the U.V.-irradiated vaccine do have an especially high affinity for the modified carbohydrate structures on irradiated forms.

In contrast, the effect of competing sugars indicates that, although vaccinated hosts do produce antibodies with specificity for carbohydrate determinants, such vaccine sera recognise the carbohydrate antigens expressed by normal schistosomula with only low affinity. This observation supports the idea that anti-carbohydrate blocking antibodies are very unlikely to modulate expression of the immunity

induced by attenuated larval vaccines. If this low affinity recognition of native carbohydrate epitopes applies to cellular as well as humoral immunity, then it seems probable that anti-polypeptide responses may dominate irradiated vaccine immunity.

Finally, the low levels of antibody induced by vaccination with U.V.-irradiated, gamma-irradiated, or Actinomycin D-treated schistosomula, and the fact that two mouse strains, only one of which was rendered resistant to challenge by the Actinomycin D vaccine, showed similar antibody responses, might argue that humoral mechanisms are not the major means by which these attenuated vaccines induce immunity.

CHAPTER EIGHT

VARIABILITY IN THE METABOLIC RESPONSE TO IRRADIATION.

8. Variability in the metabolic response to irradiation.

8.1. Variability in the response of mixed pools of cercariae.

8.1.1. Metabolic labelling after U.V.- and gamma-irradiation.

U.V.-irradiation usually resulted in a 70-90% inhibition of protein synthesis. However, in a number of experiments, considerably less inhibition was observed. This tolerance to irradiation damage could appear in parasites from snails which, on other occasions, yielded cercariae showing the predicted level of inhibition.

Figure 8.1 shows protein synthesis, on two occasions a month apart, by cercariae from two pools of snails, A and B, which were infected on different dates. In the first experiment, cercariae from the A snails showed comparatively little inhibition - 0% at 5 hours, and only 37% at 20 hours. When the same snails were shed a month later, however, inhibition reached 78% at 20 hours - about the level routinely observed. Cercariae from the B snails initially showed severe inhibition, approximately 90%, but on the second occasion, parasites from these snails showed only a 10-20% reduction in protein synthesis.

The gel of figure 8.2 illustrates the point that irradiated parasites occasionally showed very little inhibition of protein synthesis. In this experiment, the parasites showed resistance to both U.V.- and gamma-irradiation. Protein synthesis at 5 hours was not examined. Inhibition might have been detectable at this early time after irradiation. At 24, 48 and 72 hours, there appeared to be no inhibition of protein synthesis in either irradiated form, except that, at 24 hours and 48 hours, a 72 000 Mr band does appear fainter in the gamma-irradiated samples than in the normal and U.V. ones. By 96 hours, however, distinct new bands appear at Mr 65 000, 45 000, 28 000 and 23 000 in the normal track, but are not synthesized by the U.V.- and

Figure 8.1 Incorporation of ^{35}S-methionine into TCA-precipitable protein by schistosomula from two pools of snails (A, B) tested at two dates (1, 2), one month apart.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

Each bar represents the mean of 2 samples.

Deviation of paired samples from mean was ± 9.4%

On both occasions, pool A and pool B consisted of 8 snails which had been exposed to 5 miracidia each. Protein synthesis by normal and irradiated parasites from each pool was examined as follows:

PROTOCOL

Cercariae

↓

N I (U.V.-irradiate: 400 μW min cm^{-2})

Transform mechanically in GMEM w/o methionine.

Resuspend each to 4 1ml aliquots of GMEM w/o methionine/ 10% hiFCS.

Add 20μCi (740 kBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2

↓

At 5h and 20h - remove 2N and 2I samples to ice.

↓

Wash 3 times in ice-cold GMEM. Freeze for TCA-precipitation.

Uptake of free (non TCA-precipitable) ^{35}S-methionine did not vary significantly between all 4 experiments:

5h: 3136 ± 103 cpm per 100 schistosomula.

20h: 11587 ± 272 cpm per 100 schistosomula.

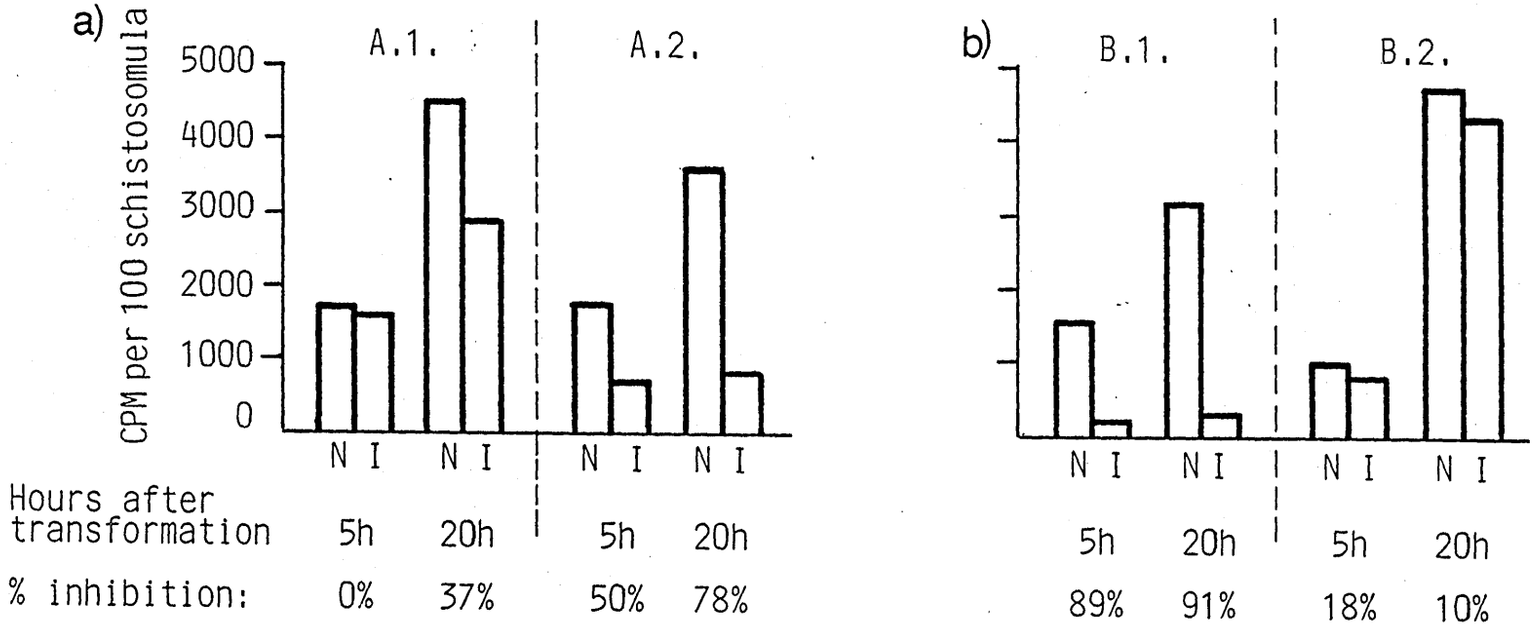

a)
A.1.
A.2.
CPM per 100 schistosomula
5000
4000
3000
2000
1000
0
N I N I N I N I
Hours after transformation
5h 20h 5h 20h
% inhibition:
0% 37% 50% 78%
b)
B.1.
B.2.
N I N I N I N I
5h 20h 5h 20h
89% 91% 18% 10%

Figure 8.2 Protein synthesis by normal, U.V.-irradiated and gamma-irradiated schistosomula from 24 to 96 hours after transformation.

N = normal

U = U.V.-irradiated (400 μW min cm^{-2})

G = gamma-irradiated (20 krad; 0.3 krad/min)

500 schistosomula/well.

3% stacking gel/10% resolving gel.

PROTOCOL:

Cercariae.

↓

Divide in 3:

↓

N — U — G

U: ↓ (U.V.-irradiate: 400 μW min cm^{-2})

G: ↓ (Gamma-irradiate: 20 krad; 0.3 krad/min).

↓

Transform mechanically in GMEM w/o methionine.

Resuspend each to 4 ml GMEM w/o methionine/10% hiFCS.

Divide into 4 1ml. aliquots.

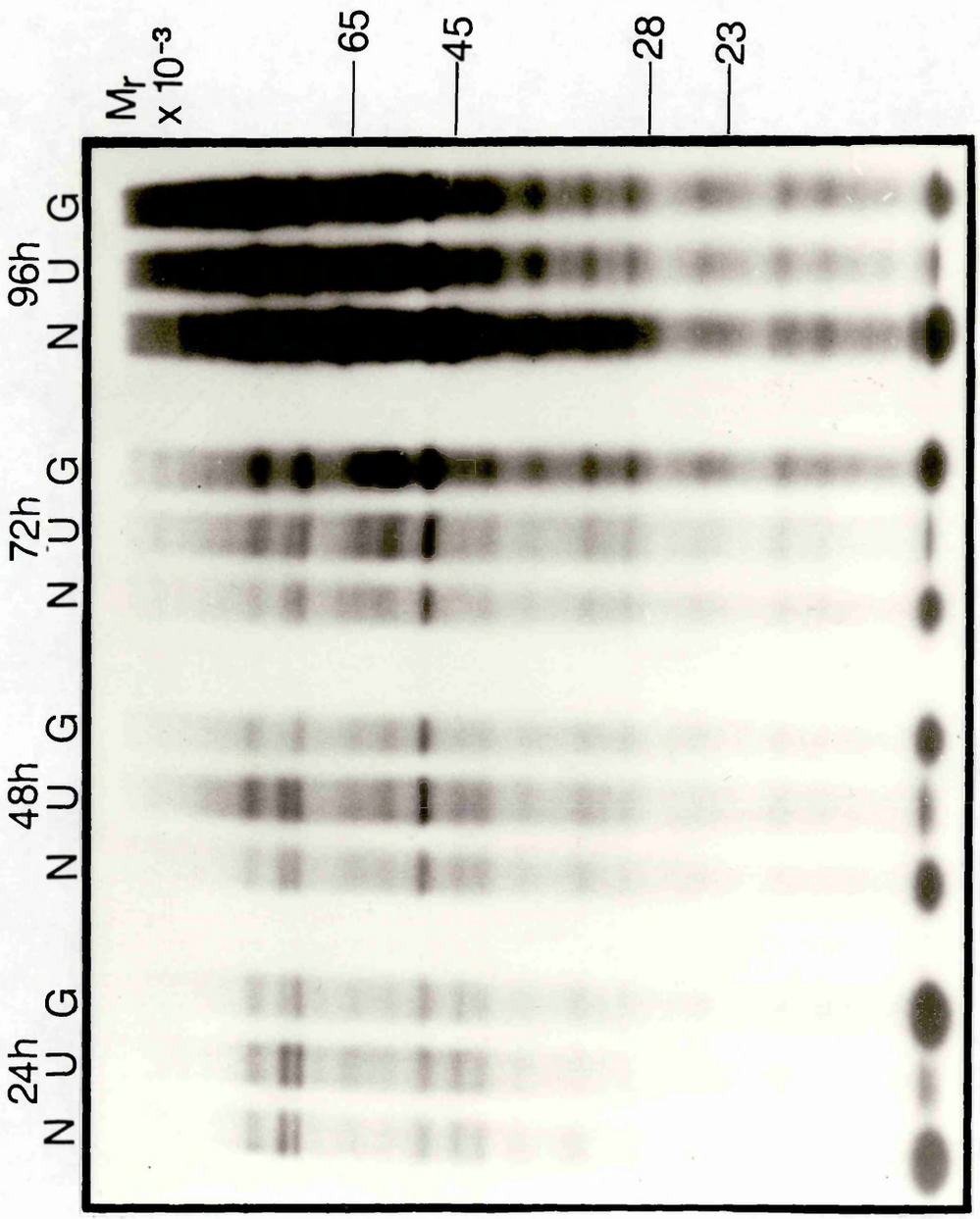
Add 20 μCi (740 kBq) ^{35}S-methionine to each.

↓

Culture at 37°C/5% CO_2.

↓

At 24, 48, 72, 96h, remove one sample from each to ice. Wash 3 times in ice-cold GMEM. Freeze for SDS-PAGE.


M_r x 10^{-3}
65
45
28
23
24h
48h
72h
96h
N
U
G

gamma-irradiated parasites. It is also noticeable that, at every time point, the normal and gamma-irradiated schistosomula, but not the U.V.-ones show an accumulation of low molecular weight radioactive material that migrates with the dye-front. This observation could indicate a failure on the part of the U.V.-irradiated larvae to synthesise these low molecular weight species: for instance, low molecular weight proteins such as ubiquitin, Mr 7 000-8 000. Alternatively, in these schistosomula, the degradative enzymes, which digest and degrade the recently-synthesized proteins to produce low molecular weight peptides, might be inactivated.

8.1.2. Schistosomula do not show a variable response to Actinomycin D treatment.

Schistosomula did not show the same variability in response to Actinomycin D-treatment as they did after irradiation. No instances of insusceptibility to Actinomycin D-induced inhibition of protein synthesis were observed. Figure 8.3 shows that schistosomula which resisted the inhibitory effects of irradiation on protein synthesis nevertheless showed the expected level of metabolic inhibition after Actinomycin D-treatment.

8.2 Sources of variability in response to irradiation.

8.2.1. Genetic sources of variability. Effect of irradiation on clones of cercariae.

In the experiments described so far, cercariae were obtained, as a mixed pool, from several snails, each of which had been exposed to 5-7 miracidia. Each miracidium which establishes successfully in the snail goes on to produce, by asexual division, a clone of genetically

Figure 8.3 Actinomycin D inhibits protein synthesis in schistosomula which resist the effects of irradiation.

N = normal

N/A = normal schistosomula, treated with Actinomycin D.

I = U.V.-irradiated schistosomula (400 μW min cm^{-2})

I/A = U.V.-irradiated schistosomula (400 μW min cm^{-2}), treated with Actinomycin D.

400 schistosomula/well.

3% stacking gel/10% resolving gel.

PROTOCOL.

Cercariae

↓

Divide in 4:

N N/A I I/A

(U.V.-irradiate: 400 μW min cm^{-2})

Transform mechanically in Elac.

Resuspend each to 1ml. Elac/10% hiFCS.

Add Actinomycin D to N/A and I/A.

Culture at 37°C/5% CO_2 for 20h.

↓

Wash 4 x in GMEM w/o methionine

Resuspend each to 1 ml. GMEM w/o methionine

Add 50μCi (1.85 MBq) ^{35}S-methionine to each.

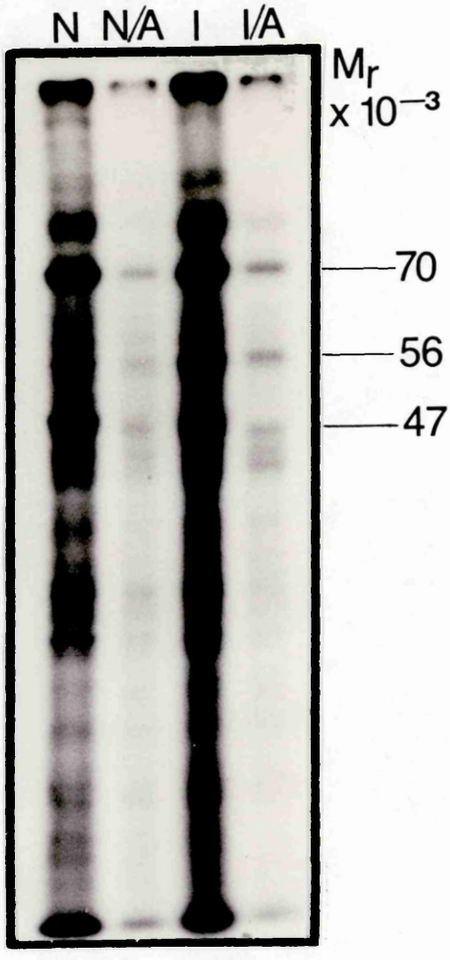
Culture at 37°C/5% CO_2 for 24h.

↓

Remove samples to ice.

↓

Wash 3 times in ice-cold GMEM. Freeze.


N
N/A
I
I/A
M_r
x 10^{-3}
70
56
47

identical cercariae. It was considered that some of the variability observed might be due to the presence, in some experiments, of a high proportion of cercariae derived from clones possessing some genetic factor which rendered them resistant to irradiation. In order to investigate this possibility, clones of cercariae were produced by infecting a number of snails with a single miracidium each. Hence, all the cercariae derived from any one snail should be genetically identical.

In figure 8.4, protein synthesis was measured in 8 clones at 5 hours and 20 hours after transformation. This figure illustrates 3 points. Firstly, there is great variability in the level of incorporation of ^{35}S-methionine into protein by the normal parasites. For instance, at 20 hours, clone 24 incorporates 5-6 times more radiolabel into protein than clone 10. It should be noted, however, that uptake of free, non-TCA precipitable ^{35}S-methionine was not measured in these experiments. Thus, normal schistosomula might incorporate different levels of radioactivity into protein because their ability to internalise free radiolabel varies. Differing concentrations of unlabelled methionine in the internal free amino acid pool could be one source of such variation in uptake of free ^{35}S-methionine. However, since previous experiments established that uptake of free ^{35}S-methionine is not inhibited by U.V.-irradiation, it is valid to compare the TCA-precipitable counts of normal and irradiated schistosomula as a measure of the extent of inhibition of protein synthesis in each clone.

Secondly, the level of inhibition of protein synthesis varies between clones. Five out of eight clones - C3, C10, C21, C23, C24 - showed the expected level of inhibition, between 70 and 90%. Clones 30A and 31A, however, showed considerably less inhibition, 23-40%, and approximately 55% respectively.

Finally, the ability of individual clones to overcome the

Figure 8.4 Protein synthesis by normal and U.V.-irradiated clones at 5 hours and 20 hours after transformation.

N = normal.

I = U.V.-irradiated (400 μW min cm^{-2}).

Each bar represents the mean of paired samples.

Deviation of paired samples from mean was ± 8.1%

PROTOCOL:

Series of clones

↓

N I ⟶ (U.V.-irradiate: 400 μW min cm^{-2})

↓

Transform mechanically in GMEM w/o methionine

Resuspend each to 4 ml GMEM w/o methionine/10% hiFCS.

Divide into 4 1ml samples.

Add 10 μCi (370 kBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2

↓

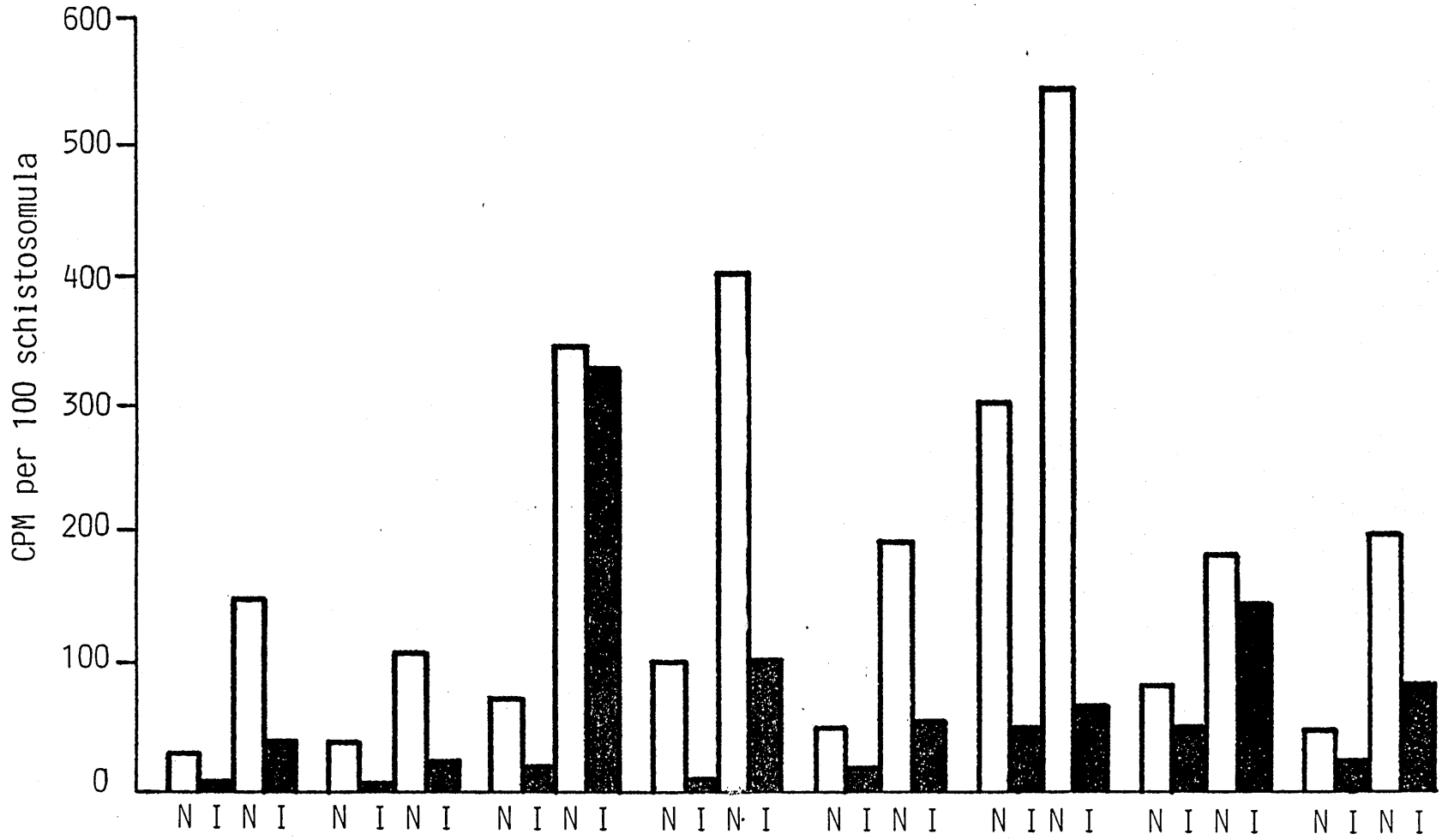
At 5h, 20h, remove 2 samples from each to ice.

↓

Wash 3 times in ice-cold GMEM.

↓

Freeze for TCA-precipitation.



Hours after transformation	5h	20h	5h	20h	5h	20h	5h	20h	5h	20h	5h	20h	5h	20h	5h	20h
Clone number	C3		C10		C20		C21		C23		C24		C30A		C31A	
% inhibition	70%	73%	79%	80%	74%	4%	89%	74%	65%	72%	83%	88%	41%	23%	50%	58%

irradiation-induced inhibition varied. Clones 3, 10, 21, 23, 24 and 31A showed no recovery at 20 hours as compared to 5 hours. Clone 20, however, showed an average level of inhibition - 74% - at 5 hours after transformation, but by 20 hours after irradiation, no inhibition was detectable. A similar recovery pattern was usual for mixed pools of gamma-irradiated parasites which showed considerable inhibition at early times after irradiation, with restoration of protein synthesis to normal levels between 24 and 48 hours (chapter 5). Clone 30A also showed some restoration of protein synthesis, though much less than C20. Inhibition for this clone was 41% at 5 hours, decreasing to 23% at 20 hours.

Figures 8.5 a) and 8.5 b) show protein synthesis by normal and irradiated members of nine clones, shed on two occasions, 10 days apart. It can be seen that the level of incorporation of radioactive amino acid into protein differs from day to day. Thus, for example, clone B shows low incorporation in a), but a high level in b). Overall, the levels of protein synthesis by normal, cloned parasites are much more similar to each other in figure 8.5 b) than in 8.5 a), or 8.4. Thus, TCA-precipitable cpm for 100 normal schistosomula vary from 105 to 540 in 8.4, 110 to 480 in 8.5 a), and 210 to 360 in 8.5 b).

As regards irradiation-induced inhibition of protein synthesis in a), seven out of nine clones show the expected levels of inhibition - 70-90%. Only D and F deviate from the average, showing 0% and 56% inhibition, respectively. In 8.5 b), only clone J shows a slightly lower level of inhibition than the others (63%). The level of irradiation-induced inhibition also differs from day to day. For instance, clone D showed no inhibition in the first experiment, and very high inhibition in the second. Inhibition in clone F was also higher in experiment b) than a), though the increase was not so striking - 89% as opposed to 56%. For clone J, however, protein synthesis was less inhibited in the second experiment than in the first

Figures 8.5 a) and b) Incorporation of ^{35}S-methionine into TCA-precipitable protein by normal and irradiated (400 μW min cm^{-2}) clones on two occasions, 10 days apart.

Incorporation of ^{35}S-methionine into protein was examined over a 24-hour period after transformation.

□ N = normal.

■ I = U.V.-irradiated (400 μW min cm^{-2}).

Each bar represents the mean of paired samples.

Deviation of paired samples from mean was ± 9.3%

PROTOCOL:

Series of Clones

↓

N I

|

U.V.-irradiate:

400 μW min cm^{-2}

↓

Transform mechanically in GMEM w/o methionine

Resuspend each to 2 ml GMEM w/o methionine/10% hiFCS

Divide into 2 1ml samples.

Add 10μCi (370 kBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2

↓

After 24h, remove samples to ice.

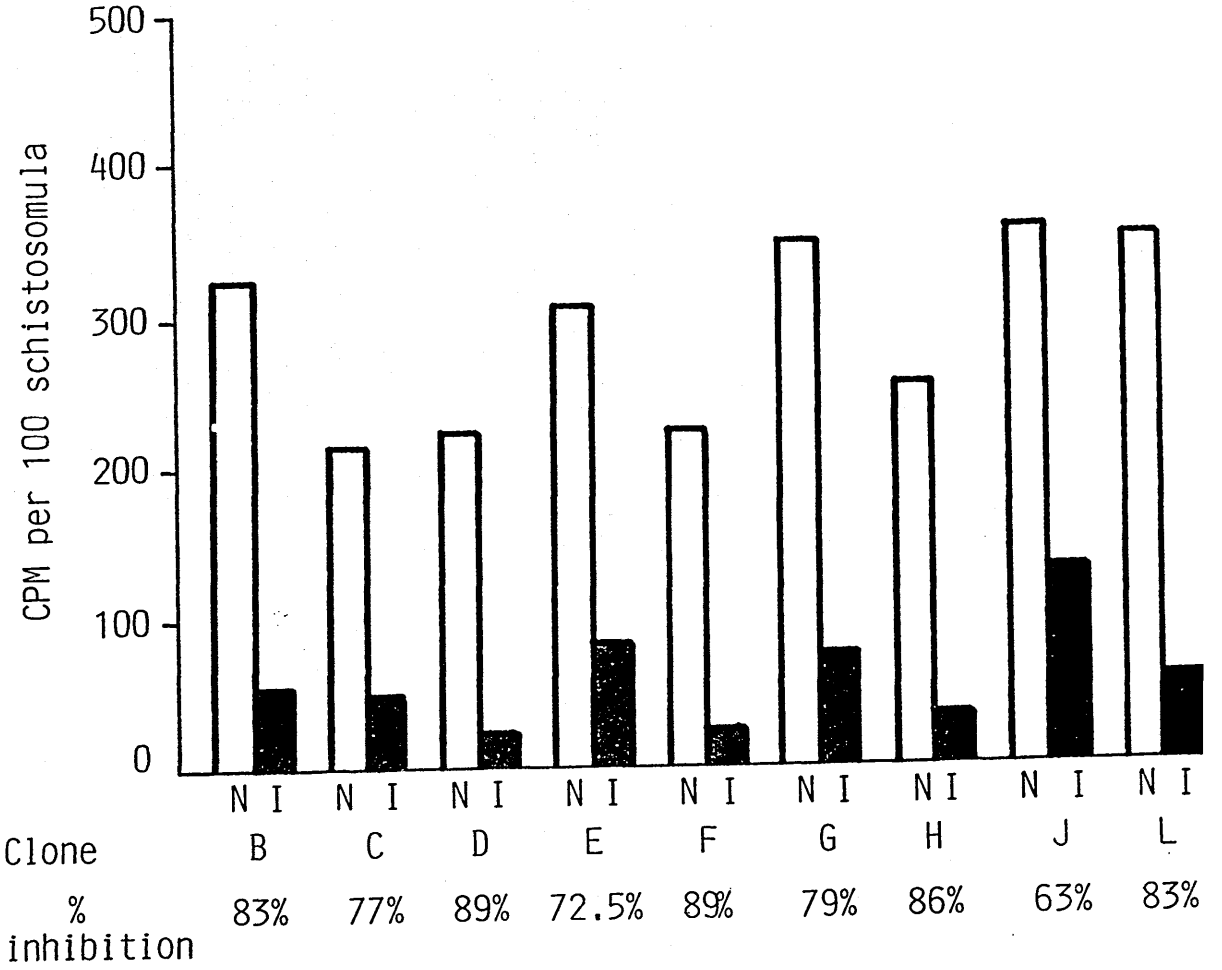
↓

Wash 3 times in ice-cold GMEM.

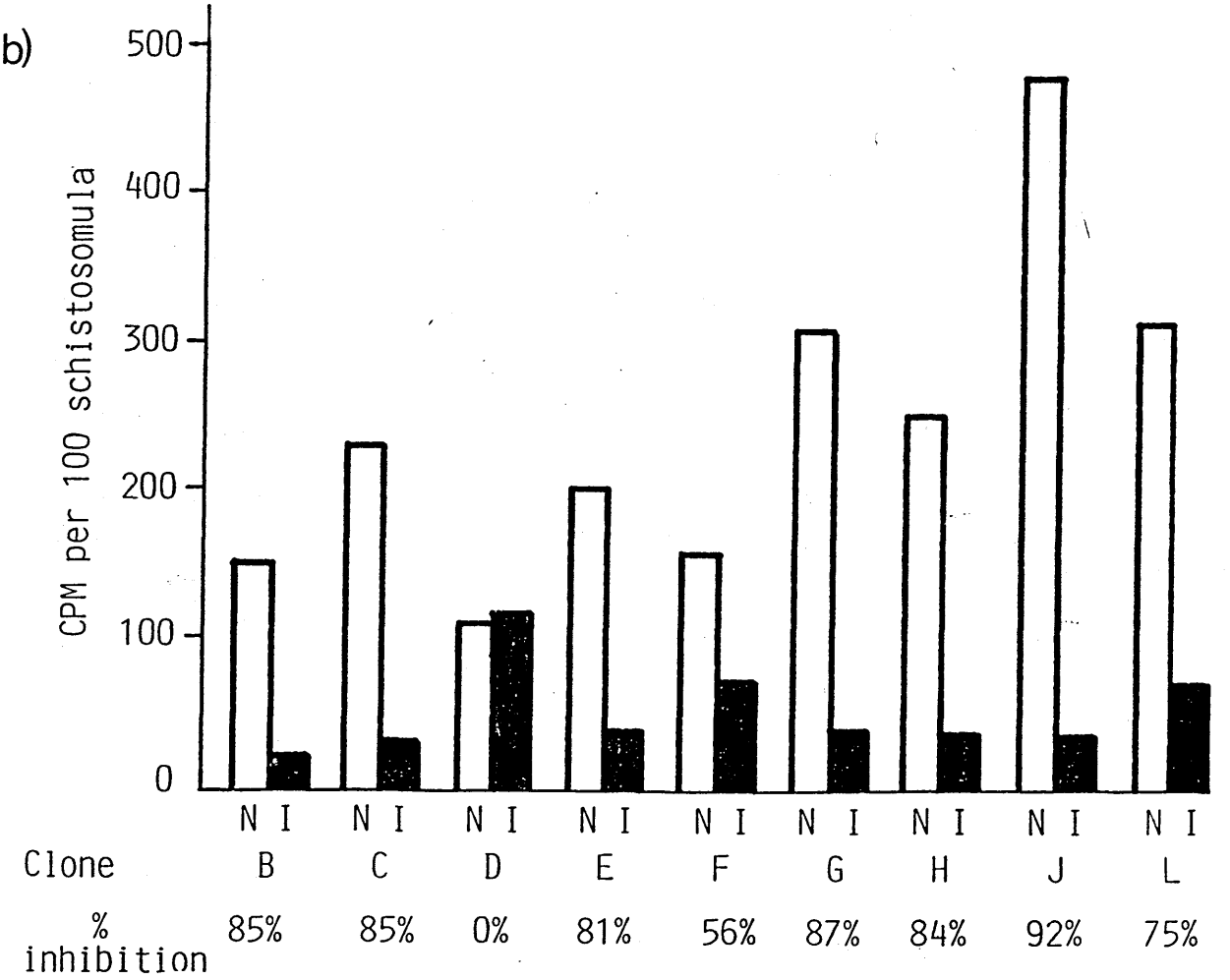
↓

Freeze for TCA-precipitation.

a)


CPM per 100 schistosomula
500
400
300
200
100
0
N I N I N I N I N I N I N I N I N I
Clone B C D E F G H J L
% inhibition 83% 77% 89% 72.5% 89% 79% 86% 63% 83%

b)


CPM per 100 schistosomula
500
400
300
200
100
0
N I N I N I N I N I N I N I N I N I
Clone B C D E F G H J L
% inhibition 85% 85% 0% 81% 56% 87% 84% 92% 75%

- 63%, compared to 92%.

In a subsequent experiment, normal and irradiated parasites from five of these clones - E, F, G, J and L - were radiolabelled for 48 hours, and protein synthesis analysed by SDS-PAGE (see figure 8.6).

In this case, there were not enough parasites to perform TCA precipitations. Only an approximate idea of the level of protein synthesis could be obtained, from the intensity of blackening of the X-ray film, which is not directly proportional to the amount of radioactivity (Laskey and Mills, 1975). As far as can be judged from examining the fluorograph, levels of protein synthesis by normal clones appear similar in this case, although L possibly shows less incorporation than the others. The levels of inhibition of protein synthesis also appear similar. As regards the proteins synthesized by clones, the same patterns were often seen with mixed cercarial pools. One difference between clones is the comparatively intense labelling of an 83 000 Mr protein by E, F and J, both normal and irradiated forms. This band is much fainter in clones G and L.

Snails F, G, J and L all died within 9 days of experiment 8.6; snails B, C, D and H died between experiments 8.5 b) and 8.6. The increase in similarity between clones in the last two experiments, in levels of both normal and irradiated protein synthesis, could reflect the fact that survival may be prolonged for only a limited population of parasites in each snail. Factors such as decreased availability of nutrients and changing hormonal levels in the aging, diseased snail, may reduce the number of parasites it can support, and may confine developing sporocysts to regions still suitable for growth, e.g. where nutrients are most readily available. Such restrictions may result in an increased similarity in metabolism between populations of cercariae derived from different snails.

Figure 8.6 Protein synthesis by normal and U.V.-irradiated members of five clones, during the 48-hour period following mechanical transformation.

N = normal.

I = U.V.-irradiated (400 μW min cm^{-2})

Approximately 500 schistosomula/well.

PROTOCOL:

Clones

↓

N I ⟶ (U.V.-irradiate: 400 μW min cm^{-2}.)

↓

Transform mechanically in Elac.

Resuspend each to 1 ml Elac/10% hiFCS.

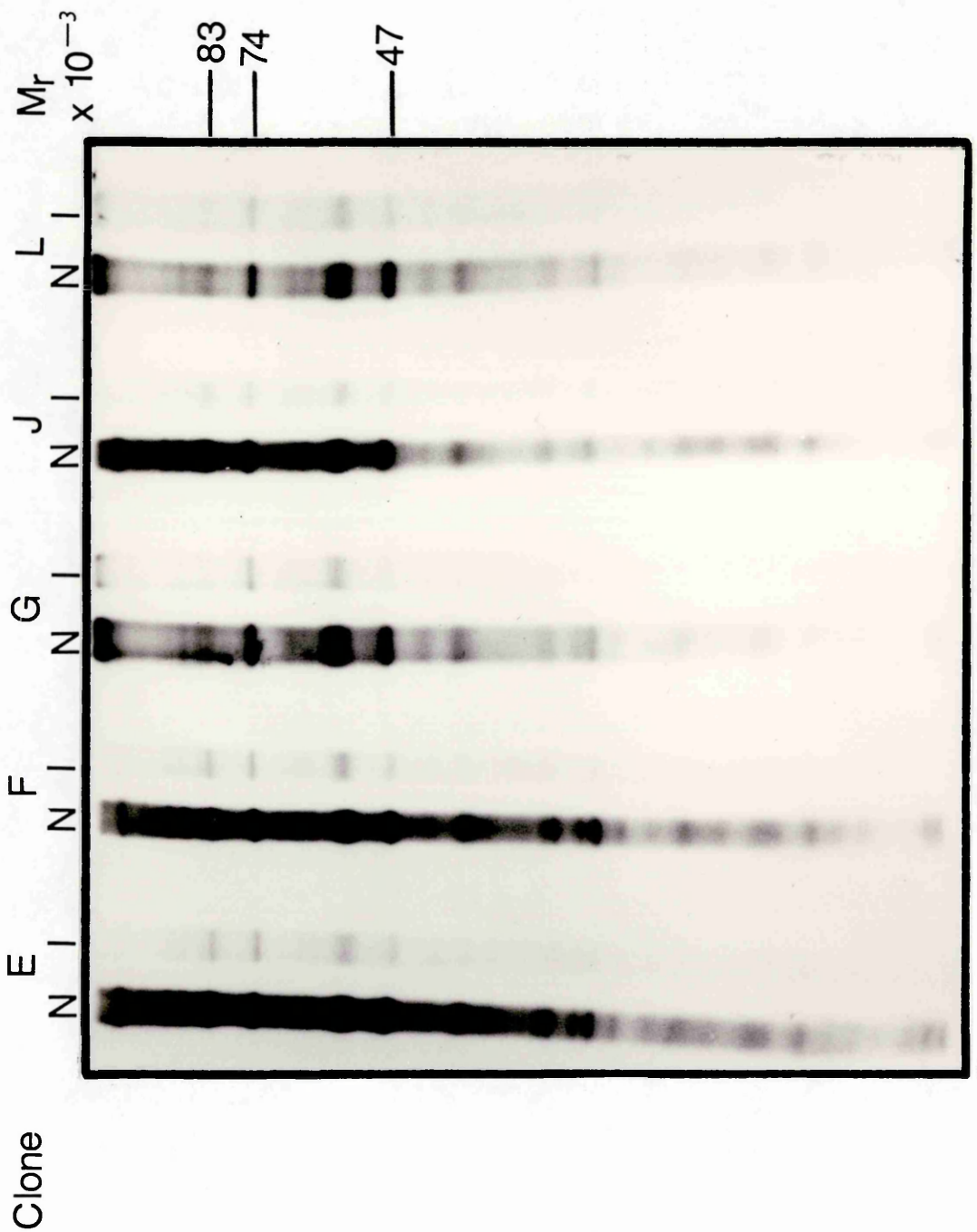
Add 50μCi (1.85 MBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2

↓

At 48h, remove to ice. Wash 3 times in ice-cold Elac.

Freeze for SDS-PAGE.


Clone
E
F
G
J
L
N
I
M_r x 10^{-3}
83
74
47

8.2.2. Environmental factors affecting the response to irradiation.

It was considered that physical variables, such as the temperature and concentration at which cercariae are irradiated, and the chemical composition of the medium in which they transform and grow, might well affect the parasites' response to irradiation. This possibility was investigated, as follows.

8.2.2.1. Temperature of irradiation. Skin versus mechanical transformation.

Cercariae were maintained at four different temperatures before U.V.-irradiation, then were transformed, either by skin penetration, or mechanically, by the syringe method. Protein synthesis in each treatment group was measured by ^{35}S-methionine incorporation, followed by SDS-PAGE and TCA precipitation (figures 8.7 a), b)).

The temperature at which cercariae were maintained prior to irradiation did not appear to influence the extent of inhibition of protein synthesis. However, all schistosomula collected after skin penetration showed significantly less irradiation-induced inhibition of protein synthesis than mechanically-transformed ones. Regardless of the temperature before irradiation, skin forms showed 55-60% inhibition of protein synthesis, as opposed to between 80 and 90% for their mechanical counterparts. This particular experiment also indicated that schistosomula obtained by the two transformation methods could synthesize different proteins, although this difference was not consistently observed in other experiments. Here, skin forms show a prominently labelled band at Mr 82 000 which is much fainter in mechanical schistosomula, while the mechanical ones show heavier labelling of a protein of Mr 30 000.

Figure 8.7 a), b) Effects of temperature, and transformation method, on protein synthesis by U.V.-irradiated schistosomula.

Labelling was for 60 hours after transformation.

N = normal. □

I = U.V.-irradiated (400 μW min cm^{-2}) ▒

MECH = mechanically-transformed.

SKIN = transformed by skin penetration.

500 schistosomula per well.

b) Accompanies a): Incorporation of ^{35}S-methionine into TCA-precipitable protein.

Each bar represents the mean of paired samples. Deviation of paired samples from mean was ± 12.3%

PROTOCOL:

Cercariae

↓

Divide in 4:

0°C 16°C 26°C 37°C

Maintain at these temperatures for 1 hour.

↓

Divide each into:

N I (U.V.-irradiate:

400 μW min cm^{-2}.)

↓

Divide each N and I sample in 2:

2000 cercariae:	Remainder:
↓	↓
Transform mechanically in GMEM w/o met.	Collect into GMEM
Incubate at 37°C for 3h.	w/o methionine at 37°C, after skin penetration for 3h.

CONTINUED ON NEXT PAGE.

PROTOCOL FOR FIGURE 8.7, CONTINUED.

Wash; resuspend each to 3 ml GMEM w/o methionine/10% hiFCS

Divide into 3 1ml samples.

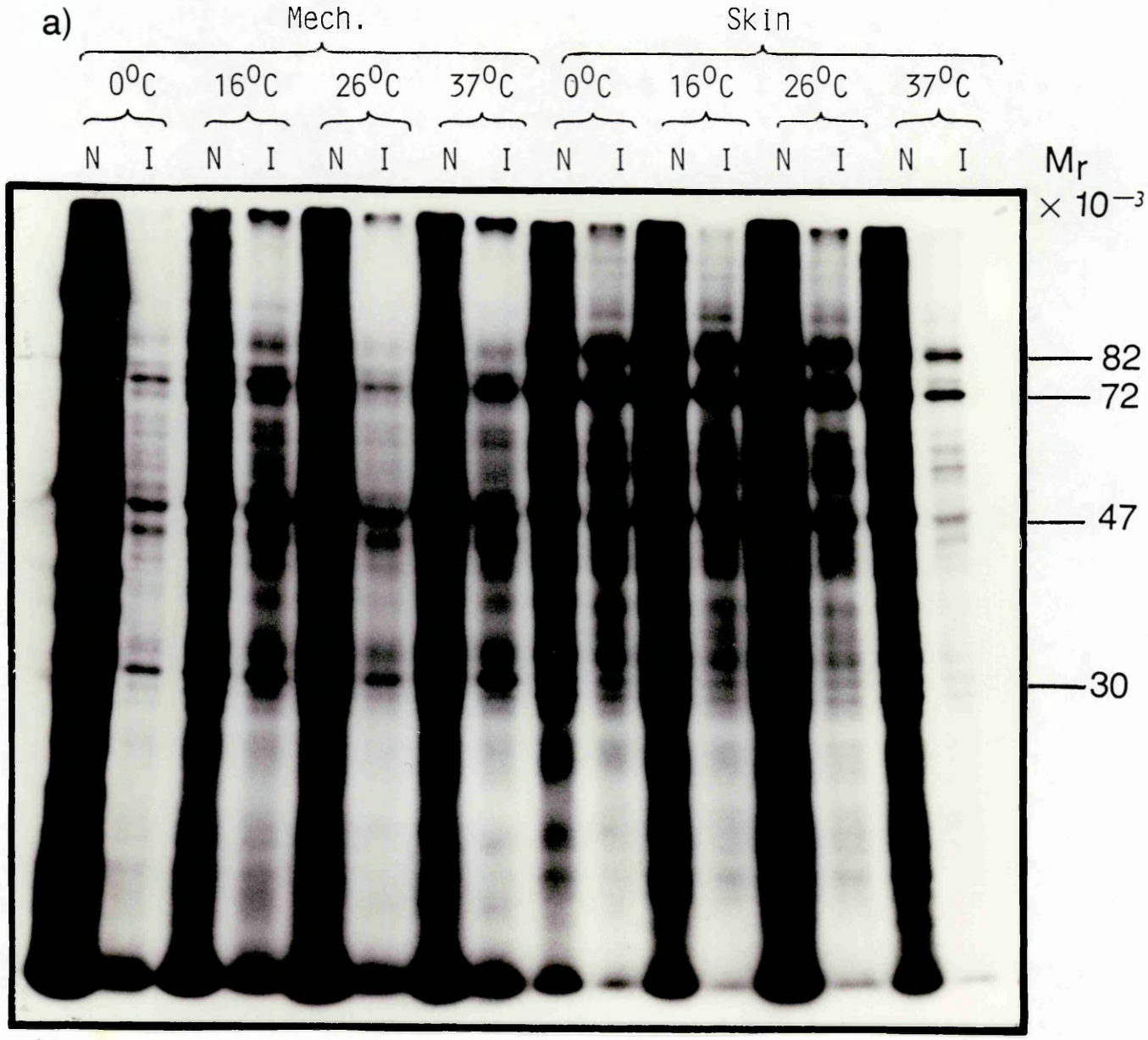
Add 10μCi (370 kBq) ^{35}S-methionine to each.

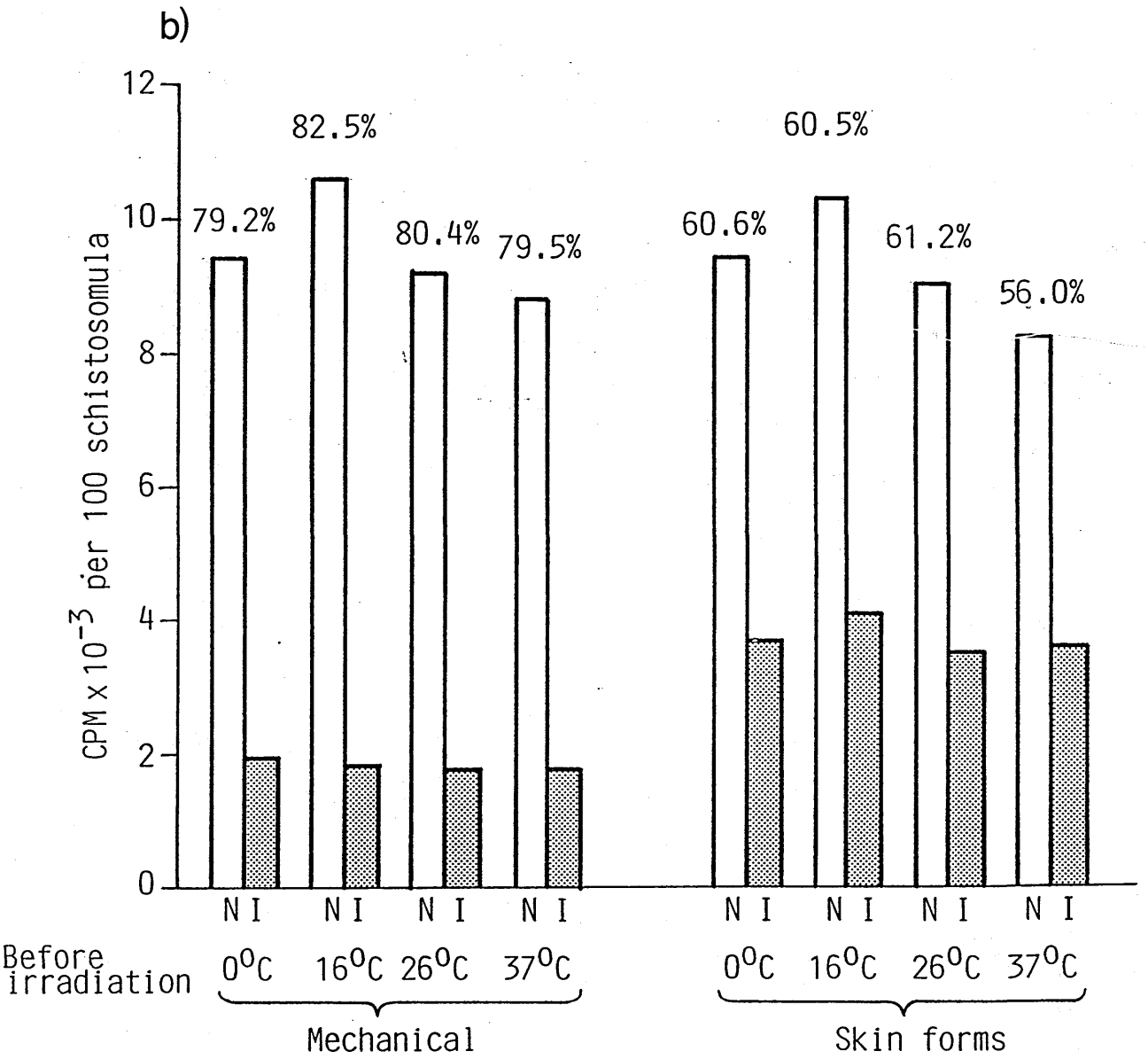
Culture at 37°C/5% CO_2

At 60h: remove samples to ice. Wash 3 times in cold GMEM. Freeze.

2 samples ⟶ TCA-precipitation

1 sample ⟶ SDS-PAGE.


a)
Mech.
Skin
0°C
16°C
26°C
37°C
0°C
16°C
26°C
37°C
N I N I N I N I N I N I N I N I
Mr
× 10⁻³
82
72
47
30


b)
12
10
8
6
4
2
0
CPM x 10^{-3} per 100 schistosomula
79.2%
82.5%
80.4%
79.5%
60.6%
60.5%
61.2%
56.0%
N I N I N I N I N I N I N I N I
Before irradiation
0°C 16°C 26°C 37°C 0°C 16°C 26°C 37°C
Mechanical
Skin forms

8.2.2.2. Effect of concentration at which cercariae are irradiated.

Cercariae from 2 separate pools of snails were irradiated at concentrations of 100/ml and 1000/ml. The extent of irradiation-induced inhibition of protein synthesis was measured at 5 hours and 48 hours after transformation (see figures 8.8 a, b).

Table 8.1 shows that, at least within this concentration range, the concentration at which cercariae were irradiated made no difference to the extent of inhibition of protein synthesis at either timepoint. However, this experiment did underline the point that different batches of schistosomula show different patterns of protein synthesis. No differences can be seen in the proteins synthesized at 5 hours, when the most prominently labelled proteins for both batches of schistosomula are at Mr 47 000 and 78 000. Fainter bands at Mr 74 000 and 70 000 are also evident in both cases, possibly representing the lower molecular weight members of the postulated heat-shock protein family between Mr 70 000 and 78 000, as suggested in chapter 3. By 48 hours, however, parasites from pool 1 show prominently labelled bands at Mr 78 000 and 74 000, with a faint one at Mr 83 000, while in pool 2, the most heavily labelled protein is at Mr 65 000. Bands at Mr 78 000 and 83 000 are also heavily labelled. Schistosomula in pool (1) also show distinct, heavy labelling of a 30 000 Mr protein which is only faintly detectable in parasites of pool (2).

8.2.2.3. Effect of age of cercariae.

Cercariae were harvested from snails which had been exposed to light for 2 hours. After collection, the cercariae were allowed to swim freely in water for varying times before irradiation. Protein synthesis by normal and irradiated parasites of different ages was then examined (figure 8.9). A lower dose of irradiation than usual - 100 μW min cm^{-2}

Figure 8.8 a), b) Effect of concentration at which cercariae are irradiated on extent of inhibition of protein synthesis, in two separate pools of parasites.

N = normal

I = U.V.-irradiated (400 µW min cm^{-2})

150 to 200 schistosomula per well. 3% stacking gels/10% resolving gels.

a): Synthesis at 5h. b): Synthesis at 48h after transformation.

PROTOCOL:

Cercariae (2 separate pools).

↓

From each pool:

2400 cercariae		7200 cercariae	
1200 = N	1200 = I	1200 = N	6000 = I
	U.V.-irradiate (400 µW min cm^{-2}) as 2 lots of 6ml at 100/ml		U.V.-irradiate (400 µW min cm^{-2}) in 6 ml, at 1000/ml
			Take 1200 cercariae (1.2 ml) for experiment

Transform mechanically in GMEM w/o methionine

Resuspend each to 6 ml. GMEM w/o methionine/10% hiFCS.

Divide into 6 1ml samples.

Add 10µCi (370 kBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2

↓

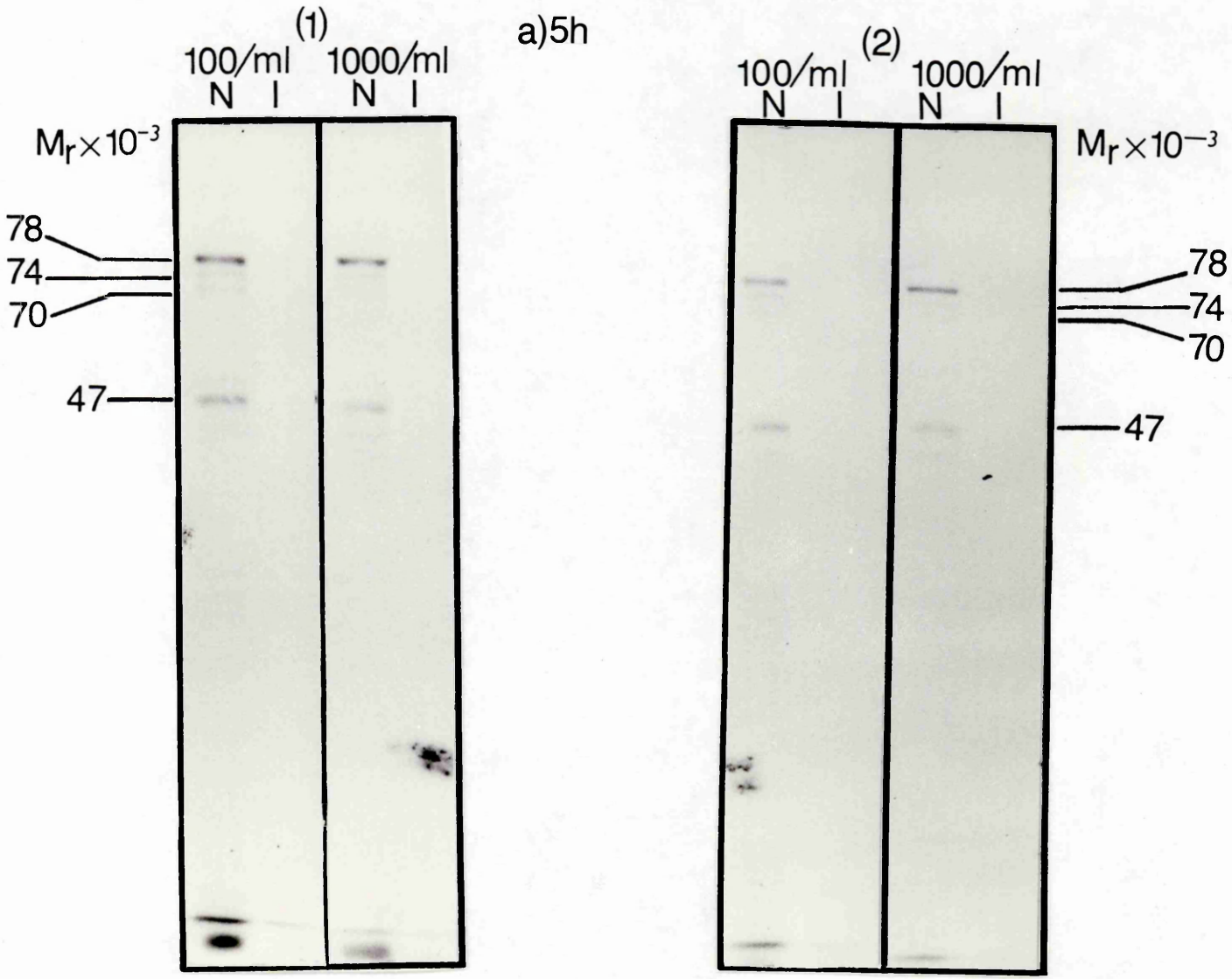
At 5h, 48h, remove 3 samples of each to ice.

↓

Wash 3 times in ice-cold GMEM. Freeze.

2 samples ⟶ TCA-precipitation.

1 sample ⟶ SDS-PAGE.


(1)
a)5h
100/ml
1000/ml
N
I
N
I
Mr×10⁻³
78
74
70
47
(2)
100/ml
1000/ml
N
I
N
I
Mr×10⁻³
78
74
70
47

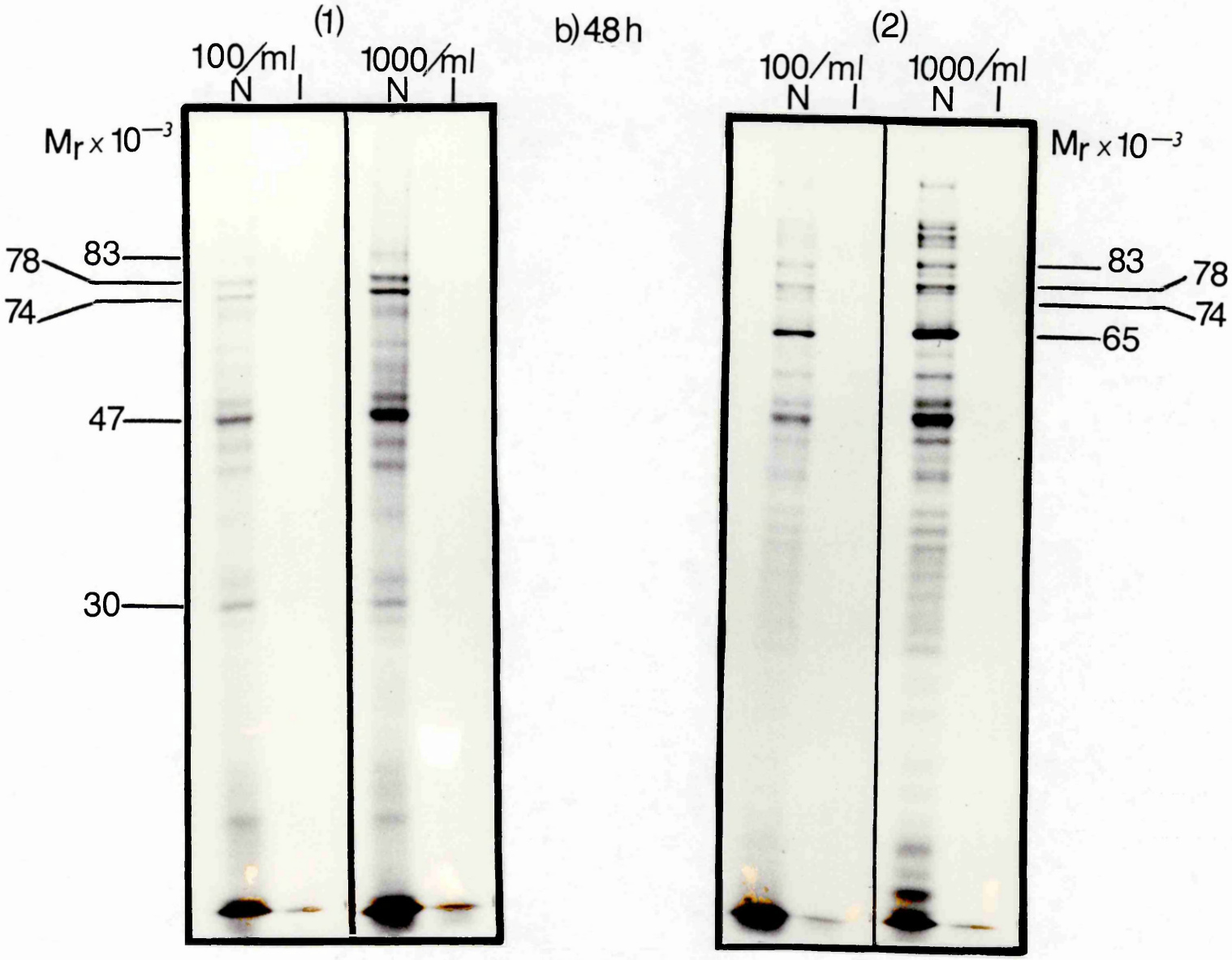

(1)
100/ml
1000/ml
N
I
N
I
Mr x 10⁻³
78
83
74
47
30
b) 48 h
(2)
100/ml
1000/ml
N
I
N
I
Mr x 10⁻³
83
78
74
65

Table 8.1 Effect of concentration at which cercariae are irradiated on extent of inhibition of protein synthesis. (Accompanies figures 8.8 a), b)).

POOL OF CERCARIAE		(1)		(2)	
TIME OF CULTURE WITH ^{35}S-methionine		5h	48h	5h	48h
		% INHIBITION OF PROTEIN SYNTHESIS.			
CONCENTRATION AT WHICH CERCARIAE WERE IRRADIATED.	100/ml	78.6	81.7	85.4	85.9
	1000/ml	86.2	77.5	71.8	89.2

Incorporation of ^{35}S-methionine into TCA-precipitable protein by paired samples of schistosomula was measured. Inhibition of protein synthesis was obtained from the means of the paired samples. Deviation of paired samples from mean was $\pm$ 10.1%

Protocol is given for figure 8.8 a), b).

- was used, to facilitate detection of any increase in the percentage inhibition.

The longer the cercariae were allowed to swim freely before irradiation, the greater was the uptake of free ^{35}S-methionine - see figure 8.9 a). Greater uptake of free radiolabel meant a higher incorporation of radioactivity into protein. In order to compare the extent of inhibition of protein synthesis by each group of parasites, it was therefore necessary to use the ratio (TCA precipitable cpm/TCA precipitable plus non-TCA-precipitable cpm), as shown in figure 8.9 b). As the cercariae aged, the inhibition of protein synthesis consequent upon irradiation increased.

8.2.2.4. Effect of composition of transformation medium.

The concentration of various nutrients in the environment surrounding schistosomula as they develop after transformation could influence their ability to overcome the damaging effects of irradiation. Protein synthesis by normal and U.V.-irradiated parasites in three media containing different nutrient supplements was therefore measured.

GMEM is the basic medium; Elac contains free amino acids from lactalbumin hydrolysate, added at 0.5% (w/v); Basch's medium contains a smaller percentage of lactalbumin hydrolysate, also added hormonal factors (see section 2.3.1.).

Figure 8.10 shows the results of this experiment. Since each track contains an equal number of schistosomula, labelling is most intense in the GMEM samples, where there were fewer amino acids to compete with the uptake of ^{35}S-methionine into the schistosomular free amino acid pool. However, irradiation-induced inhibition of protein synthesis was approximately the same for each culture medium. Nor, in this instance, was there any difference in the patterns of protein synthesis in the

Figure 8.9 a), b) Effect of age of cercariae on: a) Uptake of free (non TCA-precipitable) ^{35}S-methionine; b) Extent of protein synthesis, after U.V.-irradiation.

N = normal □

I = U.V.-irradiated (100 µW min cm^{-2}). ▒

Each bar represents the mean of 2 paired samples.

Deviation of paired samples from mean was ± 12.0%

PROTOCOL:

Cercariae (Collected by 2-hour exposure of snails to light)

↓

Divide in 3:

↓

Oh 8h 22h

At appropriate time after harvesting, divide each group in 2:

↓

N I ⟶ (U.V.-irradiate: 100 µW min cm^{-2})

↓

Transform mechanically in GMEM w/o methionine.

Resuspend each to 2ml GMEM w/o methionine/10% hiFCS.

Divide each into 2 1ml samples.

Add 100µCi (3.7MBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2

↓

After 24h of labelling, remove samples to ice.

↓

Wash 3 times in ice-cold GMEM.

↓

Freeze for TCA-precipitation.

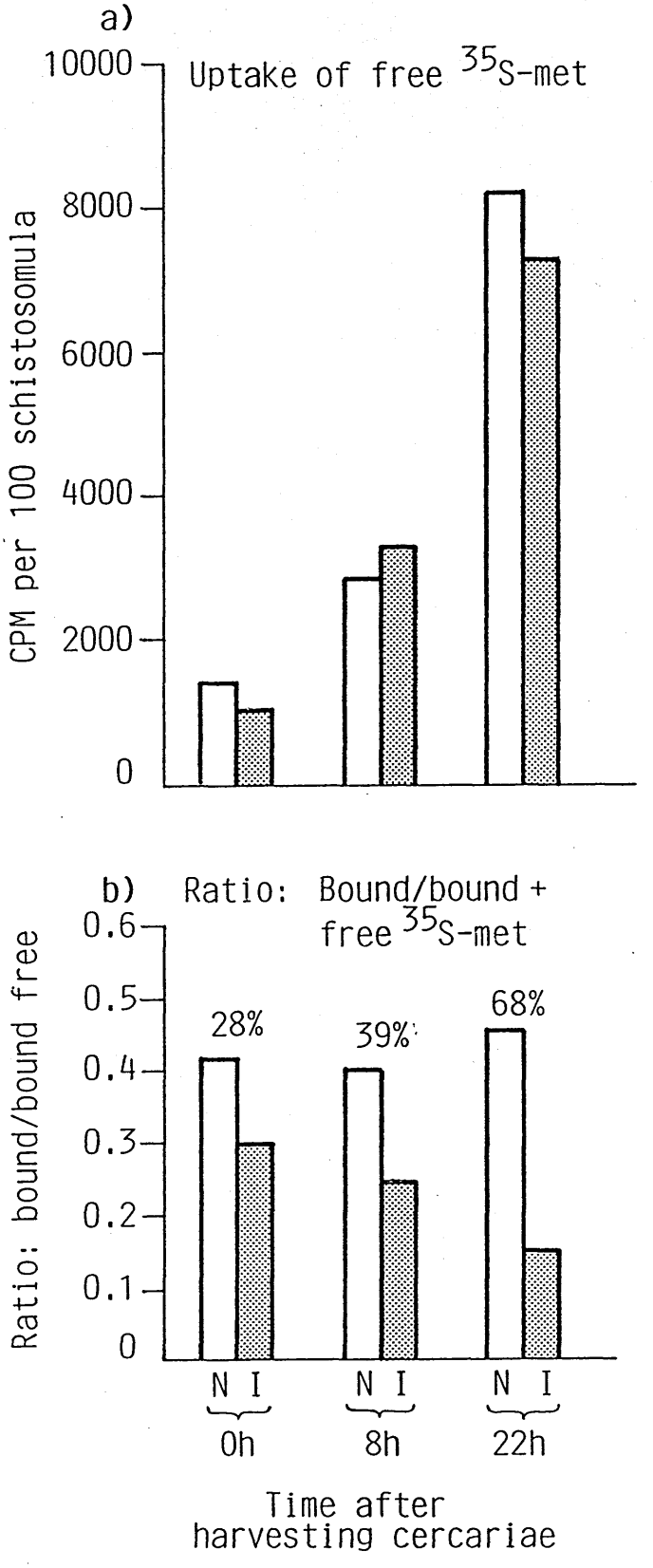

a)
Uptake of free 35S-met
CPM per 100 schistosomula
10000
8000
6000
4000
2000
0
b)
Ratio: Bound/bound + free 35S-met
Ratio: bound/bound free
0.6
0.5
0.4
0.3
0.2
0.1
0
28%
39%
68%
N I
N I
N I
0h
8h
22h
Time after harvesting cercariae

Figure 8.10 Effect of different culture media on protein synthesis by normal and U.V.-irradiated (400 μW min cm^{-2}) schistosomula.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

Approximately 300 schistosomula/well.

3% stacking gel/10% resolving gel.

Schistosomula were cultured with ^{35}S-methionine for 24h after transformation.

PROTOCOL:

Cercariae

↓

N I (U.V.-irradiate: 400 μW min cm^{-2})

Divide each in three:

Elac GMEM Basch

Transform mechanically in appropriate medium.

Resuspend to 1ml medium/10% hiFCS.

Add 50μCi (1.85 MBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2

↓

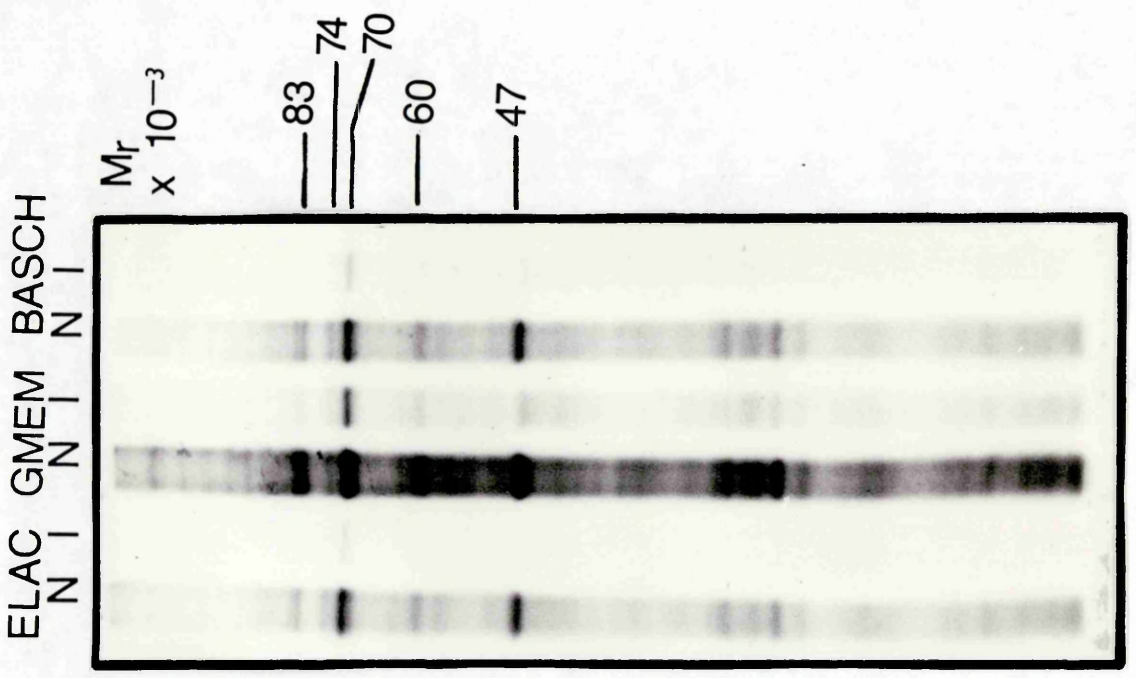
At 24h - remove samples to ice.

↓

Wash 3 times in ice-cold medium.

↓

Freeze for SDS-PAGE


ELAC
N I
GMEM
N I
BASCH
N I
Mr
x 10⁻³
83
74
70
60
47

different media.

8.2.2.5. Effect of different sera.

Foetal calf serum was the usual serum supplement, although normal human serum was used in some experiments. Using different types of sera, or even different batches of sera of the same kind, might influence the pattern of parasite protein synthesis and the response to irradiation.

In the experiment of figure 8.11, normal and U.V.-irradiated cercariae were transformed in two different media, Elac and GMEM, and cultured in the presence of FCS or NHS (both heat-inactivated). TCA precipitates indicated that inhibition of protein synthesis in response to irradiation was approximately the same in each treatment group - 73.2 $\pm$ 3.9% ($\bar{X}$ + S.E.).

Approximately equal cpm of each sample were run in every track of figure 8.11, to determine whether there were any differences in the patterns of protein synthesis by schistosomula cultured under each condition. hiFCS seemed to induce synthesis of some proteins that were not produced by parasites cultured with hiNHS. However, in this instance, in contrast to figure 8.9, the medium also appeared to influence the pattern of protein synthesis (though not the extent of inhibition). In Elac/hiFCS, both normal and irradiated schistosomula of this experiment synthesized a protein of Mr 65 000 which was not apparent under any of the other culture conditions. In GMEM/hiFCS, a 74 000 Mr band was intensely labelled, as well as the 70 000 Mr one, which was the most heavily labelled in all the other groups. The presence of hiFCS (with both Elac and GMEM) also seemed to induce quite pronounced synthesis of a 20 000 Mr protein which was not present when NHS was the serum supplement.

Figure 8.11 Influence of media and sera on protein synthesis by normal and U.V.-irradiated schistosomula.

N = normal.

I = U.V.-irradiated (400 μW min cm^{-2}).

Approximately equal cpm/track. 3% stacking gel/10% resolving gel. Schistosomula were cultured in the presence of ^{35}S-methionine for 72 hours after transformation.

PROTOCOL:

Cercariae

↓

N I ⟶ (U.V.-irradiate: 400 μW min cm^{-2})

Divide each in 4:

Elac/hiFCS GMEM/hiFCS Elac/hiNHS GMEM/hiNHS

Transform mechanically in appropriate medium.

Resuspend each to 3ml of appropriate medium/serum.

Divide into 3 1ml samples.

Add 100 μCi (3.7 MBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2.

↓

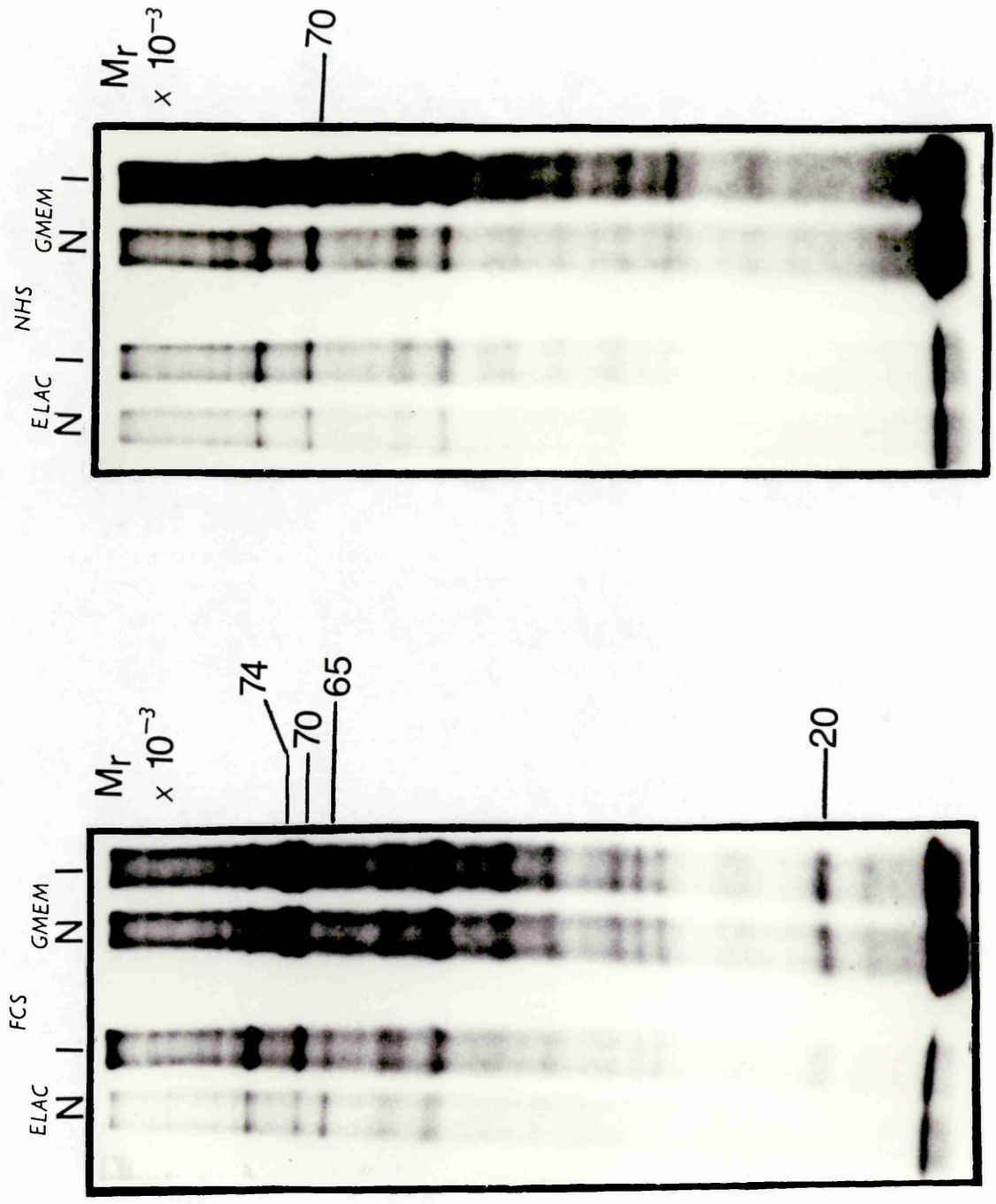
At 72h, remove samples to ice.

↓

Wash three times in appropriate medium. Freeze.

2 samples ⟶ TCA-precipitation.

1 sample ⟶ SDS-PAGE


Mr
× 10⁻³
70
NHS
GMEM
N
I
ELAC
N
I
Mr
× 10⁻³
74
70
65
20
FCS
GMEM
N
I
ELAC
N
I

Different parasites, and a different batch of FCS, were used in the experiments of figures 8.10 and 8.11; either factor may have contributed to the disparity between the two experiments. For each combination of medium and serum, however, normal and irradiated schistosomula always synthesized the same proteins.

8.2.3. Radioprotective agents. Manipulation of the metabolic response to irradiation.

8.2.3.1. Protection by SH groups.

8.2.3.1.1. Irradiation in the presence of SH groups.

The ability of SH-containing compounds to alleviate the irradiation-induced inhibition of protein synthesis was investigated by irradiating cercariae in the presence of cysteine and glutathione.

Cercariae were incubated for 3 hours in aquarium water supplemented with cysteine and glutathione, then irradiated, still in the presence of the SH groups. Initially, cysteine and glutathione were used at concentrations less than 1mM. The intensity of blackening of X-ray film after fluorography suggests that some slight lessening of the inhibition of protein synthesis might have occurred at the highest concentration used, 0.6 mM. (figure 8.12). Very high SH concentrations, 1 to 10 mM, had a marked protective effect (figure 8.13).

It is worth noting, in passing, the different patterns of protein synthesis in these two experiments. In the experiment of figure 8.12, only the 74 000 Mr member of the postulated heat-shock protein family is heavily synthesized, while a 58 000 Mr species is much more intensely labelled than in figure 8.13. In the experiment of figure 8.13, schistosomula show pronounced synthesis of all three members of the family between Mr 70 000 and 78 000, as well as a 29 000 molecular weight protein which is only faintly detectable in figure 8.12.

Figure 8.12 Effect of SH groups at concentrations less than 1mM on irradiation-induced inhibition of protein synthesis.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

SH 0.3mM, etc: Cysteine and glutathione were each present in aquarium water at concentrations of 0.3mM, etc. Supplemented water was pH'd to 7.0.

500 schistosomula per well.

3% stacking gel/10% resolving gel.

PROTOCOL:

Cercariae

↓

Divide in 3:

CONTROL 0.3mM SH 0.6mM SH

Incubate in aquarium water with appropriate supplement for 3 hours at room temperature.

↓

Divide each in 2:

N

I { U.V.-irradiate, still in presence of SH supplements (400 μW min cm^{-2})

↓

Transform mechanically in GMEM w/o methionine.

Resuspend each to 1ml GMEM w/o methionine/10% hiFCS.

Add 20μCi (740 kBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2

↓

At 24h, remove to ice. Wash 3 times in ice-cold GMEM.

↓

Freeze for SDS-PAGE.

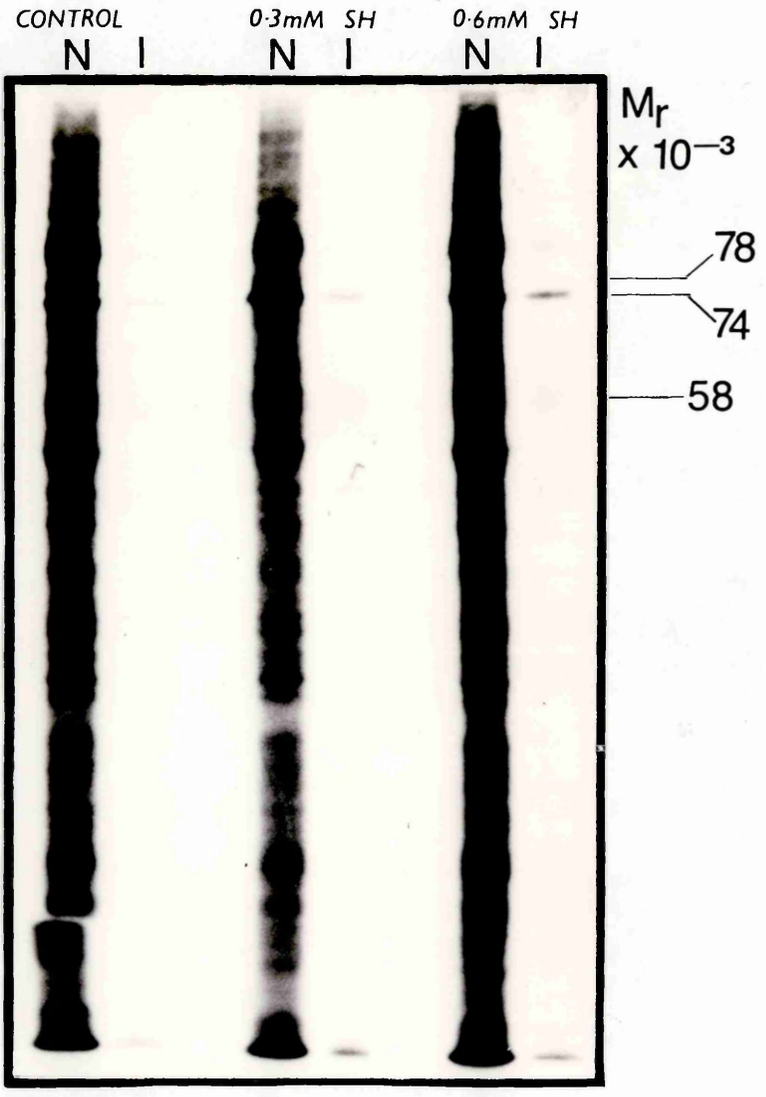
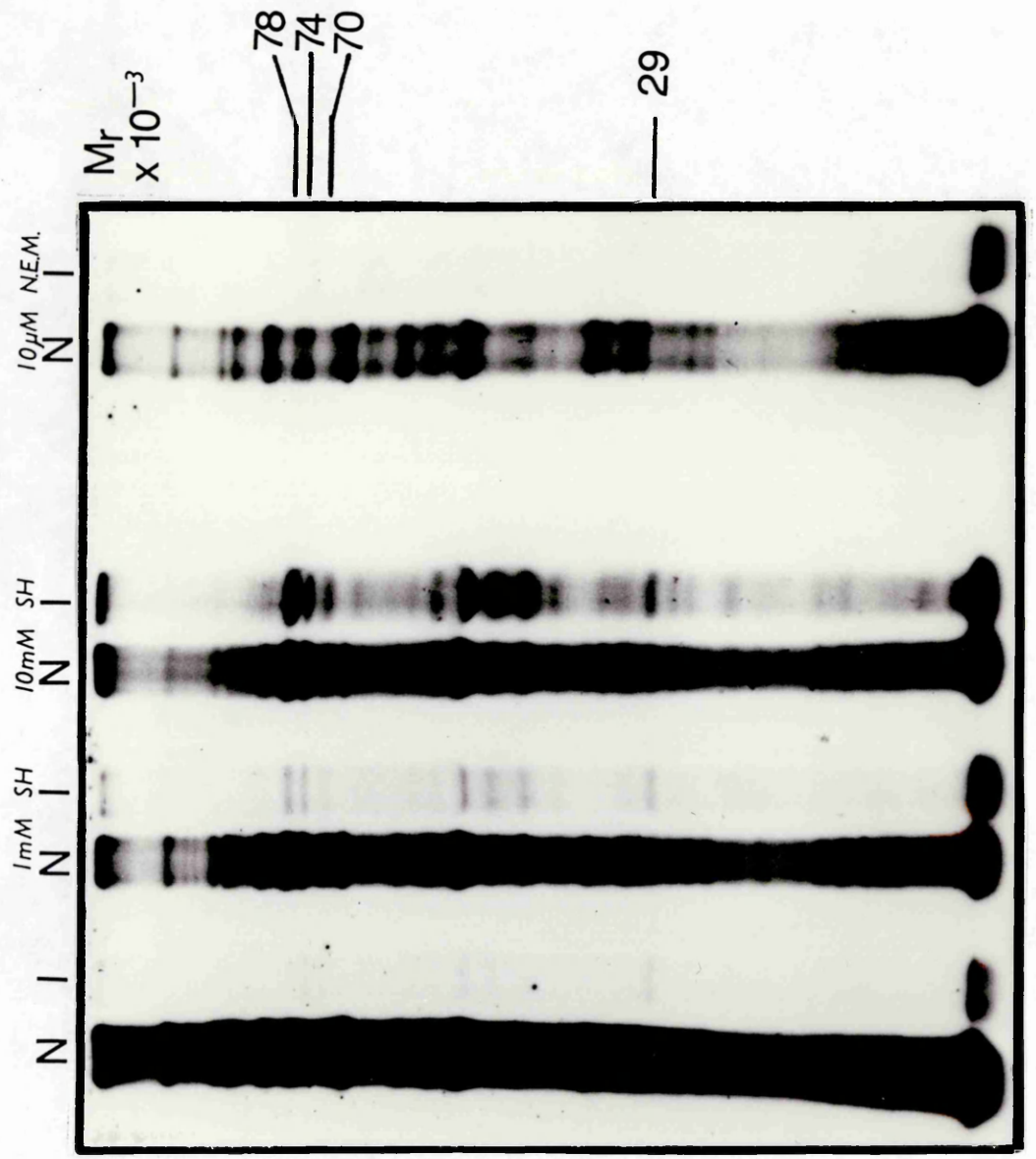

CONTROL
0·3mM SH
0·6mM SH
N I
N I
N I
Mr
x 10⁻³
78
74
58

Figure 8.13 Effect of SH groups at 1-10 mM on irradiation-induced inhibition of protein synthesis.

Abbreviations and protocol as for 8.11, but cysteine and glutathione at 1mM and 10mM instead of 0.3 mM and 0.6 mM.


Mr
x 10⁻³
78
74
70
29
10μM N.E.M.
N I
10mM SH
N I
1mM SH
N I
N I

N-ethylmaleimide (NEM) has been shown to aggravate radiation damage in various biological systems (von Sonntag, 1987). It appears to inactivate potential radioprotective mechanisms, principally by binding to SH groups. Exposure of cercariae to 10 μM NEM for 20 minutes before irradiation and transformation allowed both normal and irradiated larvae to survive for over 24 hours in culture, although they were severely damaged, as indicated by distorted shape, and granularity. However, figures 8.13 and 8.14 show that N-ethylmaleimide did not enhance radiation damage as measured by inhibition of protein synthesis. Nor was the pattern of protein synthesis affected by NEM treatment. In figure 8.13, the NEM samples, incubated at room temperature for only 20 minutes before irradiation and transformation, are not directly comparable with controls, where cercariae were allowed to swim for 3 hours before irradiation. In figure 8.14, timing was the same for control and experimental samples.

Both these experiments used a high irradiation dose, inducing almost maximal inhibition of protein synthesis in controls. With such a high baseline, it might be difficult to enhance the irradiation damage - a lower irradiation dose might reveal increased metabolic inhibition under the influence of N-ethylmaleimide.

8.2.3.1.2. Irradiation in the presence of SH groups: effect on parasite survival.

When cercariae were incubated, then irradiated, in the presence of an even higher concentration of cysteine and glutathione, 50mM each, protein synthesis during the 48 hours following transformation was restored to the same level as for normal schistosomula (figure 8.15). In order to determine whether this protection against inhibition of protein synthesis restored parasite viability to normal levels, 200

Figure 8.14 (and 8.13) Influence of N-ethylmaleimide (NEM) on irradiation-induced inhibition of protein synthesis.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2}).

Figure 8.14: 1000 schistosomula/well. 3% stacking gel/10% resolving gel.

PROTOCOL for figure 8.14:

Cercariae

↓

Divide in 2:

CONTROL — 10 μM NEM

↓

Incubate 20 minutes at room temperature.

↓

Divide each group in 2:

N — I (U.V.-irradiate: 400 μW min cm^{-2})

↓

Transform mechanically in GMEM w/o methionine

Resuspend each in 1 ml GMEM w/o methionine/10% hiFCS.

Add 20μCi (740 kBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2

↓

At 24h - remove to ice. Wash 3 times in ice-cold GMEM.

↓

Freeze for SDS-PAGE.

In figure 8.13, normal controls were incubated at room temperature for 3 hours before irradiation and transformation.

In figure 8.14 (b), gel is overexposed to highlight the proteins synthesized by irradiated schistosomula.

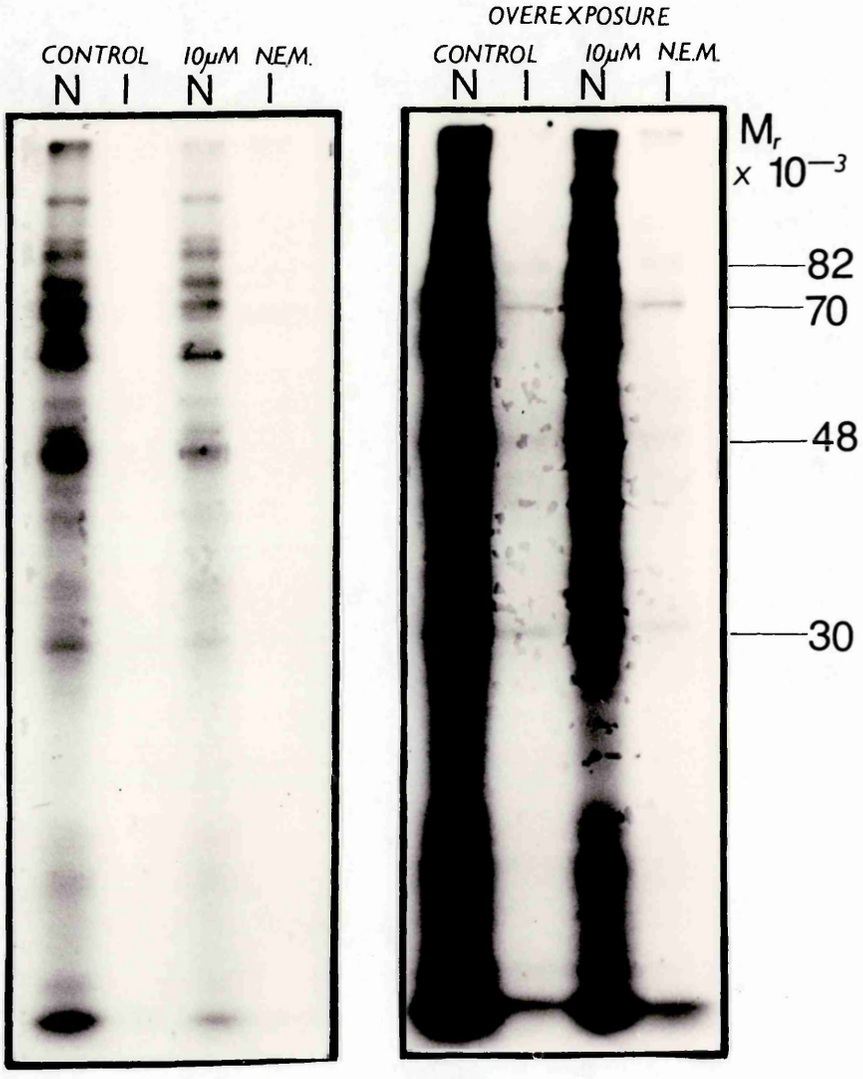

CONTROL
10μM N.E.M.
N I N I
OVEREXPOSURE
CONTROL
10μM N.E.M.
N I N I
Mr
x 10−3
82
70
48
30

Figure 8.15 Irradiation in the presence of SH at 1-50 mM; effect on irradiation-induced inhibition of protein synthesis.

N = normal.

I = U.V.-irradiated (400 µW min cm^{-2})

1500 schistosomula per well. 3% stacking gel/10% resolving gel.

PROTOCOL:

Cercariae

↓

Divide in 5:

N I I I I

1mM 10mM 50mM SH

Incubate for 3 hours at room temperature in presence of cysteine and glutathione at appropriate concentrations in aquarium water (pH'd to 7.0).

↓

U.V.-irradiate "I" samples at 400 µW min cm^{-2}.

↓

Transform mechanically in GMEM w/o methionine.

Remove 1000 schistosomula from each group; add hiFCS to 2% (v/v) final concentration.

Incubate 1h at 37°C in GMEM, then inject 200 schistosomula s.c. into 3 mice (see table 8.2).

↓

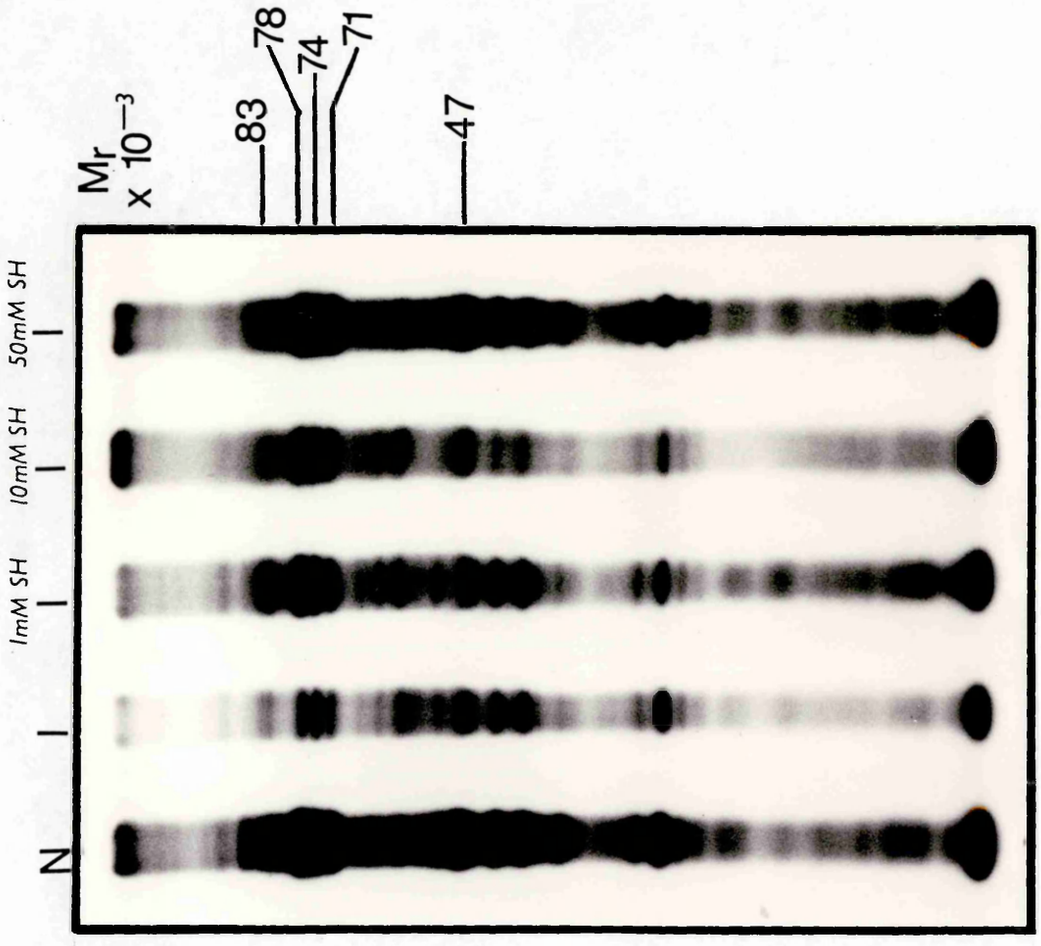
Remainder: resuspend to 2ml GMEM w/o methionine/10% hiFCS.

Add 100µCi (3.7MBq) ^{35}S-methionine

Incubate at 37°C/5% CO_2

↓

After 48h, remove samples to ice. Wash 3 times in ice-cold GMEM. Freeze for SDS-PAGE.


Mr
x 10⁻³
83
78
74
71
47
N
1mM SH
10mM SH
50mM SH

schistosomula from each treatment group of this experiment were injected s.c. into mice 1 hour after mechanical transformation. Table 8.2 shows that the presence of high concentrations of SH groups during irradiation also enhanced parasite survival *in vivo*. No stunted worms were detected in the irradiated infections, and egg granulomas were present in the livers of mice acting as hosts to irradiated and protected parasites. 10mM and 50mM SH, but not 1mM SH, improved the chances of survival for irradiated schistosomula. However, even 50mM SH, despite protecting completely against inhibition of protein synthesis over the 48 hour period examined, allowed maturation of only 15% as many parasites as survived in a normal infection.

8.2.3.1.3. Culture in presence of SH groups after transformation: effect on irradiation-induced inhibition of protein synthesis.

In the experiment of figure 8.16, cercariae were incubated in high concentrations of cysteine and glutathione (10-50mM) for 5 hours. External SH groups were then washed away before irradiation. Thus, only SH compounds which had been assimilated by the cercariae during the pre- incubation period could affect the response to irradiation. For the experiment illustrated by 8.17, the ethyl ester derivative of cysteine, a more hydrophobic compound, was used, with the aim of facilitating uptake of cysteine across the parasite membrane. Cysteine ethyl ester was washed away after a 30 minute incubation, then cercariae were irradiated. A longer incubation with this compound caused parasite death.

Figures 8.16 and 8.17 demonstrate that these attempts to increase the parasites' internal pool of SH groups did not significantly protect the schistosomula against the metabolic damage caused by irradiation, although in figure 8.16, preincubation with the highest SH

Table 8.2 Enhanced survival in mice of cercariae irradiated in the presence of SH compounds.

Female Parkes mice were used. U.V.-irradiation dose was 400 μW min cm^{-2} For each treatment group, 3 mice were exposed s.c. to 200 schistosomula at 1 hour after transformation. See figure 8.15 for preparation of parasites, and levels of protein synthesis after each treatment.

	Irradiation in presence of SH at concentration				
Treatment	None	0mM	1mM	10mM	50mM
No. of adult worms per mouse ($\bar{X} \pm$ S. E.)	77 ± 3.0	0	0	6.0 ± 2.6	14.0 ± 5.5
% male worms	61.7			66.7	42.9
Egg granulomas in liver	++++			+	++

Figure 8.16 Effect on protein synthesis of incubating cercariae in the presence of SH compounds, external SH groups being washed away before irradiation.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

Approximately 400 schistosomula/well.

3% stacking gel/10% resolving gel.

PROTOCOL:

Cercariae

↓

Divide in 3:

CONTROL 10mM SH 50mM SH

Incubate in appropriate concentration of SH (in aquarium water, pH'd to 7.0) for 5 hours at room temperature.

↓

Wash away external SH compounds on a P40 Sinterglass filter (Gallenkamp) washing through with fresh aquarium water.

↓

Divide each group in two:

N I

↓

(U.V.-irradiate:

400 μW min cm^{-2})

↓

Transform mechanically in GMEM w/o methionine

Resuspend each to 1 ml GMEM w/o methionine/10% hiFCS.

Add 20μCi (740 kBq) ^{35}S-methionine to each.

Culture at 37°C/5% CO_2

↓

After 24h, remove samples to ice.

↓

Wash 3 times in ice-cold GMEM.

↓

Freeze for SDS-PAGE.

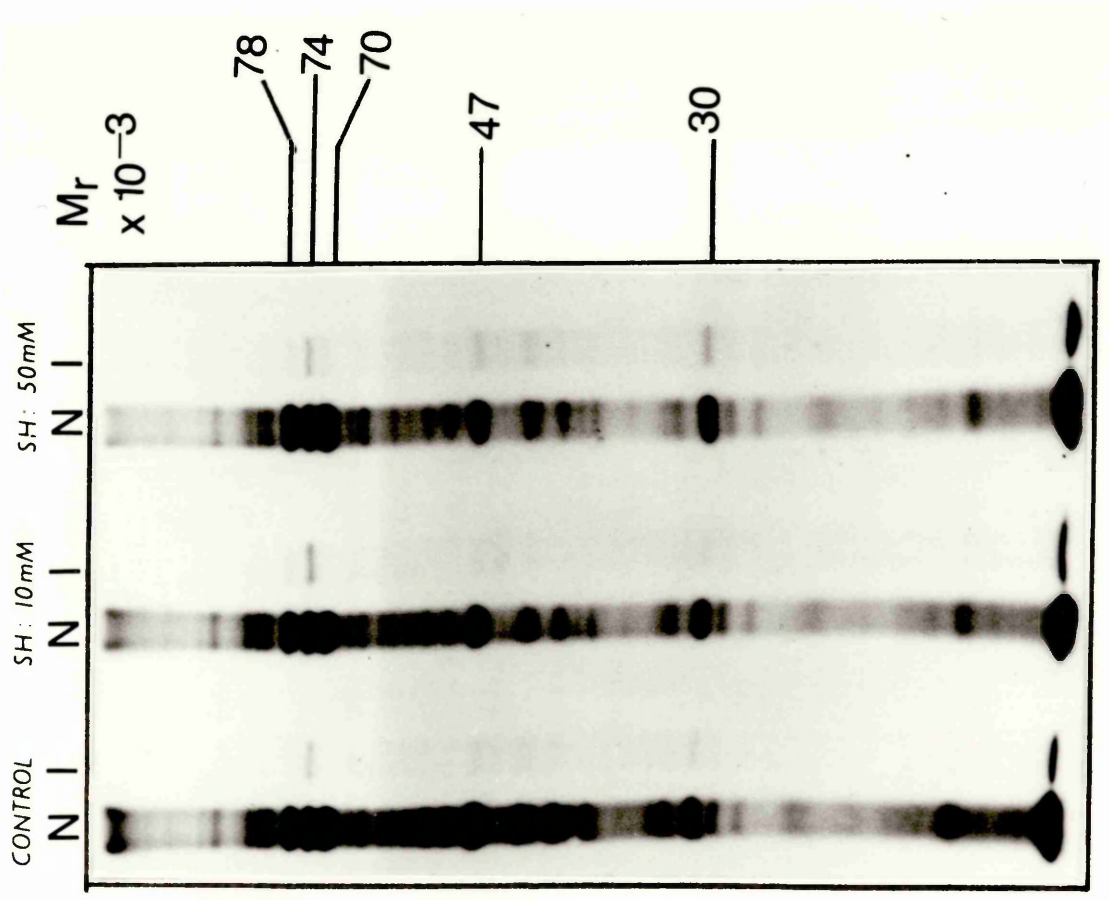

Mr
x 10−3
78
74
70
47
30
CONTROL
N I
SH: 10mM
N I
SH: 50mM
N I

Figure 8.17 Effect on protein synthesis of incubating cercariae in presence of cysteine ethyl ester before irradiation.

N = normal Cys Et = Cysteine ethyl ester

I = U.V.-irradiated (400 μW min cm^{-2})

500 schistosomula per well.

3% stacking gel/10% resolving gel.

PROTOCOL:

Cercariae

↓

Divide in 2:

CONTROL Cys Et (1.0 mM)

↓

Incubate at room temperature for 30 mins in presence or absence of cysteine ethyl ester at 1.0 mM in aquarium water, pH'd to 7.0.

↓

Remove external cysteine ethyl ester by filtration as described for figure 8.16.

↓

Divide each group in two:

N I

↓

U.V.-irradiate:

100 μW min cm^{-2}

↓

Transform, culture and label for 24 hours as described for figure 8.16.

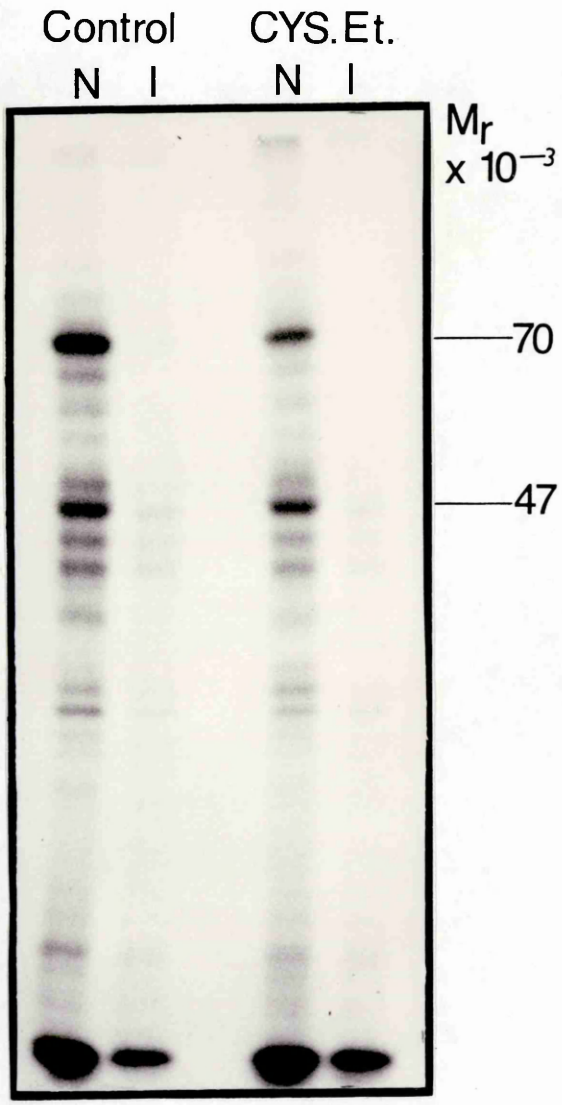

Control
CYS. Et.
N
I
N
I
M_r
x 10^{-3}
70
47

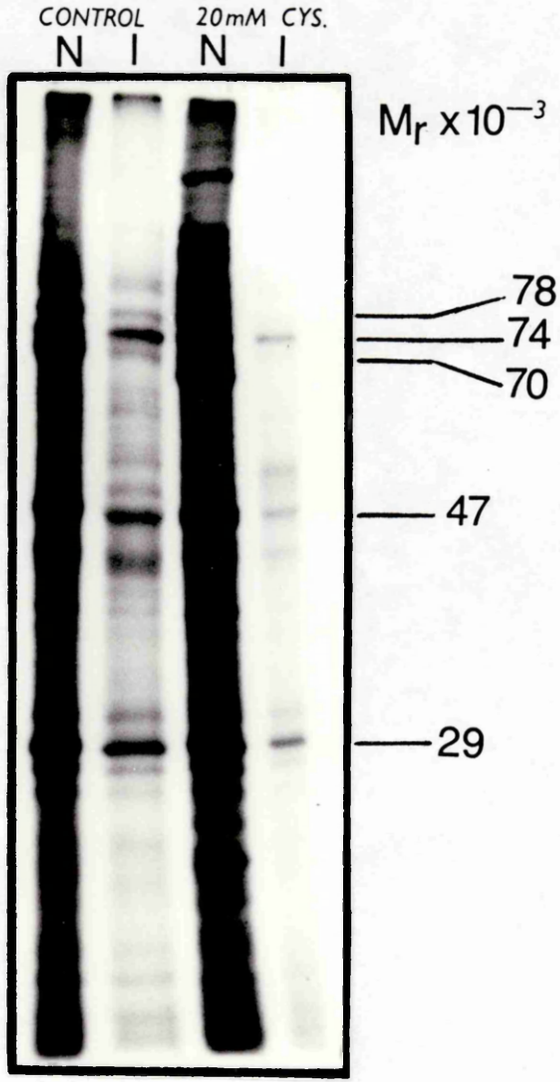
Figure 8.18 Effect on irradiation-inhibited protein synthesis of 20mM cysteine in culture medium after transformation.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

800 schistosomula per well. 3% stacking gel/10% resolving gel.

After irradiation and transformation, schistosomula were cultured at 37°C/5% CO_2 for 24h, with 50μCi (1.85 MBq) ^{35}S-methionine, in 1ml GMEM w/o methionine/10% hiFCS, in presence or absence of 20mM cysteine.


CONTROL
20mM CYS.
N
I
N
I
$M_r \times 10^{-3}$
78
74
70
47
29

concentration (50mM) may have slightly alleviated the inhibition. Nor did supplementing the medium, in which schistosomula were cultured after irradiation and transformation, with high concentrations of cysteine have any effect on the degree of inhibition, or the pattern, of protein synthesis (figure 8.18).

Although figures 8.16 and 8.17 suggest that pre-incubation of cercariae with SH compounds affords no significant protection if the SH groups are washed away before irradiation, it did seem that even when irradiation was performed in the presence of SH groups, a pre-incubation period was necessary for protection to occur. Figure 8.19 shows that irradiating cercariae immediately after addition of 1mM cysteine and/or glutathione did not lessen the inhibition of protein synthesis. However, when the parasites were irradiated in the presence of 1mM cysteine and glutathione after a 3 hour incubation with the same compounds, the metabolic inhibition was alleviated.

The blackening of the X-ray film for parasites treated for 3 hours with both cysteine and glutathione does seem more intense than for those treated with one compound only. Thus, the two reagents may have cumulative effects, although the irradiated tracks are too faint to establish this with certainty.

8.2.3.2. Effect of α-tocopherol on irradiation-induced inhibition of protein synthesis.

α-Tocopherol has been used in various biological systems to protect against irradiation damage by inhibiting lipid autoxidation (von Sonntag, 1987; section 8.3.2.5.)

Investigation of the effect of α-tocopherol was complicated by the fact that it is very insoluble in water. It was therefore used in micellar form, suspended in aquarium water or medium. The more hydrophobic derivative, α-tocopherol acetate, was used, to facilitate

Figure 8.19 Effect of time of pre-incubation with cysteine and/or glutathione on irradiation-induced inhibition of protein synthesis.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

CYS = cysteine

GSH = glutathione

500 schistosomula per well.

3% stacking gel/10% resolving gel.

PROTOCOL:

Cercariae

↓

Divide in 4:

↓

CONTROL GSH CYS GSH (each at 1mM)

Incubate in appropriately supplemented aquarium water, pH 7.0.

↓

At 0h and 3h, remove half of each treatment group.

Divide in 2:

N I

(U.V.-irradiate: 400 μW min cm^{-2})

↓

Transform mechanically in Elac.

Resuspend to 1 ml. Elac/10% hiFCS.

Add 50 μCi (1.85MBq) ^{35}S-methionine

Culture at 37°C/5% CO_2

↓

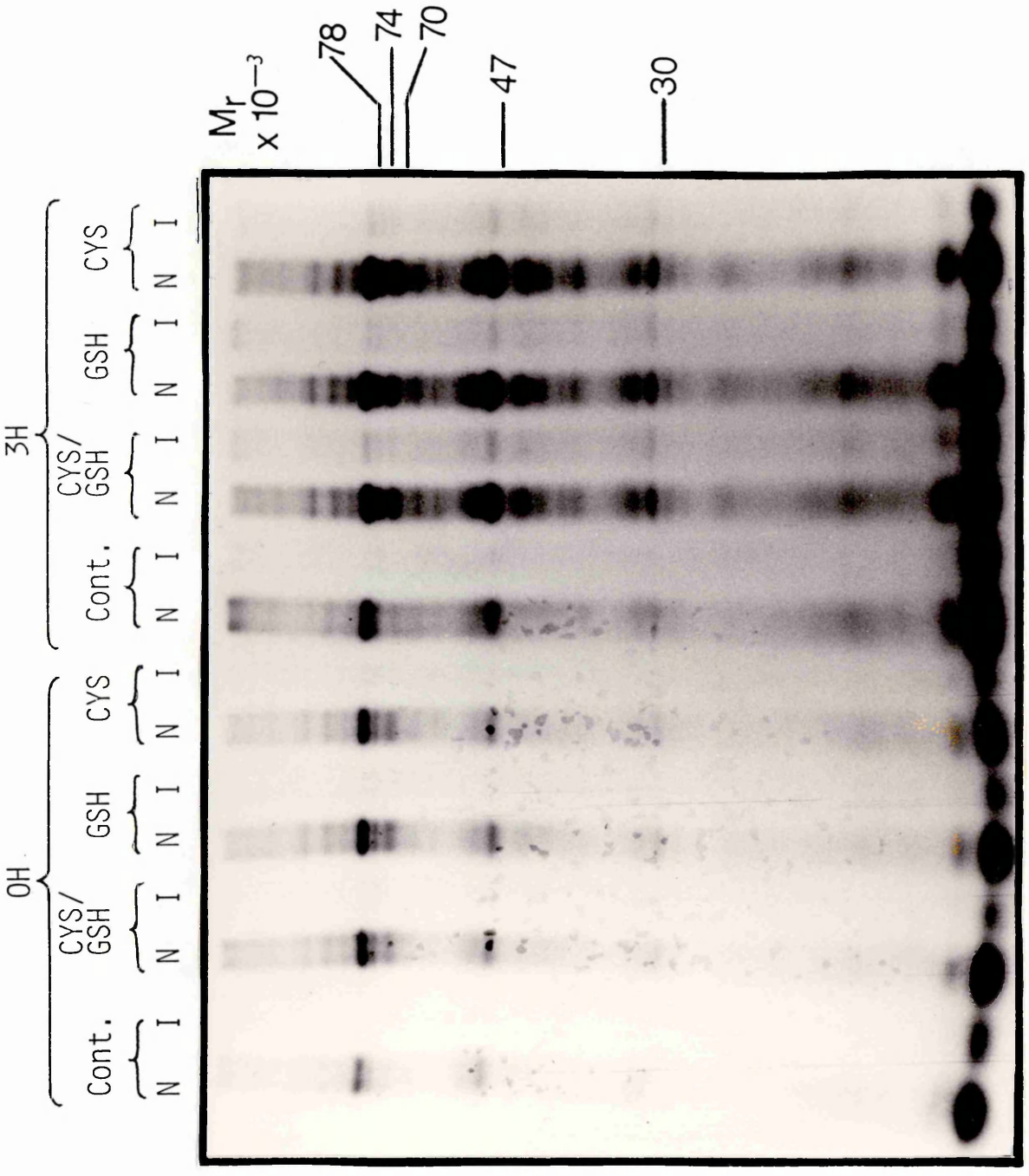
At 24h - remove samples to ice.

↓

Wash 3 times in ice-cold GMEM.

↓

Freeze for SDS-PAGE.


Mr x 10⁻³
78
74
70
47
30
OH
3H
Cont.
CYS/GSH
GSH
CYS
N I N I N I N I
Cont.
CYS/GSH
GSH
CYS
N I N I N I N I

absorption across the parasite membrane.

Figure 8.20 shows that very high concentrations of α-tocopherol (10-50mM) were able to protect against irradiation-induced inhibition of protein synthesis. In contrast to the results with cysteine and glutathione, the presence of α-tocopherol acetate in the medium after transformation seemed to afford better protection than when cercariae were preincubated, then irradiated in its presence before transformation.

8.2.3.3. SH groups and α-tocopherol protect against the effects of oxygen radicals.

When cercariae were exposed to oxygen radicals, generated by H_2O_2 in combination with ascorbic acid and copper ions, protein synthesis by schistosomula was inhibited in a concentration-dependent manner, just as previously observed with increasing doses of U.V. irradiation (fig. 8.21). Less than 10% of the schistosomula were dead 36 hours after any of the treatments. Thus, it seems reasonable to suggest that the inhibition of protein synthesis induced by irradiation may be mediated by oxygen radical attack. Presumably, therefore, cysteine, glutathione and α-tocopherol overcome this metabolic inhibition by preventing or repairing the effects of oxygen radicals.

8.3 Discussion .

8.3.1. Variable response to irradiation by different pools of cercariae.

Our observations that the inhibition of protein synthesis in response to irradiation varies from batch to batch of cercariae

Figure 8.20 Effect of α-tocopherol on irradiation-induced inhibition of protein synthesis.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

500 schistosomula per well.

3% stacking gel/10% resolving gel.

Cercariae were incubated for 3 hours at room temperature in aquarium water supplemented with α-tocopherol at various concentrations. They were then irradiated, transformed mechanically, and cultured for 24 hours in 1 ml GMEM w/o methionine/10% hiFCS with 50 μCi ^{35}S-methionine (1.85 MBq).

Other cercariae were irradiated in unsupplemented aquarium water, transformed mechanically and cultured for 24 hours in 1 ml GMEM w/o methionine/10% hiFCS, supplemented with α-tocopherol at various concentrations, and to which 50 μCi ^{35}S-methionine (1.85 MBq) had been added.

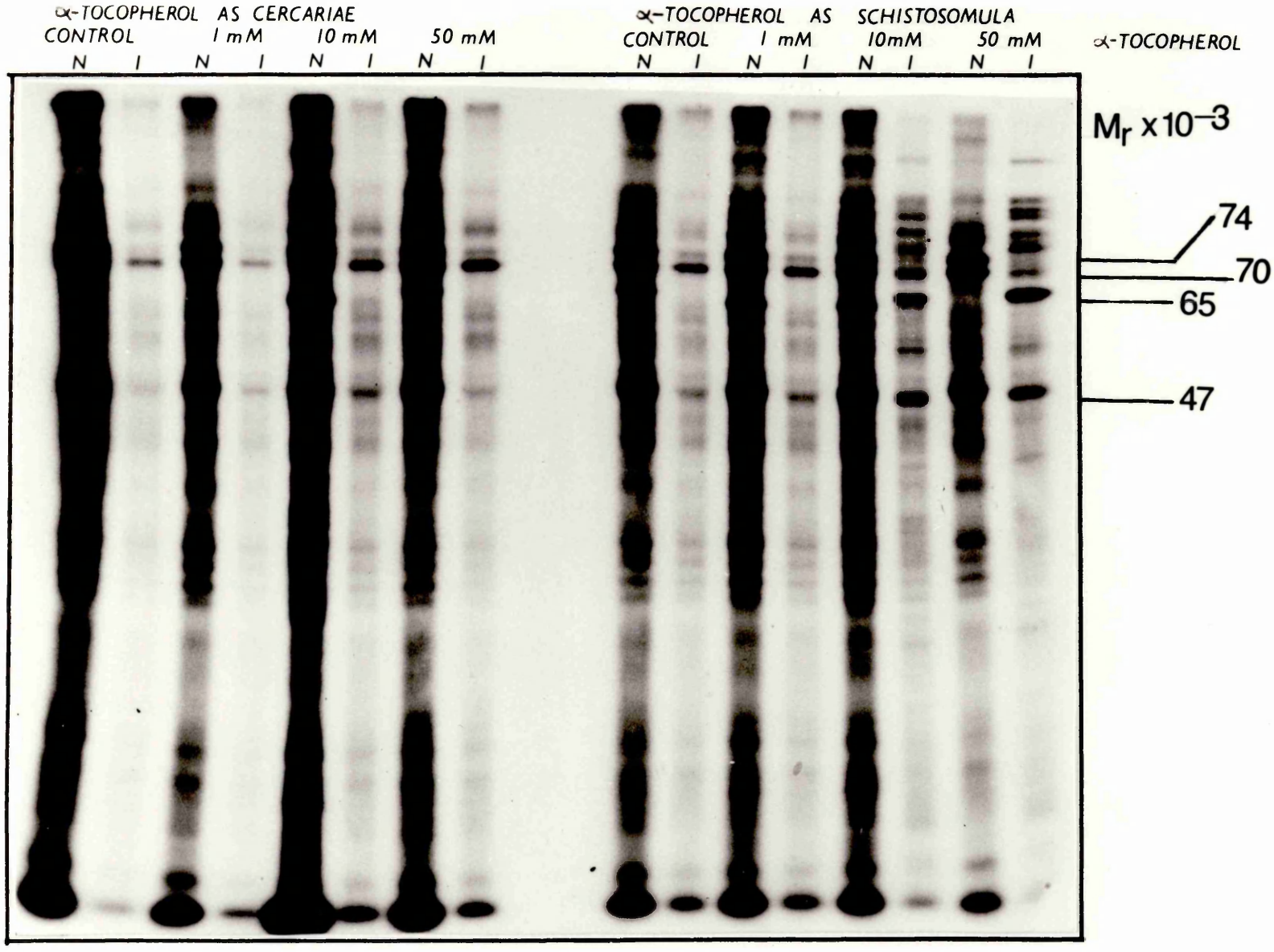

α-TOCOPHEROL AS CERCARIAE
CONTROL
1 mM
10 mM
50 mM
N I N I N I N I
α-TOCOPHEROL AS SCHISTOSOMULA
CONTROL
1 mM
10mM
50 mM
N I N I N I N I
α-TOCOPHEROL
Mr x10⁻³
74
70
65
47

Figure 8.21 Inhibition of schistosomular protein synthesis by exposing cercariae to oxygen radicals generated by H_2O_2 (after Samuni et al, 1983).

500 schistosomula per well.

N = normal

I = U.V.-irradiated (400 μW min cm^{-2})

The oxyradical generating systems were composed of:

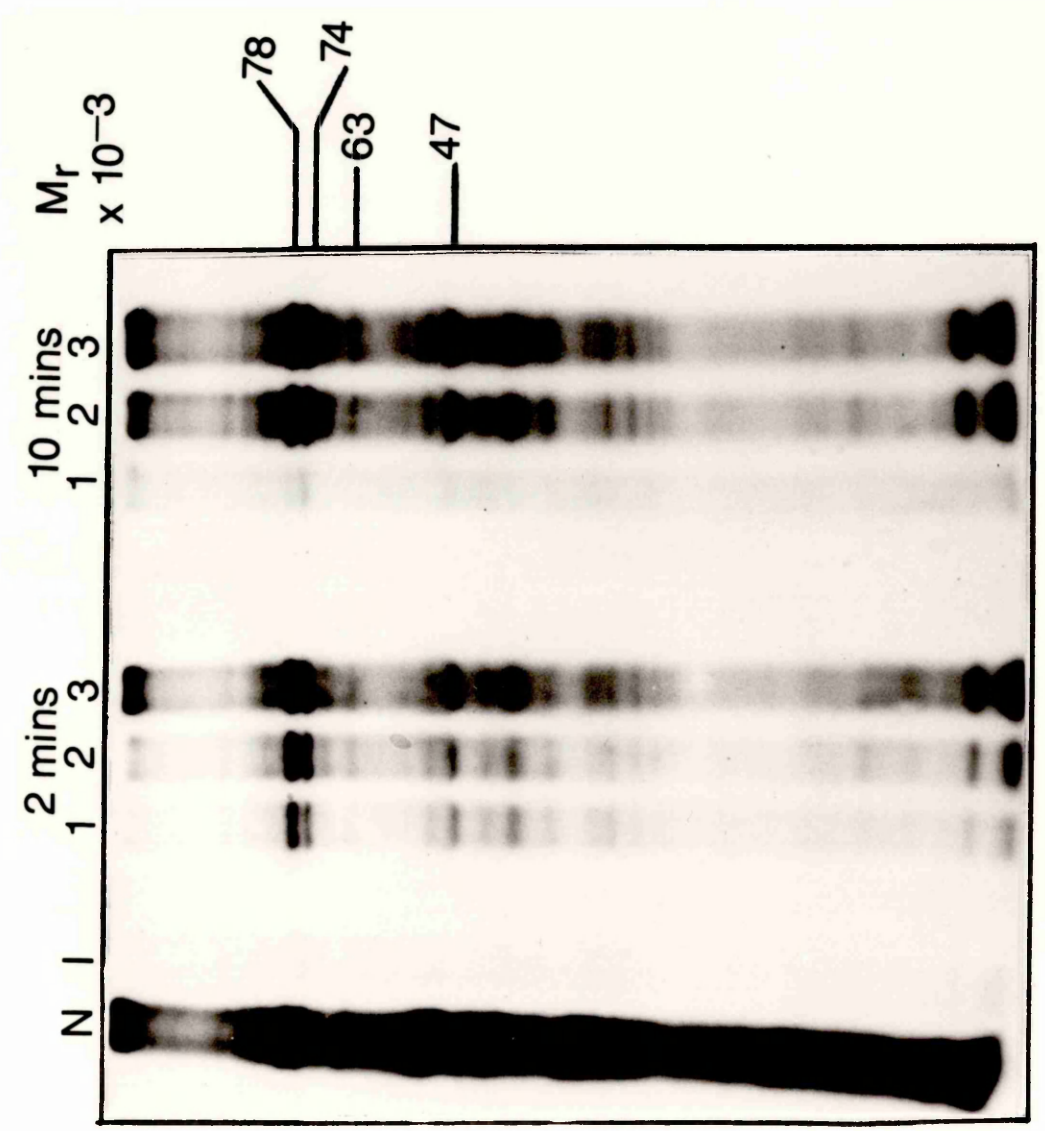
1) 1.0mM H_2O_2; 0.2M ascorbic acid; 2 x 10^{-2}mM $CuSO_4$; 10mM $MgSO_4$

2) 0.1mM H_2O_2; 0.02M ascorbic acid; 2 x 10^{-3}mM $CuSO_4$; 1mM $MgSO_4$

3) 0.01mM H_2O_2; 0.002M ascorbic acid; 2 x 10^{-4}mM $CuSO_4$; 0.1mM $MgSO_4$

The chemicals were dissolved in aquarium water and the solutions pH'd to 7.0.

Cercariae were treated for 2 minutes or 10 minutes with these solutions, then removed to ice for 20 minutes and transformed mechanically in GMEM w/o methionine. They were then cultured for 36 hours at 37°C/5% CO_2 in 1 ml. GMEM w/o methionine/10% hiFCS, in the presence of 50μCi ^{35}S-methionine (1.85 MBq).


78
74
63
47
M_r x 10^{-3}
10 mins
1 2 3
2 mins
1 2 3
N I

(figures 8.1, 8.2) find precedents in a number of reports of intraspecific variation in S. mansoni. Variability occurs at two levels. Individual cercariae within a single pool show diverse properties and separate populations of cercariae differ from each other.

Perhaps most importantly, different batches of cercariae under the same vaccination regime in an inbred mouse strain may induce quite different levels of immunity on separate occasions (Dean, 1983; Smith and Clegg, 1979). This variability applies to both the concomitant resistance and irradiated vaccine models.

As regards surface properties, Jones et al (1988) found wide variability both within and between pools of schistosomula in surface reactivity for fluorescein isothiocyanate-poly-L-lysine, which binds by charge interactions with the parasite surface, thus serving as a model for eosinophil cationic proteins. Similar variation in surface reactivity was observed for some fluorescent antibodies. By quantifying the fluorescence, this variability could be portrayed statistically in the form of frequency distributions. Some batches of schistosomula showed a normal distribution with high variances; others were not normal, but more like a Poisson distribution with a very long tail (Jones and Kusel, 1989).

Susceptibility to in vitro damage mediated by cationic proteins, assessed by uptake of the nuclear stain Hoechst H33258, also showed variability both between and within batches of schistosomula. Some parasites suffered severe damage after binding comparatively small amounts of poly-L-lysine, while others, at the opposite extreme, tolerated high amounts of poly-L-lysine, with little apparent damage. Similar observations on variation in parasite susceptibility to damage were made by McLaren et al (1981), using in vitro eosinophil killing assays. Some schistosomula always remained insusceptible to killing, even at very high cell densities. The work of Seitz et al

(1987) illustrated the same point *in vivo*. Histological studies of schistosomula in the skin of baboons showed intense cellular reactions around some schistosomula, but not around others nearby.

In an elegant series of experiments on schistosomular variability, Smith and Clegg (1979) showed that different pools of cercariae stimulated very different levels of concomitant resistance to the same challenge infection. Conversely, challenge infections drawn from different pools displayed different susceptibilities to immunity stimulated by the same primary infection. Overall, the levels of resistance observed in a single mouse strain varied from 10 to 78%.

Jones *et al* (1988) also concluded from infection data that parasites varied in their interactions with the host. When groups of inbred mice were infected with similar numbers of cercariae, the numbers of worms recovered were overdispersed, reflecting high variability in the ability of the parasites to infect hosts.

Some of the variability in parasite properties may be due to the presence of schistosomula at different developmental stages. Caulfield *et al* (1988), studying development of the cercarial glycocalyx within the snail, found that its thickness increased as the cercariae aged. This observation might help explain their previous finding (Caulfield *et al*, 1987) that two glycocalyx preparations, extracted by the same method, showed considerable variabition in their relative amounts of individual carbohydrates and amino acids. The authors suggested that less extensive post-translational modification in one instance could account for the disparity. Some of the variability in surface reactivity, described above, might have a similar basis.

Differences in developmental rates have also been demonstrated after transformation, in ultrastructural studies. Hockley and McLaren (1973) found considerable variability between parasites in timing of the transformation process, as represented by loss of the cercarial

glycocalyx and development of a heptalaminate membrane. Even at 3 hours after skin penetration, when most schistosomula possessed the typical double bilayer structure, some specimens still had a trilaminate outer membrane, appearing exactly like cercariae.

Individual schistosomula also differ markedly in the later stages of maturation, even when they are cultured in identical conditions *in vitro.* Thus, Clegg and Smithers (1972) noted that the length of the "lagphase" before growth commences, at day 4 at the earliest, in schistosomula cultured *in vitro* varies considerably between individuals.

As regards metabolic properties, Chappell's studies of free amino acid pools of larval and adult schistosomes (1974) indicated a very large variation between replicate samples, apparently due to genuine parasite variability rather than technical shortcomings.

Thus, a number of experimental approaches point to intraspecific variation in *S. mansoni.* Our own observations show that a single dose of U.V.-irradiation, which generally induces approximately 80% reduction of protein synthesis, may in occasional experiments cause very different levels of inhibition, varying from 0 to greater than 90% The level of inhibition may differ at early and later times after transformation - percentage reductions at 5 hours and 20 hours are quite different in figures 8.1 and 8.4:C20, C30A. The effects of gamma irradiation on protein synthesis (section 5.2) are also relevant here, since they indicate that schistosomula possess some capacity for tolerating, or recovering from, radiation damage by approximately 24 hours after irradiation. However, it must be borne in mind that our experiments examine parasite metabolism only over a 96 hour period (at the most) in an artificial culture medium, hence afford only limited information about events *in vivo.* Despite showing an apparent full recovery of protein synthesis by 24 to 48 hours after transformation, gamma-irradiated larvae do not survive to maturity *in vivo.*

Similarly, even when inhibition of protein synthesis in U.V.-irradiated schistosomula is abolished by treatment with high concentrations of SH compounds, only a small percentage of the parasites survive to maturity (figure 8.15, table 8.2). These observations suggest that irradiation inhibits some metabolic event, essential to schistosomular survival, other than the initial period of protein synthesis.

These observations encourage us to examine the variable response to irradiation in the light of the model already proposed (section 5.2), whereby schistosomular metabolism at successive stages in development may show different susceptibility to irradiation damage. Some of the variability observed in parasite responses to irradiation could perhaps be explained if individual batches of schistosomula show different capacities for repairing or guarding against radiation damage at the different stages in development.

This model is described below, and its relevance to the variable response to irradiation discussed.

1. During the early period after transformation (approximately 0 to 15 hours), schistosomular development relies principally on pre-formed proteins and RNA. Irradiation may interfere with translation of RNA messages, either by disrupting the mRNA itself, or by inactivating the enzymes of translation.

Measuring protein synthesis at early times (5 hours or less) after irradiation shows that this metabolic stage usually does suffer inhibition, although occasional exceptions occur, e.g. figure 8.1; A1, B2. Protection against irradiation-induced inhibition of protein synthesis at this stage might result from high intracellular levels of repair enzymes, or radioprotective species (SH-containing compounds, α-tocopherol, etc.).

2. From approximately 15 hours after transformation until growth proper

and cell division begin, at day 4 in culture, the parasite appears to require both transcription and translation of genetic messages. As already explained (chapters 3, 4), Actinomycin D only takes effect at this stage, when the parasite can no longer rely on presynthesized RNA. The transcriptionally active DNA involved at this stage is unfolded, hence, presumably, accessible to both radioprotective compounds and repair enzymes. Transcription of DNA occurs largely in the nucleoplasm (Hancock and Boulikas, 1982; Lewin, 1983) a region comparatively insensitive to irradiation damage (von Sonntag, 1987), so that transcriptional enzymes and active DNA are unlikely to suffer too severely by irradiation.

Our observations suggest that this stage is relatively insensitive to irradiation damage, for gamma-irradiated schistosomula, and, occasionally U.V.-irradiated ones, show a lessening of inhibition or complete recovery of protein synthesis during this period.

3. From day 4 after transformation onwards, a new stage of schistosomular development begins in culture, involving growth and cell division (Clegg, 1965; Clegg and Smithers, 1972).

Synthesis of new proteins, and mitosis, require activation and unfolding of formerly inert chromatin regions associated with the nuclear membrane. This stage of schistosomular development appears to be the most vulnerable to radiation damage, for gamma-irradiated schistosomula, whose protein synthesis is apparently restored to normal by approximately 24 hours, are unable to mature *in vivo.* High concentrations of SH groups may also boost protein synthesis at stage (2), but are still largely unable to permit growth and maturation. (figure 8.15; table 8.2).

Nucleic acid at the nuclear membrane does appear to be the target most susceptible to ionizing radiation damage (Munro, 1970; Datta *et al,* 1976), partly in consequence of the depth of penetration achieved

by the oxygen radicals. Moreover, the compact organisation of the DNA which is inactive at the time of irradiation, and its association with scaffolding proteins, apparently prevent access of protective molecules and repair enzymes.

Figure 8.22 illustrates this model, and the scope for variability at each stage. It may be noted that Actinomycin D causes much more reproducible inhibition of protein synthesis than irradiation (see figure 8.3). The radioprotective compounds which can defend the cell against irradiation attack will be ineffective against Actinomycin D, although intercalation of this drug into DNA could still be combated by DNA repair enzymes or gene multiplication.

8.3.2. Sources of variability in S. mansoni

8.3.2.1. Genetic variation

One potential source of variability in response to irradiation might be the existence of genetically distinct populations of parasites. We might speculate that the genetic material of some clones, but not others, could encode for synthesis of protective agents at unusually high concentrations, or for synthesis of repair enzymes of especially high efficiency. In multimiracidial infections, each snail is host to several clones of genetically identical cercariae, each derived from a single miracidium. Thus, on separate occasions, a single pool of snails could well produce batches of cercariae showing different susceptibilities to irradiation, as observed in figure 8.1.

This line of research seemed worth pursuing, since evidence does exist, albeit of a limited nature, for intraspecific genetic variation in S. mansoni. The most informative approach to characterising genetic diversity within a population has been to study protein variation by enzyme electrophoresis. In a study of 14 enzymes in

Figure 8.22

Radiosensitive stages in protein synthesis:

1. Translation of messenger RNA on ribosomes of the rough endoplasmic reticulum.
2. Transcription and processing of active DNA.

Repair enzymes, radioprotective compounds, and nucleic acid conformation can all protect against radiation damage at stages 1 and 2.

3. Activation of new genetic messages, for growth and cell division.

Inactive chromatin at the nuclear membrane is the target most susceptible to radiation damage, and its conformation renders it inaccessible to radioprotective compounds or repair enzymes.

KEY: Pathways of protein synthesis and secretion.

(a) Inactive chromatin associated with nuclear membrane.
(b) Transcriptionally active chromatin.
(c) Transcription, and processing of hnRNA in nucleoplasm.
(d) Translation of mRNA on ribosomes in rough E. R.
(e) Golgi body: proteins are packaged in membranous bodies, and "sorted" according to their various destinations.
(f) Supply of proteins to the plasma membrane.
(g) Protein secretion.

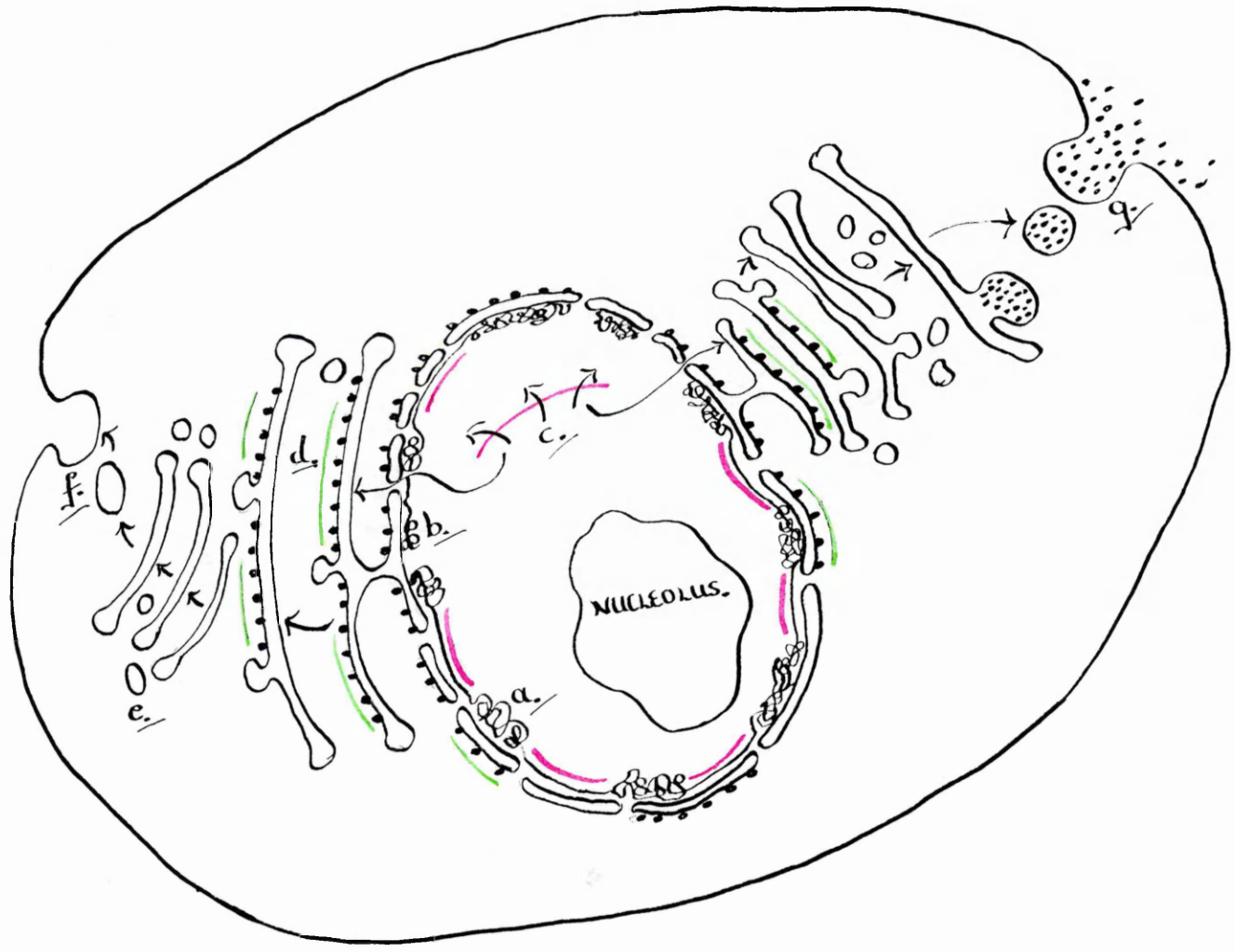
: DNA folded into heterochromatin

: DNA unfolded into active form

: RNA undergoing transcription or processing

: RNA being translated into protein

RADIOSENSITIVE STAGES IN PROTEIN SYNTHESIS: a, b, c, d.



individual adult worms representing 22 isolates of S. mansoni, Fletcher et al (1981) .recorded a range of genetic variation. Estimates of the proportion of enzyme loci that were polymorphic in a population (P) ranged from 0 to 0.33. Genetic differences between the worm populations of different hosts were indicated by the results of a study utilizing 6 separate isolates of Schistosoma haematobium from individual patients in Mauritius (Rollinson et al, 1982). Each isolate was analysed for the enzyme glucose-6-phosphate dehydrogenase, and marked differences in the frequencies of the four recognised alleles in each of the 6 isolates were observed.

Enzyme electrophoresis has also been used to identify parasites of different genotypes derived from a single snail. The most obvious interpretation of such an observation is parasitism of the snail by several sporocysts. Southgate et al (1980) isolated S. bovis from a single B. africanus snail from Tanzania, and recovered both male and female worms from animals infected in the laboratory. Out of 7 enzymes, 3 were polymorphic. Wright and Ross (1983) exposed hamsters to cercariae from each of 3 B. rohlfsi snails from a transmission site in Ghana. Subsequent enzyme analysis of the adult worms at two polymorphic loci showed that two of the snails had been infected by at least 3 miracidia, and another by 2.

Studies of this type are necessarily limited to the surviving adults of any infection - only a fraction of the larval pool from which they were derived. This original cercarial population, in turn, represents only a small percentage of the generative capacity of their founder miracidia. A more comprehensive picture of parasite variability would be obtained by studying larval, rather than adult, populations.

Further evidence for the capacity of the schistosome genome for variation comes from restriction of gene frequencies after several passages in the lab, and from comparison of the protein polymorphism of

adult worms obtained from different host species. The proportion of polymorphic loci is much higher for recently-obtained isolates than for mouse-adapted strains (Fletcher et al, 1981). Changes in the enzyme patterns of schistosomes after passage in mice were also noted by Coles (1971) and Ross et al (1978). An S. mansoni strain that had been passaged in laboratory mice showed a much lower P value (0.06) than the same isolate maintained in baboons (Fletcher et al, 1981). The possibility of host-induced selection was further examined by Loverde et al (1982). A line of S. mansoni which had originally been passaged in baboons was enzyme-typed, and subsequently passaged through murine hosts for three generations. Allele frequencies were found to drift away from frequencies for populations maintained in baboons towards the values found for parasites passaged in mice. Evidence for host selection pressures influencing expression of the parasite genome in the field was also obtained by Théron (1984), who found cercariae with distinct early or late shedding patterns in a single S. mansoni strain from the same endemic area. The shedding pattern displayed appeared to be determined by the local predominance of human (diurnal) or rodent (nocturnal) definitive hosts.

Reports of resistance to various drugs - oxamniquine, hycathone - in parasites isolated from patients (Dias et al, 1982; Yeang et al, 1987; Marshall, 1987), might also be attributable to genetic variation. Coles and Bruce (1987) achieved some limited success in selecting drug-resistant strains of S. mansoni in the laboratory. When 20 000-25 000 schistosomula treated in vitro with amoscanate, oltipraz or oxamniquine were injected in mice, a few egg-laying adults were recovered (0.018%, 0.027%, 0.022%, of the original populations respectively). Miracidia from the eggs laid by these surviving worms pairs were used to infect snails. Adults derived from the resulting cercariae showed reduced drug susceptibility, although interpretation of results was complicated by the fact that strains which had not been

drug-selected also displayed increased drug resistance. While these drugs might select for genetic variants, their mutagenic properties might equally well create, rather than select, non-responsive parasites.

We aimed to investigate the influence of such genetic variation on the schistosomular response to irradiation by selecting for particular alleles with monomiracidial infections. Different clones harvested on any one occasion did differ markedly as regards the metabolism of both normal and irradiated parasites (figures 8.4, 8.5). However, these differences were not reproducible on a day to day basis (figures 8.5 a),b)). It therefore appeared that non-genetic factors might predominate over genetic ones in generating the variable metabolic response to irradiation.

8.3.2.2. Non-genetic variation in S. mansoni.

Many lines of evidence suggest that the variability observed in parasite properties cannot all be attributed to genetic variation.

The observations presented here on the metabolism of cloned cercariae complement the work of Jones et al (1988) on their surface properties. In this study, the inhibitory effect of poly-L-lysine on aminofluorescein (5-AF) uptake was examined. Individual clones showed different levels of inhibition from day to day. Moreover, 5-AF uptake by control members of each clone, not treated with poly-L-lysine, also varied on each occasion of testing.

Antigen expression by schistosomula also does not seem to vary on a genetic basis. Smith et al (1984) employed 8 monoclonal antibodies directed against individual surface antigens of schistosomula to determine the distribution of these antigens among clones. All 8 antigens were detected on the surface membranes of 32 clones. The authors also observed that the density of separate antigens on the

surface membrane varied among individual schistosomula belonging to the same clone. Similar evidence for intraclonal variability was obtained by Vieira and Kusel (manuscript in preparation) who screened ten clones of schistosomula with four surface-specific antisera. Antiserum binding was quantified by indirect immunofluorescence, and the frequency distribution of fluorescence intensity compared for cloned cercariae and mixed populations. Variability in cloned populations always equalled, and sometimes exceeded, that for genetically heterogeneous pools.

Simpson et al (1985) also concluded that cloned parasites and mixed populations must present the same antigens, with a similar incidence of variability among individual members. Gamma-irradiated clones of cercariae protected mice against challenge by members of the same clone as effectively as against heterogeneous challenge by a mixed parasite population. Moreover, mice vaccinated with cloned cercariae produced antibodies with the same specificities as after vaccination with a mixed parasite population. A similar approach was adopted by Hackett et al (1987), who subsequently demonstrated that mice immunized with one irradiated clone were equally resistant to challenge by members of the same or a different clone.

Since the metabolic and surface properties of clones vary within and between batches just as much as for mixed populations, it is perhaps not surprising that clones protect equally well against genetically similar and dissimilar challenges. The metabolic and surface labelling studies described suggest that members of a clone harvested for challenge infection are no more likely than a mixed population to bear especial resemblance to the immunizing clone as regards either surface antigens or metabolism.

Smith and Clegg (1979) did present evidence that parasite susceptibility to host immune mechanisms might be genetically determined. Out of 15 clones, 2 showed a high level of susceptibility

to concomitant resistance induced in mice by a small bisexual infection. The other clones were not susceptible at all. However, the genetic basis of this susceptibility would have been more firmly established by assessing the results of challenge by members of each clone, shed on more than one occasion. Individual clones shed on the same day are likely to show different properties from each other (see for example, figures 8.4, 8.5), but unless these differences are consistently reproducible in day to day sheds, they are unlikely to have a genetic basis (see figures 8.5 a), b)). Vieira and Kusel (manuscript in preparation) also found that, on a given occasion, one or more clones would display a significantly different mean antibody binding (though similar variance) from the mixed population, but this result was not reproducible on a day to day basis. It seems that mixed populations obtained from different pools of snails, or from the same pool on different dates, are just as likely to show different properties as different clones (see figure 8.1).

Cohen and Eveland (1988) demonstrated that when S. mansoni clones were maintained by serial microsurgical transplantation of sporocysts from infected and uninfected snails, a number of parameters - infectivity for mice and snails, ability to induce immunity in mice after U.V.-irradiation, cercarial outputs - were consistent within each clone. However, these clones are rather distantly related to the cercariae used in our experiments. Our cercariae were obtained between 7 and 14 weeks after the original monomiracidial infection of snails, and were followed throughout the natural lifespan of the snail/parasite association. In contrast, the larvae used by Cohen and Eveland (1988) were obtained at least 3 years after the original monomiracidial infection of the parent snails. We would predict that at each successive sporocyst transplantation into new snail, there may be selection for the parasites best suited for survival in the snail host, until the cercariae finally used in the studies reported in the paper

represent only a specialized fraction of the original population, highly adapted for optimal infectivity for the snail host. Evidence in favour of such a selection process comes from the earlier observation of Cohen and Eveland (1984) that the number of snails sucessfully parasitized by transplanted sporocysts increased significantly, from 50 to 90% of the exposed snails, during the initial four passages.

Lewis et al (1985) were unable to demonstrate a genetic basis for variability in their investigation of the possibility that the incomplete immunity afforded by the irradiated vaccine might be due to genetically-determined insusceptibility in a particular schistosome sub-population. After 5 successive passages through snails and immune mice, progeny of those parasites which escaped immune killing were no more refractory to vaccine-induced resistance than the original stock, maintained in non-immune mice. Thus, variable resistance to immune killing did not appear to be genetically determined.

Similar conclusions were drawn from a subsequent study (Lewis et al, 1987), where several laboratory strains of S. mansoni were tested for relative immunogenicity, or susceptibility to anti-schistosome immunity in irradiated cercariae-immunized mice. Eleven strains and substrains were used, and cross-protection developed in all intrastrain combinations tested.

A non-genetic basis for susceptibility to immune mechanisms in vaccinated hosts was also implied by the work of Majid et al (1982) and Hsü et al (1984), who found that cattle and water buffalo displayed significant anti-schistosome resistance following immunisation with radiation-attenuated cercariae of S. bovis or S. japonicum, despite being allowed to graze freely, and thus encounter natural populations showing much greater genetic diversity than the laboratory strains.

In conclusion, it seems that variation in antigenic and metabolic properties of schistosomula is not genetically determined. However, it

should be borne in mind that the cercariae obtained from each shedding of a clone constitute only a small fraction of the total larvae which the parent miracidium has the capacity to generate. Each apparently disparate result might therefore merely represent widely-spaced points on a normal distribution curve. An indication of the average properties truly representative of all the members of each clone might be obtained by measurements on cercariae obtained from many more sheds, perhaps comparing cercariae harvested at the same point in successive sporocystogenesis-cercariogenesis cycles. The technique of transplantation of daughter sporocysts into new host snails (Jourdane and Théron, 1980), applied to cloned parasites by Cohen and Eveland (1984, 1988 - see above) could allow production of cloned cercariae in sufficient numbers for such extensive and prolonged studies, although, as already mentioned, a course of successive transplantations may tend to select for a restricted parasite population.

Nevertheless, it does seem safe to conclude that non-genetic factors contribute significantly to the variability observed both between populations of cercariae and among individuals within a single population. This non-genetic component of the variability might be generated by a number of mechanisms.

8.3.2.3. Influence of host and environmental factors on parasite biochemistry.

Some of the variability observed within and between cercarial populations may be due to development in different molluscan environments. Not only do the physiology and biochemistry of individual snail hosts differ, but, within each snail, a range of microenvironments are available to the parasite.

Laboratory stocks of B. glabrata display considerable genetic variability in enzyme electrophoretic analysis (Mulvey and Vrijenhoek,

1981). Further evidence for genetic variation in the snail host comes from the frequent occurrence of spontaneous morphological mutants in laboratory- maintained B. glabrata (Richards, 1970; Richards and Merritt, 1972; Lie et al, 1979). Lie et al (1979) also traced the inheritance of resistance to S. mansoni infection, a genetically-determined characteristic, in inbred lines developed from the same stock B. glabrata strain, 10-R2. This line is strongly resistant to S. mansoni. Three separate subpopulations, however, showed, respectively, slight loss of resistance, 50% susceptibility, and retention of the original resistance. Since no selection for resistance was involved in the breeding protocol, the genetic changes were apparently random ones that became established in the separate inbred substrains. The authors concluded that spontaneous mutations had exposed recessive susceptibility factors present but unexpressed in resistant snails.

Thus, the genetic constitution of individual snails of the same strain could produce quite different environments. Some examples of genetically-determined molluscan factors which might crucially affect parasite biochemistry could be levels of organic and inorganic nutrients, rates of metabolism and respiration, hormone concentrations, rates of snail maturation. Of course, external factors, such as diet, water pH and temperature, number of snails in a given volume, will also influence the composition of snail body fluids, and hence cercarial development. The capacity of the parent miracidium for sporocystogenesis and cercariogenesis may vary extensively (reports for productivity of a primary sporocyst range from 34 to 625 daughter sporocysts (Jourdane and Théron, 1987) and will also influence the amount of nutrients, etc. available to individual parasites.

Variability among cercariae produced by any one snail may result, in part, from the local microenvironment in which individual sporocysts develop. Initially, miracidia of S. mansoni develop into mother

sporocysts in the subepithelial region of the head-foot. Differentiation into daughter sporocysts occurs, and between days 10 and 17 these young sporocysts migrate to the digestive gland. Two routes of migration seem to be used - either actively through the snail's loose connective tissues, or passively via the circulatory system. Some sporocysts do not reach the digestive gland, but develop around the kidney or the rectal ridge. Mature, daughter sporocysts are then faced with three developmental choices. Some go on to produce new sporocysts directly. These replicating sporocysts are generally smaller in size than the first generation, with fewer young sporocysts in their cavity. Unlike first generation daughter sporocysts, they tend to be located in the head-foot. Other daughter sporocysts initially produce cercariae, producing a new generation of sporocysts after degeneration of the cercarial embryos. In this case, the replicating sporocysts are fully developed, and generally located in the digestive gland of the snail. Sporocystogenesis after cercariogenesis occurs at precise points in the parasitic process, followng a circamensual rhythm. The least common pattern of multiplication is simultaneous production of new sporocysts and cercariae (reviewed by Jourdane and Théron, 1987). Clearly, individual cercariae, even from a clone, will have quite different developmental histories, and have developed in a range of ecological niches, likely to differ in concentrations of, for example, CO_2, glucose, amino acids, hormones.

The variation in metabolic and surface properties between and within mixed batches of cercariae, and between different clones shed on a single occasion may be partially attributable to such environmental sources of variability. These different environmental factors could be reflected directly in nutrient and energy levels in the cercariae, or could influence expression of the parasites' genetic messages.

However, the chronobiology of cercarial development may be

especially important in inducing variation on a day to day basis in parasites from the same snails, even when the cercariae shed on separate occasions are genetically identical (figures 8.5 a), b)). Evans and Stirewalt (1951) originally observed fluctuations in cercarial infectivity according to the age of parasitosis in monomiracidial infections. They suggested that these fluctuations in cercarial properties might be directly related to the physiological condition of the snail host. This hypothesis, that cercarial and molluscan physiology are interrelated, and both conditioned by the stage of development of the parasitic infection, was later modified and extended by Théron (1981) and Théron and Moné (1984). These authors demonstrated that cercarial production and infectivity for mice during monomiracidial infections both followed a circamensual rhythm, reaching a maximum at the same points throughout the course of the parasite infection. These cyclic variations in productivity and infectivity originated from the regular renewal of cercarial generations and from the synchronism of development of intrasporocyst larval stages. The troughs in production and infectivity involved cercariae shed at the end of a generation of daughter sporocysts, when the germinal cell stock was being renewed. The peak periods, on the other hand, corresponded with maturation and first shedding of cercariae of the same generation. Interestingly, the infected snails also showed a rhythmic pattern of growth, negatively correlated with the variations in cercarial productivity. High cercarial productivity periods matched periods of slow growth by the mollusc, and, conversely, during low cercarial productivity periods, the snails showed faster growth. These results seem to indicate metabolic exchanges between host and parasite. Thus, cercariae released at different time points during the lifespan of a single generation may have reached a different state of biochemical and physiological development, varying, for instance, in the levels of nutrients assimilated from the snail.

The importance of the energy levels of the cercariae in determining the parasites' response to irradiation is suggested by the results of figure 8.9. Cercariae allowed to swim freely in water for many hours before irradiation and transformation show enhanced inhibition of protein synthesis. This increase in susceptibility may be due in part to depletion of glycogen reserves, which decline with time after shedding (Olivier, 1966). Depletion of the internal free amino acid pool is suggested by the increased uptake of exogenous, radioactive amino acid by aging cercariae. Utilization of organic nutrients may leave less energy available for repair of radiation lesions. Alternatively, potential radioprotective compounds - glutathione, cysteine, α-tocopherol - may either have been assimilated into macro- molecules or catabolized to provide energy. Lawson and Wilson (1980b) reported a 3-fold variation in the glycogen content of different batches of cercariae (4.8 - 14.2 ng/cercaria). They suggested that this variation might reflect the nutritional state of the snail host, as previously postulated by Becker (1971). Such variation in cercarial energy levels might contribute to the variable response to irradiation. However, some of the increased responsiveness to irradiation which we observed after a long interval of free swimming might also be due to accumulation of oxygen metabolites during this period of aerobic metabolism.

We might speculate on other, age-related alterations operating at the level of both parasite and host to induce variability between sheds from the same snail, on different dates. Towards the end of each sporocyst generation, the cercariae may be more likely to show chromosomal aberrations and damage. Similarly, as the sporocysts themselves age, aging phenomena are more likely to alter the genetic material of the germinal cells. Hence, each new generation of cercariae may not, in fact, be genetically identical to the previous one. Finally, as the snail itself ages, the concentrations of

nutrients and hormones in the parasite environment will change. Such alterations may, in turn, affect the snail's ability to support a parasite burden, and alter the biochemical characteristics of the developing cercariae.

It should not be discounted that generation of variability might even be a purposeful survival strategy which is advantageous to the parasite. Only a small fraction of the total miracidial and cercarial populations go on to mature in molluscan and vertebrate hosts, respectively. It is conceivable that the pronounced amplification of parasite numbers during egg production and cercariogenesis might be directed towards creating diversity. In this way, the probability might be optimised that at least a proportion of the miracidia or cercariae will find a new host suited to support their maturation and reproduction. Genetic mechanisms for generation of protein diversity are well established in systems such as immunoglobulin production by B-lymphocytes. These mechanisms include availability of multiple germ-line genes, recombination of gene segments, recombinational inaccuracies, DNA regions especially prone to somatic point-mutations, and variable association of protein subunits to form the final multimeric structure. It might be worth investigating whether generation of variability might be sufficiently important to the parasite population to warrant the existence of some similar systems for creating enzyme or antigen diversity.

8.3.2.4. Influence on radiation response of environmental factors encountered after transformation.

After transformation, the initial variability intrinsic to the cercarial population will be amplified as the parasites encounter a wide variety of microenvironments and host signals in mammalian skin, and during subsequent migration to and through the lungs and liver.

Limited studies were performed on the influence of some of the factors encountered after transformation.

The temperature to which parasites are exposed before or after irradiation may have some effect on the extent of metabolic inhibition. Cercariae irradiated at different temperatures (0-37°C) show the same degree of metabolic inhibition, provided they are transformed by the same method. However, skin forms suffer less metabolic inhibition, than mechanical ones (figures 8.7 a), b)). This difference may arise from the fact that, during mechanical transformation, cercariae are removed to ice immediately after irradiation, whereas in skin penetration, most cercariae transfer from room temperature to 37°C in the skin within 10 to 30 minutes. We could envisage that, whereas at the higher temperatures involved in skin transformation, repair enzymes are active, at 0°C, the initial radiolesions become permanently fixed. Alternative explanations could also be proposed, however. For instance, cercariae are known to incorporate linoleate, a radioprotective agent (Norman et al, 1988) during skin penetration (Haas and Schmitt, 1978). Finally, the schistosomula collected after 3 hours of skin penetration are a selected subpopulation of the total parasite pool. It is quite possible that those parasites which penetrate the skin first could be those possessing especially high levels of nutrients and protective agents, hence best fitted to overcome radiation damage. The time of residence in the skin for different schistosomula varies widely; $t_{½}$ for exit from mouse skin has been estimated at 88 hours (Miller and Wilson, 1978). Exposure to factors in the host skin for different times is likely to incur further variability in schistosomular properties. With mechanical forms, no such host factors influence parasite properties; moreover, we see only the average response over the whole schistosomular pool.

A number of authors have reported that sera from different hosts, even of the same animal, the type of medium, and the culture vessel,

can all influence the response to irradiation in cellular systems (lymphocytes; Smith et al, 1985; fibroblasts; Nagasawa and Little, 1988). However, the experiments presented here found no difference in the extent of inhibition of protein synthesis in response to irradiation in different media or sera, although different culture conditions could induce slightly different patterns of protein synthesis by both normal and attenuated parasites.

8.3.2.5 Mechanisms of protection against radiation damage.

Some of the variability observed in the radiation response by schistosomula must be due to their relative capacity to forestall or repair radiation damage. When we consider that the doses of both U.V.- and gamma-irradiation required to attenuate cercariae are considerably greater than required to achieve cell death in mammalian cells or bacterial systems (table 8.3), it does seem very probable that S. mansoni larvae must possess some highly effective radioprotective mechanisms. Since free-swimming cercariae are normally exposed to U.V.-radiation in sunlight, and show a powerful positive phototactic response, it is perhaps not surprising that their metabolism should display such resistance to radiation damage. Such protection might perhaps be mediated either by radioprotective compounds, eg. SH-containing compounds, or by radiation repair enzymes.

The anti-oxidant enzymes superoxide dismutase, cytochrome-c peroxidase and glutathione-S-transferase have been found in significant concentrations in adult S. mansoni (Mkoji et al, 1988; Callahan et al, 1989; Mitchell, 1989). It has been proposed that these anti-oxidant systems protect the adult worm against the reactive oxygen species produced by activated host phagocytes. The same enzymes are present, though at lower levels, in schistosomula (Mkoji et al, 1988). We might predict that such systems could enable free-swimming

Table 8.3 Comparison of lethal radiation doses for *S. mansoni* cercariae, and cellular systems.

Attenuating dose for *S. mansoni* cercariae	Lethal doses in other cellular systems.
U.V.-irradiation.	
400 μW min cm^{-2} = 240 Jm^{-2} (240 Joules of energy incident on an area of $1m^2$)	CHO cells: 90% cell death at 10J m^{-2} (Friedberg, 1985).
	Human skin fibroblasts: 90% cell death at 14Jm^{-2} (Weichselbaum *et al,* 1978)
	E. coli: 99% death at 110Jm^{-2} (Gross and Gross, 1969)
Gamma-irradiation.	
20 krad. = 0.2 Joules of energy absorbed per gram of absorbing material.	Human skin fibroblasts: 90% dead at 800 rad (8×10^{-3} Jg^{-1}) (Taylor *et al,* 1975).
	Other human cell lines (Li 106, AG 1518, GM 1381, EX25: one lethal event per cell at an average of 147 rad. (1.47×10^{-3} Jg^{-1}) (Weichselbaum *et al,* 1978)

cercariae to tolerate the oxidising effects of U.V. rays in sunlight. It would be interesting to compare the concentrations of these enzymes in different batches of cercariae with their susceptibility to radiation damage.

Note 8.1 summarises the mechanisms by which these different anti-oxidant enzymes may scavenge or quench the reactive oxygen species produced by irradiation.

As well as enzymatic repair, chemical repair by various chemical species may protect against radiation damage. Compounds containing free thiol groups are effective protective agents. Free radicals may be produced in DNA, proteins or lipids either by direct absorption of radiation energy (reaction 1, followed by reaction 2), or by OH radicals from the radiolysis of water (reaction 3). SH compounds repair the target radical T· by donating an H-atom (reaction 4).

$$TH \xrightarrow{h\nu} TH^{+\cdot} \qquad (1)$$

$$TH^{+\cdot} \longrightarrow T\cdot + H^{+} \qquad (2)$$

$$TH + \cdot OH \longrightarrow T\cdot + H_2O \qquad (3)$$

$$T\cdot + SH \longrightarrow TH + S\cdot \qquad (4)$$

In order to act as a radiation protector according to reaction 4, a compound must be sufficiently mobile. Among the various intracellular RSH compounds, high molecular weight peptides containing thiol groups are therefore usually neglected, and only the low molecular weight RSH is taken into account. Cysteine and cysteamine are potent radioprotectors, but intracellular levels are usually low (Jocelyn, 1972) and most of the free RSH in cells is stored in the form of (reduced) glutathione (GSH):

Note 8.1 Actions of the oxyradical scavenging enzymes of schistosomes.

(von Sonntag, 1987).

1. Superoxide dismutase.

$$E^{\circ} + O_2^{\cdot-} \longrightarrow E^{-} + O_2$$

$$E^{-} + O_2^{\cdot-} \cdot (+2H^{+}) \longrightarrow H_2O_2$$

E° = native enzyme.

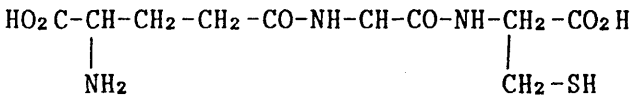
E^{-} = enzyme in reduced form.

2. Cytochrome-c peroxidase.

Cytochrome-c in its oxidised form reacts rapidly with solvated electrons, $O_2^{\cdot-}$, and many organic radicals, via its active centre, iron within a porphyrin system, and also via adducts of the peptide envelope.

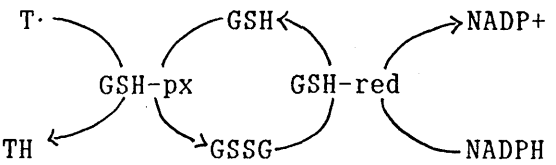
3. Glutathione-S-transferases.

Glutathione-S-transferases protect cells from toxic damage by conjugating reduced glutathione to the electrophilic centres created in various molecules as a result of free radical attack.



Reduced glutathione (GSH).

Normal intracellular concentrations of glutathione as high as 10mM have been reported (Jocelyn, 1972). The importance of naturally occurring glutathione in radiation protection has been demonstrated by enhancement of cell survival and reduction of DNA strand breakage in several kinds of experiments. Cells with different GSH contents have been compared (Revesz et al, 1963), or the same cell line has been compared in exponential phase of growth, and at plateau phase, when the GSH content is half of the former (Cullen et al, 1980). Certain drugs, such as buthione sulphoxime, have been added, which reduce the level of GSH within the cell (Shenoy and Singh, 1985). Use has also been made of mutants lacking certain enzymes for the biosynthesis of glutathione (Revesz, 1985). During repair of target radicals, intracellular glutathione alternates between reduced and oxidised forms, with the mediation of the enzymes glutathione peroxidase (GSH-px) and glutathione reductase (GSH-rd):



The importance of GSH in protecting schistosomes against free-radical damage is suggested by experiments performed on adult worms by Mkoji et al (1988). Depletion of GSH with buthione sulphoxime rendered a significantly increased proportion of the adult worms susceptible to oxidant killing in a xanthine-xanthine oxidase system.

It would be interesting to determine how the variable response to irradiation by schistosomula correlates with intracellular levels of

free SH groups.

In our experiments, it was possible to manipulate the degree of irradiation-induced inhibition of protein synthesis by addition of exogenous thiols, in the form of cysteine and glutathione, at concentrations greater than or equal to 1mM. The two compounds apparently exerted a cumulative protective effect (figure 8.19). They seemed to act predominantly at the cercarial surface, and to quench radicals as they were formed, during, or just after, the irradiation itself, since attempts to protect against radiation damage by increasing intracellular levels of SH before irradiation or after transformation were ineffective (section 8.2.3.1.3.). No measurements of the efficiency of uptake were made, hence it is not clear by how much, if at all, intracellular levels of SH groups were increased in these experiments.

However, despite the fact that absorption of cysteine and glutathione into the intracellular pool does not appear to protect in this system, a pre-incubation period of several hours does seem to be necessary for protection to occur (figure 8.19). We might speculate that this period allows the SH compounds to become enmeshed in the glycocalyx network, and associate closely with the surface membrane. The targets of radiation damage in both these regions could then be repaired immediately damage occurs. Moreover, to some extent, cascade reactions transferring free- radical damage into more internal regions may be prevented.

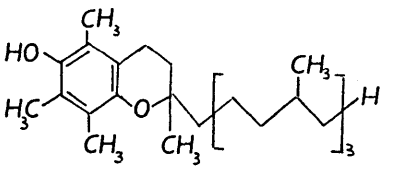
N-ethylmalemide, (NEM) on the other hand, sensitizes cells to radiation damage (Han et al, 1976; Debieu et al, 1985), apparently by two mechanisms involving competition with the repair acitivites of SH groups (Mullenger and Ormerod, 1969). The mechanisms envisaged are:

i. Reaction with free SH groups which would otherwise protect a radio-biologically important site.

ii. Interference with post-irradiation repair processes, most likely by binding to critical SH-containing enzymes concerned with repair of radiation damage.

In our experiments, NEM did not enhance irradiation-induced inhibition of protein synthesis. However, this could be due to the fact that the radiation dose employed already achieved almost maximal inhibition. It might be possible to detect some effect of NEM at lower radiation doses.

The lipophilic antioxidant α-tocopherol (vitamin E) was also studied. α-Tocopherol is an antioxidant whose lipophilicity renders it specific for membranes:



α-Tocopherol.

It inhibits radiation-induced peroxidation of unsaturated fatty acids more efficiently than either glutathione or cysteamine (Konings and Drijver, 1979). It is believed that it acts according to reactions 5 and 6 (Burton et al, 1980):

$$TO_2^{\bullet} + R\text{-}OH \longrightarrow TOOH + RO\cdot \qquad (5)$$

$$RO\cdot + TO_2^{\bullet} \longrightarrow \text{NON-RADICAL PRODUCTS} \qquad (6)$$

ROH = α-tocopherol.

$TO_2^{\bullet}$ = Target radical.

Thus, one molecule of α-tocopherol consumes two peroxyl radicals.

High concentrations of α-tocopherol (10-50mM) apparently reduce

radiation damage to cercariae (figure 8.20). Interestingly, unlike cysteine and GSH, culture with α-tocopherol after transformation also appears to protect against radiation-induced inhibition of protein synthesis. This may be due to the ease with which the highly hydrophobic α-tocopherol is assimilated into membrane structure. It would be worth determining whether α-tocopherol protects against parasite death more effectively than the thiol compounds. We might speculate that, if α-tocopherol has a high affinity for the nuclear membrane as well as the surface, some of the damage to normally inaccessible regions of chromatin at the nuclear membrane might be prevented or repaired.

Thus, the use of protective or sensitizing agents such as thiols and α-tocopherol allows us some insight into the mechanisms by which irradiation attenuates schistosomula and enhances their immunogenicity in the host. This approach also indicates some possible sources of variability in the parasites response to irradiation, namely different intracellular concentrations of radioprotective compounds.

In conclusion, a combination of genetic and non-genetic factors may contribute to the variable levels of metabolic inhibition suffered by schistosomula after irradiation. This variation in susceptibility seems to be limited to the initial stages after transformation, approximately 96 hours, when metabolic demands are comparatively low, and no reorganisation of DNA to activate new regions, required for growth or cell division, is necessary. Regardless of their capacity to override irradiation-induced inhibition of protein synthesis during this early stage, no schistosomula are able to achieve the activation of new genetic messages required for growth and cell division in later development. Further investigation of the causes of variability in response to irradiation, and, in particular, of their relation to the considerable variation in resistance induced by the same vaccination protocol, seems advisable.

CHAPTER NINE

IMMUNISATION OF MICE WITH IRRADIATED AND ACTINOMYCIN D-TREATED LARVAE OF SCHISTOSOMA MANSONI.

9. IMMUNISATION OF MICE WITH IRRADIATED AND ACTINOMYCIN D-TREATED LARVAE OF SCHISTOSOMA MANSONI.

Having established that Actinomycin D treatment could mimic the effects of irradiation on schistosomular protein, RNA, glycoprotein and phospholipid synthesis (chapters 3, 4) the role of such metabolic inhibition in rendering schistosomula immunogenic was tested in animal protection experiments.

9.1 Comparison of normal, U.V.- and gamma-irradiated cercariae as agents for protective immunisation of NIH/Ola mice.

Initially, the protection afforded by U.V.-irradiated cercariae was compared with the more fully researched concomitant immunity and gamma-irradiated vaccine models. NIH mice were chosen as experimental hosts, since Dean et al (1983) had previously demonstrated significant protection in this mouse strain after immunisation with U.V.-irradiated cercariae.

In our experiments, both U.V.- and gamma-irradiated cercarial vaccines induced significant immunity to subsequent cercarial challenge (groups 2, 3, 4; table 9.1). The resistance afforded by 400 μW min cm^{-2} U.V.-irradiated cercariae was significantly greater than 20 krad gamma-irradiated or 800 μW min cm^{-2} U.V.-irradiated cercariae ($P<0.01$ in each case). 400 μW min cm^{-2} was therefore the U.V. dose routinely used in metabolic and surface studies. However, none of the vaccinated groups was as resistant to challenge as those mice receiving an initial normal infection (group 1). With both doses of U.V.-irradiation, a small fraction of worms (0.08-0.2%) survived to adulthood.

Overall, these results seemed to justify study of U.V.-irradiated larvae as an attenuated vaccine offering protection equal to or exceeding the gamma-irradiated model.

Table 9.1. Comparison of normal, U.V.- and gamma-irradiated cercariae as agents for protective immunisation of NIH/Ola mice.

NIH/Ola male mice, 8 weeks old at the start of the experiment, were used.

Table 9.1.

GROUP (number of mice)	INFECTION SCHEDULE		No. OF ADULT WORMS RECOVERED (5-6 weeks POST-CHALLENGE) $\bar{X} \pm$ S.E.	No. OF SURVIVING CHALLENGE WORMS $\bar{X}_A - \bar{X}_B$	% REDUCTION	P
	INITIAL INFECTION (p.c.)	CHALLENGE (6 weeks later) (p.c.)				
1A (4)	150 NORMAL CERCARIAE	150 NORMAL CERCARIAE	10.5±4.5	2.9±4.5	95.7	<0.001
1B (5)	150 NORMAL CERCARIAE	/	7.6±2.0	/		
2A (5)	500 GAMMA-IRRADIATED (20 krad) CERCARIAE	150 NORMAL CERCARIAE	22.8±3.5	22.8±3.5	66.0	<0.001
2B (5)	500 GAMMA-IRRADIATED (20 krad) CERCARIAE	/	0	/		

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Table 9.1. continued

GROUP (number of mice)	INFECTION SCHEDULE: INITIAL INFECTION (p.c.)	INFECTION SCHEDULE: CHALLENGE (6 weeks later) (p.c.)	No. OF ADULT WORMS RECOVERED (5-6 weeks POST-CHALLENGE) $\bar{X} \pm$ S.E.	No. OF SURVIVING CHALLENGE WORMS $\bar{X}_A - \bar{X}_B$	% REDUCTION	P
3A (5)	500 (400 μW min cm^{-2}) U.V.-IRRADIATED CERCARIAE	150 NORMAL CERCARIAE	14.8±5.5	14.4±5.5	78.5	<0.001
3B (5)	500 (400 μW min cm^{-2}) U.V.-IRRADIATED CERCARIAE	/	0.4±0.4	/		
4A (5)	500 (800 μW min cm^{-2}) U.V.-IRRADIATED CERCARIAE	150 NORMAL CERCARIAE	24.2±6.2	23.2±6.2	65.4	<0.001
4B (5)	500 (800 μW min cm^{-2}) U.V.-IRRADIATED CERCARIAE	/	1±0.6	/		
5(7) challenge control	/	150 NORMAL CERCARIAE	67.1±6.7	67.1±6.7		

9.2 U.V.-irradiated cercariae do not protect BALB/c mice against challenge.

No resistance to cercarial challenge was observed when BALB/c mice were exposed to U.V.-irradiated cercariae according to the immunisation régime which stimulated such potent protection in the NIH strain. This unresponsiveness to the U.V.-irradiated vaccine occurred in 4 successive experiments; table 9.2 shows the results of two. Even when a few worms survived irradiation, as in experiment 2, they did not render the mice significantly resistant to challenge. By contrast, the usual concomitant immunity model seemed to be fully effective in BALB/c mice.

Table 9.2. Comparison of normal and U.V.-irradiated cercariae as agents for the protective immunisation of BALB/c mice.

Mice were BALB/c females.

N.S. = not significant

EXPERIMENT NUMBER	GROUP (number of mice)	INFECTION SCHEDULE		No. OF ADULT WORMS RECOVERED (5-6 weeks POST-CHALLENGE) $\bar{X} \pm$ S.E.	No. OF SURVIVING CHALLENGE WORMS $\bar{X}_A - \bar{X}_B$	% REDUCTION	P (Student's t-test)
		INITIAL INFECTION (p.c.)	CHALLENGE (p.c.) (6 weeks after initial)				
1.	1A (4)	150 normal cercariae	150 normal cercariae	94.0±12.8	-2.7±14.1	109	<0.025
	1B (4)	150 normal cercariae	/	96.7±14.1	/		
	2A (5)	500 (400 μW min cm^{-2}) U.V.-irradiated cercariae	150 normal cercariae	25.8±7.7	25.8±7.7	6.9	N.S.
	2B (4)	500 (400 μW min cm^{-2}) U.V.-irradiated cercariae	/	0	/		
	3 (10) challenge control		150 normal cercariae	27.7±6.6	27.7±6.6		

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Table 9.2.

EXPERIMENT NUMBER	GROUP (number of mice)	INFECTION SCHEDULE		No. OF ADULT WORMS RECOVERED (5-6 weeks POST-CHALLENGE) $\bar{X} \pm$ S.E.	No. OF SURVIVING CHALLENGE WORMS $\bar{X}_A - \bar{X}_B$	% REDUCTION	P (Student's t-test)
		INITIAL INFECTION (p.c.)	CHALLENGE (p.c.) (6 weeks after initial)				
2.	1A (4)	150 normal cercariae	150 normal cercariae	28.8±7.1	-2.9±7.3	105	<0.001
	1B (3)	150 normal cercariae	/	31.7±7.3	/		
	2A (5)	500 (400 µW min cm^{-2}) U.V.-irradiated cercariae.	150 normal cercariae	41.8±10.5	41.4±10.5	26	N.S.
	2B (5)	500 (400 µW min cm^{-2}) U.V.-irradiated cercariae.	/	0.4±0.4	/		
	3(10) challenge control	/	150 normal cercariae	55.9±10.2	55.9±10.2		

Table 9.2., continued

9.3 Actinomycin D-treated schistosomula protect against cercarial challenge.

Three separate experiments with NIH mice demonstrated that 500 Actinomycin D-treated schistosomula, injected subcutaneously in NIH mice, induced significant resistance to percutaneous challenge with 100 or 150 cercariae, six weeks after immunisation (tables 9.3, 9.4, 9.5). The levels of immunity achieved, 41.6-77.1%, rival those induced by irradiated cercariae, applied percutaneously (Dean, 1983). No Actinomycin D-treated schistosomula survived to maturity.

These experiments provided some other interesting information about immunity stimulated by Actinomycin D- and U.V.-attenuated vaccines. CBA mice, in contrast to NIH, did not seem to be protected by vaccination with Actinomycin D-attenuated schistosomula (table 9.4). However, only one experiment was performed in this mouse strain. Moreover, this experiment seems somewhat unsatisfactory in two respects. Firstly, a very low proportion of the challenge worm dose survived, even in control animals - only 7 to 8 from the original 100 cercariae. Secondly, in contrast to all the other experiments, no concomitant immunity was evident in either NIH or CBA mice on this occasion. Nevertheless, the conditions of the experiment were sufficient to demonstrate highly significant protection in Actinomycin D-immunised NIH mice.

The experiment of table 9.5 indicates that, whereas 20-hour old, Actinomycin D-treated schistosomula, injected subcutaneously, induced significant resistance to challenge in NIH mice, U.V.- or gamma-irradiated parasites of the same age, injected by the same route, did not stimulate any immunity in this mouse strain.

Table 9.3. Comparison of normal and Actinomycin D-treated 20-hour schistosomula as agents for protective immunisation of NIH mice.

Mice were NIH/Ola females, 8 weeks old at the start of the experiment.

PREPARATION OF IMMUNISING SCHISTOSOMULA.

Cercariae were transformed mechanically in Elac, and cultured at 37°C/5% CO_2 for 20 hours in Elac/10% hiFCS in presence or absence of Actinomycin D. They were then washed, resuspended in Elac/2% hiFCS, and injected at the appropriate concentration in a volume of 0.5 ml.

Table 9.3.

GROUP (number of mice)	INFECTION SCHEDULE		No. OF ADULT WORMS RECOVERED (5-6 weeks POST-CHALLENGE) $\bar{X} \pm$ S.E.	No. OF SURVIVING CHALLENGE WORMS $\bar{X}_A - \bar{X}_B$	% REDUCTION	P
	INITIAL INFECTION (p.c.)	CHALLENGE (p.c.) (6 weeks after initial)				
1A (10)	150 NORMAL 20h. SCHISTOSOMULA	150 NORMAL CERCARIAE	14.9±3.3	0.6±3.3	97.4	<0.005
1B (10)	150 NORMAL 20h. SCHISTOSOMULA.	/	14.3±3.0	/		
2A (10)	500 ACTINOMYCIN D-TREATED 20h. SCHISTOSOMULA	150 NORMAL CERCARIAE	8.6±1.9	8.6±1.9	62.1	<0.05
2B (10)	500 ACTINOMYCIN D-TREATED 20h. SCHISTOSOMULA	/	0	/		
3(10) CHALLENGE CONTROL	/	150 NORMAL CERCARIAE	22.7±5.4	22.7±5.4		

Table 9.4. Comparison of normal and Actinomycin D-treated 20-hour schistosomula as agents for protective immunisation of NIH/Ola and CBA/Ca/Ola mice.

Mice were females, 8 weeks old at the start of the experiment.

Immunising schistosomula were prepared as described for table 9.3.

N.S. = Not significant.

Table 9.4.

MOUSE STRAIN	GROUP (number of mice)	INFECTION SCHEDULE		No. OF ADULT WORMS RECOVERED (5-6 weeks POST-CHALLENGE) $\bar{X} \pm$ S.E.	No. OF SURVIVING CHALLENGE WORMS $\bar{X}_A - \bar{X}_B$	% REDUCTION	P
		INITIAL INFECTION (s.c.)	CHALLENGE (6 weeks later) (p.c.)				
NIH/Ola	1A (10)	150 NORMAL 20h. SCHISTOSOMULA	100 NORMAL CERCARIAE	22.6±3.8	6.7±3.8	4.3	N.S.
	1B (10)	150 NORMAL 20h. SCHISTOSOMULA	/	15.3±2.7	/		
	2A (10)	500 ACTINOMYCIN D-TREATED 20h. SCHISTOSOMULA	100 NORMAL CERCARIAE	1.6±0.7	1.6±0.7	77.1	<0.01
	2B (10)	500 ACTINOMYCIN D-TREATED 20h. SCHISTOSOMULA	/	0	/		
	3(20) challenge control	/	100 NORMAL CERCARIAE	7.0±1.6	7.0±1.6		

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Table 9.4. continued.

MOUSE STRAIN	GROUP (number of mice)	INFECTION SCHEDULE		No. OF ADULT WORMS RECOVERED (5-6 weeks POST-CHALLENGE) $\bar{X} \pm$ S.E.	No. OF SURVIVING CHALLENGE WORMS $\bar{X}_A - \bar{X}_B$	% REDUCTION	P
		INITIAL INFECTION (s.c.)	CHALLENGE (6 weeks later) (p.c.)				
CBA/Ca/Ola	1A (10)	150 NORMAL 20h. SCHISTOSOMULA	100 NORMAL CERCARIAE	28.6±5.3	7.5±5.3	3.8	N.S.
	1B (10)	150 NORMAL 20h. SCHISTOSOMULA	/	21.1±3.7	/		
	2A (10)	500 ACTINOMYCIN D-TREATED 20h. SCHISTOSOMULA	100 NORMAL CERCARIAE	9.1±2.0	9.1±2.0	-16.7	
	2B (10)	500 ACTINOMYCIN D-TREATED 20h. SCHISTOSOMULA	/	0	/		
	3(20) challenge control	/	100 NORMAL CERCARIAE	7.8±1.9	7.8±1.9		

TABLE 9.5. Comparison of normal, gamma-irradiated, U.V.-irradiated and Actinomycin D-treated 20-hour schistosomula, injected subcutaneously, as agents for protective immunisation of NIH/Ola mice.

Mice were females, 8 weeks old at the start of the experiment.

Immunising schistosomula were prepared as described for table 9.3.

N.S. = not significant.

Table 9.5.

GROUP (number of mice)	INFECTION SCHEDULE		No. OF ADULT WORMS RECOVERED (5-6 weeks POST-CHALLENGE) $\bar{X} \pm$ S.E.	No. OF SURVIVING CHALLENGE WORMS $\bar{X}_A - \bar{X}_B$	% REDUCTION	P
	INITIAL INFECTION (s.c.)	CHALLENGE (6 weeks later) (p.c.)				
1A (10)	150 NORMAL 20h. SCHISTOSOMULA	150 NORMAL CERCARIAE	58.6±11.7	49.7±11.7	31.8	<0.05
1B (7)	150 NORMAL 20h. SCHISTOSOMULA	/	8.9±1.9	/		
2A (10)	500 20 krad GAMMA-IRRADIATED 20h. SCHISTOSOMULA	150 NORMAL CERCARIAE	58.5±6.4	58.5±6.4	19.8	N.S.
2B (10)	500 20 krad GAMMA-IRRADIATED 20h. SCHISTOSOMULA	/	0	/		

Continued on next page.

Table 9.5 continued.

GROUP (number of mice)	INFECTION SCHEDULE		No. OF ADULT WORMS RECOVERED (5-6 weeks POST-CHALLENGE) $\bar{X} \pm$ S.E.	No. OF SURVIVING CHALLENGE WORMS $\bar{X}_A - \bar{X}_B$	% REDUCTION	P
	INITIAL INFECTION (s.c.)	CHALLENGE (6 weeks later) (p.c.)				
3A (10)	500 (400 μW min cm^{-2}) U.V.-IRRADIATED 20h. SCHISTOSOMULA	150 NORMAL CERCARIAE	86.1±6.7	86.1±6.7	-18.1	N.S.
3B (10)	500 (400 μW min cm^{-2}) U.V.-IRRADIATED 20h. SCHISTOSOMULA	/	0	/		
4A (9)	500 ACTINOMYCIN D-TREATED 20h. SCHISTOSOMULA	150 NORMAL CERCARIAE	42.6±7.8	42.6±7.8	41.6	<0.01
4B (10)	500 ACTINOMYCIN D-TREATED 20h. SCHISTOSOMULA	/	0	/		
5(20) CHALLENGE CONTROL	/	150 NORMAL CERCARIAE	72.9±7.1	72.9±7.1		

9.4 DISCUSSION

9.4.1. Protection induced by U.V.-irradiated and Actinomycin D-treated larvae. Comparison with reported levels for irradiated vaccines.

In the NIH mouse experiment of table 9.1, cercariae U.V.-irradiated at 400 μW min cm^{-2} induced significantly better protection against challenge than 20 krad gamma-irradiated cercariae: 78.5% compared to 66.0% $P < 0.01$. Cercariae U.V.-irradiated at 800 μW min cm^{-2} induced about the same degree of protection as gamma-irradiated ones (65.4%). These levels of resistance, and those obtained with Actinomycin D-treated schistosomula, 41.6-77.1%, are comparable with those reported in the literature for gamma- and X-irradiated cercariae, where protection varies from 30 to 90% in individual experiments. (Dean, 1983; section 1.9.2.3.) Note 9.1 compares the optimal attenuating doses of ultraviolet and gamma radiation.

Since this project is concerned mainly with the U.V.-irradiation model, which is less fully researched than the gamma-irradiated vaccine, it is worth comparing in some detail the results of our protection experiments with published data. Dean et al (1983) exposed mice percutaneously to approximately 500 U.V.-irradiated cercariae, and challenged four to five weeks later with 150-200 normal cercariae. In two successive experiments, the irradiation doses inducing optimal resistance were 330 μW min cm^{-2} and 440 μW min cm^{-2}, respectively. The levels of protection obtained were 68.8% and 52.2%. Slightly greater protection was observed in our experiment with a comparable radiation dose of 400 μW min cm^{-2}. Dean et al (1983) found that an attenuating dose of 880 μW min cm^{-2} induced significantly less protection - 14.9% and 26.6% in the two experiments. We also observed a decrease in protection, though much less striking, at the higher

Note 9.1. Comparison of optimal attenuating doses of ultraviolet and gamma radiation for cercariae of S. mansoni.

Optimal attenuating dose of gamma radiation is usually 20 krad.
1rad. is defined as 10^{-5} Joules of absorbed energy per gram of absorbing material.
Hence, 20 krad = 0.2 Joules of absorbed energy per gram of parasite tissue.

Optimal attenuating dose of U.V. radiation is 400 μW min cm^{-2}.
1kWh is equal to 3.6×10^{6} Joules.
Hence, 400 μW min cm^{-2} = 24×10^{-3} Joules applied per cm^2.

Half-value thickness of tissue for 254 nm. ultraviolet radiation is approximately 15 μm (Figure for penetration of human skin:. Environmental Health Criteria 14, 1979)
Hence, 12×10^{-3} Joules of ultraviolet energy, applied over an area of $1cm^2$, are dissipated in a depth of 15 μm.
ie., 12×10^{-3} Joules are dissipated in a volume of $15 \times 10^{-3} cm^3$.
Assuming density of parasite tissue = 1g cm^{-3},

then $12 \times 10^{-3}/15 \times 10^{-3}$ Joules = 0.8 Joules of ultraviolet energy are absorbed per gram of parasite tissue.
Thus, it seems that 4 times as much ultraviolet radiation energy as gamma radiation energy is required to achieve the same attenuating effect in cercariae of S. mansoni.

irradiation dose of 800 μW min cm^{-2}.

Our results differ most markedly from those of Dean's group in that, with both doses of irradiation, a small percentage of worms survived to adulthood. It cannot be excluded that these surviving worms might produce eggs, and make some contribution to the protection observed. However, the small number of worms make it unlikely that this is a major effect. Moreover, when similar numbers of parasites survived irradiation in BALB/c mice, as in experiment 2 of table 9.2, they did not render the mice significantly resistant to challenge. We might argue that the similar small number of surviving adults in NIH mice would be insufficient to stimulate the high degree of immunity observed. Since we used U.V. doses and irradiation techniques very similar to those of Dean et al (1983), it seems possible that our S. mansoni strain may be the more resistant to U.V.-irradiation. By contrast, on no occasion did any worms survive gamma-irradiation or Actinomycin D- treatment.

The studies of Cohen and Eveland (1988) on immunisation with U.V.-irradiated cercariae are perhaps most noteworthy for the variability in levels of protection achieved in different experiments. The workers immunized CD-1 mice by percutaneous tail exposure with both clones and mixed pools of cercariae, U.V.-irradiated at 300 μW min cm^{-2}. When immunisation was with clones, levels of protection were highly variable, ranging from minus 17.0% (no protection) to plus 55.6% ($P<0.001$). Considerable variability was also observed when immunisation was with a mixed population. Two groups of CD-1 mice immunised with the same mixed pool of U.V.-irradiated cercariae displayed strikingly different levels of resistance to separate batches of challenge cercariae. Mice challenged with one pool of cercariae showed no significant resistance, whereas other mice, immunised with the same larvae, but challenged with a second pool of cercariae, showed high levels of protection (67.7%, $P<0.001$).

Two other, less extensive, published experiments describe attempts to protect mice by prior exposure to U.V.-irradiated cercariae of S. mansoni. In these experiments, mice were exposed percutaneously to 500-600 U.V.-irradiated cercariae, and challenged four weeks later with unirradiated cercariae. In the first experiment, when immunising cercariae were irradiated at 100 ergs/sec for three minutes (= 3 μW mins), approximately 25 out of 500 parasites survived to adulthood. Challenge adult worm recoveries were reduced by 66% compared to unimmunised controls (Murrell et al, 1975). In the second experiment, (Ghandour and Majid, 1978) 28% resistance to challenge was induced, but the dose of irradiation was not reported, and no data was provided for survival of immunising, irradiated worms. Thus, neither of these studies exclude the possibility of a significant contribution to resistance by egg-laying adult worms.

U.V.-irradiated cercariae of S. japonicum have also been used in mouse protection experiments. Moloney et al (1985) employed a much higher irradiation dose than for S. mansoni - 40 seconds at an intensity of 42.2 ± 1mW cm^{-2} = 28.1mW min cm^{-2} - to prevent parasite maturation and induce optimal resistance. Percutaneous exposure to 300-800 irradiated cercariae induced 31-57% resistance to percutaneous cercarial challenge four weeks later. Cercariae irradiated for just 10 seconds longer (35.2mW min cm^{-2}) induced much poorer resistance.

Overall, then, the levels of protection observed in our experiments with U.V.-irradiated cercariae compare favourably with those reported in the literature.

9.4.2. Model for induction of immunity by irradiated larvae of S. mansoni.

Our protection experiments yielded three unexpected results.

Firstly, U.V.-irradiated cercariae induced immunity to challenge in NIH mice, but not in the BALB/c strain. Secondly, 20-hour old, Actinomycin D-treated schistosomula, injected subcutaneously, rendered NIH, but not CBA, mice resistant to cercarial challenge. Finally, in contrast to Actinomycin D-treated parasites of the same age, 20-hour old U.V.- or gamma-irradiated schistosomula, injected subcutaneously, did not protect NIH mice against infection. Examination of the literature suggests a possible model for induction of immunity by irradiated larvae, which might help to reconcile these apparently conflicting results.

9.4.2.1. Two stages in induction of immunity by irradiated cercariae.

A number of lines of evidence suggest that irradiated cercariae of S. mansoni could be considered to induce immunity in two distinct stages. We would suggest that antigens released by irradiated larvae as they migrate through, or die in, the skin, have the potential to induce skin-specific immune mechanisms, which subsequently act against challenge parasites in cutaneous tissues. When the attenuated larvae reach the lungs, where the majority die, the antigens released may activate a second class of immune mechanisms. These mechanisms, in turn, are predicted to operate against challenge schistosomula migrating through the lungs.

Autoradiographic tracking studies with ^{75}Se-labelled, irradiated cercariae have shown that, in C57BL mice at least, gamma-irradiated parasites, despite being retained in the skin for slightly longer than normal ones, almost all go on to reach the lungs. The majority of gamma-irradiated schistosomula die in the lungs; lower proportions en route from lungs to liver (Dean et al, 1981c; Mastin et al, 1983; Mangold and Dean, 1984; Mastin et al, 1985b; Wilson and Coulson, 1986). However, even highly-irradiated cercariae (90 krad) that did not

migrate out of the skin induced some resistance to challenge - approximately half that afforded by parasites irradiated at the optimal 20 krad dose which allowed survival to the lung stage (Mangold and Dean, 1984). This observation is consistent with induction of a substantial share of the total potential immunity by irradiated cercariae within the skin, though the lower level of protection might also be attributable to the direct effects of such high radiation doses on parasite immunogenicity.

Comparison of the levels of immunity induced by irradiated cercariae, as opposed to schistosomula, and by immunisation routes that include or bypass the skin, support the idea that the irradiated vaccine may induce immunity in two stages - firstly, by cercariae in cutaneous tissue; secondly, by dying schistosomula in the lungs. When the vaccination protocol interferes with either of these stages, resistance is generally poorer than when the attenuated larvae are allowed to accomplish both phases of migration. Bickle et al (1979) compared the resistance induced by 20 krad gamma-irradiated, 1 hour-old schistosomula administered to mice by intramuscular, intradermal and intravenous routes. Immunisation by all three routes induced levels of resistance of 20-40%, compared with 60-80% after percutaneous exposure to irradiated cercariae. Sher and Benno (1982) found that 30 krad gamma-irradiated cercariae, applied percutaneously, induced on average 63.0% resistance to challenge. In contrast, irradiated day 0 skin penetrants and irradiated 4-5 day-old lung schistosomula, injected intramuscularly, induced only 42.8% and 24.8% immunity, respectively. Dean et al (1981c) found that lung-stage schistosomula exposed to 50 krad induced significant resistance to challenge after i.v., i.m. or i.p. injection. In one experiment, irradiated cercariae by p.c. infection induced significantly greater resistance than schistosomula administered by the other 3 routes, but in a second experiment, both larval stages induced similar levels of immunity. These observations of

Dean et al (1981c) might indicate that the contributions of the two stages of immunity to overall resistance could vary from batch to batch of immunising cercariae. Dean et al (1981c) also investigated the immunising potential of irradiated day 21 and day 28 liver-stage worms. Levels of resistance were very low - 22.7% on average.

Interestingly, subcutaneous injection seems to be the one route which completely disallows induction of immunity by either irradiated cercariae or schistosomula. Bickle et al (1979), using 1-hour old irradiated schistosomula, Savage and Colley (1980), using irradiated cercariae, and Eveland and Morse (1978), using schistosomula irradiated at low doses, 3-6 krad, were all unable to stimulate resistance by the s.c. route. These observations support our own experiments (table 9.5) where U.V.- and gamma-irradiated, 20-hour schistosomula failed to induce immunity by subcutaneous injection.

We could speculate that other vaccination methods showing a preference for inoculation via cutaneous or non-cutaneous tissue might function by the mechanisms characteristic of the first (skin-associated) or second (lung-associated) stages in induction of immunity by irradiated cercariae. Thus, the non-living vaccine of James (1985) and Sher et al (1986), consisting of frozen-thawed schistosomula, or a paramyosin-containing soluble adult worm fraction in combination with BCG, only protects against challenge when injected intradermally. I.v. injection does not induce immunity. Resistance induced by this vaccination régime is generally less than or equal to 50%. The requirement for intradermal administration, and this comparatively low level of protection, suggest that this vaccine may induce only the first, skin-associated stage of immunity as defined above for the irradiated vaccine model. In contrast, the vaccine consisting of irradiated, cryopreserved schistosomula (James, 1981; Stirewalt et al, 1984), is most effective by intramuscular injection. Levels of resistance are again relatively low, 20-50%. We might suggest,

therefore, that this vaccine functions in the same way as the second, post-skin stage of immunity stimulated by irradiated (unfrozen) cercariae.

Evidence to support the concept of induction of immunity in two stages by irradiated cercariae has also come from experiments investigating the relationship between survival of irradiated S. mansoni larvae and the degree of resistance stimulated. Immunising parasites are treated with schistosomulicidal drugs, or the site of infection excised, at intervals post-vaccination. Immunity to challenge infection is then assessed. Bickle (1982) found that elimination of irradiated parasites at one week after vaccination, just as arrival in the lungs began, allowed development of 26-37% resistance against challenge - about half that observed in vaccinated, untreated hosts (51-71%). Curtailing the lifespan of the vaccinating cercariae in this way may permit induction of only the first, skin-associated stage of immunity. The second stage, induced by irradiated parasites migrating through the lungs and vascular system, is prevented.

9.4.2.2. Two stages in challenge attrition by hosts immunised with the irradiated vaccine.

The two phases postulated for induction of immunity by irradiated cercariae seem to be reflected in elimination of challenge parasites at two stages - firstly, in the skin; secondly, in the lung or during migration from lung to liver.

Miller and Smithers (1980), using tissue mincing and incubation techniques to determine the recovery of 2-day old skin schistosomula, 5-day old lung worms and 21-day old liver worms from challenged, vaccinated mice, concluded that the majority of challenge parasites are killed before day 5, ie. pre-lung stage, only a small proportion (17%) of the total attrition occurring in the lungs or later. With the same

technique, Hsü et al (1979) obtained similar results in mice vaccinated with cercariae attenuated by exposure to X- rather than gamma-radiation. Ghandour and Majid (1978) also found a significant reduction in recovery of lung schistosomula at day 6 after challenge of U.V.-irradiated cercariae-vaccinated mice, and concluded that the major phase of attrition occurred in the skin. Histopathological studies similarly led Hsü et al (1983) to conclude that the skin is an important site of challenge attrition in X-irradiated cercariae-immunised mice.

In contrast, Minard et al (1978b) and Stek et al (1980) concluded from tissue recovery techniques that, at most, half the challenge attrition in mice immunised with gamma-irradiated cercariae occurred in the skin. They favoured the lung as the major site for elimination of challenge parasites. Mastin et al (1983) claimed from serial sectioning techniques that, since virtually all challenge schistosomula reached the lungs of gamma-irradiated cercariae-immunised mice, the skin could not be an important site of attrition in this host. As McLaren et al (1985) pointed out, however, the absence of dead parasites in cutaneous tissues does not necessarily mean that the parasites are killed solely at post-lung sites. The challenge schistosomula may be lethally damaged in the skin, but still be able to migrate to post-skin sites before dying.

The most striking evidence for 2 distinct phases of challenge attrition, in the skin and lungs respectively, comes from the autoradiographic tracking technique, which allows the fate of challenge schistosomula to be conclusively determined. Different investigators have demonstrated challenge attrition at one or the other stage.

In CBA/Ca mice, the major phase of immune-dependent challenge elimination seems to occur in the cutaneous tissues. Autoradiographic tracking of radiolabelled challenge cercariae (Kamiya et al, 1987) indicated that 58.5% of challenge larvae failed to reach the lungs of

vaccinated CBA/Ca mice. Lung phase challenge attrition was calculated as 25%. Moreover, vaccine-induced immunity in this mouse strain could be abrogated by some 67% following administration of a monoclonal antibody that ablates cutaneous effector cells, provided the monoclonal was injected on or close to the day of challenge (McLaren et al, 1987).

Conflicting results were obtained by groups studying irradiated vaccine immunity in C57BL mice. Dean et al (1984) and Wilson et al (1986), using the autoradiographic tracking technique, recorded only 27% and 10% parasite loss in the skin of this mouse strain. According to these authors, the majority of challenge attrition in C57BL mice occurs in the lungs. However, it was noted that, despite going on to reach the lungs, challenge parasites were delayed some 2-3 days longer in the skin of vaccinated as compared to naive mice. No such retardation was recorded by Kamiya et al (1987) for the minority of parasites that did go on to reach the lungs of CBA mice in their studies. Kamiya et al (1987) therefore suggested that similar anamnestic inflammatory reactions may be induced by irradiated larvae in the skin of vaccinated CBA and C57BL mice; they are, however, less efficient in parasite killing in the latter.

Thus, it seems that challenge parasites may be eliminated by immune mechanisms operating against either skin or lung stages. In CBA mice, mechanisms antagonistic to skin schistosomula are more effective; in C57BL mice, those operating against lung forms predominate. However, the routes of challenge infection used by the different groups could contribute, in part, to this anomaly in site and mechanism of challenge attrition. The CBA/Ca mice were immunised on the ear, and challenged on the abdomen, whereas the C57BL ones were challenged via the tail. Rodent tail skin is deficient in Langerhans cells (Bergstresser et al, 1980), whose antigen presenting properties may be essential to stimulate a skin-associated response against challenge parasites. Thus,

challenge larvae administered via the tail may not stimulate skin-associated immune effector mechanisms very effectively.

Stage-dependent elimination of a challenge infection has also been investigated using challenge régimes that permit sequential elimination of successive sites and stages in schistosomular development. Vaccinated rats eliminated both percutaneously applied cercariae, and lung stage parasites introduced directly into the lungs, bypassing the skin (McLaren et al, 1985). Vaccinated guinea-pigs were able to eliminate challenge schistosomula, not only in the skin and lungs, but also when they were introduced into the liver circulatory system. Consistently with the autoradiographic tracking data, vaccinated CBA mice were only significantly resistant to a percutaneously applied cercarial challenge, showing only 15% immunity against lung worms. Similarly, Miller et al (1981) reported that mice vaccinated with irradiated cercariae were immune to cercarial challenge (43-54%) but not significantly resistant to a day-5 lung worm challenge injected into the tail vein (15-26%). In contrast, Dean et al (1981c) found, in a similar system, that vaccinated mice were 45-60% resistant to a percutaneously applied cercarial challenge, and were also significantly, though slightly less, resistant (32-44%) to challenge infection by 6- or 12-day old lung worms. Skin-associated immune effector mechanisms may be the most important in the studies of McLaren et al (1985) and Miller et al (1981), while lung-associated mechanisms predominate in the experiments of Dean et al (1981c). Again, it is worth noting that, in those experiments showing challenge attrition in the skin, initial infection was on the ear pinna, and cercarial challenge via the abdomen, whereas in the study by Dean et al (1981c), showing significant resistance operating against lung-stage schistosomula, both initial immunisation and cercarial challenge were by tail immersion. As suggested above, the deficiency of Langerhans cells in rodent tail skin may prevent stimulation of

skin-associated immunity so that lung-specific attrition becomes of first importance.

The non-living, BCG - associated vaccine developed by James (1985) may operate by the first, skin-specific stage of immunity defined by the irradiated vaccine model. Resistance seems to be induced, and challenge larvae eliminated, in the skin (James, 1987). Moreover, mice immunised with this vaccine were immune to cercarial challenge on the abdomen, back or footpad, but unable to resist cercarial challenge by tail exposure. As explained above, immunisation or challenge infection via the tail (Langerhans cell-deficient) seems to forestall induction or expression of skin-associated immunity.

In summary, it seems that two distinct immune mechanisms may be induced by the irradiated vaccine. One operates in the skin; the second in the lung. Some insight into the nature of these mechanisms has been obtained by characterising the immune responses to the irradiated vaccine by various mouse strains.

9.4.2.3. Response to the irradiated vaccine by different mouse strains.

A number of published reports agree that NIH and C57BL mice consistently develop high levels of immunity in response to gamma-irradiated cercariae, while CBA strains are comparatively low responders. (Murrell *et al,* 1979c; Lewis and Wilson, 1982; James *et al,* 1981). P-strain and A/J mice either show no vaccine immunity or else only marginal resistance (Murrell *et al,* 1979c; James and Sher, 1983). Sher *et al* (1982) showed that the MHC complex exerted some control over induction of vaccine immunity. Inbred mice bearing either *b* or *d* MHC haplotypes developed higher levels of vaccine-based resistance than mice with other MHC haplotypes. Correa-Oliveira *et al* (1986) subsequently demonstrated that the defective immunity which characterises P-strain mice is inherited in a fully recessive manner,

controlled by a single locus, Rsm-1, not associated with the MHC.

The similarity between the first stage in induction of immunity by irradiated cercariae, and the non-living, BCG-associated vaccine of James (1985) may be extended to the response stimulated in different mouse strains. C57BL and CBA mice both develop the partial (30-50%) resistance typical of this model. As demonstrated for the gamma-irradiated vaccine, P mice do not develop immunity. Nor do BALB/c mice, although this mouse strain does show protection in the gamma-irradiated model. We could suggest that BALB/c mice do not express skin-associated immune responses, but that lung stage resistance still operates in this strain.

9.4.2.4. Immune mechanisms in the two stages of attrition.

Table 9.6 summarises which of the two phases of immunity postulated for the irradiated vaccine model - skin or lung - may be induced by different immunisation and challenge infection routes, and may be associated with different mouse strains.

Our model postulates that metabolic inhibition is partially responsible for expression of antigens in modified, highly immunogenic conformations by irradiated larvae. During the initial few days after transformation, when the schistosomula are in the skin, metabolic inhibition is, as a rule, more severe and long-lasting for U.V.-irradiated than gamma-irradiated larvae (chapters 3 to 5). Thus, it would seem reasonable to predict that the early, skin-associated stage of immunity may be more significant in the action of the U.V.-irradiated than the gamma-irradiated vaccine. The second, lung-associated stage of immunity is likely to be induced by both U.V.-irradiated and gamma- irradiated vaccines, as the dying or dead larvae are carried to the lungs. However, the fact that BALB/c mice, which apparently express only lung-stage immunity, display resistance

Table 9.6.

1. Immunisation or challenge infection route.	Associated stage of immunity.
i.v.	L
i.m.	L
tail	L
p.c.	S
i.d.	S
(non-living vaccine; James, 1985)	
2. Mouse strain.	
CBA	S
C57BL	S + L
NIH	S + L
BALB/c	L
P	neither

Stages of immunity in the irradiated vaccine model stimulated by various immunisation or challenge infection routes, and associated with different mouse strains.

S = induction of immunity, or challenge attrition, occurs in the skin.

L = induction of immunity, or challenge attrition, occurs chiefly in the lung.

to challenge in response to immunisation with gamma-irradiated, but not U.V.-irradiated larvae, suggests that the U.V.-attenuated forms might be less effective in inducing this second stage of immunity. Alternatively, U.V.-irradiated, but not gamma-irradiated, schistosomula, might activate some immune suppressor mechanism in BALB/c mice. Cohen and Eveland (1988) did induce immunity in CD-1 mice with U.V.-irradiated cercariae administered percutaneously via the tail. Since this immunisation route is believed largely to bypass the skin stage of induction of immunity, due to a deficiency of Langerhans cells in rodent tail skin, it does seem that U.V.-irradiated larvae can stimulate the second, lung-associated phase of immunity. However, as explained in section 9.4.1., levels of protection in this study were extremely variable, ranging from minus 17.0% to plus 67.6%. This extensive variability suggests that the U.V.-irradiated vaccine may not induce immunity in the lung.

We would postulate that attenuated larvae in the skin may stimulate potent protective immunity by means of antigens in altered conformations, released by secretion, to be processed in a novel way by antigen presenting cells. Comparatively few schistosomula die in the skin, at least in the gamma-irradiated model. On reaching the lungs, however, the irradiated larvae die and undergo autophagocytosis (Mastin et al, 1983, 1985b). At this stage, the modified antigens which have accumulated in the body and at the surface of the parasite may be made available for interaction with the host immune system (see chapter 10).

As described above, experiments involving different routes of immunisation with irradiated larvae have provided evidence for the hypothesis that two distinct stages may operate in irradiated vaccine immunity. Greene and Benacerraf (1980) outlined how antigens administered by different routes may stimulate the immune system in different ways. They used the model originally proposed by Medawar (1958) which defines two separate components of the immune system -

central and peripheral. The central immune system comprises the bone marrow, thymus and white pulp of the spleen; the peripheral system is composed of the lymph nodes around the respiratory or enteric organs. Antigens administered via the skin are delivered to the draining lymph nodes, thus stimulating the peripheral immune system, while antigens injected intravenously accumulate in the spleen, activating the central compartment of the immune system. The central immune system appears to be primarily concerned with regulation of the immune response. Experimentally, administration of large amounts of antigen directly into the central compartment by the i.v. route may lead to a preponderance of regulatory and suppressor cells. The peripheral mode of antigen presentation, on the other hand, generally stimulates potent cellular immunity. As regards schistosome immunity, invading cercariae traverse the skin, then enter the blood vascular system. Hence, they can interact with both components of the immune system at different stages in their development.

Greene and Benacerraf (1980) proposed that the basis for differences in response seen when preferentially activating the central or peripheral compartments reflected the nature and function of antigen presenting cells in each of these areas. The skin seems to possess its own characteristic antigen presenting cells; possibly even a complete self-sufficient immune system.

Streilen (1983) first proposed the concept of "skin-associated lymphoid tissue" (SALT). Bos and Kapsenberg (1986) subsequently modified and extended this model in their description of the "skin immune system" (SIS). Antigens appear to be accepted, processed and presented chiefly by antigen presenting cells unique to the skin - Langerhans cells - though other dendritic cells, and macrophages, may also be involved. Using the hapten dinitro-chlorobenzene, Macher and Chase (1969) demonstrated that, for effective induction of immunity, the site of antigen application on the skin had

to remain intact for at least 24 hours after initial contact. Presumably, this time was required for Langerhans cells trafficking through the skin to recognise the antigen. This observation may also help explain the inability of Bickle (1982) and Bickle and Andrews (1985) to demonstrate vaccine immunity when irradiated cercariae were drug-killed, or the immunisation site excised, at 24 hours after initial exposure. Langerhans cells expressing antigen in immunogenic form flow to the draining lymph nodes, where they activate paracortical T-cells to proliferate and differentiate. Most develop into memory cells; some into effector cells. Although these activated lymphocytes do disseminate systemically, a high proportion display special affinity for the epidermis, where the effector cells may directly attack the antigenic foreign bodies. On secondary challenge, memory cells, either already present in the skin or rapidly recruited to it, are activated. Keratinocytes secrete interleukin-1, stimulating these lymphocytes to proliferate and differentiate, and attracting effector cells in defence against the foreign organisms.

Clearly, this model could readily be applied to induction and expression of skin-stage immunity in the irradiated vaccine for schistosomiasis. It has already been suggested (section 9.4.2.2.) that the scarcity of Langerhans cells in rodent tail skin may adversely affect activation of skin immune mechanisms when immunisation or challenge is by this route.

Under certain conditions, it is also possible for cutaneously applied antigen to induce a suppressive immune response. It has been suggested that a second class of skin-specific antigen presenting cells, Granstein cells, may act in opposition to Langerhans cells, interacting with suppressor rather than helper T-lymphocytes. Thus, an antigen processed and presented preferentially by this class of antigen presenting cells might tend to induce antigen-specific immuno-suppression.

Specific unresponsiveness to a particular antigen also occurs in a more indirect way for certain antigens which are only immunogenic when presented by Langerhans cells in the epidermis. When Langerhans cells are depleted from skin, by UVB irradiation or by repeated stripping with cellophane tape, cutaneously applied antigen not only fails to sensitize, but evokes profound and specific unresponsiveness. This route to induction of tolerance was initially demonstrated in a contact hypersensitivity model: Streilen et al (1980) found that, when mice were painted with the hapten dinitrofluorobenzene on a section of skin artificially depleted in Langerhans cells, contact hypersensitivity was not induced. Moreover, the animals were unable to mount effective hypersensitivity reactions to this hapten when the Langerhans cell population was restored, and the mice presented with a normal immunogenic regimen. Interestingly, hapten-specific immunity in BALB/c mice was not suppressible by depletion of Langerhans cells in this way. In accordance with our own conclusions, drawn from available data on immunity induced by irradiated cercariae, this observation suggests that normal skin-associated immune mechanisms do not function in BALB/c mice.

Thus, the skin immune system may be quite self-contained (see Streilen, 1983), so that presentation of antigens released by attenuated schistosomula, stimulation of immunological memory upon challenge, and subsequent activation of inflammatory and effector cells, all take place in the skin. However, the antigens derived from parasite death and disintegration in the lung presumably activate the central immune system, being conveyed by antigen presenting cells to the spleen, where they induce B- and T-cell proliferation and systemic distribution of memory cells. When a secondary response is activated by challenge schistosomula migrating through the lungs, inflammatory and effector cells will be attracted to the site of challenge. Attrition against challenge larvae apparently occurs by a combination of: (1)

inflammatory responses which impede schistosomular migration, and (2) direct effector cell damage to the parasites (section 1.9.2.3.7; figure 1.5).

Actinomycin D-treated schistosomula, being injected subcutaneously, have some opportunity to stimulate the skin-associated, peripheral immune system. However, Langerhans cells in the subcutis are very rare (Bos and Kapsenberg, 1986), hence this vaccine is unlikely to stimulate the same immune mechanisms as irradiated cercariae traversing the Langerhans cell-rich epidermis. Actinomycin D-treated schistosomula apparently do not protect against challenge in CBA mice (table 9.4), hosts in which the skin-specific phase of irradiation vaccine immunity seems to predominate. This observation supports the idea that Langerhans cells and their associated immune network may play a crucial role in this first, skin-specific stage of immunity induced by irradiated cercariae. Assuming that the Actinomycin D-attenuated schistosomula, and antigens derived from their secretion or disintegration, penetrate into the vascular system and reach the lungs, we would predict that these drug-attenuated larvae activate predominantly the second, lung-associated stage of immunity, involving the central immune system.

In contrast to Actinomycin D-attenuated larvae, gamma and U.V.-irradiated, 20-hour schistosomula, injected subcutaneously, do not appear to induce immunity to cercarial challenge (table 9.5; section 9.4.2.1.). One interpretation of these results could be that the irradiated forms expose some immunosuppressive antigen which Actinomycin D-treated schistosomula have either lost, or express in a non-suppressive conformation. The most obvious candidate for such an antigen is some element of the cercarial glycocalyx. Since metabolism in Actinomycin D-treated schistosomula continues almost as normal for some 15 to 20 hours after exposure to the drug, these parasites have as much opportunity as unattenuated ones to cast off, degrade, or alter

the conformation of glycocalyx antigens. However, these manoeuvres, which require metabolic activity, will be severely restricted in irradiated schistosomula, whose metabolism is usually inhibited for at least 24 hours after treatment. The potential of the glycocalyx for suppression of lymphocyte proliferation has been demonstrated *in vitro* (Vieira *et al*, 1986). Alternatively, rather than direct immunosuppression, glycocalyx antigens may require processing by Langerhans cells in order to be rendered immunogenic. As demonstrated by Streilen *et al* (1980) in the contact hypersensitivity model described above, if such antigens encounter the immune system otherwise than via Langerhans cells, specific unresponsiveness results. Thus, according to this model, mice exposed to glycocalyx antigens on s.c.-injected, irradiated parasites, that do not traverse the epidermis, would be as susceptible to challenge infection as naive hosts. As mentioned in section 9.4.2.1, other authors have similarly observed that irradiated cercariae, or schistosomula just after mechanical transformation, do not induce immunity by subcutaneous inoculation.

While it does seem that both induction of immunity by irradiatd cercariae, and challenge attrition in vaccinated hosts, can be dissected into two distinct phases, skin- and lung-associated, the variable nature of both immunising and challenge pools of cercariae may mean that the contribution of each stage to overall immunity will vary considerably in different studies. Thus, according to the response of immunising cercariae to irradiation, they may be better able to stimulate one or other stage of immunity. The susceptibility of challenge larvae to each phase of immunity is also likely to vary from experiment to experiment. Thus, for instance, little protection would be evident in hosts immunised with irradiated cercariae which preferentially activate lung- stage immunity, but challenged with larvae which are more susceptible to skin-specific immune mechanisms.

Such an explanation could help to account for the fact that both skin- and lung-stage challenge attrition have been demonstrated to predominate in separate immunisation experiments performed under identical conditions (sections 9.4.2.1., 9.4.2.2.), and for observations such as that by Cohen and Eveland (1988) that a single pool of immunising, U.V.-irradiated cercariae may induce good immunity against one batch of challenge cercariae, but no resistance at all against a second batch.

In summary, it is proposed that induction and expression of irradiated vaccine immunity may occur in two distinct phases, one operating in the skin, the other principally in the lung. Induction of immunity at both stages is postulated to depend on expression of antigens in modified conformations, such that they are presented to the host immune system in a novel way, to stimulate a potent protective response.

CHAPTER TEN.

GENERAL DISCUSSION: IRRADIATED VACCINE IMMUNITY.

10. General Discussion: Irradiated vaccine immunity.

Our studies on metabolism and antigen expression by irradiated *S. mansoni* larvae suggest a possible model for induction of high levels of protective immunity against cercarial challenge. Parasite antigens are apparently presented in highly immunogenic forms at a number of clearly identifiable stages in the abbreviated lifespan of the attenuated larvae. These stages will be described in the following overview. The presentation of parasite antigens in such a way as to stimulate a highly protective host response will then be discussed.

10.1. Definition of the stages in induction of protective immunity.

The comparisons of metabolism and antigen expression by normal, U.V.-irradiated, gamma-irradiated and Actinomycin D-treated schistosomula presented in the preceding chapters form a basis for an initial outline of the various stages in induction of immunity by radiation-attenuated cercariae, and open the way for further testing and better definition of this model. Each stage is characterised by distinct metabolic changes, and expression of different immunogens. We would speculate that, in consequence of induction of immunity by several successive stages in larval development in this way, the protective response should comprise many potential mechanisms of immune attack, directed against challenge parasites at successive points in their migration and development.

10.1.1. Stage 1: 0 to 40 minutes after transformation.

a) Normal schistosomula

It is proposed that the predominance of negatively charged

phospholipids (PS, PI, PG) in the surface membrane of cercariae in fresh water may cause intermolecular repulsion and lateral expansion, tending to create domains of weakly associated lipids. This fluid organisation of the membrane makes it very permeable to solutes (section 3.10.1; figure 3.26; section 4.2.3.).

Electron microscopy shows that, at 30 minutes after entering the skin, the parasite membrane is still trilaminate, i.e. the composition remains characteristic of cercariae (Hockley and McLaren, 1973). It is likely that the higher ionic strength and osmolarity of the mammalian environment, as compared to fresh water, may facilitate closer associations between lipids, producing a more rigid arrangement of lipids in this trilaminate membrane as soon as the parasite enters the skin. This rigid surface forms a tight seal against solute entry. We might speculate that immobilisation of antigens in "crystalline" domains could perhaps enhance their availability for interaction with the host immune system at this time.

Synthesis of one or more members of the Mr 70 000-78 000 postulated hsp family is immediately induced by the stresses of transformation (sections 3.1.2., 3.3.2.). At this very early time after transformation, before the heptalaminate membrane of the schistosomulum appears, the most important function of these heat-shock proteins is likely to be to remove and renature molecules whose conformation and activity have been disrupted by the natural forces - osmotic stress, temperature change, proteolytic activity - brought to bear on the parasite during transformation.

(b) U.V.-irradiated schistosomula.

A decrease in permeability to ^{35}S-methionine is observed on transfer of U.V.-irradiated as well as normal cercariae from fresh water to medium (figure 3.7 and section 3.10.1), suggesting that

"crystallisation" of the membrane also occurs in irradiated parasites immediately after transformation.

Binding of infected human serum, anti-CMAG antiserum, and the A3 monoclonal to U.V.-irradiated cercariae, or to schistosomula 30 minutes after transformation is enhanced (figure 6.9). These observations have been interpreted as indicating that radiation-induced disruption of the glycocalyx may expose new antigenic determinants, both within the glycocalyx and in the underlying membrane.

Synthesis of the Mr 70 000-78 000 hsp's is severely inhibited in U.V.-irradiated forms (section 3.3.2.). Denatured material, resulting from the direct effects of irradiation itself on protein and carbohydrate structure (section 1.10), as well as from the stresses naturally associated with transformation - increased temperature and osmotic pressure; mechanical shearing stress; protease action - is therefore expected to accumulate. It is proposed that antigens whose conformations are modified in this way may interact especially effectively with antigen presenting cells to stimulate helper T-cells. Increased binding of 6-IAF to irradiated cercariae (figure 6.7) may result from exposure of new (SH-containing) peptide determinants in this way.

(c) Gamma-irradiated schistosomula.

We would predict that the characteristics of antigen expression by gamma- and U.V.-irradiated schistosomula should be very similar at this time. Synthesis of proteins, including the postulated heat-shock proteins, is similarly inhibited in both types of irradiated larvae during this early period (sections 5.1, 5.2). Thus, just as for the U.V.-irradiated schistosomula, antigens may be expressed in aberrant conformations, allowing activation of the host immune system in a novel, highly effective manner.

(d) Actinomycin D-treated schistosomula.

Actinomycin D specifically inhibits DNA-dependent transcription of RNA. Synthesis of schistosomular proteins, including the heat-shock proteins, during approximately the first 15 hours after transformation seems to depend on previously-transcribed mRNA (figure 3.18; section 3.10.3.5). Hence, we would expect that protein synthesis and antigen expression by Actinomycin D-treated schistosomula should be identical to normal parasites at this early time after transformation.

10.1.2. Stage 2: 40 minutes to 6 hours after transformation.

a) Normal schistosomula.

During this period, the cercarial trilaminate membrane, rich in negatively charged phospholipids, is shed in the form of microvilli, together with some 70% of the glycocalyx (Samuelson and Caulfield, 1982, 1985; Hockley and McLaren, 1973). The cercarial surface is replaced by the heptalaminate membrane typical of blood-dwelling helminths. This new double bilayer is PC-rich (section 4.2.3), and appears to be considerably more fluid than the trilaminate cercarial membrane in the isotonic host environment (Foley et al, 1988).

A number of lines of evidence lead to the conclusion that, during this stage of development, schistosomula depend largely on proteins, carbohydrates and phospholipids pre-synthesized and stored by cercariae. Firstly, very low levels of incorporation of radioactive precursors into proteins and surface phospholipids have been detected at this time in both our experiments and those of other workers (sections 3.10.2; 4.2.3; Nagai et al, 1977; Yuckenberg et al, 1987). Secondly, U.V.-irradiated schistosomula synthesize a complete

double bilayer, despite the inhibition of protein synthesis, phospholipid synthesis and protein glycosylation (section 6.7). The heat-shock proteins do require to be synthesized immediately after transformation, but even they are apparently derived from pre-existing mRNA transcripts (section 3.10.3.3; figure 3.18).

Judging by their roles in cellular systems (Pelham, 1986; 1988), we would speculate that, during this phase of development, heat-shock proteins are essential in assisting both membrane-associated and cytosolic proteins to achieve their final, mature conformations (Pelham, 1986; 1988). The association of these hsp's with the surface membrane of schistosomula (figures 3.24, 3.25) suggests that they may be involved in inserting the proteins in their correct conformations into the new schistosomular heptalaminate membrane. In addition, the heat-shock proteins will be required for removal or renaturation of any denatured material.

b) U.V.-irradiated schistosomula.

Enhanced binding of anti-coat 1 and anti-haemolymph antisera to U.V.-irradiated schistosomula during this period is detected by immunofluorescence, suggesting that new antigenic determinants may be exposed on the glycocalyx, either during its disaggregation and removal, or on the residual glycocalyx material remaining on the parasite surface after transformation (figures 6.1, 6.6; section 6.9.1).

U.V.-irradiated schistosomula do complete the double bilayer, apparently relying on proteins and lipids synthesized and stored by cercariae (sections 6.7; 6.9.4.2.).

The continued absence of heat-shock proteins, due to general inhibition of protein synthesis, would be expected to lead to persistence and accumulation of antigens in the aberrant conformations

induced directly by irradiation and by the stresses of transformation. Without hsp's to ensure correct folding and membrane insertion, any pre-synthesized proteins entering the heptalaminate membrane are likely to assume abnormal conformations. Increased binding of 6-IAF to exposed SH groups on the 3-hour schistosomular surface (figure 6.7) may reflect expression of new epitopes in this way.

c) Gamma-irradiated schistosomula.

Inhibition of protein and hsp synthesis is still evident at this time. Thus, antigen expression presumably follows the same pattern as for U.V.-irradiated forms.

d) Actinomycin D-treated schistosomula.

Reliance on pre-formed proteins means that antigen expression by normal and Actinomycin D-treated schistosomula should still be identical.

10.1.3. Stage 3: 6 to 15 hours after transformation.

a) Normal schistosomula.

The rate of ^{35}S-methionine incorporation into protein increases during this period (Nagai et al, 1977; Yuckenberg et al, 1987). This increase in metabolic activity might imply that, by this stage, the cercarial store of pre-synthesized macromolecules is exhausted. Thus, schistosomula must now depend on de novo synthesis of proteins, phospholipids and glycoproteins. However, Actinomycin D-treated schistosomula still show very little inhibition of protein synthesis at this time (figure 3.18; section 3.10.3.5), suggesting that synthesis

may now occur on pre-existing mRNA templates, still not necessitating de novo transcription from DNA. As before, the 70 000-78 000 Mr hsp's may assist in correct folding and insertion of cytosolic and membrane proteins.

The antigenic profile of the schistosomular surface is altered by incorporation of host lipids into the membrane (Rumjanek and McLaren, 1981; section 4.2.2.2), and by acquisition of host molecules which are believed to help prevent the host immune system from recognising parasite antigens as foreign (McLaren, 1980; section 1.5.7).

b) U.V.-irradiated schistosomula.

Irradiation-induced disruption of messenger RNA, or of transcriptional enzymes, means that synthesis of proteins and phospholipids, and protein glycosylation, occur at very low levels during this period (sections 3.3.2; 3.4; 3.10.3.5; 4.2.1.; 4.2.3; 4.3.1.). Inhibition of glycosylation is likely to alter the conformation of glycoproteins, exposing cryptic determinants in the polypeptide core, and altering the pattern of proteolytic digestion by antigen presenting cells (section 4.3.2.; Feizi and Childs, 1987; Alexander and Elder, 1985; Olden et al, 1982; Kornfield, 1986). Altered antigen processing is predicted to lead, in turn, to presentation of novel antigenic determinants which stimulate potent protective immunity.

Since carbohydrate moieties act as signals to direct denatured material to lysosomes for degradation, aberrant glycosylation may also aggravate the persistence of antigens, in non-native conformations, which would normally be scheduled for breakdown (Rothman and Kornfield, 1986; Lenard, 1984).

The altered membrane lipid composition of U.V.-irradiated forms, resulting from inhibition of new phospholipid synthesis, while uptake

of neutral host lipids continues (sections 4.2.2.2.; 4.2.4) may also lead to exposure of antigens in abnormal conformations, since many integral membrane proteins have a very precise requirement for a lipid environment which complements and maintains their native structure (section 4.2.4; Houslay and Stanley, 1982).

Any synthesis which does occur on the damaged and altered mRNA templates is likely, in the absence of heat-shock proteins, to lead to further accumulation of denatured and aberrant antigens. Figures 3.24 and 3.25 suggest that only a very small proportion, if any, of the proteins which are synthesized by attenuated schistosomula are inserted into the surface membrane, presumably due to the lack of heat-shock proteins, which assist in insertion of integral membrane proteins. However, protein secretion does not seem to be inhibited in irradiated larvae.

On all these counts, then, U.V.-irradiated schistosomula are likely to express antigens in modified conformations which allow antigen presentation to the host immune system in such a way as to stimulate potent protective immunity.

c) Gamma-irradiated schistosomula.

As a rule, inhibition of protein synthesis is still evident at this time (sections 5.1; 5.2), implying that antigen expression by gamma- and U.V.-irradiated schistosomula continues to be very similar.

d) Actinomycin D-treated schistosomula.

Since this stage of development apparently depends on pre-formed mRNA, Actinomycin D-treated schistosomula should remain comparable to normal forms.

10.1.4. Stage 4: 15 to 96 hours after transformation.

a) Normal schistosomula.

During this period, protein synthesis by schistosomula is at last dependent on de novo transcription of RNA messages from DNA. The similarity in the pattern of proteins synthesized at this time and during the preceding 15 hours, when the parasite was dependent on mRNA stored by cercariae, suggests that the same DNA sequences were transcriptionally active in cercariae.

Synthesis of proteins and phospholipids, and protein glycosylation, now proceed at comparatively high rates. Uptake of host lipids and acquisition of host antigens continue to modify the nature of the parasite surface.

b) U.V.-irradiated schistosomula.

The preferential absorption of 254 nm U.V. light by DNA generally results in disruption and inactivation of the genetic messages responsible for synthetic activity and development by schistosomula. Accumulation and presentation of denatured antigens, as described in 10.1.3b), continues. Modifications in the structure or conformation of carbohydrate antigens expressed by 20-hour U.V.-irradiated, as opposed to normal, larvae, were indicated by the studies on antibody binding affinity described in chapter 7. In culture, schistosomula whose metabolism is incapacitated in this way become increasingly granular, and distorted in shape. 100% death is usually evident in vitro by 96 hours.

It may be noted, however, that occasional exceptions to this rule do occur, when protein synthesis by U.V.-irradiated schistosomula seems to be restored to normal levels during this period (sections 8.1;

8.2.1).

c) Gamma-irradiated schistosomula.

Transcriptionally active DNA in the nucleoplasm appears to be relatively insusceptible to gamma-irradiation damage (sections 5.1; 5.2; 8.3.1.). Since it is unfolded for transcription, this DNA should also be accessible to radioprotective compounds and repair enzymes. Presumably for these reasons, synthesis by gamma-irradiated schistosomula, during this period of dependence on DNA that is already transcriptionally active, is restored to normal levels (section 5.1).

It therefore seems likely that gamma- and U.V.-irradiated larvae will differ markedly at this point in development. The antigens expressed by gamma-attenuated schistosomula at this time are predicted to become more similar to those presented by normal larvae, although the carbohydrate antigens of 20-hour, gamma-irradiated schistosomula do seem to be in an altered, abnormal conformation (see chapter 7). We might also speculate that the resumption of normal metabolic activity during this period may give the gamma-irradiated parasites a new lease of life, perhaps enabling them to survive in the host for longer than is usual for U.V.-irradiated forms.

d) Actinomycin D-treated schistosomula.

Intercalation of Actinomycin D into DNA means that, at this stage, transcription of genetic messages, and, in consequence, protein synthesis, are finally inhibited in Actinomycin D-treated forms. As protein and phospholipid synthesis and protein glycosylation all decrease, while heat-shock proteins and degradative enzymes already present are depleted or inactivated, aberrant and non-native structures would be expected to accumulate over a period of days. Thus,

presentation of antigens in modified, highly immunogenic conformations may also account for induction of immunity by Actinomycin D-attenuated larvae (sections 3.8; 4.2.1; 4.3.1). Actinomycin D treatment, like the two types of radiation attenuation, seems to result in modification of the carbohydrate antigens expressed by 20-hour larvae (see chapter 7).

10.1.5. Stage 5: 96 hours onwards.

a) Normal schistosomula.

It is postulated that new regions of DNA, formerly condensed into inactive heterochromatin, associated with the nuclear membrane, must be activated, preparatory to growth and cell division, which occur from day 4 onwards in culture (Clegg, 1965; Clegg and Smithers, 1972). Synthesis of new proteins by normal schistosomula at this stage may be evident at 96 hours in gel 8.2.

b) U.V.-irradiated schistosomula.

The culture conditions used in these experiments do not allow us to determine what proportion of U.V.-irradiated schistosomula usually survive past the 96-hour stage *in vivo*. The pronounced susceptibility of DNA to damage by U.V. radiation of 254 nm strongly suggests that any schistosomula which do survive radiation injury up to 96 hours will be unable to decondense and activate heterochromatin at this time. The dying or dead schistosomula, carried to the lung, would be predicted, as they disintegrate, to make available to the host immune system the modified antigens which have accumulated at the surface and in the parasite body.

c) Gamma-irradiated schistosomula.

As described above, it is postulated that radiation-induced disruption of heterochromatin at the nuclear membrane prevents its activation, as is necessary for growth and mitosis from day 4 onwards. As described in (b) above, it is predicted that, as the schistosomula die in the lungs, presentation of antigens expressed in modified, highly immunogenic conformations may again occur at this stage.

d) Actinomycin D-treated schistosomula.

Actinomycin D intercalates into heterochromatin as well as transcriptionally active DNA. Thus, mitosis will be inhibited, and disruption of synthetic activity, with expression of antigens in non-native conformations, is presumed to continue. Internal and surface antigens will be released when the metabolically-inhibited schistosomula finally die.

10.1.6. Parasite distribution at each of these stages.

According to the time course of parasite migration derived from autoradiographic tracking data in mice (Wheater and Wilson, 1979; Wilson and Lawson, 1980), normal (non-attenuated) parasites successfully entering the skin reach the base of the epidermis within 30 minutes. Only a small minority enter the dermis at this time, however. For the rest of the schistosomula, the basement membrane of the epidermis impedes progress. In the hamster cheek pouch, schistoso- mula remain epidermal for about 40 hours before beginning to enter the dermis. Once in the dermis, a schistosomulum takes about 10 hours to locate a post-capillary venule, and about 8 hours to penetrate the venule wall. Once inside the lumen of the blood vessel, the parasite is

rapidly carried away by blood flow. Normal schistosomula are first detected in lungs on day 3 post-infection in hamsters, mice and rats, rising to a peak at day 6 in mice (Miller and Wilson, 1980). At day 6 to 7, schistosomula are found in the pulmonary capillaries, causing considerable distention, and even erupting into the alveoli (Crabtree and Wilson, 1986; Mastin et al, 1985b). Thus, stages (1) to (3) and part of stage (4) in schistosomular development are predicted to occur predominantly in the epidermis, exposing the Langerhans cells of this tissue to a wide range of parasite antigens. Parasites at stage (4) will also be found in the dermis, and blood vessels of the dermis and subcutis. Stage (5) will occur principally in the lungs.

At least for gamma-irradiated schistosomula, parasite death in vivo appears to occur predominantly in the lungs (Mastin et al, 1983; 1985b) i.e. at stage (5). We would postulate that, during the skin-associated stages of parasite development, immunity may be stimulated chiefly by the antigens released by the damaged, but still living, schistosomula. It has been suggested that schistosomula may shed large membrane fragments (Kusel and MacKenzie, 1975), thus preserving the antigens in their original environments. The modified conformation of these released antigens is postulated to result in an altered pattern of antigen processing, and presentation to the host immune system in such a way as to stimulate potent protective immunity.

The protracted stay of irradiated schistosomula in the epidermis (Mastin et al, 1983) will allow Langerhans cells to process and present antigens released by the attenuated forms at stages (1), (2), (3), and part of stage (4) in development. According to the model of Streilen (1983), the skin immune system is self-sufficient, with its own antigen presenting cells, quiescent T-cells, immune memory cells, and effector cells.

When the irradiated larvae die, and undergo autophagocytosis in the lung (stage (5)), as described by Mastin et al (1985b), the

partially denatured, aberrant antigens which have accumulated in the body and surface of the attenuated parasites should become available for presentation by other antigen presenting cells (chiefly macrophages) to the systemic immune system.

Table 10.1 and figures 10.1 a), b) summarise the proposed model, outlining how U.V.-irradiated, gamma-irradiated and Actinomycin D-treated schistosomula may express antigens in highly immunogenic conformations at different stages in their migration and development.

In summary, U.V.-irradiated, gamma-irradiated and Actinomycin D-treated larvae all die before maturity, and all induce protective immunity in an appropriate host. A common factor in all three treatments is inhibition of protein synthesis. It is proposed that U.V.-, gamma-, and Actinomycin D-treated parasites all express antigens in modified conformations at definite stages in their curtailed development. These "immunogenic phases" are different for each type of attenuation. Antigens from U.V.-irradiated schistosomula are postulated to be especially competent in inducing immunity at stages (1) to (4), chiefly in the skin. Stage (5), in the lung, may make a smaller, less consistent contribution to U.V.-irradiated vaccine immunity. Gamma-irradiated schistosomula are predicted to be immunogenic at stages (1), (2) and (3) in the skin, and stage (5), in the lung. Actinomycin D-treated schistosomula, according to the present model, may interact effectively with the host immune system to provoke a protective response, from 15 hours after transformation onwards - stages (4) and (5) - that is, possibly during migration from skin to lung, but predominantly within the lung.

10.1.7. Challenge attrition in the three attenuated vaccine models.

This discussion has concentrated on expression of antigens by attenuated parasites, rather than analysing the host immune responses

Table 10.1 Expression of antigens in modified, highly immunogenic conformations at several distinct stages in development by irradiated and Actinomycin D-treated schistosomula.

Table 10.1.

STAGE (TIME AFTER TRANSFORMATION)	LOCATION OF PARASITES	DESCRIPTION (NORMAL SCHISTOSOMULA).	EXPRESSION OF ANTIGENS IN IMMUNOGENIC CONFORMATIONS BY SCHISTOSOMULA: U.V.-IRRADIATED	GAMMA-IRRADIATED	ACTINOMYCIN D-TREATED
(1) 0 to 40 mins	Epidermis	-Trilaminate (cercarial) membrane, rigid lipid domains. -Induced synthesis of hsp's begins.	Yes -Exposure of new antigenic sites in glycocalyx and underlying membrane. -Denatured antigens produced by direct effects of irradiation and by stresses of transformation persist. -no hsp's to clear denatured material.	Yes	No
(2) 40 mins to 6 hours	Epidermis	-Loss of cercarial membrane and glycocalyx. -Completion of double bilayer (fluid). -New membrane is composed of phospholipids and proteins pre-synthesized and stored by cercariae.	Yes -New glycocalyx antigens exposed. -Lack of hsp's causes accumulation of antigens in non-native conformations.	Yes	No
(3) 6 to 15 hours	Epidermis	Protein and phospholipid synthesis, also protein glycosylation, depend on pre-synthesized mRNA. Uptake of host lipids and acquisition of host antigens occur.	Yes -Accumulation of denatured, modified antigens continues, due to lack of hsp's. -Reduced and aberrant synthesis of new proteins and phospholipids, together with continued acquisition of neutral host lipids, causes major changes in the conformation of parasite antigens.	Yes	No

Table 10.1 continued.

STAGE (TIME AFTER TRANSFORMATION)	LOCATION OF PARASITES	DESCRIPTION (NORMAL SCHISTOSOMULA).	EXPRESSION OF ANTIGENS IN IMMUNOGENIC CONFORMATIONS BY SCHISTOSOMULA: U.V.-IRRADIATED	GAMMA-IRRADIATED	ACTINOMYCIN D-TREATED
(4) 15 to 96 hours	Epidermis, dermis blood vessels of dermis, subcutis; en route to lung.	Synthesis depends on transcription of RNA messages from DNA already transcriptionally active in cercariae.	Yes Non-native material, as described in (3), accumulates further.	No Transcriptionally active DNA in the nucleoplasm of cercariae suffers little permanent damage from gamma-irradiation.	Yes Actinomycin D disrupts DNA messages. Hence, synthesis of proteins (and hsps), glycoproteins and phospholipids is inhibited. Expression of antigens in non-native conformations therefore occurs, as described in (3).
(5) 96 hours onwards	Lungs	Previously inactive heterochromatin must be decondensed and activated, as new proteins are required for growth and mitosis.	Yes	Yes Heterochromatin at nuclear membrane is especially susceptible to radiation damage. Hence, metabolic activity is again disrupted.	Yes
			As schistosomula die and disintegrate, the modified antigens which have accumulated in the parasite body and surface become available to the host immune system.		

Figure 10.1 a) accompanies table 10.1, showing the organisation of the parasite surface antigens at each stage.

 = Antigen in native conformation.

 = Antigen in non-native conformation.

——— = protein or lipid synthesized and stored by cercariae.

——— = protein or lipid synthesized after transformation, on mRNA transcripts stored by cercariae.

——— = synthesis requires de novo transcription of mRNA from DNA.

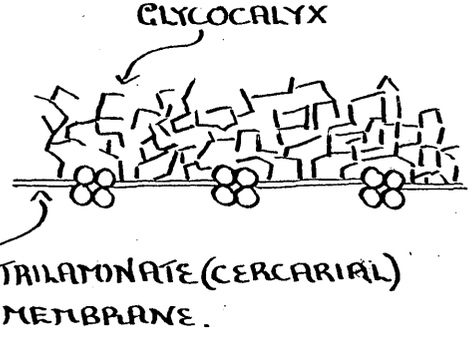
 = host lipid acquired by schistosomula.

Induction of immunity in the skin (stages (1)-(3), part of (4)) is postulated to be attributable chiefly to release by schistosomula of antigens in modified, highly immunogenic conformations. In the lungs (stage (5)), as the attenuated schistosomula die and disintegrate, the denatured antigens in the parasite surface and body may be made available to antigen presenting cells.

FIGURE. 10.1 a); (1)-(5).

(1) 0-40 MINUTES AFTER TRANSFORMATION.

NORMAL OR ACTINOMYCIN D-TREATED.

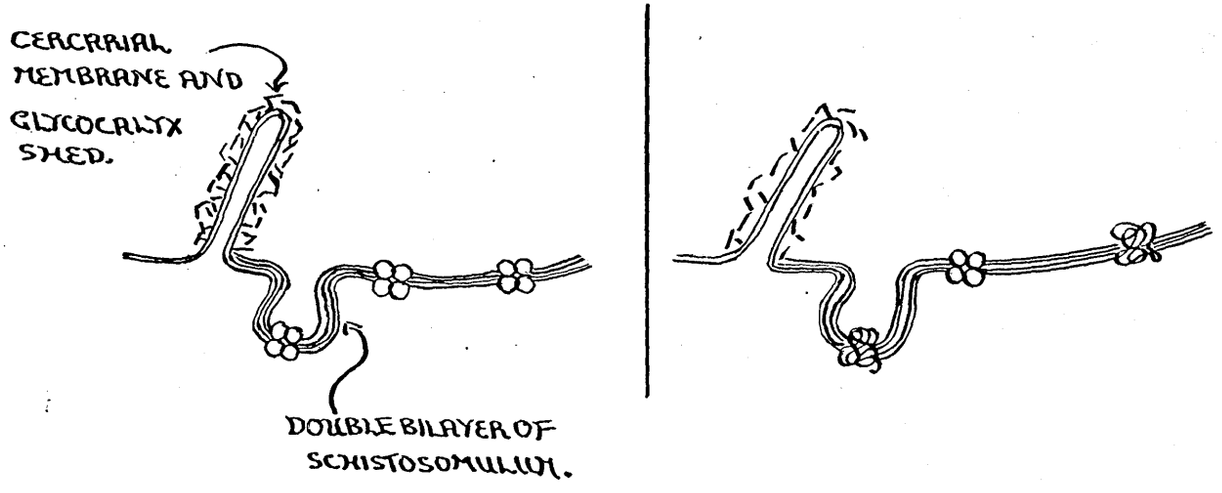


U.V.- OR GAMMA-IRRADIATED.

(2) 40 MINUTES TO 6 HOURS AFTER TRANSFORMATION.

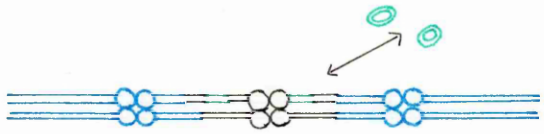
NORMAL OR ACTINOMYCIN D-TREATED.

U.V.- OR GAMMA-IRRADIATED.

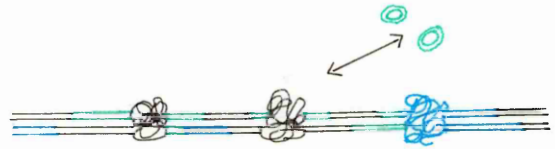


(3) 6 TO 15 HOURS AFTER TRANSFORMATION.

NORMAL OR ACTINOMYCIN D-TREATED.

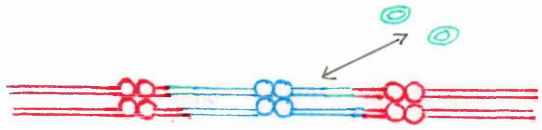


U.V.- OR GAMMA-IRRADIATED.

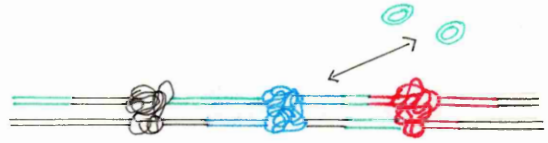


(4). 15 TO 96 HOURS AFTER TRANSFORMATION.

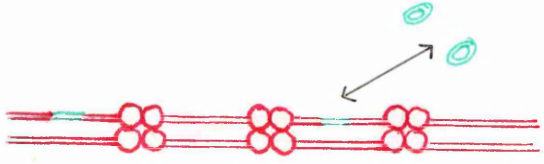
NORMAL.



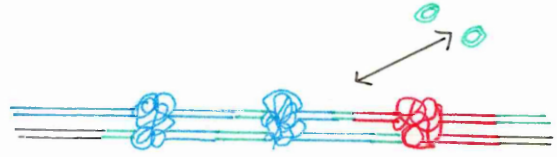
U.V.- IRRADIATED.



GAMMA-IRRADIATED.

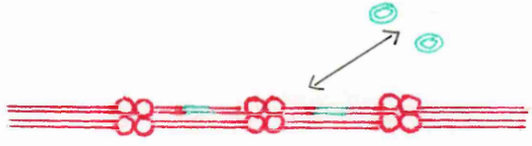


ACTINOMYCIN D-TREATED.

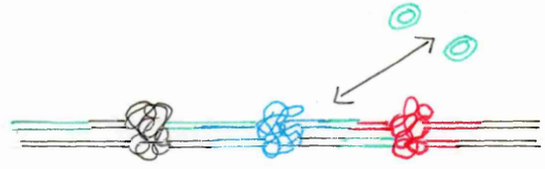


(5). 96 HOURS AFTER TRANSFORMATION ONWARDS.

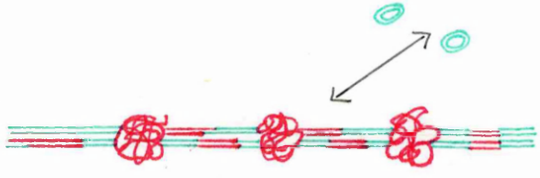
NORMAL.



U.V.-IRRADIATED.



GAMMA-IRRADIATED.



ACTINOMYCIN D-TREATED.

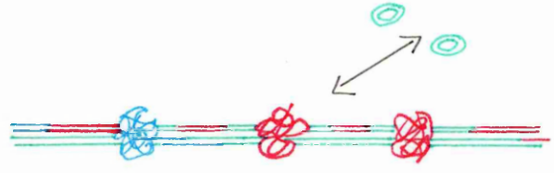
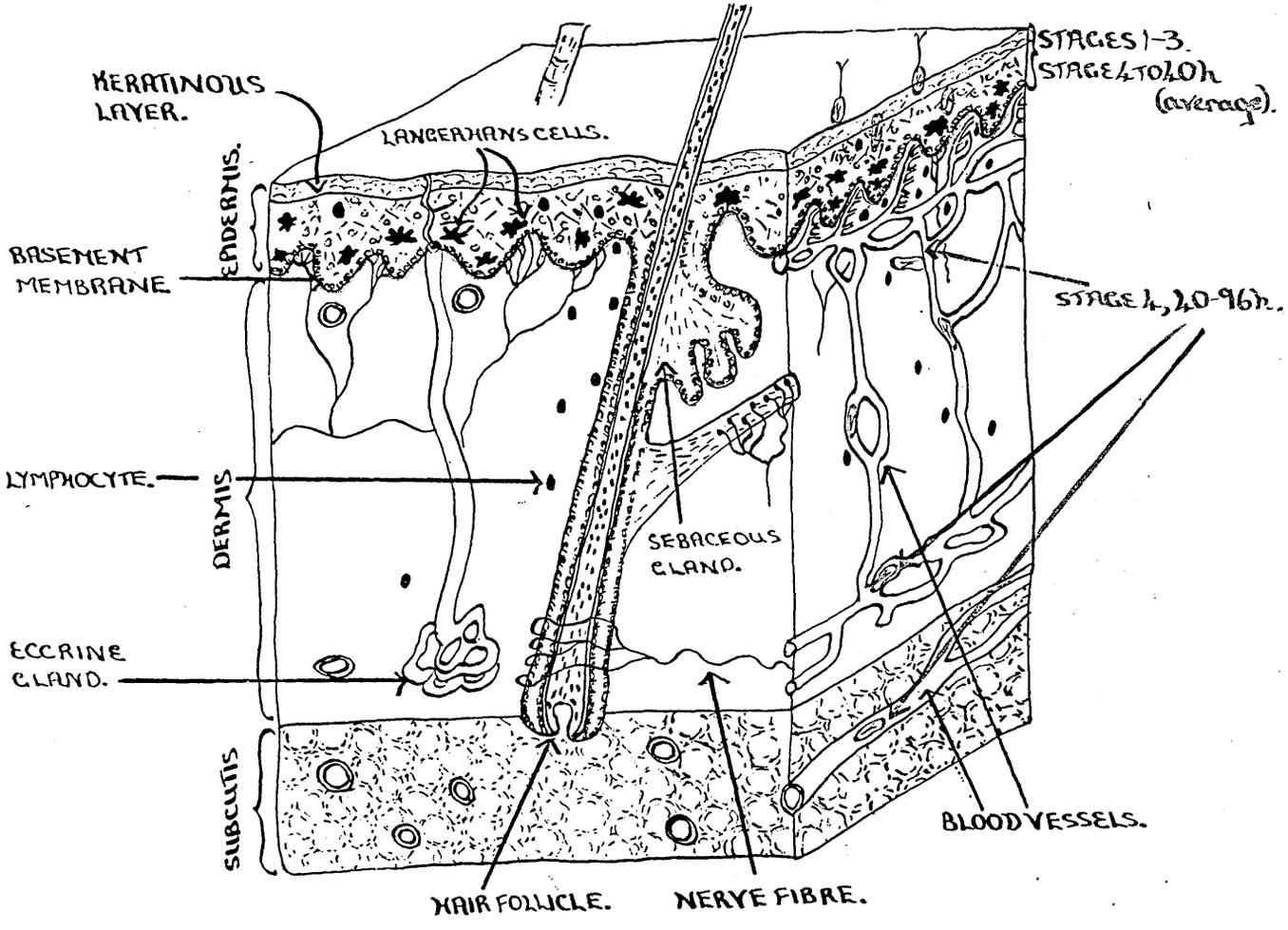
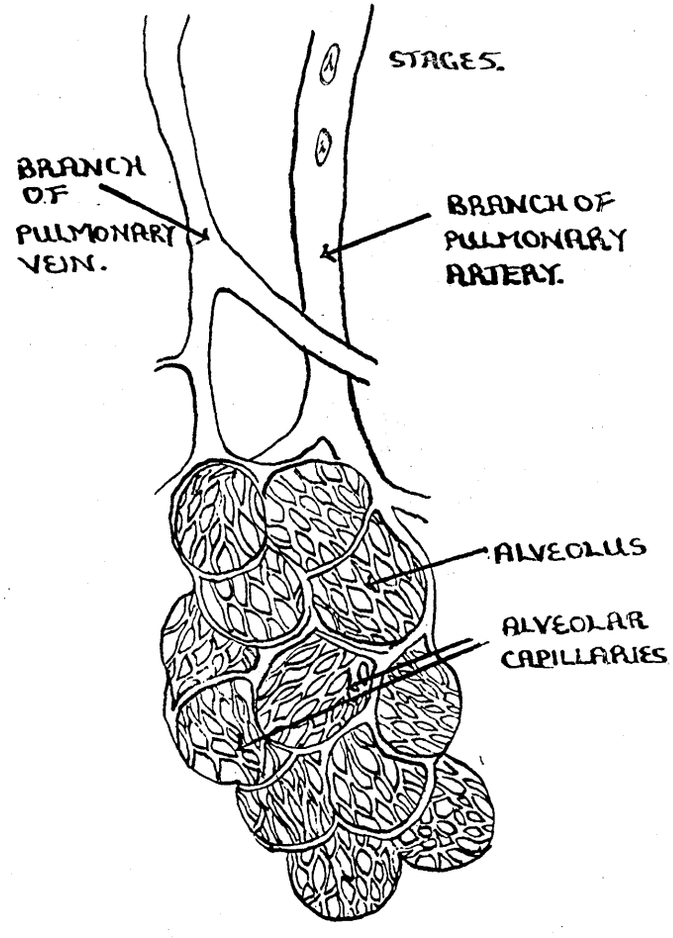


Figure 10.1b) shows the host environment in which the parasite is located at each of these stages. The basement membrane of the epidermis (marked in red) forms a barrier to parasite migration for some 40 hours after skin penetration (mouse model; Wilson and Lawson, 1980). The Langerhans cells of the epidermis are therefore exposed to parasite antigens at several of the stages in larval development.

FIGURE 10.1b).



THE ANATOMY OF SKIN (based on Streilen, 1983).



BLOOD VESSELS OF THE LUNG (based on Vander et al, 1980).

to the different vaccines. However, some predictions may be made about the sites and targets of immune attrition.

The sites of challenge elimination seem to coincide with the sites of induction of immunity. Thus, since gamma-irradiated parasites, according to our model, express antigens in immunogenic form in the skin and lungs, at stages (1), (2), (3), and (5), challenge schistosomula, in turn, die in both the skin and the lungs (sections 9.4.2.1., 9.4.2.2.). Actinomycin D-treated schistosomula, injected subcutaneously, are postulated to stimulate a host immune response predominantly in the lung (stage 5). Since systemic responses and skin-associated immunity seem to function largely independently of each other (section 9.4.2.4.), we would expect that challenge larvae in "Actinomycin D-immunised" mice should activate immunological memory and attrition mechanisms as they migrate through the lung, rather than in the skin. U.V.-irradiated cercariae fail to induce immunity in BALB/c mice, which appear to be deficient in skin-specific immune responses, but which do effect systemic and lung-associated attrition (sections 9.4.2.3; 9.4.2.4.) We would therefore predict that cutaneous responses may be of primary importance in U.V.-irradiated larval vaccine immunity. Challenge attrition should therefore occur predominantly in the skin. The work of Cohen and Eveland (1988), described in section 9.4.1., suggests that U.V.-irradiated larvae may also be capable of activating immune mechanisms in the lung (i.e. at stage (5)), but lung-specific immunity in the U.V.-irradiated vaccine model appears to be very inconsistent, and generally to reach only low levels.

10.1.8. Variations on the basic model.

The model outlined in the preceding sections for induction and expression of irradiated vaccine immunity applies to an idealized situation, where the attenuated larvae are capable of inducing each of

the postulated stages of immunity; the challenge parasites, in turn, are susceptible to expression of immune effector mechanisms at each of these stages, and the host is capable of mounting the appropriate immune response at each stage.

The studies on parasite variability described in chapter 8 make it clear that the response to irradiation does vary considerably in different batches of cercariae. While, in general, U.V.-irradiated larvae showed a 70 to 80% inhibition of protein synthesis during the 96 hours following transformation, exceptions to this rule were not infrequent. Levels of inhibition of only 20 to 40% were sometimes observed, as was recovery of protein synthesis to near normal levels after an initial period of inhibition. There was even an occasional instance where no inhibition of protein synthesis could be detected at all throughout the course of the experiment (96 hours maximum). In such cases, we might speculate that the attenuated schistosomula will fail to induce one or more of the skin-associated stages of immunity (stages (1)-(3); part of (4)). The susceptibility of challenge parasites to particular immune effector mechanisms also seems to vary considerably for different batches of cercariae. Thus, Smith and Clegg (1979) demonstrated that hosts infected with a single pool of cercariae (either a mixed population or a clone) displayed different levels of concomitant resistance to challenge with separate batches of parasites. Cohen and Eveland (1988) drew the same conclusion from their studies on the effectiveness of immunity induced by a single population of U.V.-irradiated larvae against challenge by different pools of cercariae. McLaren et al (1981), Seitz et al (1987) and Jones et al (1988) demonstrated that schistosomula varied considerably in susceptibility to eosinophil killing, and in binding of poly-L-lysine, a model for eosinophil cationic protein. These observations could be related to the variation observed in parasite susceptibility to immune attrition in vivo . We would suggest that in those cases where no resistance was

observed by Smith and Clegg (1979) and Cohen and Eveland (1988), the immunising larvae may have stimulated immune responses to which the majority of parasites in some challenge batches were not susceptible.

Such variability in both the immunising potential of radiation-attenuated larvae and the susceptibility of challenge parasites to the various immune effector mechanisms might help account for the broad variation in levels of protection (30 to 90% in individual experiments) stimulated by irradiated cercariae, and for the fact that some 10% of challenge infection has always been found to survive in even the most effectively immunised host (Dean, 1983; section 1.9.2.3.). The extremely high protection (just over 90%) which Hsü et al (1979, 1981, 1982) were able to stimulate by multiple (five) inoculations of irradiated larvae might be explained if so many vaccinations with different batches of cercariae ensured that all the potential mechanisms of immunity were induced. Thus, despite variation in susceptibility to particular immune effector responses, very few challenge larvae were likely to evade all the mechanisms of attrition activated by the host.

It may be noted that Actinomycin D-treated schistosomula, unlike irradiated ones, did not vary significantly in the level of inhibition of synthetic activity. Thus, the immune mechanisms stimulated by Actinomycin D-treated larvae should not vary so much between different batches of parasites. However, we would speculate that hosts immunised with the Actinomycin D-treated vaccine may be quite strictly limited in their ability to eliminate challenge larvae. Successful attrition may be dependent upon challenge parasites being highly susceptible to the immune mechanisms stimulated, in the lung, at stages (4) and (5) according to our model.

We would suggest that, in order to overcome the problem of variation in susceptibility of challenge parasites to different immune effector mechanisms, an effective vaccine might require to induce a

wide range of immune responses, as achieved by the multiple immunisations of Hsü et al (1979, 1981, 1982), described above. The model, presented here, of discrete stages in induction of immunity by attenuated larvae, could provide a basis for development of such a multiple component vaccine, which would activate host immunity against challenge parasites at successive stages of development.

As discussed in sections 9.4.2.1; 9.4.2.2., routes of immunisation and challenge will also influence the efficacy of the various potential immune effector mechanisms. Thus, animals immunised intravenously or intramuscularly, to bypass the skin, will not activate skin-associated immune mechanisms against challenge cercariae. Immunisation or challenge via the tail, an area deficient in the Langerhans cells which are an essential component of the skin immune system, will also prevent optimal expression of cutaneous immunity.

The ability of the host to mount appropriate immune responses will also be important in determining the mechanisms of challenge attrition. Thus, CBA mice vaccinated with gamma-irradiated cercariae seem to eliminate challenge parasites chiefly in the skin (Kamiya et al, 1987; McLaren et al, 1987; section 9.4.2.2.), while gamma-irradiated cercariae-vaccinated BALB/c mice are unable to perform immune attrition in the skin, but appear to be competent in lung-stage elimination (sections 9.4.2.3; 9.4.2.4.). C57BL and NIH mice immunised with gamma-irradiated cercariae seem to express both skin- and lung-associated immunity, while P-strain mice are incapable of performing immune attrition at either stage.

Mouse strain considerations are also important in determining the effectiveness of the U.V.-irradiated and Actinomycin D-treated larval vaccines. Thus, as stated above, BALB/c mice, being deficient in skin-mediated immune responses, are not protected against challenge by immunisation with U.V.-irradiated cercariae. On the other hand, CBA mice, which apparently express skin- but not lung-associated immunity,

are not resistant to challenge after vaccination with Actinomycin D-treated schistosomula, which, according to our model, stimulate immune responses in the lung.

10.1.9. Predictions and tests of the model.

According to the model presented here, each of the three types of attenuated larvae: U.V.-irradiated, gamma-irradiated and Actinomycin D-treated, interacts with the host immune system in a number of different ways, at successive stages in their migration and development. In consequence, the vaccinated hosts effect challenge attrition against several schistosomular stages, and by a variety of mechanisms. The model predicts that elimination of any one of the immunising larval stages (1) to (5) should result in failure to induce the protective responses unique to that stage. Thus, when irradiated cercariae are prevented from migrating out of the skin, eliminating stages (4) and (5), immune effector mechanisms should be stimulated against skin-, but not lung-stage, challenge parasites. Conversely, animals immunised with irradiated larvae which bypass the skin and are delivered directly to the lungs, should perform only lung-stage challenge attrition.

To some extent, these predictions have already been tested for the gamma-irradiated vaccine, though not with this model of immunity in mind. Section 9.4.2. describes how the relative importance of skin- and lung-stage challenge attrition after vaccination with gamma-iradiated cercariae varies with different mouse strains and immunisation routes. The observations, by a number of workers, that gamma-irradiated larvae restricted to either the skin or the lungs induced approximately half the resistance stimulated by irradiated cercariae allowed to accomplish both stages of development are consistent with this hypothesis of immunity operating in several stages, although the site of challenge attrition was not determined in these studies (Bickle et al, 1979;

Sher and Benno, 1982; Dean et al, 1981c). Bypassing the skin immune system by allowing cercariae to penetrate via the tail (deficient in Langerhans cells) seemed to induce only lung-stage challenge attrition in the studies of Dean et al (1984), and Wilson et al (1986). In contrast, McLaren et al (1985) did observe killing of challenge parasites in the skin of mice of the same strain when immunising cercariae were administered percutaneously via the abdomen, thus stimulating the skin immune system.

These results all agree with the prediction that immunity is induced by, and, in turn, acts against, a number of discrete stages in parasite development. However, more precise studies, using tissue recovery techniques, or the more accurate autoradiographic tracking approach, to assess migration of both attenuated and normal, challenge larvae, would be required for full confirmation of this model. The predictions of the model could also be tested by restricting the immunising larvae to the skin (stages (1) to (3)) by drug clearance after the immunising infection, or excision of the infection site, as described by Bickle (1982). Alternatively, they could be restricted to the systemic and lung stages (stages (4), (5)) by intravenous injection. Tissue recovery or autoradiographic tracking techniques might then be used to determine whether the site of attrition of a challenge infection coincided with the site of induction of immunity.

Other testable predictions of our model are:

(1) Percutaneous immunisation with U.V.-irradiated cercariae should provide less protection against challenge with lung forms than against cercariae administered percutaneously.

(2) U.V.-irradiated cercariae administered percutaneously via the tail should stimulate only lung-stage challenge attrition, since rodent tail skin is deficient in essential components of the skin immune system (Langerhans cells). Induction of immunity by U.V.-irradiated cercariae could also be limited to the lung-stage by U.V. exposure of mouse skin

before percutaneous immunisation. U.V.-light inactivates Langerhans cells (Streilen, 1983).

(3) U.V.-irradiated cercariae applied percutaneously are postulated to induce predominantly skin-associated immunity. However, it may be possible to elicit very high levels of lung-stage immunity with U.V.-irradiated parasites by inoculating the U.V.-irradiated schistosomula intravenously, so that they are delivered directly to the lungs. In this way, the presentation of novel epitopes from antigens in aberrant or denatured conformations, which usually occurs in the skin, could take place in the lung.

(4) Actinomycin D-treated schistosomula are postulated to act only by the immune mechanisms specific for stages (4) and (5) in parasite development. Hence, they would be predicted to effect challenge elimination predominantly in the lung.

(5) Larvae protected from irradiation damage by SH groups or α-tocopherol, as described in sections 8.2.3.1., 8.2.3.2., would be expected to induce immunity in the same way as Actinomycin D-treated parasites. As judged by their normal levels of protein synthesis during 24 to 48 hours after transformation, these protected schistosomula do not appear to suffer radiation damage during the early, skin-associated stages of development. Nevertheless, the majority of radioprotected schistosomula do not survive to maturity (table 8.2). This observation is interpreted as indicating that radiolesions in DNA in condensed, inactive conformation at the time of irradiation (heterochromatin) are sufficiently severe to prevent cell division and growth, despite the presence of radioprotective compounds which prevent or repair damage to the transcriptionally active DNA. Accordingly, such "radioprotected" parasites should exhibit metabolic disruption and surface disorganisation only at stage (5) in development. They would therefore be predicted to induce only lung-specific immunity.

10.1.10 Potential for application of this model to an anti-schistosome vaccine.

The most successful human vaccines in use at present are attenuated viruses - smallpox, yellow fever, poliomyelitis, measles, rubella. These vaccines owe their success (greater than 90% effectiveness: Ada, 1986) chiefly to generation of long-lived memory B-lymphocytes. In this way, upon encountering a wild-type infectious agent, rapid production of antibody, which prevents infection or neutralises the action of a toxin, is ensured (Ada, 1986). In contrast, for a metazoan parasite as complex and well-adapted as the schistosome, a direct effect of antibody against the target is not sufficient for protective immunity. The many attempts to induce resistance in experimental hosts with defined antigens - the Mr 38 000 glycoprotein, Mr 26 000 and 28 000 glutathione-S-transferases, paramyosin, or the various species identified by protective monoclonals (section 1.8.3.) generally stimulate partial protection against challenge, but do not consistently induce the high levels of immunity necessary for a commercial vaccine. Together with the wide spectrum of potential immune effector mechanisms identified both *in vitro* and *in vivo* in the concomitant and vaccine models of resistance, these observations suggest that, in order to be consistently and highly effective against schistosomiasis, a vaccine must comprise multiple components, stimulating a variety of immune mechanisms which are effective against several successive stages in parasite development. Vaccination with live, attenuated schistosomula apparently does allow the host immune system to interact with the whole complex antigenic profile of the young schistosomulum, and induces a concerted response comprising several effector mechanisms, active against different larval stages. Nevertheless, although the levels of protection induced by gamma-irradiated, U.V.-irradiated, gamma-irradiated and cryopreserved,

or Ro-11 3128-treated larvae are generally 50% or greater (Dean, 1983; Bickle and Andrews, 1985; section 1.9), immunity still does not reach the consistent, very high (greater than 90%) levels demanded by an effective commercial vaccine. This lack of consistency may be attributable to variation, in immunogenicity, or in susceptibility to immune mechanisms, on the part of the immunising and challenge parasites, respectively. Ideally, a defined vaccine should induce high levels of responsiveness at each of the stages where infecting, challenge parasites may be partially susceptible to immune attrition. The present model proposes one scheme for dissecting induction of immunity into discrete stages, at each of which different antigens are presented to specific compartments of the host immune system. Such a model could perhaps act as a starting-point for identifying the various antigens and immune mechanisms which contribute to effective immunity. It is postulated that antigens of the cercarial glycocalyx and trilaminate membrane (at stages (1) and (2)), also secreted proteins and glycoproteins of irradiated schistosomula during the initial 96-hour period after transformation (stages (2), (3) and (4)), stimulate the skin immune system. As the attenuated parasites are trapped and die in the lungs, surface and internal body proteins are made available to the host's systemic immune system (stages (4) and (5)). Perhaps the most important aspect of our model is that stimulation of a protective immune response at each of these stages is highly dependent on effective antigen presentation.

10.2 Induction of protective immunity depends on presentation of schistosomular antigens.

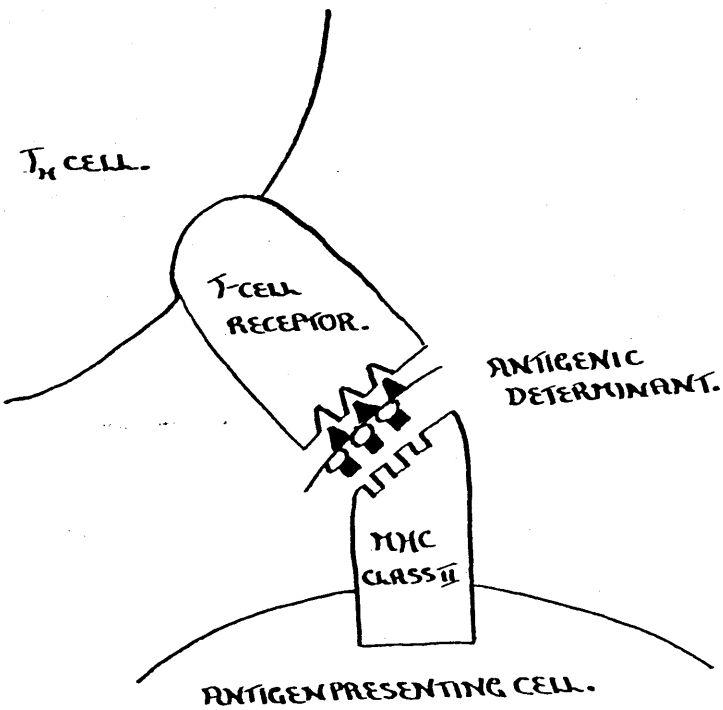
The characteristics of the immune response to foreign antigens - for instance, predominance of helper versus suppressor cells, and of humoral versus cellular responses, as well as specificity and affinity

of antigen recognition, are, in general, consequent upon initial recognition of antigen by T-cells. The antigen-specific receptor of T-cells recognises a bimolecular ligand composed of foreign antigen, usually in non-native form, in association with an MHC class II antigen. This complex is expressed at the surface of an antigen presenting cell (APC).

In general, processing of the original antigen by APC's is necessary to produce a conformation recognisable by T-lymphocytes. The conformation of the immunogenic peptide must allow contact with both the T-cell receptor and the MHC class II molecule. (Heber-Katz et al, 1983; see figure 10.2). Allen et al (1987) identified these two contact sites for the antigen hen egg lysozyme. Each site was composed of three residues interspersed in the primary amino acid sequence. Modelling studies revealed that the antigenic peptide concerned assumes an α-helical conformation which allows the residues that interact with the T-cell receptor and with the MHC class II antigen to segregate on opposite sides of the helix. The α-helix appears to be a common component of the secondary structure recognized by T-cells (Pincus et al, 1983; Sponge et al, 1987). Allen (1987) defined antigen processing as "the change in an antigen which affords it the conformational freedom to form a secondary structure which allows contact with both the T-cell receptor and class II molecule".

From the data available on the processing requirements of different antigens, Allen (1987) proposed that there are at least three types of antigen processing requirement, which depend upon the antigen and T-cells involved. In some cases, processing describes only unfolding of the polypeptide chain to expose the immunogenic determinants. Examples of antigens of this type have been found in lysozyme, ribonuclease, and myoglobin. Other determinants, on these same three proteins, and also on ovalbumin, insulin, and cytochrome c, require proteolytic cleavage to supply the conformational flexibility

Figure 10.2. Recognition of the antigen-MHC class II complex by a T-cell receptor.



The conformation of the antigenic determinant must allow interspersed amino acids in the protein sequence to form contacts with both the MHC class II molecule (■) and the T-cell receptor (▲).

(Based on diagram from Allen, 1987).

needed to form structures which can contact both the MHC class II antigen and the T-cell receptor. A third class of antigenic epitopes seems to be located on a portion of the polypeptide chain which has sufficient conformational freedom in its native state to form the necessary contacts, and does not require further processing. Some determinants on fibrinogen, and, possibly, on some listerial proteins, belong to this class.

We would suggest that the denatured and deglycosylated antigens from irradiated larvae may be processed by antigen presenting cells differently from the corresponding native molecules. Thus, new determinants may be expressed in association with the MHC class II antigen at the antigen presenting cell surface. The bimolecular complex exposed for interaction with helper T-cells therefore may comprise novel epitopes which are not made available after processing the native antigen. As a result, the T-cells activated by processing of these modified antigens should differ from those stimulated during a normal infection. It is postulated that, in this way, the altered processing and presentation of non-native antigens from attenuated larvae may induce a highly potent protective immune response.

Proteins already converted to non-native conformations by direct radiation damage (section 1.10) or indirectly as a result of radiation-induced metabolic inhibition may be able to interact with the MHC and T-cell receptors without uptake and further processing by antigen presenting cells. Dendritic cells, which apparently present antigen without prior endocytosis and intracellular processing (Kapsenberg et al, 1986) might be especially likely to interact with released, denatured antigens in this way. If the modified antigens from irradiated larvae do interact with different antigen presenting cells from normal ones, this might well influence the resulting T-cell response. Allen et al (1985a) demonstrated that different antigen-presenting cells directed activation of particular T-cell

clones. Two T-cell clones responded very differently when the same antigen was presented to each by different antigen presenting B-cell hybridomas.

Presentation of larval antigens during a normal infection does not induce high levels of specific immunity. Hence, during immunisation with irradiated larvae, the parasite antigens might be presented in a novel manner which renders them highly immunogenic. Yet, at the same time, the determinants against which the immune response is directed must be sufficiently similar to the native antigens from normal larvae to elicit an effective memory response upon challenge with normal parasites. We would postulate that irradiation doses which induce optimal resistance may strike the ideal balance between these two aspects of protective immunity, producing antigens which depart sufficiently from normal conformation to induce potent immunity, yet recall the original native determinants effectively enough for immunological memory to be reactivated upon exposure to the normal challenge infection. This explanation might account for the fact that stimulation of immunity by both U.V.- and gamma-irradiated larvae is highly dependent on radiation dose. Below and above the optimal radiation dose (300-400 μW min cm^{-2}; 20 or 50 krad), significantly poorer immunity is induced (Dean et al, 1983).

Our results clearly indicate that irradiated or Actinomycin D-treated larvae do express modified antigens, since the glycocalyx structure is disrupted, and proteins and glycoproteins are denatured and deglycosylated. Nevertheless, the surface antigens expressed by attenuated schistosomula remain sufficiently similar to untreated ones to sustain normal levels of binding by various lectins and antisera (figures 6.10, 6.11, 6.12; sections 6.6, 6.8, 6.9.4.3, 6.9.5). These apparently contradictory findings could support the proposal that the attenuated larvae may present determinants common to native antigens, but in the context of novel, highly immunogenic epitopes generated by

radiation damage.

For instance, the antigens presented to T-lymphocytes after either denaturation or proteolysis may express some normal epitopes, but in association with aberrant sequences generated by irradiation or drug-treatment. Alternatively, the entire immunogenic peptide expressed at the antigen presenting cell surface could be present in the original, native protein, but not correspond precisely to the sequence produced by processing of the normal antigen. This situation could arise when aberrant protein conformation and deficient glycosylation modify the pattern of proteolysis or unfolding by antigen presenting cells. Expression of these altered immunogenic determinants will stimulate different T-cell clones, producing an overall immune response of a different character from that induced by normal larvae. Even if the actual structures which contact the T-cell receptor and MHC class II molecule are conserved in antigens from attenuated schistosomula, differences in the sequence or conformation of regions adjacent to the contact sites could markedly influence the subsequent stimulation of T-lymphocytes. Thus, Allen et al (1985b) observed that two T-cell hybridomas recognising the same peptide determinant in hen egg lysozyme showed very different responses when residues were added synthetically to the amino terminus of this peptide.

The receptors on lymphocytes originally activated by partially modified antigens must retain the capacity to bind native antigens encountered upon challenge with normal cercariae. Subsequent activation and proliferation of memory lymphocytes presumably initiate a potent immune effector response which accomplishes challenge attrition. The atomic mobility of polypeptides might also be important in inducing the anamnestic response to challenge infection. Proteins in solution possess regions of great conformational freedom, which exist in dynamic equilibrium between native and denatured forms (Tainer et al, 1984, 1985; Westhof et al, 1984). Hence, although the antigens presented

during a normal challenge infection exist predominantly in native configuration, they may briefly assume non-native conformations, exposing internal epitopes to which host lymphocytes were primed by immunisation with attenuated larvae. Figure 10.3 describes the proposed models for stimulation of effective immunity by irradiated or Actinomycin D-treated parasites, and expression of this immune response upon challenge infection.

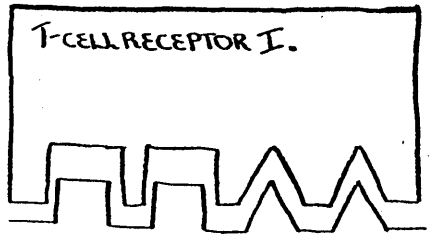
This discussion concentrates on the nature of the antigens which stimulate immunity, rather than attempting to characterise in detail the host immune response to vaccination. However, the observations on polypeptide and carbohydrate epitopes of normal and attenuated schistosomula, presented in chapter 7, do indicate one way in which the response to challenge may differ from that in normally infected hosts, due to vaccination with antigens in a partially modified conformation. Antibodies produced in response to the disrupted carbohydrate sequences on the surface of irradiated schistosomula bind with only low affinity to the native carbohydrate antigens of normal parasites. Assuming that this observation also holds true for receptors of T-lymphocytes activated by vaccination with attenuated larvae, then anti-carbohydrate reactivity is likely to make only a small contribution to the anamnestic response induced by challenge larvae. Thus, as previously concluded by Omer-ali et al (1986; 1988), Yi et al (1986), and Smithers et al (1987), anti-carbohydrate antibodies should not prevent expression of irradiated vaccine resistance by blocking antibody-dependent cellular cytotoxicity or suppressing cell-mediated immunity.

10.2.1. Summary

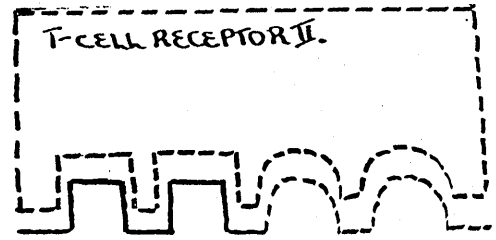
In summary, our data suggest that induction of high levels of

Figure 10.3. Four models for induction of an immune response by antigens from irradiated parasites, and reactivation of that response upon challenge with normal schistosomula.

The term "antigen" here refers to the structure formed by interaction of the MHC class II receptor and the determinants produced by antigen processing. This bimolecular ligand is expressed at the surface of an antigen presenting cell.



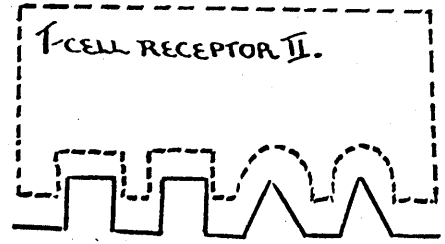
NATIVE ANTIGEN.



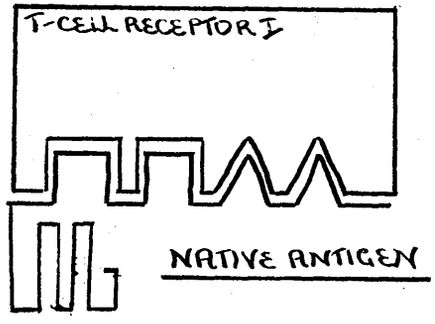
IRRADIATED ANTIGEN.

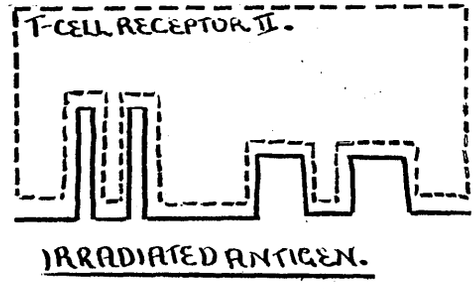
1. INDUCTION OF IMMUNITY: IRRADIATED PARASITE ANTIGEN EXPRESSES SOME NATIVE EPITOPES (⊓⊔) AND SOME MODIFIED ONES (⌒⊔).

RESPONSE TO CHALLENGE:
NATIVE EPITOPES (⊓⊔) CAN ACTIVATE T-CELL II.



NATIVE ANTIGEN.

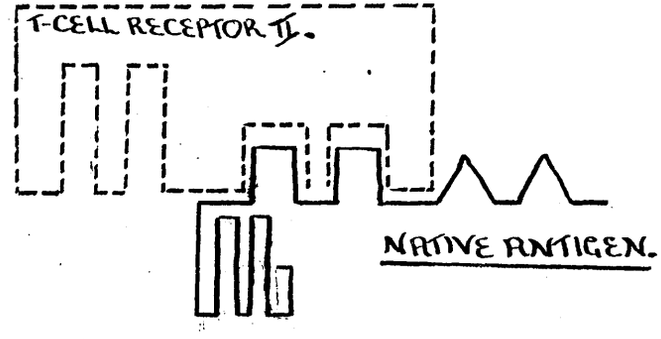


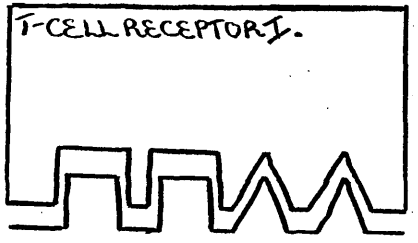


2. INDUCTION OF IMMUNITY: IRRADIATED ANTIGEN EXPOSES EPITOPES (⊓⊔) PRESENT IN THE NATIVE ANTIGEN, BUT FOLDED INTO THE INTERIOR.

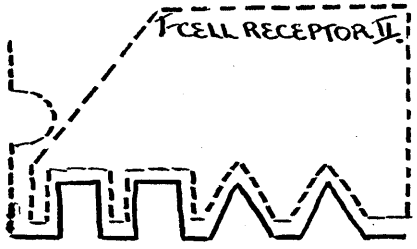
RESPONSE TO CHALLENGE:

EXPOSED EPITOPES ON NATIVE ANTIGEN (⊓⊔) CAN ACTIVATE T-CELL II.





NATIVE ANTIGEN.

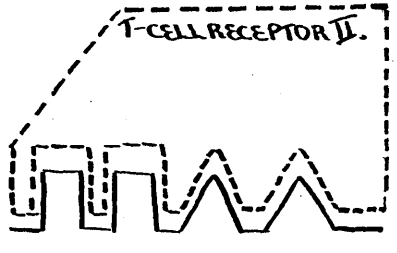


IRRADIATED ANTIGEN.

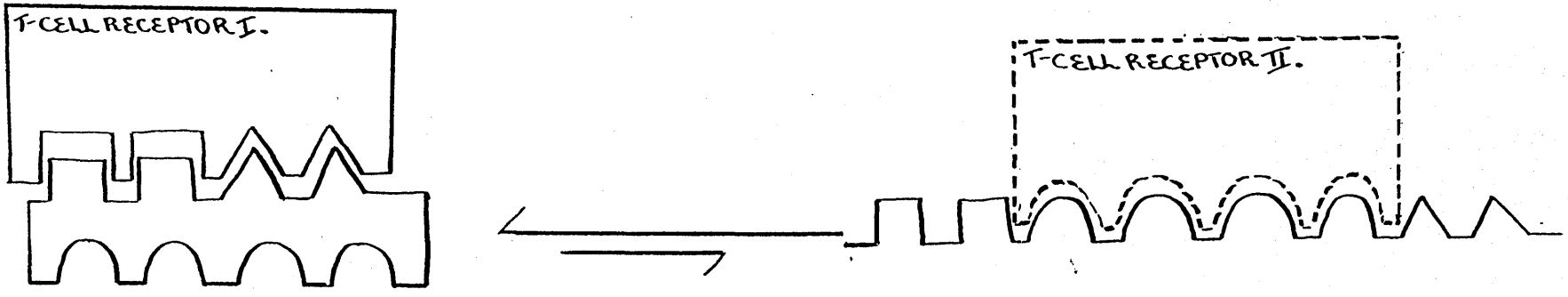
3/ INDUCTION OF IMMUNITY: T-CELL RECOGNISES THE SAME EPITOPES ON IRRADIATED AND NATIVE ANTIGENS. HOWEVER, THE MODIFIED CONFORMATION OF AN ADJACENT PEPTIDE SEGMENT (b), NOT INVOLVED IN CONTACT WITH THE T-CELL RECEPTOR, CAUSES ACTIVATION OF A DIFFERENT T-CELL CLONE FROM THAT RESPONDING TO THE NATIVE ANTIGEN.

RESPONSE TO CHALLENGE:

NATIVE EPITOPES ACTIVATE T-CELL II.



NATIVE ANTIGEN.



NATIVE ANTIGEN.

DENATURED ANTIGEN, e.g. AFTER IRRADIATION.

4. T-CELL II IS ACTIVATED BY INTERNAL EPITOPES (⌒) EXPOSED ON DENATURED ANTIGEN BY IRRADIATED PARASITE.

NORMAL ANTIGEN IS IN DYNAMIC EQUILIBRIUM BETWEEN NATIVE STATE AND THE UNFOLDED FORM, WHICH CAN STIMULATE IMMUNOLOGICAL MEMORY DURING THE CHALLENGE INFECTION.

immunity against schistosomiasis may require participation by a number of antigens, from several different stages in larval development. Potent immunogens may be contained within material derived from the secretions, glycocalyx, surface membrane, or internal proteins of irradiated parasites at each stage. Irradiation seems to alter the structure of these antigens in such a way that they are processed differently from normal ones by antigen presenting cells, and novel epitopes are presented to host T-lymphocytes. Native antigenic determinants common to normal schistosomula expressed in this context of new, highly immunogenic epitopes induce a highly protective immune response against challenge larvae.

It may be noted that other workers, using different vaccination systems - Ro-11 3128 - killed schistosomula (Bickle et al, 1985), and frozen-thawed schistosomula or paramyosin in association with BCG (James, 1985, 1986) - have also suggested that the method of antigen presentation may be the key to inducing high levels of resistance.

10.2.2. Relevance to human immunity.

In areas endemic for schistosomiasis, the total adult worm burden of an individual seems to be acquired as a result of multiple small (trickle) cercarial exposures over an extended period of time (Warren, 1973; Wilkins, 1977). In contrast, the effectiveness of vaccination procedures in the studies presented here was tested by collecting and counting adult worms following a single mass exposure of immunised mice to a large number of cercariae (100-150). It would be important to determine the relevance of these test infections to the situation which would occur in the field, where immunity must be effective against repeated trickle exposures.

Out of the mass exposures commonly used in the laboratory, a high proportion of parasites die before maturity, for reasons other than

protective immunity. Even in naive mice, it is rare for more than half of an original infection of 150 to 200 cercariae to survive to adulthood. Use of large mass challenges may give a false picture of the immune status of a vaccinated host in face of natural infection if the immune mechanisms under study depend on initial exposure of internal antigens by dead and disintegrating schistosomula. This seems to be the case for the immunisation procedures involving paramyosin or glutathione-S-transferases as target antigens (section 1.5.7). Inflammatory responses to these internal antigens, released by damaged or dying schistosomula of a challenge infection, apparently impede migration of the surviving parasites. According to the model proposed here, part of the resistance to challenge observed in experimental hosts immunised with irradiated or Actinomycin D-treated schistosomula may be based on recognition of internal antigens. However, there are so many other antigens which can initially induce immunity and whose recognition will activate immunological memory - glycocalyx, surface membrane or secreted products of living schistosomula - that exposure of internal antigens by dying parasites may not be an essential preliminary to expression of an effective immune response.

The field tests of irradiated vaccines in livestock (Majid et al, 1980; Hsü et al, 1984; section 1.9.2.2.) gave promising results for their effectiveness under conditions of natural exposure. Immunised animals allowed to graze alongside unimmunised controls over a period of months showed levels of resistance at least as high as immunised cattle receiving a mass cercarial exposure at a single time under controlled laboratory conditions.

Thus, there does seem reason to hope that definition of the immune mechanisms operating in the irradiated vaccine model in animal hosts may be relevant to induction of immunity in humans in endemic areas.

10.2.3. Tests and further development of the antigen presentation hypothesis.

Our hypothesis that antigens from irradiated schistosomula may be processed and presented in such a way as to expose novel, highly immunogenic epitopes, might be confirmed and extended by asking the following questions:

1) Is it possible to detect expression by irradiated schistosomula of new antigenic determinants, exposed on antigens in modified conformation?

2) Can such abnormal epitopes induce immunity to cercarial challenge in experimental hosts, or activate helper T-cells in vitro?

3) How do irradiated larvae interact with antigen presenting cells? Can processing and presentation of modified antigens to produce novel epitopes be detected?

4) What are the relative roles of radiation-induced pertubation of the surface membrane, and the metabolic inhibition consequent upon nucleic acid damage, in modifying the conformation of schistosomular antigens to produce potent immunogens?

We might propose to investigate these questions in the following ways.

1). Expression of denatured antigens by irradiated schistosomula.

A number of approaches could be adopted to detect expression of antigens in non-native conformations by irradiated schistosomula. Covalent binding of radiolabelled N-ethylmaleimide, iodoacetamide or p-mercuriphenylsulphonate to the irradiated schistosomular surface, or to secreted material, followed by SDS-PAGE and autoradiography, should identify those antigens whose SH groups are exposed by denaturation (section 6.2). Peptide mapping of these covalently labelled proteins

(Cleveland et al, 1977) should help define especially immunogenic polypeptide sequences. Proteins identified in this way could then be purified by ligand affinity chromatography using, for instance, iodoacetamide coupled to sepharose (Heftman, 1975). A similar approach could be extended to other reagents which covalently label regions of integral membrane proteins exposed at cell surfaces. Such reagents could include acetamido-isothiocyanate stilbene disulphonate (SITS), which labels amino, tyrosyl, histidyl and SH groups, or galactose oxidase in combination with KB^3H_4, to label terminal galactose residues.

Homogenates of schistosomular proteins denatured with urea, or even intact larvae treated with urea as described by Kusel (1970b; 1971) could be used to produce antisera in rabbits or mice. Our hypothesis predicts that such antisera might react preferentially with antigens expressed in denatured conformations by irradiated schistosomula. Such antigens may be cytoplasmic, surface-associated, or secreted. By comparing the immunoblotting patterns of proteins from normal and irradiated parasites with antisera raised against normal or denatured material, antigens preferentially recognised by the latter may be identified. These selected antigens may then be subjected to protease digestion, and the peptides separated by SDS-PAGE (Cleveland et al, 1977). Immunoblotting of the resulting peptide maps should help define the location of the immunogenic epitopes more precisely.

Ideally, such approaches to detect expression of novel, highly immunogenic epitopes should be applied separately to each of the five stages in induction of immunity outlined in 10.1.

2) The role of denatured antigens in stimulation of immunity.

The importance of epitopes expressed on non-native antigens of irradiated parasites in inducing resistance to cercarial challenge

might be assessed by immunizing mice with peptides excised from SDS gels after identification as in (1). Resistance to cercarial challenge would be measured at 4-6 weeks after immunisation.

Alternatively, mice could be immunised with schistosomula exposed to concentrations of urea (less than 1M) which allow the parasite to remain active for a considerable period before death (Kusel, 1970b; 1971). This immunisation procedure should be analogous to the irradiated vaccine in that denatured antigens should be presented by attenuated schistosomula during their curtailed lifespan.

The role of denatured antigens in stimulating immunity could also be verified in vitro, by using irradiated or urea-treated schistosomula, or isolated surface antigens, denatured with urea, in blastogenesis assays with lymphocytes from spleen lymph nodes.

According to our model, antigenic determinants exposed within the irradiated glycocalyx should be important in inducing immunity at stages (1) and (2) in schistosomular development. The immunogenicity of the irradiated glycocalyx could be compared with normal glycocalyx material in lymphocyte proliferation assays, firstly as a crude homogenate, and then as individual fractions separated chromatographically according to molecular weight.

3) The interaction of normal and attenuated schistosomula with antigen presenting cells.

Our hypothesis that irradiated schistosomula are highly immunogenic because they expose antigens in non-native conformations to antigen presenting cells urges that we investigate the molecular pathways of processing and presenting parasite antigens. We could compare the interactions of normal, irradiated and urea-treated schistosomula with different antigen presenting cells. Mouse peritoneal macrophages, readily obtained in quantity, could serve as a prototype

for antigen presenting cells. However, localized parasite-cell interactions might be especially informative. For instance, as described above, invading cercariae must present a major antigenic challenge to the Langerhans and dendritic cells of the skin, while the Kupffer cells of the liver process and present most circulating antigens. Techniques for isolation and study of Langerhans and Kupffer cells are well established (Steinman and Nussenzweig, 1980; Smedsrod et al, 1985).

Both in vivo and in vitro analyses using these antigen presenting cells would be possible. Normal and attenuated larvae could be radiolabelled, either metabolically with ^{35}S-methionine, or on the surface with ^{125}I. Mice could be exposed to such radioactive parasites, or the schistosomula could be cultured in vitro with isolated antigen-presenting cells. Direct autoradiography of excised exposure sites, or harvested cells, would trace the fate of labelled parasite antigens. Immunoprecipitation, using schistosome-specific antiserum, or monoclonal antibodies specific for discrete epitopes, of labelled surface and internal components of antigen-presenting cells, should indicate parasite antigens selected for processing. Techniques of this type, for analysis of processing of radiolabelled antigens by macrophages, have been developed for the intracellular parasite Listeria monocytogenes, allowing antigen presentation to T-cells to be defined in considerable detail in this system (Allen et al, 1984).

4) The contributions of nucleic acid and membrane damage in enhancing the immunogenicity of irradiated schistosomula.

Denatured or aberrant antigens may arise and persist as the result either of metabolic inhibition in irradiated schistosomula or as a direct effect of oxygen radicals perturbing the parasite surface

membrane.

Just as Actinomycin D has allowed us to study in isolation the consequences of DNA damage for schistosomular immunogenicity, it may be possible to determine the effects of radiation injury at the surface alone. It would be most valuable to devise radiomimetic systems which inflict oxygen radical damage principally at the outer surface - for instance, hydrogen peroxide in combination with reducing agents, or lactoperoxidase with an appropriate substrate. Figure 8.21, in section 8.2.3.3., suggests that it may indeed be possible to develop a H_2O_2 - based system which causes O_2 radical damage at the surface but does not inhibit protein synthesis. We could assess whether oxygen radical damage at the schistosomular membrane stimulates interaction with the immune system by testing the treated parasites in vitro in lymphocyte proliferation assays and in vivo in mouse protection experiments.

Another approach to studying how factors other than nucleic acid damage contribute to the immunogenicity of irradiated schistosomula could involve the use of Fenfluramine (Janet Jones, unpublished data). This drug specifically inhibits phospholipid synthesis (section 4.2.1), hence might allow us to study the effect of disruption of membrane lipid organisation independently of inhibited protein synthesis.

10.3 Overview: Induction of immunity by irradiated larvae of Schistosoma mansoni.

This thesis has aimed to relate the biochemical effects of irradiation on larvae of S. mansoni to the enhanced immunogenicity of irradiated cercariae. The studies described indicate that antigen presentation may be the key to induction of potent protective immunity.

Observations on protein, glycoprotein and phospholipid synthesis, and antigen expression, suggest that irradiated schistosomula express antigens in modified, non-native conformations. Such denatured antigens may be generated by normal environmental stresses, by direct radiation damage to parasite molecular structures, and as a result of radiation-induced disruption of parasite synthetic activity. Irradiated parasites synthesize severely reduced amounts of the heat-shock proteins which assist non-native proteins to achieve their mature conformations, or else ensure their removal and degradation. Hence, these aberrant antigens are predicted to persist and accumulate in irradiated schistosomula.

It is proposed that the modified conformation of such antigens alters the pattern of processing by host antigen presenting cells. Thus, novel determinants, not presented after processing of the native molecules, are made available to helper T-lymphocytes. As a result, the T-cells activated by antigens from normal and irradiated larvae are expected to differ in both antigen specificity and affinity. In this way, the characteristics of the immune response stimulated by irradiated schistosomula may ensure especially effective attrition of challenge infection.

Our results indicated that, despite variation between separate batches of parasites, the abbreviated lifespan of radiation-attenuated schistosomula could be divided into several stages, each characterised by a different state of synthetic activity and antigen expression. It

is inferred that, at each stage, the irradiated parasites may expose different antigens for interaction with separate compartments of the host immune system. Antigens from the cercarial glycocalyx and trilaminate membrane and from schistosomular secretions released during the initial period following transformation may be processed and presented in the skin. Later, as the attenuated schistosomula die and disintegrate in the lung, antigens in the parasite surface and body may interact with antigen presenting cells migrating through this tissue. In this way, a broad spectrum of immune responses should be activated, capable of effecting attrition of challenge schistosomula at successive stages in their migration and development.

This model for induction of immunity by irradiated larvae of S. mansoni may suggest considerations for future vaccine development. A variety of larval antigens might be required to activate a broad spectrum of immune mechanisms, capable of accomplishing attrition at several stages in migration and development of challenge schistosomula. Effective presentation of immunising antigens at each stage appears to be essential to stimulate a highly protective immune response.

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