Dynamic aspects of the nematode surface

A thesis presented for the degree of
DOCTOR OF PHILOSOPHY
by

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ABBREVIATIONS

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

cAMP	cyclic adenosine monophosphate
AC	adenylate cyclase
AF	aminofluorescein
Biot/strep	biotin/streptavidin
BME	ß-mercaptoethanol
BSA	bovine serum albumin
Con A	concanavalin A
CTAB	cetyltrimethylammonium bromide
DAG	diacylglycerol
DEC	diethylcarbamazine
DiI18	1,1-di (octadecyl)-3,3,3',3'-tetramethylindocarbocyanine perchlorate
D_L	lateral diffusion coefficient
DMSO	dimethylsulphoxide
DSC	differetial scanning calorimetry
DTAF	dichlorotriazinylaminofluorescein
F	fluorescence
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FM	fluorescein-5-maleimide
FRAP	fluorescence recovery after photobleaching
GC	guanylate cyclase
cGMP	cyclic guanosine monophosphate
Gp	G-protein
H_{II}	hexagonal phase (lipid)
HHS	hyperlipidaemic human sera
IAF	6-iodoacetamidofluorescein
IBMX	3-isobutyl-1-methyl-xanthine

IMP	intra-membranous particle
IP_3	Inositol trisphosphate
L1, L2, L3 & L4	first, second, third and fourth larval stage
LYH	lucifer yellow hydrazide
MEM w/o methionine	minimal essential medium without methionine
Mc540	merocyanin 540
mf	microfilaria(e)
M_r	relative molecular weight
MSL	muscle-stage larva(e)
NBD	nitrobenzoxadiazole
NBL	new-born larvae
NHS	normal human serum
NJS	normal jird serum
NR	nile red
NRS	normal rat serum
N2	wild-type strain of *Caenorhabditis elegans*
PAGE	polyacrylamide gel electrophoresis
PA-TSC-SP	periodate-thiosemicarbazide-silver proteinate
PBS	phosphate-buffered saline
PIP_2	phosphatidylinositol bis-phosphate
PKC	protein kinase C
PLC	phospholipase C
PMT	photomultiplier tube
PMSF	phenylmethylsulphonylfluoride
PNA	peanut agglutinin
R	receptor
%R	percentage recovery
RH	rhodamine
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N',-tetramethylenediamine

t.l.c.	thin-layer chromatography
TG	triglyceride
TMB-8	3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester
TPA	12-0-Tetradecanoylphorbol 13-acetate (phorbol myristate acetate)
U.V.	ultra-violet
W-5	N-(6-aminohexyl)-1-naphthalenesulphonamide
W-7	N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide

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Summary

The biophysical properties of the surface of a range of nematode species and their developmental stages were examined in an attempt to understand the nature of the host-parasite interface. Fluorescent probes were used to label the nematode surface, and fluorescence recovery after photobleaching (FRAP) was used to measure the freedom of each probe to diffuse in the plane of the surface. These methods were applied to living, intact parasites, and the analysis confined to the outermost surface.

In all cases, surface lipid was unusual in its selectivity for the insertion of the fluorescent lipid probes. In adults, it appears that the major restriction to insertion of lipids is a charge effect such that negatively-charged probes and neutral probes are easily inserted, whereas lipid probes with a positive charge are excluded. It is thought that this may be caused by exposure of positively-charged amino acids on the surface by a protein associated with the epicuticle.

A large, non-diffusing fraction of lipid was detected on the surface of all the adult parasitic nematodes examined using the anionic lipid probe 5-N-(octadecanoyl) aminofluorescein (AF18) and the FRAP technique. The general immobility of AF18 in parasitic forms was in stark contrast to the surface of the adult free-living nematode *Caenorhabditis elegans*, in which this probe diffuses rapidly and with a large mobile fraction. This fundamental difference in lipid diffusibility prompted the suggestion that immobility of a proportion of surface lipid is an adaptation to physicochemical and biological environments encountered upon infection. It was therefore interesting to find that certain anthelmintics (CGP 20376 and Suramin) could induce mobility in otherwise immobile surface lipid.

The suggestion that the nematode epicuticle may contain a heterogeneous lipid layer was prompted by results which showed unrestricted diffusion of a fluorescent cholesterol probe (NBD-chol). In addition, certain probes are internalized after a period of time, whereas others remain on the surface. Differential mobility of fluorescent lipids in FRAP experiments suggests the presence of lipid domains. It is

proposed that the gel-phase or solid domain is maintained by underlying proteins, e.g. cuticlin, and that the fluid-phase domain is from host-derived lipids, e.g. serum cholesterol. Several alternatives to this hypothesis exist, including one where lipid domains are maintained by lipid-lipid interactions leading to phase separations.

The change of physicochemical environment, encountered by the infective larva upon transition from vector or free-living environment to the mammalian host, was found to have rapid effects on the biophysical properties of the larval surface. In all cases studied, there was a rapid increase in lipophilicity of the surface for the AF18 probe. In *Strongyloides ratti* this increase in lipophilicity was accompanied by the release of anionic material labelled with fluoresceinated cationised ferritin. In *Trichinella spiralis*, changes in structural organization were monitored using differential scanning calorimetry (D.S.C.) and repeated with adults for comparison.

The physical changes which take place at the surface to allow this increase in lipophilicity remain a matter of speculation. However, the importance of this change is outlined by experiments which appear to demonstrate uptake of host lipids, and experiments which point out candidates for the infection stimuli.

It has become clear that parasitic nematodes present a highly unusual surface to their hosts, and that this extends to the characteristics of its lipid. This is most notable for the transition to the mammalian host environment, and could provide important clues as to the resistance of parasitic nematodes to host defence mechanisms.

Chapter One

General Introduction

1. GENERAL INTRODUCTION

1.1 The Nematodes

Nematodes represent a distinctive phylum of metazoan organisms which have been able to exploit nearly every conceivable aquatic and terrestrial habitat in the world. Within this phylum, the life-style ranges from the completely free-living to the complex parasitism of plants and vertebrates. The lack of interspecific morphological variation between different nematodes belies their ecological diversity and success. A schematic classification of the nematodes of major interest as medical or veterinary parasites, or as experimental models is shown in table 1.1.

A typical nematode is an unsegmented, elongated, cylindrical worm which is encased in a very tough, semi-transparent extracellular cuticle (figure1.1). Resistance of the intact nematode to desiccation strong chemicals (e.g. glutaraldehyde), detergents, and extremely low temperatures (Cassada and Russell, 1975) has been attributed to the special properties of the cuticle. Moulting of the cuticle is associated with changes in developmental stage, a process which may be similar in many respects to that of the Insecta. This complex process normally occurs four times before the adult stage is reached (figure 1.2).

Successful completion of the life-cycle to adult parasites normally demands that L3 infective larvae experience severe environmental shifts, going for example, from the free-living environment to the homeostatic mammalian host environment. The mode of infection (figure 1.3) determines the type of environmental stresses involved, and specific physical and chemical signals of the new host environment provide cues for further developmental changes (Lackie, 1975).

The emphasis of this study is to investigate the properties of the cuticle in a variety of nematodes. First, I would like to deal with the nematodes themselves, and then the

Table 1.1 : A schematic classification of the nematodes of major interest as medical or veterinary parasites, or as experimental models (adapted from Maizels & Selkirk, 1988).

Phylum Nematoda

Adenophorea

Trichinella
Trichuris

Secernentea

ASCARIDS
Ascaris
Anisakis
Toxocara

FILARIAE
Acanthocheilonema
Brugia
Dirofilaria
Litomosoides
Onchocerca
Wuchereria

RHABDITIDS
Caenorhabditis
Strongyloides

STRONGYLIDS
Ancylostoma
Dictyocaulus
Haemonchus
Necator
Nippostrongylus
Ostertagia
Trichostrongylus

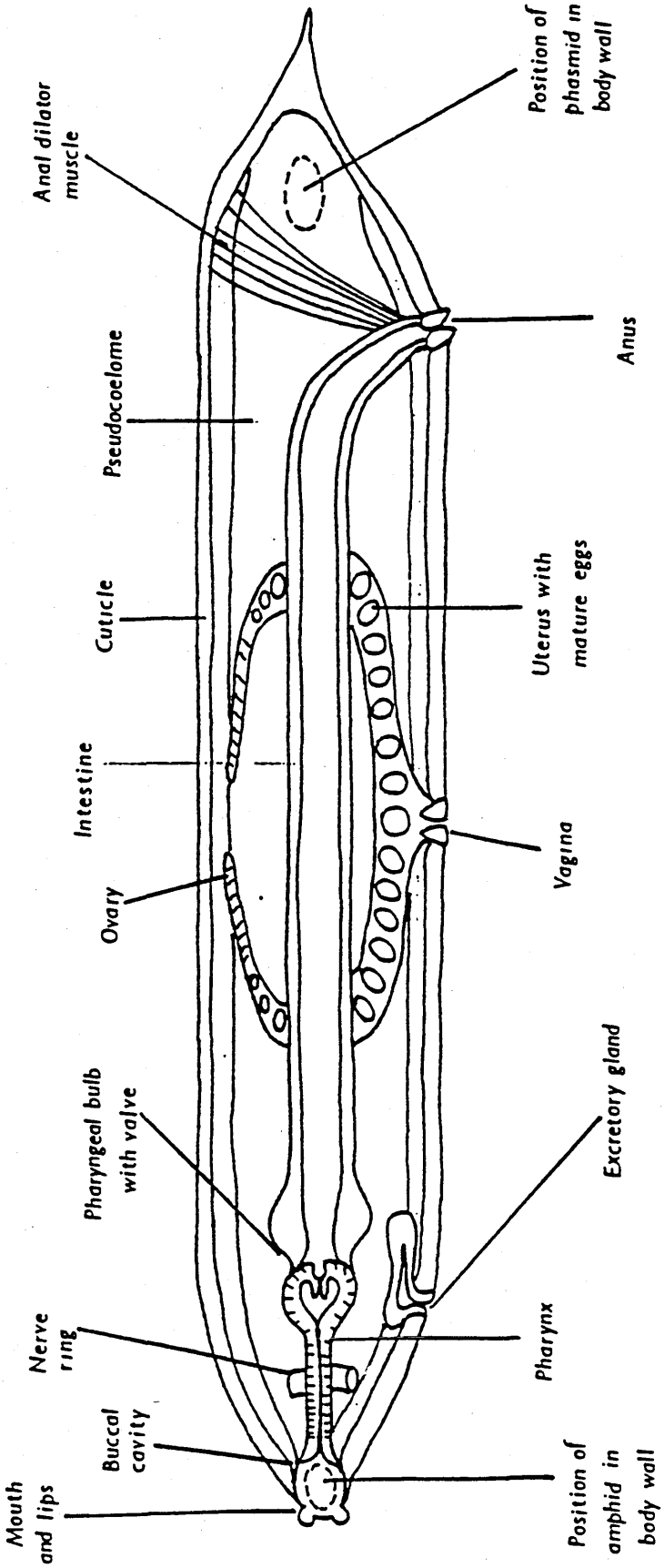


Figure 1.1 Schematic diagram of nematode morphology.

Figure 1.2 Generalized nematode life-cycle

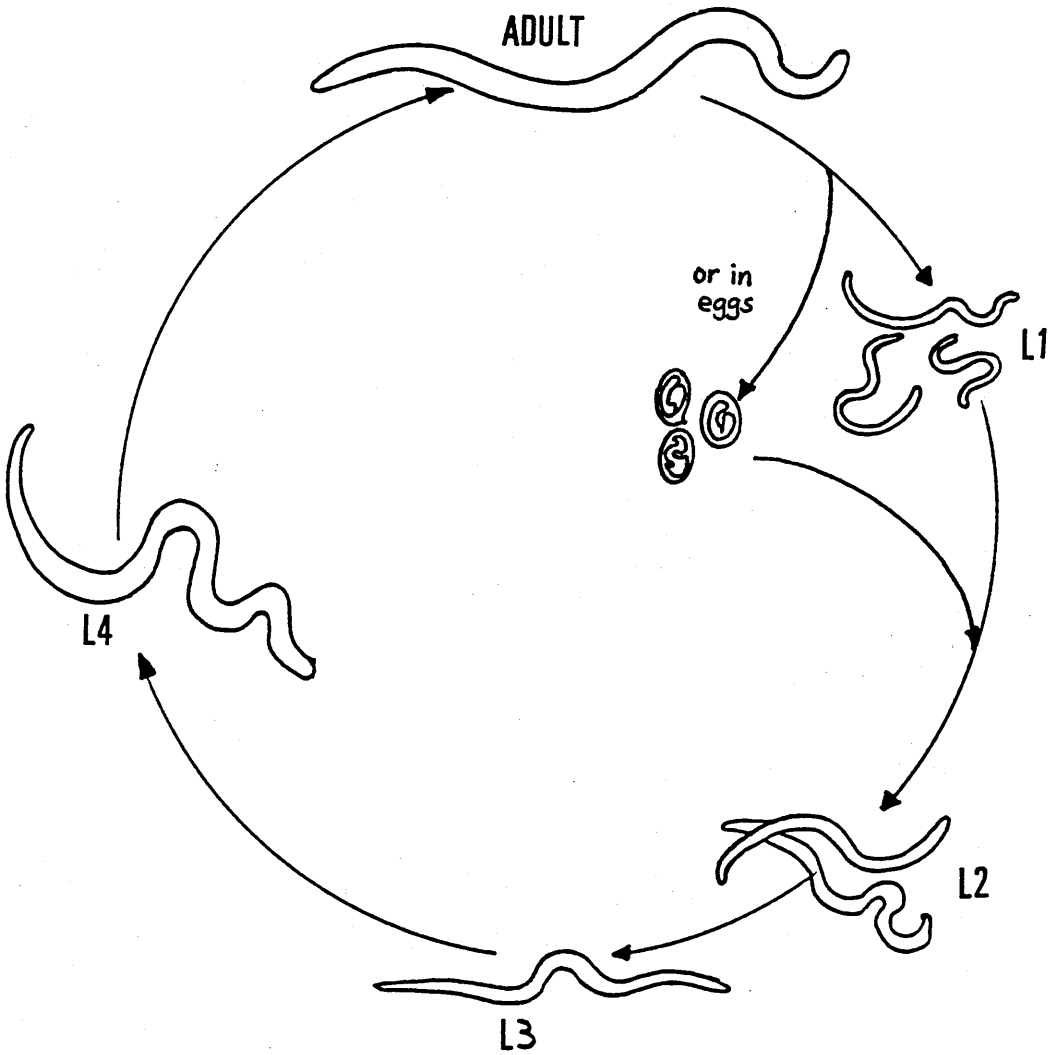
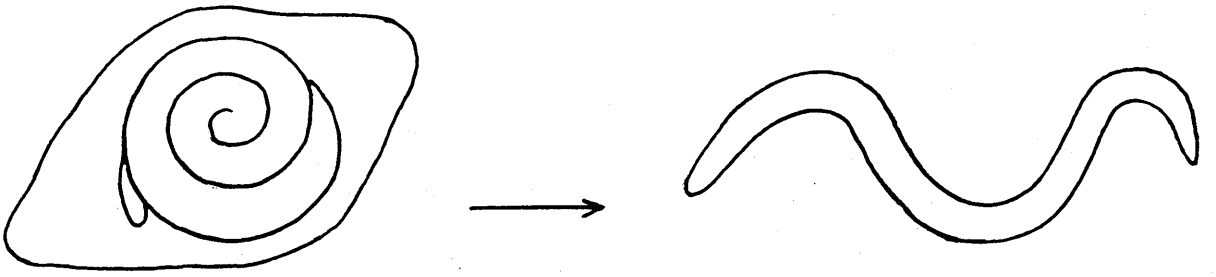


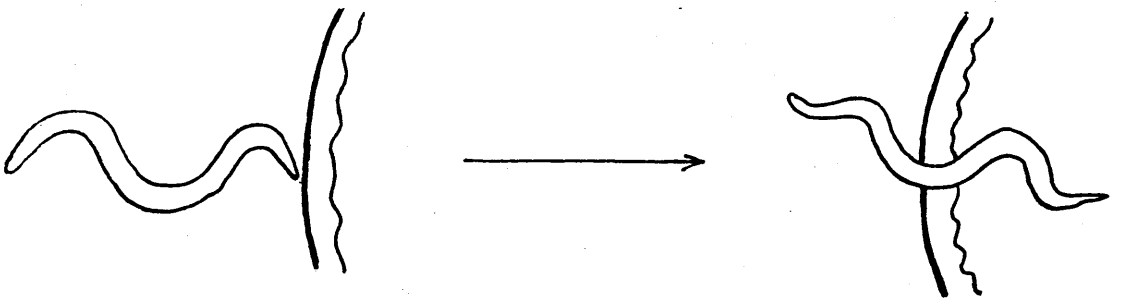
Figure 1.3 **MODE OF INFECTION**:

(a) INGESTION e.g. ***Trichinella spiralis***



INFECTED MUSCLE → STOMACH Pepsin & HCl → INTESTINE Bile & Trypsin → SNAKE-LIKE MOVEMENT

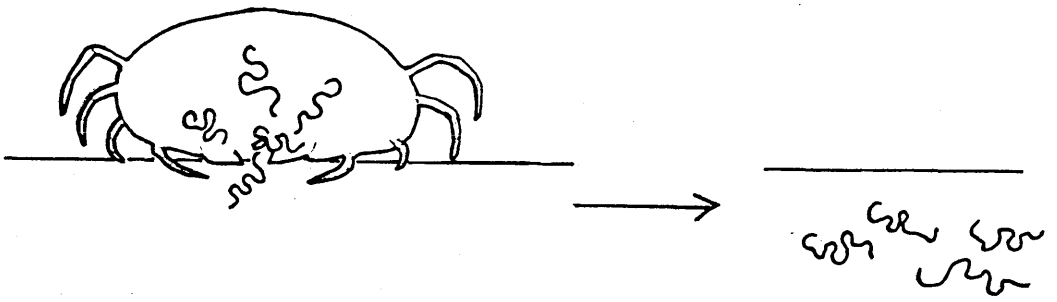
(b) SKIN PENETRATION e.g. ***Nippostrongylus brasiliensis***



FREE-LIVING IN RAT FAECES → PENETRATES

(c) MIGRATION FROM ARTHROPOD VECTOR INTO WOUND SITE.

e.g. ***Acanthocheilonema viteae***



BLOOD-FEEDING TICK → LARVAL MIGRATION

general structure and properties of the cuticle, with particular emphasis on the outermost layer, the epicuticle.

1.2 Parasitic nematodes: their life-cycles & diseases

1.2.1 Ascarids

(i) ***Ascaris***: *Ascaris lumbricoides* is the largest of the intestinal nematodes of man, the female being 30 cm long, with several hundred million people infected globally. The extent of transmission is largely determined by human faecal disposal practices. The closely related animal parasite, *A. suum*, is a very common nematode of pigs and this is the parasite which is most often used in experimental studies of *Ascaris* and the infection.

(ii) ***Toxocara***: Toxocariasis in humans is a zoonotic infection brought about by the accidental ingestion, usually by children, of infective eggs of *Toxocara canis* and less commonly, *T. cati*. Children become infected by ingesting eggs which are promiscuously distributed by the dog. Cats are incriminated to a lesser extent, presumably because these animals are somewhat more fastidious in defaecation habits. The normal life-cycle of the parasite is carried out in the canine or feline female and her off-spring.

Infection with *Toxocara* in man is referred to as a paratenic infection. This infection may result in two different manifestations of the disease: visceral larva migrans, and ocular toxocariasis. Visceral larva migrans is the term which was proposed by Beaver (1952) for the prolonged migration of *Toxocara* larvae in tissues other than skin with the formation of small widespread granulomas. The disease is most often found in young children who typically have a marked eosinophilia and an enlarged liver. Lesions are most common in the liver and lungs but also occur in brain and eyes. Ocular toxocariasis is the condition causing severe pathological effects in the eye, often resulting in blindness.

1.2.2 *Trichinella*

Unlike most human helminths, this one is almost entirely absent from the tropics; it is primarily a parasite of Europe, the United States, and arctic regions. In domestic cycles throughout the world man usually becomes infected by eating cyst-containing pork from domesticated pigs (figure 1.4). In feral cycles meat from wild boar or bears can give rise to infections.

Trichinosis, caused by *Trichinella spiralis*, is a nematode disease with very low vertebrate-host specificity, and unusual mode of transmission. Short-lived adult infections in the guts of a wide range of carnivorous and omnivorous mammals give rise to large numbers of invasive new-born larve (NBL) which migrate to voluntary muscle sites throughout the bodies of these same hosts. They have been found in practically every organ of the body but will only become encysted in voluntary muscle. After entering muscle fibres the worms grow rapidly to a length of 1mm, ten times their original size, and become sexually differentiated. They finally roll themselves into a spiral and are infective after about 18 days.

The main pathogenic phase of the *T. spiralis* life-cycle in man is the population of migrating and encysting larvae. The clinical course of trichinosis is very irregular, with gastro-intestinal symptoms or fever initially, and then there is usually puffiness of the face and more serious cardiovascular and neurological problems.

1.2.3 Filariae

Adult filarial nematodes are thin, thread-like worms, between 4 and 40cm long. They live, for long periods of time, in the connective tissues of the vertebrate host where they produce the first of the four stages in the life-cycle, the microfilariae (L1's) which may

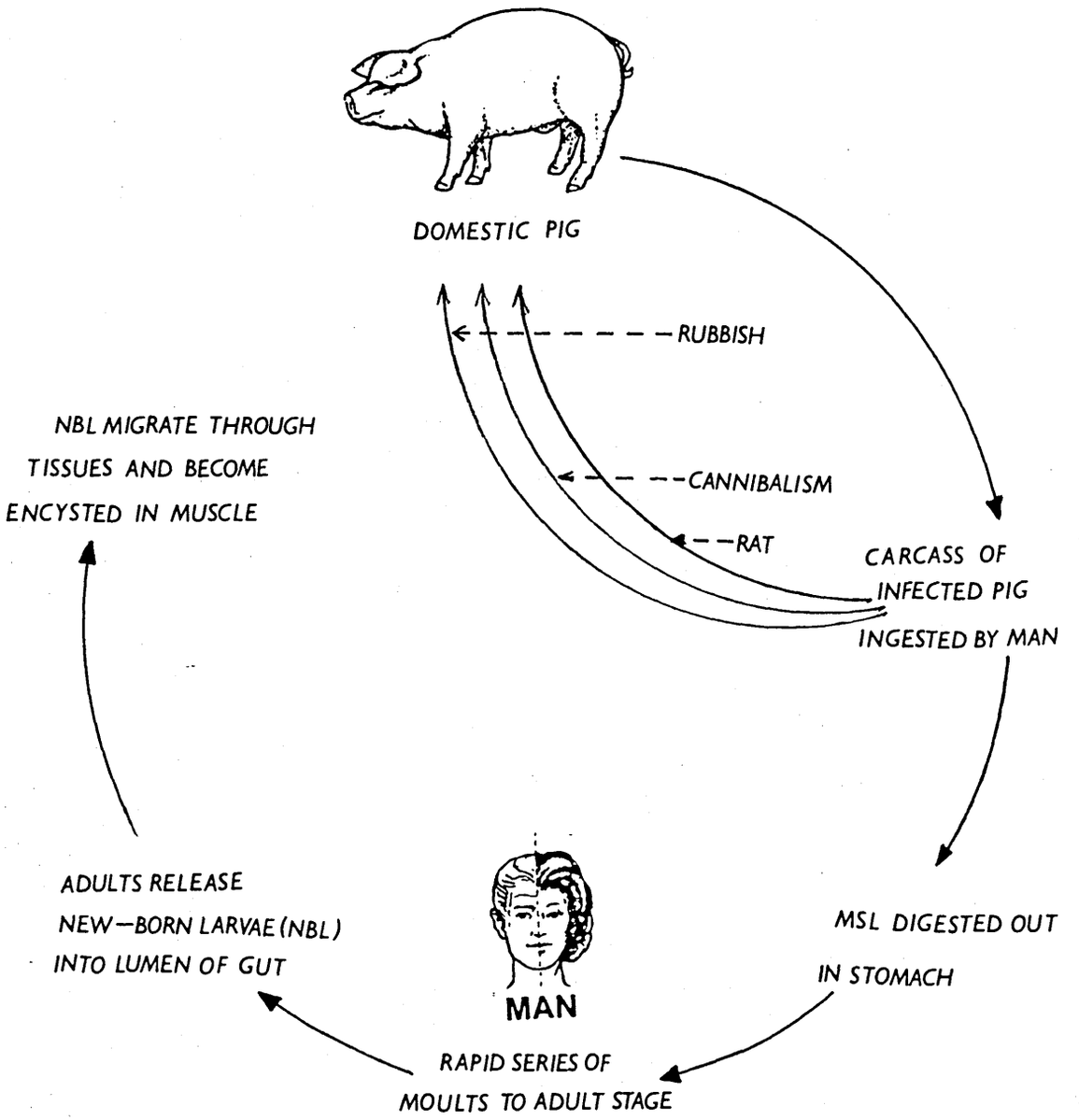


Figure 1.4 Domestic cycle of transmission of *Trichinella spiralis*

be sheathed or unsheathed. These are then so distributed that they can be ingested by the appropriate blood-sucking arthropods. For the next stage of their development, L2's moult to infective L3's and are re-introduced into the vertebrate host in a subsequent blood meal, after which development proceeds through the L4 to the adult stage.

(i) Lymphatic filariasis

Two filarial species *Wuchereria bancrofti* and *Brugia malayi*, which are very similar morphologically, cause bancroftian and brugian lymphatic filariasis, respectively. Bancroftian filariasis probably affects over 250 million people in the tropics and subtropics of the world, whereas brugian filariasis is mainly restricted to rural areas in S.E. Asia because of its restriction to *Mansonia* mosquito vectors. *W. bancrofti* is transmitted most commonly by species of *Culex*, *Anopheles* and *Aedes* mosquitoes.

During development to adults, which takes a few months, the host is in a state of developing sensitivity and allergy, and may develop eosinophilia and skin rashes. The presence of adults in the lymph glands may produce allergic flares (due to the presence of adult filarial antigens), accompanied by lymphangetic fever, leaving residual swellings. The pathological changes in the lymphatic system are usually slow and depend on a cumulative input of adult worms, since one L3 can only result in one adult. In its later stages, lymphatic filariasis is associated with gross swellings of the limbs and genitalia known as elephantiasis.

Experimental models for use in the laboratory include *Brugia pahangi*, and *Litomosoides carinii*. *L. carinii* is not closely related to the other filarials causing lymphatic filariasis, but does cause a natural infection in cotton rats. Both sexes of adult *L. carinii* live in the pleural cavities of the rat and sometimes in the peritoneal cavity. Sheathed microfilariae are in the blood (day and night). A major difference, however, is in the development of the *L. carinii* microfilaria to the infective stage, which is through the tick

Ornithonyssus.

(ii) Onchocerciasis

Infective L3's of *Onchocerca volvulus* are transmitted to humans by the blackfly, *Simulium* (the buffalo gnat) which breeds in fast-flowing rivers. The disease caused by *O. volvulus* called onchocerciasis, causes considerable morbidity across Africa and relatively minor areas of infection in Brazil, Colombia, Guatemala, Venezuela, Mexico and Yemen.

The most notable feature of onchocerciasis is the development of fibrous nodules enclosing the worms. These occur on bony prominences such as the iliac crests, the head and the knees. Adult worms release unsheathed microfilariae which are found in the skin, and it is these microfilariae which cause the severe pathological disease. Microfilariae escaping from nodules on the head can invade the tissues of the eye, leading to blindness (river blindness). About 340,000 people suffer from this extreme form of onchocerciasis, and in some Ghanaian villages 15 per cent of the population is permanently blind. Another severe effect of microfilarial migration is onchodermatitis, which is due to changes in the skin which leave it thin, inelastic, fibrous and painful.

Laboratory investigations of onchocerciasis are hindered because, unfortunately, there is no convenient laboratory host for *O. volvulus*. However, there is a parasite called *Acanthocheilonema viteae* (previously known as *Dipetalonema perstans*)which has some similarities with *O. volvulus*, primarily in the fact that it releases unsheathed microfilariae. *A. viteae* is found naturally infecting the Libyan jird *Meriones*. A major difference, however, is in the development of the *A. viteae* microfilaria to the infective form, which takes place through the argasid tick *Ornithodorus* rather than the blackfly. A generalized life-cycle of *O. volvulus* is shown in figure 1.5, with *A. viteae* (the laboratory model) for comparison.

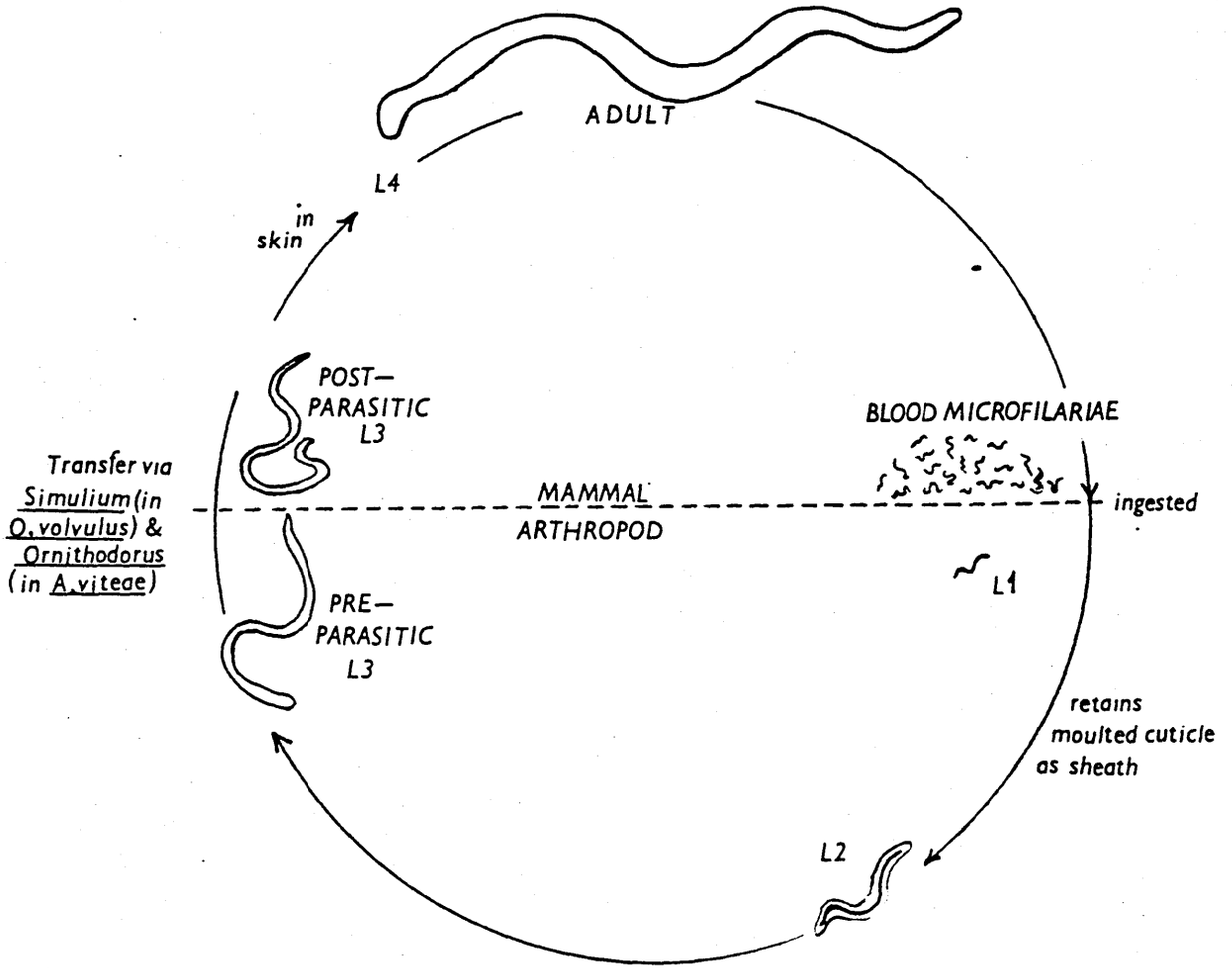


Figure 1.5 Generalized life-cycle of *Onchocerca volvulus* with *Acanthocheilonema viteae* (a laboratory model) for comparison.

1.2.4 Rhabditids

The order *Rhabditida* is of particular interest from an evolutionary point of view since it contains nematodes showing every imaginable gradation from free-living forms to strict parasites. Members of the family *Strongyloididae* have bridged the gap between free-living and parasitic existence by a method peculiar to themselves - a true alternation of generations, so that some L3's develop into free-living adults, and others infect a suitable host and develop into adult parasites.

Infection with *Strongyloides stercoralis* occurs either when the L3 larvae penetrate the skin, or when they are ingested and develop directly within the gut. The life-cycle is complicated by the existence, in some circumstances, of an entirely free-living cycle of sexually reproducing adult forms and their larvae in the soil. This alternative cycle can produce the so-called heterogonic L3 larvae that are also able to infect man.

After skin-penetration, some larvae remain in the skin for a long time, causing redness and intense itching. However, from the third day onward most of the larvae migrate to the lungs where they may cause acute inflammation. Development to adult form is in the lungs, after which they migrate to the gut via the trachea and throat. Unlike most other nematode diseases, strongyloidosis can be lethal, especially in those patients who have a reduced ability to mount cell-mediated immunity.

1.2.5 Strongylids

The large order *Strongylida* includes the blood-feeding hookworms of man, *Ancylostoma duodenale* and *Necator americanus*. The skin-penetrating L3 larvae cause an irritating 'cutaneous larva migrans' and migrate via the heart and lungs to the gut where they develop into the adult form. Anaemia is a prominent symptom of infection due to blood-

feeding by adults.

The trichostrongylids predominate as nematode parasites of veterinary importance. These include *Dictyocaulus viviparus*, which is a lung parasite, and *Ostertagia ostertagi* and *Haemonchus contortus* which are blood-feeding stomach parasites.

Nippostrongylus brasiliensis is a skin-penetrating nematode with adults parasitic in the rat intestine. This is a much used laboratory nematode in immunological, developmental and physiological studies.

1.3 The Nematode Surface

1.3.1 The Cuticle

The nematode surface is comprised mainly of a tough, but flexible extracellular cuticle which is secreted by the hypodermis (Bonner and Weinstein, 1972; Martinez-Palomo, 1978; Bird, 1980). A number of surface invaginations of the nematode body wall are also cuticle-lined. Hence, cuticle covers the inner surfaces of the buccal cavity, pharynx, excretory pore, vulva, chemosensory pits and rectum. Normally there is little variation in the gross features of the cuticle in animal parasites (with the exception of the copulatory bursa), although some plant parasites and some free-living species have elaborate protuberances which may have a sensory function.

The transverse grooves in the nematode cuticle give them their segmented appearance and are known as annulations. It may be that transverse annulations are present in varying degrees in all nematodes and are associated with their characteristic dorsoventral undulatory movement. Longitudinal markings usually take the form of longitudinal ridges or of alae. Functionally, they probably assist locomotion and may permit slight changes in the width of the animals.

Considerable variation in cuticle structure exists between different nematode species and between different stages of the same species (reviewed by Bird, 1980). Despite this variation, a basic pattern exists (see figure 1.6), in which the outermost layer, the epicuticle, is always present. The three main layers or zones of the cuticle are the cortical, the median and the basal. Bird (1984) prefers the use of the term "zone" since often there are no distinct boundaries between the so-called layers, and the term " zones" emphasizes that the cuticle is a closely-linked living structure.

The cortical zone is often divided into an external cortical zone and an internal cortical zone. The outermost surface of the external cortical zone consists of the epicuticle, an osmiophilic layer which often appears as a triple-track structure, but occasionally due to overstaining, the three layers can appear equally electron dense (Bird and Rogers, 1965; Bird, 1968). The triple-track structure is most often seen in infective-stage larvae, but difficult to resolve in adult nematodes (Storey et al, 1988). In infective-stage nematodes, the epicuticle can be divided into an outer membrane of about 7nm, a middle, relatively non-osmiophilic layer of about 20nm and an inner osmiophilic layer which varies from about 10 to 30nm. The external cortical zone is also thought to contain proteins linked by non-reducible bonds which would make it a highly resistant layer (Fujimoto and Kanaya, 1973; Betschart and Jenkins, 1987). The inner cortical zone appears to be biochemically active in some nematodes as both enzymes and RNA have been detected in it (Anya, 1966).

The median zone usually appears homogeneous and devoid of structure, but sometimes it is traversed by thin columns of material which connect the cortical layer to the basal layer. It is difficult to resolve in some nematodes and appears to be absent in others such as the dauer larva of *C. elegans* (Bird, 1980).

The basal zone is perhaps the most interesting and structurally complex of the three main zones of the nematode cuticle. As illustrated in figure 1.6, it may be made up of striations which are thought to be blocks of protein separated by membranes (Bird, 1980).

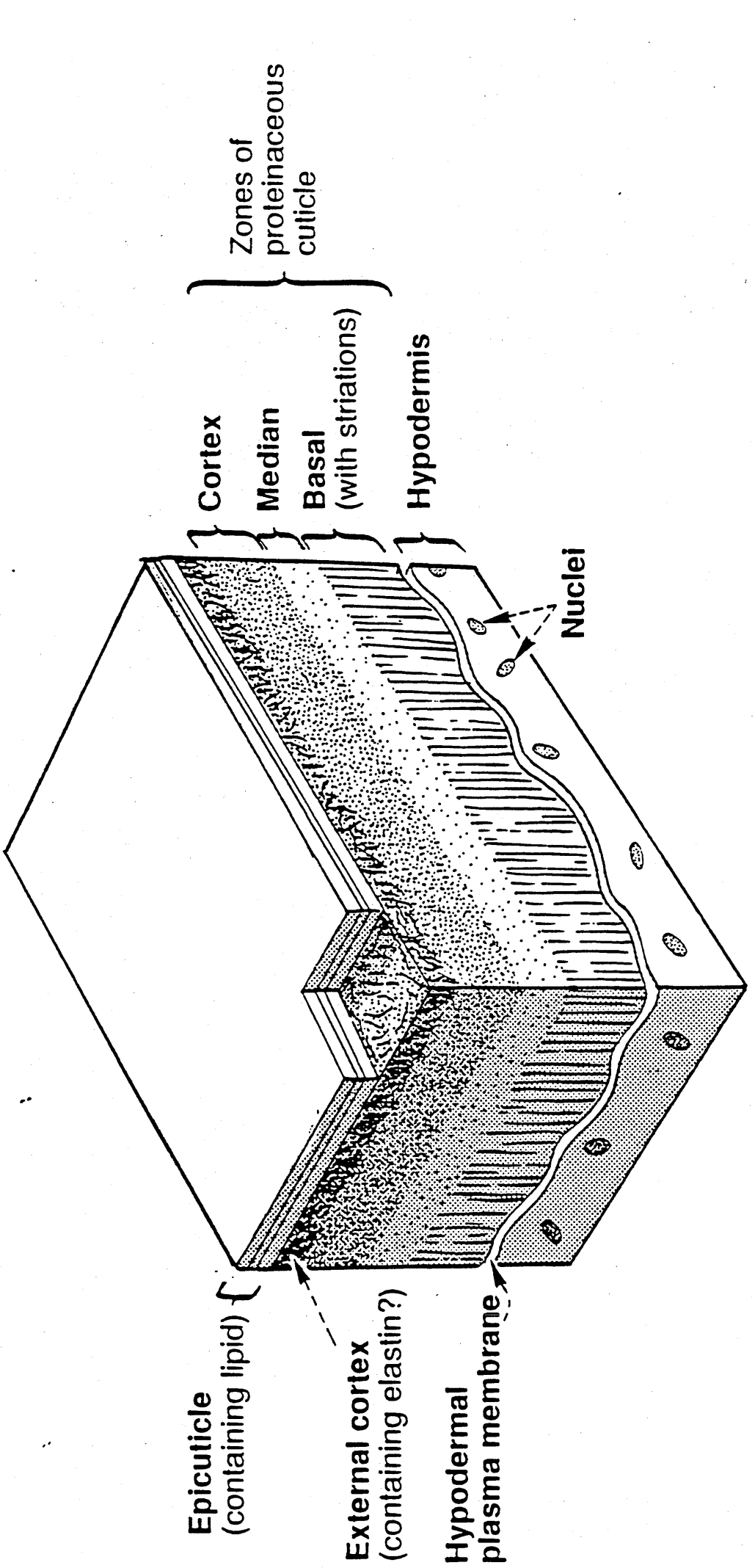


Figure 1.6 Generalized diagram of cuticle structure.

Their function is not yet understood but they may have something to do with survival mechanisms since this pattern is usually found in infective-stage larvae and in dauer larvae of *C. elegans*.

Variability in cuticle morphology is often, but not always, associated with parasitism. At one extreme, the cuticle is virtually non-existent in adult females of *Bradynema* (Riding, 1970) where the hypodermal membrane covers the surface in the form of differently shaped microvilli; and at the other extreme, the multilayered cuticle of the animal parasite *Strongylus equinus* is up to 100 μm in thickness (Bird, 1958).

1.3.2 Chemistry of the cuticle

Detailed information on the chemistry of the nematode cuticle has been obtained from research on the larger ascarids such as *A. lumbricoides* (Bird, 1957; Anya, 1966; McBride and Harrington, 1967). The reason for this is, of course, that the majority of nematodes are far too small for microdissection of cuticles free of adhering material. These workers established that, apart from water, the cuticle consists predominantly of protein with traces of lipid and carbohydrate.

Recent research on the cuticles of the free-living nematode *Caenorhabditis elegans* (Cox et al, 1981a,b) and the parasitic nematodes *Acanthocheilonema viteae* (Betschart and Jenkins, 1987) and *Onchocerca volvulus* (Titanji et al, 1988) have shown that the bulk of the cuticle contains collagen-like proteins held together by disulphide bridges and small amounts of carbohydrate. The work on *C. elegans* was extended to include the cuticles of various developmental stages including the dauer larva (Cox et al, 1981b). Dauer larvae are formed in response to adverse environmental conditions and are in a quiescent state. The basal zone of the dauer larva contains a striated or striped layer about 84nm thick, resembling that found in the cuticles of many infective larvae and thought to be made up of a

lattice of regularly arranged vertical rods or striations (Bird, 1971). The solubilizing effect of ß-mercaptoethanol (BME) was tested on four different stages of *C. elegans*. The dauer larva cuticle is only slightly affected by BME, whereas all the other stages tested had most of the cuticle dissolved away, with the exception of the epicuticle.

The most consistent structural pattern in the nematode cuticle is at the outermost surface. This structure, which can most conveniently be called the epicuticle, will now be considered.

1.4 The Epicuticle

As the major interface with the environment, the epicuticle is potentially important in the nutrition of the parasite and its protection against immunological assault. The ability of parasitic nematodes to survive for prolonged periods in immunocompetent hosts testifies to the success with which they have adapted to a potentially hostile environment. The inherent resistant properties of the nematode surface probably contribute to this success. Many researchers in the fields of immunology and chemotherapy believe that the epicuticle is the primary site for attack by both humoral and cellular components of the immune system (Mackenzie, 1978; Maizels and Selkirk, 1988). It is, therefore not surprising that interest is being drawn to the possible dynamic properties of the surface, their biological significance and their mechanisms.

1.4.1 What is the epicuticle?

In the past, the epicuticle has been compared to a highly modified plasma membrane, largely because of its transmission electron microscopical resemblance to this structure

(Bird,1980). However, it does have properties which distinguish it from such a membrane and it has been compared to an extracellular envelope (Locke, 1982) and to the cuticulin membrane of insects (Howells and Blainey, 1983) which are thought to be inert structures.

In recent years, the comparison between the epicuticle and inert biological structures has not been held unanimously. Jain (1988) stated that " In the hierarchy of biological organization, the structure and function of membranes lies somewhere between macromolecules and cells. As non-covalently aggregated macroscopic structures arising from amphipathic molecules, membranes in general have their unique characteristics that are not shared by other molecules and their aggregates". This quotation could be seen to defend the inclusion of the epicuticle in the class of structures to which plasma membranes belong, given that 'membranes' vary widely in their properties. For example, the plasma membrane of fertilized Xenopus eggs is peculiar in various respects including a very high specific resistance (De Laat et al, 1973), lack of selectivity to cations (De Laat et al, 1975), insensitivity to cytochalasins (De Laat and Bluemink, 1974), possession of large intra-membranous particles (IMP's) (Bluemink et al, 1976) and has a substantial fraction of immobilized lipid (Dictus et al, 1984).

Current concepts of membrane structure and function are based largely on information derived from a limited number of microorganisms and mammalian cell types. Consideration of other organisms, such as parasites, might alter the consensus of what constitutes a 'membrane'(Lumsden, 1975). It might then be that that the epicuticle will be considered a structure apart from other biological surfaces, or that it represents an extreme in a limited but continuous range of specialisations of a basic membrane structure.

1.4.2 **The epicuticle as a membrane.**

Membranes are lipid bilayers containing proteins and glycoproteins, forming a structure capable of controlling the internal cellular environment by selective interactions

with the external environment. The question is whether the epicuticle is capable of such selective interactions. What is certain, however, is that proteins and glycoproteins are associated with the surface (reviewed by Maizels and Selkirk, 1988) and could play a role in internal homeostasis or nutrient acquisition. The uncertainty lies in the precise location and function of these proteins and whether they are integral to the epicuticle. Devaney (1988) and Scott (1988) noted that the major surface-associated peptides of adult *Brugia pahangi* and Dirofilaria immitis were extracted in buffer without the aid of detergents. This would indicate that these components are not anchored on the surface of the worm in the manner of a conventional integral membrane component. The presence of integral proteins is believed to be one of the factors associated with the appearance of intra-membranous particles (IMPs) on freeze-fracture electron microscopy (Verkleij and Ververgaert, 1978). For nematodes, contrasting results for different species and stages have left us with no consensus as to whether a characteristic of the epicuticle is a lack of IMPs or whether they occur variably. This could in part be due to technical difficulties in obtaining large fracture faces in the epicuticle, and that the plane of fracture commonly occurs between the epicuticle and the rest of the cuticle, rather than within the epicuticle itself . IMP's have been reported in the epicuticle of *Nippostrongylus* (Lee and Bonner, 1982) and *Meloidogyne* (Bird, 1984), but are reported to be absent in microfilariae of *Onchocerca volvulus* (Martinez-Palomo, 1978) and in various stages of *Trichinella spiralis* (Lee et al,1984,1986). The absence of IMPs does not preclude integral membrane proteins because some atypical membranes, such as the myelin sheath, tend to fracture smoothly without particles (Branton and Deamer, 1972), although integral proteins are known to be present (Boggs and Moscarello, 1978). This is also true for the outer membrane of Gram-negative bacteria (Lugtenberg and van Alphen, 1982; Nikaido, 1989) which only produces small fracture faces due to its unusual bilayer organization.

The trans-epicuticular penetration of many polar compounds (Howells et al, 1983) is

thought to be evidence that the epicuticle is not an uninterrupted lipid bilayer, but one punctuated by components involved in nutrient translocation. However, the precise site of this activity is not known. Rutherford et al (1977) showed that sites for amino acid transport were probably located in the epicuticle of *Mermis nigrescens*, a larval parasite which has a much reduced cuticle. In the majority of nematode species, however, it is probable that nutrients are transported at the hypodermal membrane (Howells, 1987) and that the epicuticle and cuticle are selectively permeable to such nutrients.

By way of comparison, the bacterial outer membrane is able to transport nutrients to the cytoplasmic membrane despite its apparent impermeability (Nikaido, 1989). An analogy could, therefore, be drawn between the outer membrane or envelope of the *Enterobacteriaceae* and the nematode epicuticle in that both form the physical and functional barrier between internal membranes and the environment. The bacterial cytoplasmic membrane (c.f. nematode hypodermal membrane) is responsible for the metabolic functions, including biosynthetic activities, while the major known functions of the outer membrane (c.f. nematode epicuticle) are primarily physical. In *Escherichia coli* the low permeability of the outer membrane is modulated by porins, a class of 30kD-40kD proteins that produce water-filled, non-specific, trans-membrane diffusion channels (Nikaido and Vaara, 1985; Benz, 1985). This is in the same molecular mass range as the predominant surface-associated (glyco)protein of the surface of filarial nematodes (Selkirk et al, 1986; Devaney, 1988; Scott et al, 1988). As mentioned above, this group of parasites is known to absorb nutrients transcuticularly, but there is no evidence as yet for porin-like molecules.

The protective capacity of the epicuticle is impressively demonstrated by dauer larvae of *Caenorhabditis elegans* which survive for prolonged periods in 2% SDS (Cassada and Russell, 1974). Likewise, *E. coli* can grow in the presence of as much as 5% SDS (Lugtenberg and van Alphen,1983). This could be taken to indicate that the bacterial outer membrane and the nematode epicuticle do not have a typical membrane structure, or that a conventional membrane structure is shielded from the environment, perhaps by glycoconjugates.

1.4.3 The epicuticle as an envelope

Many workers now believe that there is no homology between the epicuticle and plasma membranes and that it may be an 'envelope' structure. Locke (1982) stated that "some cells in almost all groups from bacteria to vertebrates have the ability to secrete an envelope directly through or above their plasma membranes, and that envelopes are not plasma membranes". In the present context, an envelope would form a barrier to the environment that allows the hypodermis to control theintervening space, that is, the cuticle. It is true to say, however, that in parasites, hypothetical envelope structures must vary greatly in function. For instance, Podesta et al (1982) described the surface layer of the adult schistosome as an envelope overlying a membrane, although many workers have described this as a double membrane (Kusel, 1972; Hockley and McLaren, 1973; Foley et al, 1986). At the other extreme, there is the cystacanth envelope of the acanthocephalan parasite *Moniliformis* (Lackie and Rotheram, 1972; O'Brien, 1988) which is considered to be a stable, almost inert, structure.

1.4.4 Current consensus

There is still considerable confusion as to whether the outermost surface of the nematode cuticle should be considered a membrane, an envelope, or some type of intermediate structure. This has led to double-think in the literature, as exemplified by reference to the 'surface membrane' of the infective larva but to the 'epicuticle' in adults, sometimes in the same paper (Storey et al, 1988). Such confusion is understandable in the light of current knowledge and an attempt has recently been made to reach a consensus in which it was proposed that the epicuticle be regarded as the "trilaminate differentiation containing lipids" (Wright,1987). We will, however, retain the use of the term epicuticle here, and it is the purpose of this chapter to review its known structure, then concentrate on

the biophysical properties of its lipid components.

1.5 Biochemical nature of the nematode epicuticle

Very little is known about the biochemical composition of the epicuticle, mainly due to difficulties in its isolation or solubilisation by methods conventionally employed for membranes (Bird, 1957; Cox et al, 1981; Murrell et al, 1983; Betschart and Jenkins, 1987). In spite of this difficulty, surface labelling with extrinsic agents, for example with radio-iodine, has provided important details on surface-associated proteins and glycoproteins, many of which are antigenic (reviewed by Philipp and Rumjaneck, 1984; Maizels and Selkirk, 1988). Recently, attention has turned towards the non-proteinaceous components of the epicuticle (Kennedy et al, 1987a; Scott et al, 1988), and it is hoped that, in this thesis, their importance will be established.

1.5.1 Lipids

Evidence for cuticular lipids is as follows. Firstly, many workers noted the dark, osmiophilic layer on the outermost surface of the cuticle which would become much lighter after extraction with organic solvents, and Bird (1957) had noted that sudan-black-stainable lipid was extracted from *Ascaris* when they were dipped in ether. Secondly, Scott et al (1988) isolated glycolipid from the surface of adults of the filarial nematode *Dirofilaria immitis*. This lipid was observed in the low molecular-weight region on an SDS-PAGE gel, after radio-iodination of the intact surface and subsequent extraction. The glycolipid was thought to be potentially important in the provision of a non-immunogenic, protease-resistant barrier between the cuticular surface and the host environment. Epicuticular lipid might then be vital for the maintenance of an effective defence in an immunocompetent host.

Indirect evidence for the presence of lipid in the epicuticle comes from the

observation that certain fluorescent lipid probes can be inserted into the epicuticle (Kennedy et al, 1987a). This established its lipophilic nature, although the authors noted that the insertion of lipid probes was highly selective and that this could be related to the length of the acyl chain and to the general physico-chemical characteristics of the probe. Further indirect evidence comes from the observation that the permeability of the surface of the anhydrobiotic nematode *Ditylenchus dipsaci* increases sharply between 40°C and 50°C (Wharton et al, 1988), suggesting that this is caused by some phase change or transition in epicuticular components, probably lipid.

1.5.2 Surface-associated proteins

The epicuticle and cortical layers of the cuticle contain proteins which are extensively cross-linked by non-reducible bonds (Cox et al, 1981; Betschart and Jenkins, 1987). This insoluble matrix may be common among nematode surfaces and could have similarities with the non-collagenous cuticlin of *Ascaris* cuticle, described by Fujimoto and Kanaya (1973). Cuticlin is the protein remaining after extraction of the *Ascaris* cuticle with SDS detergent and ß-mercaptoethanol and is resistant to solubilization by urea, NaOH and formic acid. A cuticlin-like matrix could account for the remarkable detergent resistance properties of the intact epicuticle in many species of nematode (Pritchard et al, 1985; Mok et al, 1988).

In addition to the structural components of the epicuticle, there are a small number of proteins which are soluble under non-reducing conditions, many of which are antigenic (see Maizels and Selkirk, 1988 for a review). The extent of detergent solubilization of surface antigenic material depends on the type of detergent employed and the nematode species and stage. The cationic detergent cetyltrimethyl-ammonium bromide (CTAB) is effective in the removal of antigens from both *Necator* (Pritchard et al, 1985, 1988) and *Trichinella* (Grencis et al, 1986), but precisely which part of the cuticle from which these proteins are derived is controversial and possibly variable, but a strong possibility is that their immediate source is the surface coat.

1.5.3 Surface coat

Under transmission electron microscopy, an amorphous, glycocalyx-like layer has been observed immediately overlying the epicuticle (Himmelhoch and Zuckerman, 1978). This layer is particularly prominent in the infective larvae of *Toxocara canis* (Badley et al, 1987), and is thought to be composed of antigenic material which is continuously released from the surface (Smith et al, 1981; Maizels et al, 1984). In this parasite, the coat can be observed under scanning electron microscopy to break away from the epicuticle, potentially effecting the removal of immunoglobulin, eosinophils and toxins (Badley et al., 1987). A surface coat is apparent in the infective stage of most parasitic species (Grove et al, 1984, Lee et al, 1986; Abraham et al, 1988) and in free-living nematodes (Zuckerman et al, 1979), but adult parasitic nematodes do not appear to have a surface coat. Lee et al (1986) noted that adult *Trichinella spiralis* lacked one, and the same has been noted by Kieffer et al (1989) for adult *Acanthocheilonema viteae.*

A remarkable feature of some nematode parasites is that sugar residues are apparently not exposed on the intact surface for lectin-binding. For instance, the 47kDa antigen of *Trichinella spiralis* (Parkhouse et al,1981; Ortega-Pierres et al,1984), the 30kDa antigen of *Brugia pahangi* (Devaney, 1988), and the 49kDa antigen of *Dirofilaria immitis* (A.Scott, personal comm.) have carbohydrate determinants which are not available for lectin binding on the intact surface. Several possibilities exist to explain this: firstly, glycoproteins are near to, but not exposed on the surface, yet are iodinatable; secondly, they are positioned such that lectin-binding is sterically hindered; or, thirdly, that glycoproteins are inserted with their sugar side chains in inverted lipid micelles (Wright and Hong, 1988). The last explanation would require an unusual lipid composition and/or phase which will be discussed later.

1.5.4 Glycoconjugates

Sugars are apparently exposed on the intact surface of the infective larva of the dog ascarid *Toxocara canis*, to which anti-carbohydrate monoclonal antibodies can bind (Maizels et al,1987). Such monoclonals also bind to *Toxocara cati* but which of these are exposed, hidden or present at all differs between the two species (Kennedy et al, 1987b).

Certain microfilariae, such as those of *Wuchereria*, *Brugia* and *Litomosoides*, possess a sheath of a different origin from that of the L3 larval sheath, which has ultrastructural characteristics of the egg-shell membrane (Zaman, 1987). The exposed sugars of this sheath are of particular interest as the carbohydrate antigenic determinants on it could be the primary targets of the host's immune response. The microfilarial sheath prevents entry of immunoglobulins which could potentially damage the epicuticular surface, but will allow the entry of smaller proteins, such as the 36kD wheat-germ lectin (Devaney et al, 1985). Wheat-germ agglutinin (WGA) is specific for the sugar N-acetylglucosamine which is present in the egg-shell chitin. Sheathed microfilariae fluoresce very strongly after labelling with this lectin (Kaushal et al 1984; Paulson et al 1988). Paulson et al (1988) studied the lectin-binding of six species of microfilariae and found that only the sheath, and not the larval surface bound lectins. In addition, all lectin binding of *in utero*-derived *Onchocerca volvulus* larvae was associated with the sheath because hatched microfilariae showed no ability to bind any of the lectins tested.

It is particularly notable that sugars have not been found exposed on many of the stages which migrate through mammalian tissues (*T. canis* being a major exception), since we know that other tissue-dwelling parasites such as trypanosomes (Vickerman, 1974) and schistosomes (Lumsden, 1975; Podesta et al, 1982) have polyanionic carbohydrate in their surface coat. It is possible that parasitic nematodes conceal their surface sugars in order to reduce antigenicity, or that this is a surface specialization related to protection of structural

proteins from parasite or host-derived proteinase. How this concealment could be achieved by non-antigenic material is unknown, but lipids, especially those derived from the host, is a possibility.

1.6 Dynamics of the nematode surface

It is now clear that the nematode surface is not merely an inert extracellular covering; surface-associated antigens can be rapidly shed and can alter radically without a moult taking place (Philipp et al,1980; Smith et al, 1981; Maizels et al, 1983, 1984; Marshall and Howells, 1986; Carlow, 1987). The process of antigen shedding perhaps best illustrates the dynamic properties. The release of surface antigens of the second larval stage of *Toxocara canis* is rapid, and Maizels et al (1984) showed that larvae release about 25% of the extrinsically radiolabelled surface components in less than one hour *in vitro*. Smith et al. (1981) demonstrated that this shedding is an energy-dependent process which is inhibited in the presence of antimetabolites or if the larvae are maintained at a low temperature. Whether this energy dependency is associated with the shedding process itself or is related to worm motility remains to be established.

In *Brugia pahangi*, it appears that the surface of the third-stage larva is more dynamic than that of the fourth-stage larva or the adult in that the turnover of radiolabelled proteins in the third-stage is considerably faster (Marshall and Howells,1986). In in vitro experiments with L3s of this parasite, a monoclonal IgM against surface component(s) was lost from the surface in minutes (Carlow et al, 1987). It is possible that in most species, antigens which are lost from the surface can be rapidly replaced, although Ibrahim (1989) suggests that in *Brugia* at least, this is not the case.

1.7 The value of biophysical techniques in the investigation of nematode surface dynamics

Many of the techniques which are currently used to study the cuticle, first require the use of strong detergents or potent chemicals for the purpose of extraction. The resultant extract of nematode molecules is further purified and subjected to sophisticated methods of analysis, and conclusions are made about the possible function of these molecules and how they might be organized in the surface. However, discrepancies may arise when there is some doubt as to the 'extractability' of the various regions of the cuticle. For example, how could we assure ourselves that our extract is derived from the epicuticle and not the hypodermal membrane or other parts of the cuticle? One encouraging possibility is to use non-invasive biophysical techniques which deal with the live, intact parasite. Such techniques involve either the insertion of a surface probe whose physico-chemical properties are influenced by its environment, or the measurement of some physical property inherent within the organism. An example of the first type of biophysical technique is fluorescence recovery after photobleaching (FRAP) which measures the lateral diffusion of fluorescent markers of surface molecules (for reviews see Peters, 1981; Wolf, 1988). An example of the second type of biophysical technique is differential scanning calorimetry (DSC) which measures the change in heat capacity of a system with temperature, and can detect gross structural changes in membranes (Sturtevant, 1974).

Non-invasive biophysical techniques are commonly used for the study of biological membranes but could also be used to investigate the organization of the nematode epicuticle. This could provide important information to many areas of nematode research which rely on an understanding of the sometimes paradoxical host-parasite interface. As has been discussed previously, there is a lack of information about the dynamic properties (or otherwise) of the nematode surface and it is the purpose of this study to investigate these properties using, primarily, biophysical methods.

1.8 Biophysical studies of biological membranes

For many years the predominant concept of membrane organization has been embodied in the fluid mosaic model of Singer and Nicolson (1972). This model was proposed to stress the dynamic aspects of membrane structure, although it belies the complexities now recognized as features of many membranes. Rather than being a homogeneous "sea" of lipid, membrane lipids exist in discrete domains which may differ greatly in their composition and properties. Radda (1975) stated that, "even a given membrane cannot be described by a single structure but at any one instance must be considered as an ensemble average of several rapidly interconvertible forms".

The sheer diversity of membrane lipids suggests that the original fluid mosaic model does not provide a complete picture, and to completely understand how membranes function it will be necessary to determine how membrane components interact to produce their characteristic physical and biochemical properties. It is, therefore, not surprising that, methods for studying membrane dynamics and organization have evolved which exploit the preferential solubilities of amphiphiles in different lipids and lipid phases (Shimshick and McConnell, 1973; Klausner and Wolf, 1980). That is, to introduce spectroscopic "handles" (spectroscopic probes) into the membrane. The success of such an approach depends on our ability to introduce the probe in a desired region of the membrane and to observe some readily detectable and interpretable signal from it. Of course, the immediate interest in this study is how the organization and dynamics of the nematode surface could be examined using the same biophysical techniques. The following section will concentrate on the use of fluorescent probes.

1.8.1 Insertion of fluorescent lipid probes in membranes

Fluorescent lipid probes can be obtained with different fluorescent organic chromophores and lipidic moities of different structure and acyl chain length (Figure1.7). From the point of view of orientation in the membrane or the epicuticle, the physico-chemical properties of the probe are most important (Radda, 1975). Molecules that contain both charged and hydrophobic residues such as the fatty acid analogues 5-(N-octadecanoyl)aminofluorescein (AF18) and octadecyl rhodamine B (RH18) , would normally occupy the polar-nonpolar interface region of a membrane so that different charged probes would have slightly different vertical partitioning. It should be emphasized that insertion could be influenced by interfacial interactions with a polyanionic surface coat.

Lack of charge in the nitrobenzoxadiazolamine (NBD) fluorophore permits sensing of the hydrophobic interior of membranes. It also has useful spectral properties in being essentially non-fluorescent unless incorporated into a lipid layer, in which case the emission is similar to fluorescein. Nonpolar lipid probes such as NBD-methylamino-cholesterol (NBD-chol) will likely be distributed in the hydrophobic interior of a membrane, with little or no interaction with a charged surface coat. The depth of insertion of these different fluorescent probes can be estimated by measuring the extent by which a non-permeant molecule included in the medium (Trypan Blue) can quench the fluorescence of the probe by Forster resonance energy transfer (Foley et al,1986).

1.8.2 Fluorescence Recovery After Photobleaching (FRAP)

The freedom of a fluorescent lipid probe to diffuse within the plane of a surface can provide important information on the physical state of the lipid in its immediate environment. The biophysical technique of fluorescence recovery after photobleaching (FRAP) is used extensively in the study of lateral diffusion of plasma membrane components (for reviews see Peters, 1981; Wolf, 1988). In all versions of FRAP, the lateral mobility of molecules in the membrane surface is determined by measuring the rate of

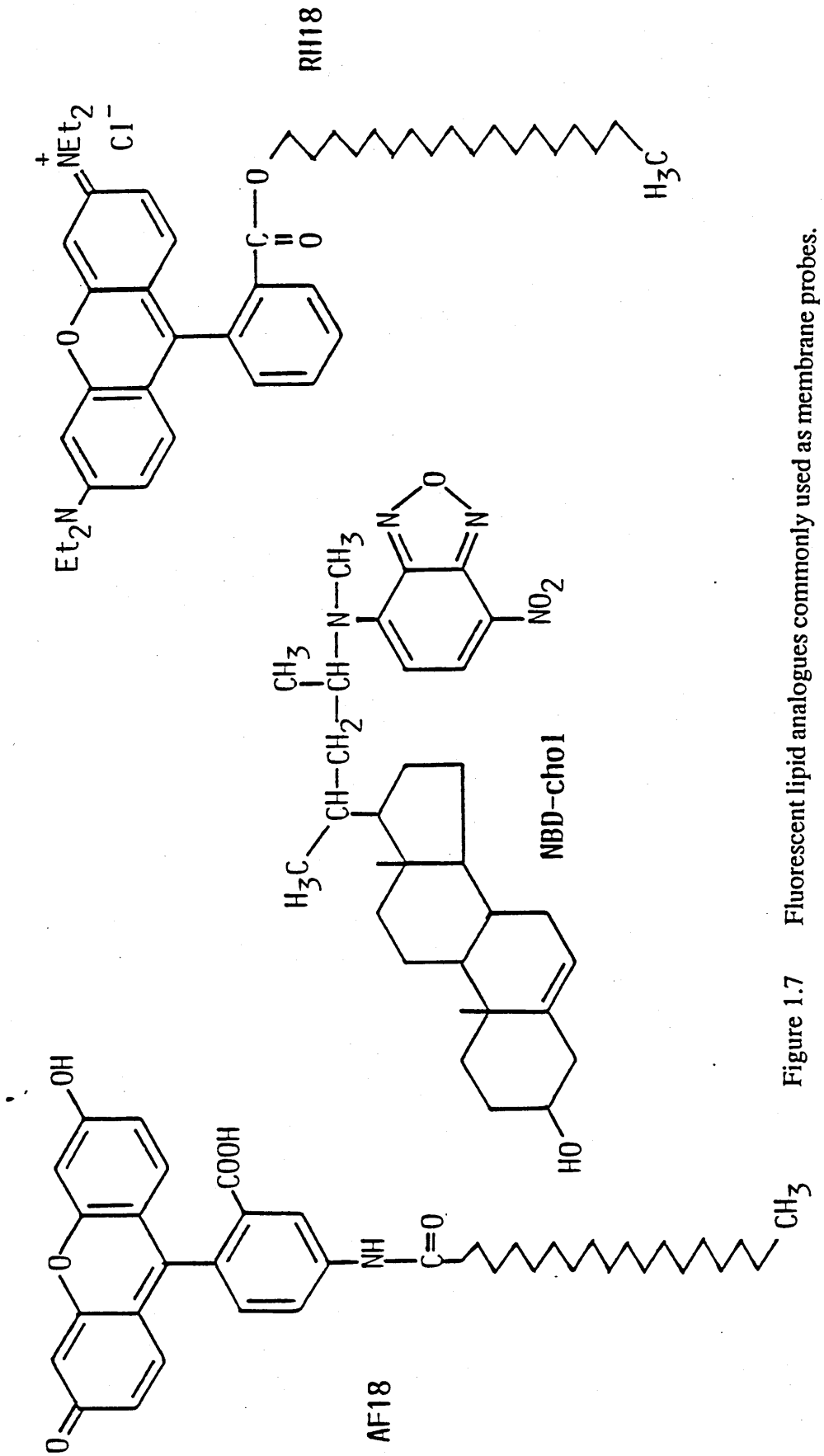


Figure 1.7 Fluorescent lipid analogues commonly used as membrane probes.

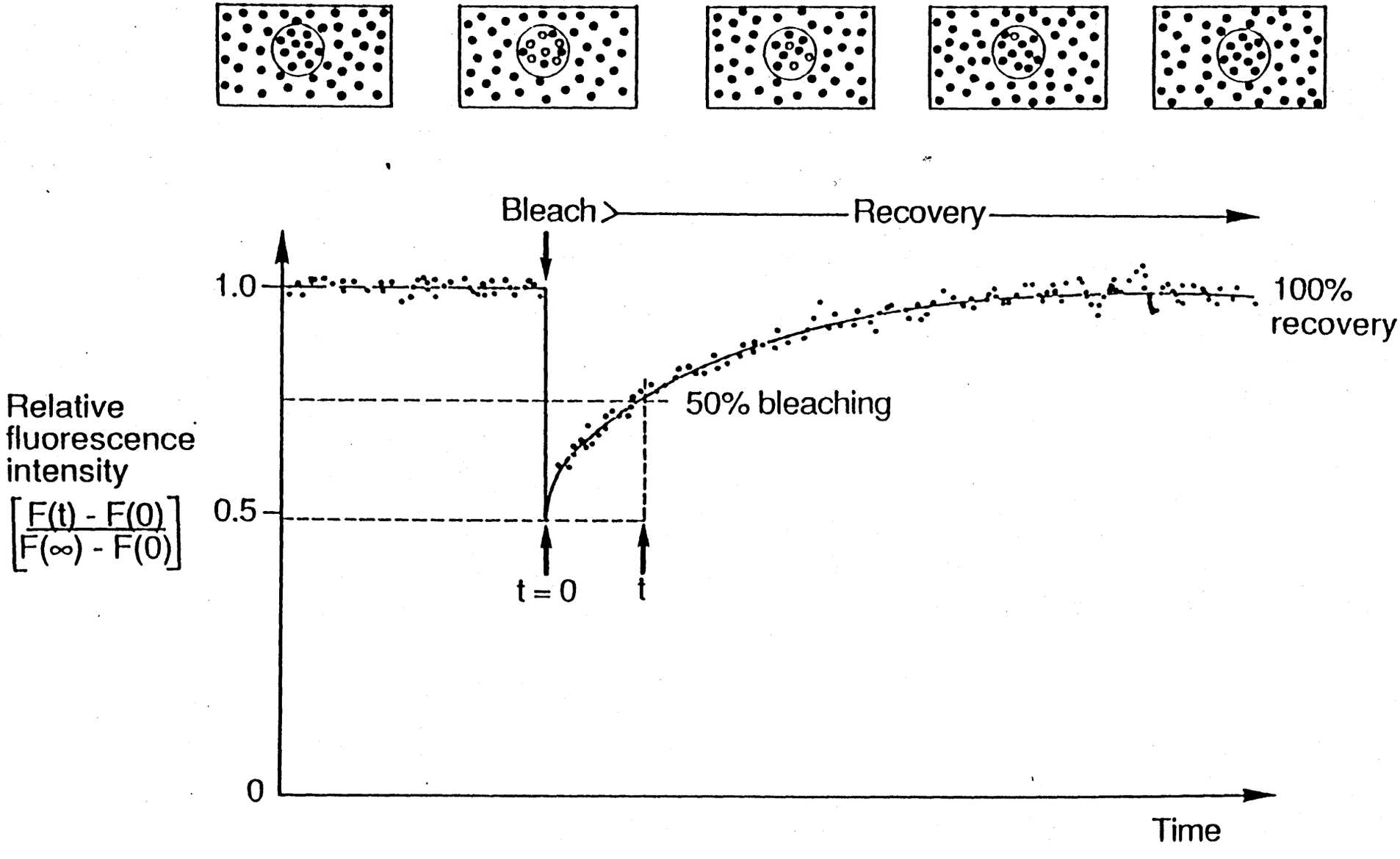


Figure 1.8 Typical fluorescence recovery after photobleaching (FRAP) curve.

local changes in the concentration of fluorescent markers (Figure 1.8) after photobleaching a small area of the membrane with a laser (see Materials and Methods section 2. for a more detailed description). FRAP provides us with two measures of diffusion: first, the fraction of the probe that is free to diffuse (per cent recovery, %R) and second, the lateral diffusion coefficient (D_L) of that fraction (Axelrod et al, 1976).

FRAP studies of lipid probe diffusion in typical eukaryotic plasma membranes show rapid lateral diffusion ($D_L = 10^{-6}$ to$10^{-7} cm^2 s^{-1}$) with 90% to 100% of the probe free to diffuse. However, it appears that a significant fraction of plasma membrane lipid is not free to diffuse in certain specialised membranes such as those of Xenopus eggs (Dictus et al,1984), sea-urchin eggs (Peters and Richter,1981), spermatozoa (Wolf and Voglmayr,1984; Wolf et al,1988), and in certain other differentiating or highly polarized cells.

Most membrane proteins diffuse about fifty times slower than lipid molecules ($D_L \sim 4 \times 10^{-10} cm^2 s^{-1}$), and a substantial fraction of membrane proteins are immobile (Schlessinger, 1983). However, in the absence of a restraining network, such as the cytoskeleton, it is possible for membrane proteins to diffuse with $D_L = 5 \times 10^{-9} cm^2 s^{-1}$ (Peters, 1981; Saffman and Delbruck, 1975).

One potential problem in the application of these techniques to nematodes is that the surface of the nematode is not planar or even cylindrical. However, diffusion coefficients are thought not to be significantly affected for curved or annulated surfaces such as those of concern here (Aizenbud and Gershon, 1982). For instance, diffusion rates of a lipid analogue in a microvillous surface is similar to that in featureless cell surfaces (Wolf et al,1982).

1.8.3 **Differential scanning calorimetry (DSC).**

As our knowledge of biomolecular structure gets ever more detailed, it becomes increasingly important that we study the basic physical forces between and within macromolecules in sufficient detail that we might be able to understand biological processes at the molecular level. Differential scanning calorimetry (DSC) involves measurement of heat energy uptake (heat capacity) during changes of temperature, and is typically used for studying thermal unfolding processes of proteins and nucleic acids or phase transitions of lipid systems (Sturtevant, 1974). A more detailed explanation of this biophysical technique is given in the Materials and Methods section 2.15 .

Lipid transitions have been detected in cell membranes using DSC. Steim (1968) demonstrated the occurrence of co-operative thermotropic transitions in the membranes of the unicellular organism *Acholeplasma laidlawii* using DSC. Since the first observation, it has now been found that thermotropic transitions occur in a wide variety of cell membranes which contain little or no cholesterol including both gram-positive and gram-negative bacteria and membranes of sub-cellular organelles, in particular, mitochondria and endoplasmic reticulum (Steim, 1972).

1.9 Significance of the nematode surface in developmental signalling mechanisms.

1.9.1 **Transmission to the mammalian host environment**

During its life-cycle, a parasite may exist in different environments in which there are several physical and chemical stimuli to which it may respond. In the parasitic stages, some adult nematodes can be characterized by the restricted sites they occupy, while others have complex migrations during and after maturation (Crompton, 1973). In free-living

stages, the biological imperative is to find and infect the next host. Certain 'trigger cues' act on important physiological processs to signal infection which may include: temperature, pH, osmotic pressure, pO_2, pCO_2, changes in ionic balance and changes in the availability of nutrients. Metabolic switches reflect the changes in the physico-chemical properties of the environment and the changing roles of the various different stages in the life-cycle.

Entry of the parasite into a potential host animal may take one of three basic routes:

(1) the parasite is ingested by the potential host in the form of infective eggs, sheathed larvae, or muscle-encysted larva, e.g. *A. lumbricoides*, *O. ostertagi* and *T. spiralis*;

(2) the larval stage is passed into the wound of the potential host during blood-feeding by an arthropod vector, e.g. *A. viteae* and *B. pahangi*; or,

(3) active skin-penetration of the potential host by the free-living larval stage, e.g. *S. ratti* and *N. brasiliensis*.

1.9.2 **The surface as a sensory structure**

Most of the research on infection has concentrated on behavioural aspects (Sukhdeo and Mettrick, 1987) or the nature of host signals (Sommerville and Rogers, 1987). However, very little is known about the mechanisms for signal transduction across the cuticle which might initiate development to the parasitic stage. Since nematodes are well endowed with sensory structures and exhibit complex patterns of behaviour, it seems likely that they would be able to 'sense' their new host environment by nerve transmission.

Parasitic and free-living nematodes possess essentially identical nervous systems, consisting of a circum-oesophageal nerve ring, on which are located varying numbers of ventral, dorsal and lateral ganglia. Using a variety of techniques, it has been shown that nematodes are abundantly supplied with free nerve endings, have a peripheral nervous

system, and are sensitive to changes in light and heat in parts of their anatomy which are lacking in complicated sensory structures. Variously named structures such as the hemizonids, cephalids, and caudilids are thought to be sensory structures whose cuticular differentiation is visible under the light microscope (Bird, 1971).

The function of some of the surface sensory structures must be the detection of a temperature gradient, as skin-penetrating nematodes such as *Necator* and *Nippostrongylus* possess behavioural patterns that are clearly adaptations for locating a warm-blooded animal in a terrestrial environment. Infective larvae of *Nippostrongylus* wave around in the air trying to find the homeothermic host, the rat , and when they do, they spiral their way around a hair and rapidly penetrate the skin.

It is clear that nematodes, in possessing a highly sophisticated sensory system, are able to respond rapidly to their new host environment so that the approriate developmental signals may be generated.

1.9.3 **Biochemical signalling in development**

Infective-stage larvae are in a state of hypometabolic dormancy in which the intake of nutrients, growth and development are suspended. Entry into the appropriate host causes the immediate initiation of developmental changes which are necessary for adaptation to the new host environment, therefore it is assumed that the mechanisms involved are already present. One might then automatically assume that developmental signals to the hypodermal cells arise solely from nervous stimulation, however, it is possible that molecular signalling plays a more important role within, and across the cuticle itself.

In mammalian cells, it is well established that external signals are often amplified by second messengers such as 3', 5'-cyclic-AMP (cAMP) , whereby Ca^{++} mobilization as well as inositol lipid turnover, protein kinase activation and frequently cGMP production are induced (Nishizuka, 1984, 1986) (see figure 1.9). In this regard, there is little information

Table 1.2 : **Chemicals affecting the activity of cellular signalling systems**

Chemical	Action or Effects
cAMP & TPA	Activators of protein kinase C (Nishizuka, 1984a,b)
Forskolin & serotonin	Activators of adenylate cyclase (Nishizuka, 1984a,b; Kawamoto et al,1989).
Imidazole & NH_4Cl	Phosphodiesterase activators which deplete levels of cAMP/cGMP (Heyworth et al, 1984; Kawamoto et al, 1989).
cGMP	Possible mediator of G-protein interactions (Garbers, 1989).
Nitroprusside	Potent activator of guanylate cyclase (Schultz et al, 1977; Katsuki et al, 1977).
IBMX	Inhibitor of cAMP/cGMP phosphodiesterases (Kawamoto et al, 1989).
A23187, TMB-8 & W-7 (Campbell, 1989).	A potent Ca^{++} ionophore, inhibitor of Ca^{++} release from internal sources, and calmodulin inhibitor, respectively 1983; Tanabe et al,
Monensin	Sodium ionophore (Siffert and Akkerman, 1987).
Amiloride	Inhibitor of Na^+/H^+antiport (Moolenaar, 1983; Thomas, 1984; Frelin et al, 1988; Madshus, 1988).
Ouabain	Inhibitor of Na^+/ K^+ -ATPase (Skou, 1965).

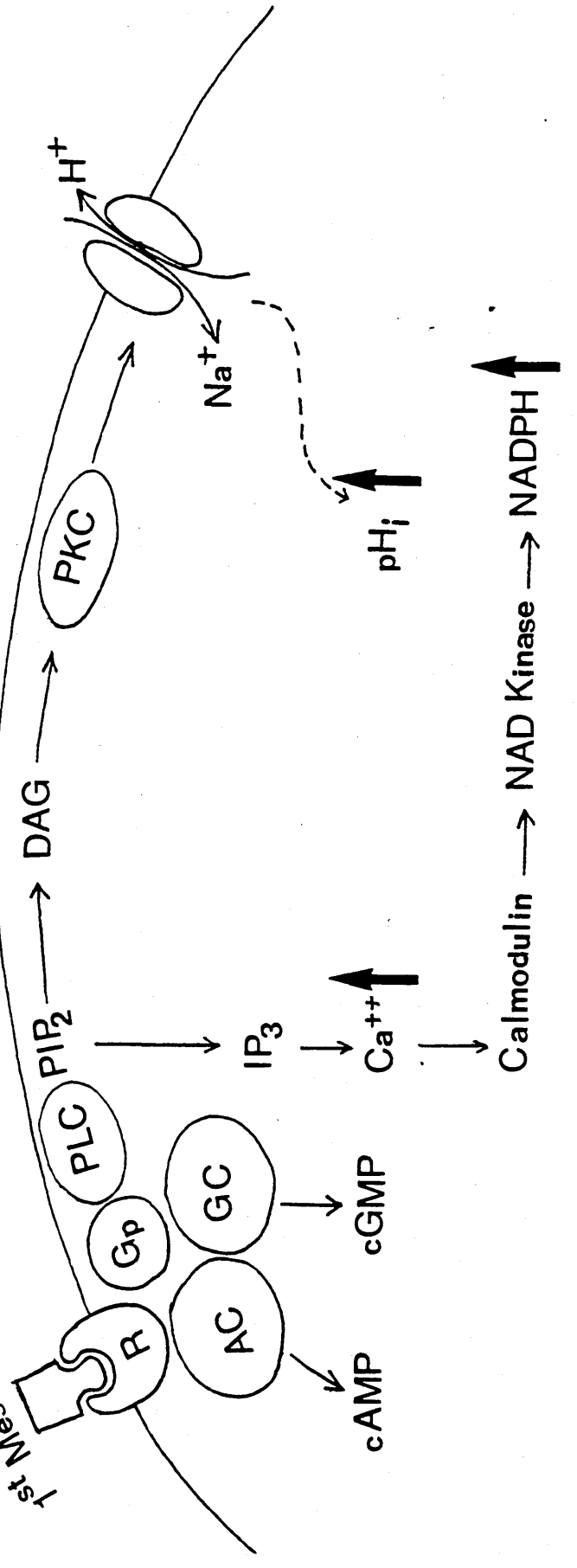


Figure 1.9 Schematic diagram of cellular signalling mechanisms.

on the roles of these systems in nematode development. However, a number of hormones that affect mammalian metabolism, such as serotonin, adrenalin and noradrenalin, and the enzyme adenylate cyclase, have been found in helminths (Mansour, 1984; Estey and Mansour, 1987). There are also extensive reviews on helminth behaviour (Sukhdeo and Mettrick, 1987) and the nature and action of host signals (Sommerville and Rogers, 1987), which might provide clues for further research into nematode-host interactions. Recent findings that certain drugs, which can activate or interfere with signalling mechanisms (table 1.2), can actually alter development in schistosomes (Kawamoto et al, 1989) and *Plasmodium* (Kawamoto et al, 1990 in press) parasites encourages the belief that these mechanisms exist in nematodes, perhaps extending to the cuticle.

Chapter Two

Materials & Methods

2.1 Biological Materials

2.1.1 The Parasites

(i) *Toxocara*:

The infective-stage larvae of *Toxocara canis* were obtained from the Scottish Parasite Diagnostic Laboratory, Stobhill General Hospital, Glasgow.

(ii) *Trichinella*:

The life cycle of *Trichinella spiralis* is maintained in mice at the Wellcome Laboratories for Experimental Parasitology, University of Glasgow.

(iii) *Ostertagia*:

The life cycle of *Ostertagia ostertagi* is maintained at the Wellcome Laboratories for Experimental Parasitology, University of Glasgow. Continuous passage of infective larvae through 3-4 month old calves is used to maintain the life-cycle.

(iv) *Acanthocheilonema*, & *Litomosoides*:

The life cycles of *Acanthocheilonema viteae* and *Litomosoides carinii* were a kind gift from Dr. William Harnett and Dr. Michael Worms of the Division of Parasitology, National Institute for Medical Research, London.

Adult worms of *A. viteae* were harvested from the connective tissue of the subcutis of infected jirds, *Meriones unguiculatus*, *M. libycus*, *M. shawi* or the rat, *Mastomys natalensis*. Adult worms of *L. carinii* were harvested from the peritoneal cavity of the cotton rat, *Sigmodon hispidus* or the jird, *M. unguiculatus*. The intermediate host, *Ornithodorus tartakovskyi* was used to obtain infective-stage larvae of *A. viteae* (London), however another source of *A.viteae* L3's was also used, i.e. *Ornithodorus moubata* from the Institute of Tropical Medicine, University of Heidelberg, West Germany.

(v) *Brugia*:

The life cycle of *Brugia pahangi* is maintained at the Liverpool School of Tropical Medicine, Liverpool, and at the Wellcome Research Laboratories, Beckenham, Kent.

Adult *B. pahangi* were harvested from the peritoneal cavity of jirds, *Meriones unguiculatus*.

Infective third-stage larvae were harvested from *Aedes egypti* (strain RE^{fm}) mosquitoes, which were a kind gift from Dr. Eileen Devaney, Department of

Parasitology, Liverpool School of Tropical Medicine.

(vi) *Strongyloides*:

L3's: *Strongyloides ratti* (homogonics and heterogonics) were a kind gift from Dr. Jenkins, Department of Biological Sciences, Portsmouth Polytechnic, Portsmouth.

(vii) *Nippostrongylus*

The life cycle of *Nippostrongylus brasiliensis* is maintained in rats at the Wellcome Laboratories for Experimental Parasitology, University of Glasgow.

2.1.2 Free-living model

The free-living (non-parasitic) nematode, *Caenorhabditis elegans* was used as an experimental model for the parasitic forms:

(i) strain N2 (wild-type)

(ii) srf-2 (yj262), reference allele of srf-2.

(iii) srf-3 (yj10). Only allele of srf-3.

C.elegans was maintained on agar plates carrying a lawn of OP50, a uracil requiring strain of *Escherichia coli* (Brenner, 1974).

Mutants of *C.elegans* were a kind gift from Dr. S. Politz of the Worcester Polytechnic Institute, Worcester, Massachusetts.

2.2 Preparation and culture of nematodes

2.2.1 Plasticware & Glassware

All plasticware from Sterilin. Microscope slides and coverslips from Chance Proper Ltd.

2.2.2 Culture Media

(i) RPMI 1640 medium Flow

The major ingredients of RPMI 1640 are shown in table 2.1

(ii) M199 medium Flow

Table 2.1 Major ingredients of Grace's insect tissue culture medium and RPMI 1640 mammalian tissue culture medium

Ingredients	**Grace's** (pH 6.0) mg l^{-1}	**RPMI 1640** (pH 7.4) mg l^{-1}
$NaHCO_3$	350	2000
NaCl	-	6000
KCl	2240	400
$NaH_2PO_4.2H_2O$	1140	-
Na_2HPO_4	-	800
D-sucrose	26680	-
D-fructose	400	-
D-glucose	700	2000
$CaCl_2.2H_2O$	1325	-
$Ca(NO_3)_2$	-	400
$MgSO_4.7H_2O$	2780	100
$MgCl_2.H_2O$	2280	-

Grace's and RPMI 1640 also contain varying amounts of amino acids and vitamins.

(iii) Minimum essential medium without methionine
(MEM w/o methionine) Flow

(iii) Grace's Insect tissue culture medium Flow
The major ingredients of Grace's insect medium are shown in table 2.1.

(iv) Artificial stomach solution:
0.9% saline
0.5% pepsin Sigma
0.5% HCl

(vi) Wash RPMI:
RPMI 1640 +
nystatin (6 I.U./ml) Sigma
fluorocytosine (1ng/ml) Sigma

(vii) Nutrient agar:
'Lab-Lemco' Powder 1.0g
yeast extract 2.0g
peptone 5.0g
sodium chloride 5.0g
Agar No. 3 15.0g
(pH 7.4) Oxoid.

2.2.3 Sera

(i) Foetal calf serum (FCS): Northumbria Biologicals Ltd.
(heat-inactivated).

(ii) Human, cortisol-deficient serum
Essentially lipid-free, from Sigma.

(iii) Hyperlipidaemic Human serum (HHS)

Sera from several different hyperlipidaemic patients (elevated cholesterol and triglyceride levels) were a kind gift from the Cardiovascular Research Unit, Edinburgh.

(ii) Normal jird serum (NJS):

This was a kind gift from Dr. Eileen Devaney, Liverpool School of Tropical Medicine, Liverpool, U.K.

(iii) Hyperlipidaemic jird sera

These were a kind gift from Dr. Michael Worms, National Institute for Medical Research, Mill Hill, London.

2.3 Biological Methods

2.3.1 Harvest and culture of parasites

(i) *Toxocara canis*

Adult worms were recovered from the intestines of infected dogs at necropsy. A large number of eggs can be harvested from female worms, and these eggs can be artificially hatched using 2% sodium hypochlorite. Emerged infective-stage (L2) larvae were cultured in RPMI 1640 medium at 37°C.

(ii) *Trichinella spiralis*

Adults: The infected mouse was killed by cervical dislocation and swabbed with ethanol before making a transverse incision in the abdomen. The small intestine was then removed by cutting at a point below the stomach and again at the other end of the intestine. Sharp scissors were inserted into one open end, and the intestine was cut lengthways. Baermann extraction of the adult worms involved folding the opened intestine into a piece of fine-mesh netting, which barely touched the surface of warm medium (RPMI at 37°C), and adult worms were attracted through the mesh into the medium within 2h. The adult worms were cultured in RPMI with or without FCS, depending on the experiment.

Muscle-stage larve (MSL): The infected mouse was killed by cervical

dislocation, and the extremities (head, limbs and tail) were removed with sharp scissors to leave behind the body. The remaining skin was removed carefully (wearing gloves) and the body was eviscerated. The carcass was then placed over the blades of a blender and 200ml artificial stomach solution was poured on top. The carcass was then homogenised for 1min. on half speed and then for a further 1 min. on full speed. The homogenate was then poured into a 1 litre flask and incubated for 3h at 37°C with agitation (outlet from aquarium pump). After this period of incubation/agitation, the homogenate was filtered on a 50µm metal filter in a plastic funnel. The extract was left to settle in a tall cylinder and any residue was washed off with saline. The surface liquid was then poured off and the 100ml, or so at the bottom containing the larvae was settled in a pharmacological glass funnel.

New-born larvae (NBL): NBL were obtained from the culture supernatant of adult females, cultured in RPMI at 37°C.

(iii) *Ostertagia ostertagi*:

L3's: These were recovered from faecal egg cultures by baermannization through a layer of 25 µm pore nylon cloth. They were not refrigerated. Exsheathed larvae were obtained, either by treatment with 1.5% sodium hypochlorite in saline for no longer than 15 minutes, or by incubation in RPMI with excess CO_2 bubbled through it for 1h.

L4's & Adults: These were recovered (at necropsy) from the abomasal glands of the rumen of a calf.

(iv) (a) *Acanthocheilonema viteae*

Adults: The infected jird was killed, a longitudinal incision was made in the abdominal skin, and the connective tissue was cut back to reveal the adult worms situated in the subcutis. These worms could then be lifted up on a cat's whisker, and transferred to wash medium for washing in anti-bacterial and antifungal agents, before culture in RPMI+FCS at 37°C.

mf: Microfilariae were harvested from the culture supernatant of adult females.

L3's: Ticks (*O. moubata* or *O. tartakovskyi*) were handled with care as they can cause human infestation. Butyl phthalate (mite repellant) was always used on the outside of disposable gloves, and a white background with vaseline at the boundaries was required to halt escapees. A heavy coverslip was used to crush *O. tartakovski* against a glass slide. The crushed tick and its dark-coloured fluid contents were then washed, with the appropriate medium (Grace's insect medium, or RPMI medium, depending on experimental conditions) into a watch-maker's glass. After 1h in Grace's at 27°C or 1h in RPMI at 37°C, the L3's migrated out of the tick into the medium. They could then be washed several times in medium.

O. moubata were treated in essentially the same way, although because of their greater size, they must be opened up with a scalpel blade.

(b) *Litomosoides carinii*

Adults: The infected cotton rat was killed, and a longitudinal incision was made in the abdominal skin. Adult worms were recovered from the pleural cavity, transferred to the wash RPMI, and then they were then cultured in RPMI+ FCS at 37°C.

mf: Microfilariae were harvested from the culture supernatant of adult female worms.

(v) *Brugia pahangi*

Adults & mf: The infected jird was killed, and a longitudinal incision was made in the abdominal skin. The skin was pulled back to reveal the peritoneal membrane. Approximately 5ml of PBS was injected into the peritoneal cavity. The cavity was lavaged to release the microfilariae. A small inscision was made in the peritoneal wall and the tip of a pasteur pipette was inserted to remove the fluid containing microfilariae. The peritoneal wall was then cut further to expose the adult worms. They were removed with fine-tipped forceps into the wash RPMI before culture in RPMI+FCS at 37°C.

L3's: Infected mosquitoes were aspirated into a long piece of plastic tubing where they were concentrated at a piece of netting near the top, and stunned by hitting the tube sharply against the bench. Heads were used to obtain infective larvae as the rest of the mosquito contains mainly pre-infective and L2-stage larvae. The heads were

gently teased apart with needles in a watch-makers glass containing Grace's insect medium or RPMI, depending on the experimental conditions.

(vi) *Strongyloides ratti*

L3'S: (Homogonic and heterogonic larvae).

From day 7 post-infection, *Strongyloides* eggs appear in the faeces of infected rats. Rats were kept in wire-bottomed cages and faeces was collected on a moist paper covered tray beneath. The faeces was then distributed on raised pieces of filter paper (9cm diam.), and incubated in the dark at 28°C. Eggs hatched, and larvae moulted twice over the following six days. Larvae migrated to the edges of the filter paper from where they could be harvested.

(vii) *Nippostrongylus brasiliensis*

L3's: method as described above for *S. ratti* with minor variations.

2.3.2 Culture of the free-living nematodes.

Caenorhabditis elegans were maintained on a lawn of *E.coli* OP50 strain on 9cm sterile agar plates. The plates of worms were kept in plastic boxes at room temperature. High humidity was maintained by placing moist tissues at the base of the box. If the plates became overcrowded, or if starvation occured, the nematodes entered the resistant dauer form. To avoid this, most plates had to be sub-cultured onto a fresh lawn of *E. coli* every 2 weeks. The worms were transferred by means of a platinum wire pick which had been flamed and cooled.

When dauer larvae were specifically required, they could be obtained by placing worms on an unseeded (no *E. coli*) agar plate which had a circle cut out of the middle. Worms fall into the circle where conditions become rapidly overcrowded and dauer larvae are formed.

2.4 General reagents

All of the following chemicals were obtained from Sigma Chemical Company Ltd :

Bovine serum albumin (BSA)
Coomassie brilliant blue R250
Trypan blue
Chloramphenicol; Cycloheximide; Nystatin; 5-Fluorocytosine
Orcinol-ferric chloride (Bial's reagent)
Sodium taurocholate; Sodium deoxycholate; Cetyltrimethyl ammonium bromide (CTAB); Nonidet P 40; n-Octyl b-D-glucopyranoside
Bile (bovine mixture of free and conjugated acids)

Albumin, bovine-fluorescein isothiocyanate (FITC-albumin)
Glutathione (reduced form)
Methionine (non-radioactive)
Tyrosine

Protease (type XXV); Trypsin (type XII); Pepsin; Galactose oxidase (type V); Elastase (type III); Heparinase (from *Flavobacterium heparinum*); Phospholipase C (type IX); Lipase (from *Chromobacterium viscosum*).

Sodium nitroprusside
cAMP; 8-bromo cAMP; cGMP; 8-bromo cGMP
forskolin
Imidazole
5-hydroxytryptamine (serotonin)
N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7)
N-(6-aminohexyl)-1-naphthalenesulphonamide (W-5)

3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8)	
Nicardipine (1,4-dihydro-2,6-dimethyl-4-[3-nitrophenyl]methyl-2-[methyl(phenylmethyl)amino]-3,5-pyridinedicarboxylic acid ethyl ester).	
3-Isobutyl-1-methyl-xanthine (IBMX)	
Monensin	
Ouabain	
Heparin	Flow
7.5% Sodium bicarbonate	
Hoechst Dye No. 33258 (Bisbenzimide)	Calbiochem (La Jolla, Ca.)
Ferritin, cationised, fluorescein conjugate (FITC- cationised ferritin)	Molecular Probes.
Collagenase (from *Clostridium hystolyticum*)	Boehringer Mannheim.
Hyaluronidase (from ovine testes)	
TCA	BDH Chemicals.
90% formic acid	
Silguard (resin)	
Ecoscint A	National Diagnostic.
sulpho-NHS-biotin	
Iodogen	Pierce.

The following antihelminthic drugs were a kind gift from David Hayes of the

Wellcome Research Laboratories, Beckenham, Kent.

Amoscanate; CGP 6140; CGP 20376; Diethylcarbamazine citrate (DEC); Albendazole; Furapyrimidone; Levamisole HCl; Suramin; Rafoxamide. An outline of the different types of anthelmintics and their possible sites of action is shown in table 2.2.

2.5 Fluorescence Labelling Reagents

2.5.1. Fluorecent lipid probes

All the following fluorescent lipids were obtained from Molecular Probes, Inc. with the exception of NBD-PC, which was obtained from Sigma Chemical Co. Ltd :

(i) Fluorescent amino lipids

AF 12: 5-N-(dodecanoyl)aminofluorescein, [EX. 495; EM. 521].

AF 16: 5-N-(hexadecanoyl)aminofluorescein,[EX. 495; EM. 521].

AF 18: 5-N-(octadecanoyl)aminofluorescein, [EX. 496; EM. 517].

These fatty acids are more polar than phospholipids, and bind to membranes with the anionic fluorophore at the aqueous interface and the fatty tail embedded in the membrane.

(ii) Carbocyanine lipids

DiI 18: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, [EX. 547; EM. 571].

The orientation of probe insertion in the erythrocyte membrane is with the two alkyl chains in the lipid layer and the cationic fluorophore relatively parallel to the surface (Axelrod,1979).

Table 2.2 Anthelmintics and their sites of action

Anthelmintic Agent	Sites of Action
Diethylcarbamazine (DEC)	Neuromuscular system, cuticular surface, carbohydrate and folate metabolism, and host immune factors.
Suramin	Carbohydrate and folate metabolism, protein kinases, intestinal epithelium.
Ivermectin	Neuromuscular system, host immune factors.
Benzimidazoles	Assembly of microtubules.
Isothiocyanates & derivatives	Cuticular surface, carbohydrate metabolism, cAMP-phosphodiesterase, 5'-nucleotidase, amino acid t-RNA ligases.
Levamisole	Neuromuscular system, carbohydrate metabolism.

(iii) **Rhodamine**

RH 18: octadecyl rhodamine B, chloride salt [EX. 556; EM. 577].

In typical membranes, the cationic fluorophore is situated at the aqueous interface while the fatty tail is embedded in the membrane.

(iv) **Phospholipids**

NBD-PE: N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-a-phosphatidylethanolamine, [EX. 460; EM. 534]. Also obtainable from Sigma.

NBD-PC: 1-hexadecanoyl-2-[N-(-7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminohexanoyl]-sn-glycero-3-phosphocholine. Also obtainable from Sigma.

(v) **Miscellaneous lipid probes**

Merocyanine 540: This is a probe of membrane lipid asymmetry that appears to bind preferentially to loosely packed membranes with high disorder in the fluid phase (Williamson et al, 1983).

Nile Red: The dye is almost non-fluoresent in water and polar solvents but undergoes large absorption and emission shifts to shorter wavelengths on transfer to a lipophilic environment, and can therefore, be used to stain lipid and lipid droplets.

2.5.2 Chemically-Reactive Fluorescent Probes

(a) **Sulphydryl-group reactive probes**

Fluorescein maleimide and fluorescein iodoacetamide were obtained from Molecular Probes (Oregon). Both probes are brightly fluorescent, water soluble thiol reagents.

(b) **Amino-group reactive probes**

Many proteins do not have free thiols, but virtually all have lysines and most have a free amino terminus. Isothiocyanates (R-N= C =S) form thioureas on reaction with amines. Fluorescein isothiocyanate (FITC) was obtained from Sigma.

Succinimidyl esters are good reagents for amine modification, since the amide products are very stable. Biotin derivatives such as N-hydroxysuccinimide (NHS)-

biotin and sulpho-NHS-biotin were obtained from Pierce Europe B.V., the Netherlands. FITC-streptavidin, which is then coupled to the biotin complex, was obtained from Sigma.

Dichlorotriazinylaminofluorescein (DTAF) from Sigma has high reactivity with amines and alcohols, and works well for the nematode surface in the presence of 0.2M sodium borohydride (Sigma).

(c) **Carbohydrate reactive probe**

Lucifer yellow is a fluorescent hydrazide, from Aldrich Chemical Co. Nematode surfaces oxidized with $NaIO_4$ or galactose oxidase can be reacted with this probe.

2.5.3 Fluorescent lectin-binding studies

All the fluorescein isothiocyanate-conjugated lectins, and the sugars which were used to inhibit them were obtained from Sigma.

Lectin	Inhibitory sugar(s)
Con A	α-Methyl-α-D-mannopyranoside
PNA	D(+)-galactose
WGA	(1)N,N',N''-acetylchitotriose (2)N-acetyl-D-glucosamine.

Each inhibitory sugar was made up at a concentration of 1.0M and used at a final concentration of 150mM.

2.5.4 Immunofluorescence studies

Caenorhabditis elegans

Rabbit polyclonal anti-surface IgG

(i) Unadsorbed antiserum - 100µl in PBS; 3µl was sufficient to saturate binding to approx. 50 adults.

(ii) Antiserum adsorbed with N2 adults - 325µl in PBS; 95µl of this serum is equivalent to 3µl of unadsorbed serum.

Second antibody

FITC-conjugated goat anti-rabbit IgG, from Sigma.

2.6 Methods for fluorescence-labelling of the nematode surface

2.6.1 Insertion of fluorescent lipids

The dry, powdered form of the fluorescent lipid analogues AF12, AF16, AF18, RH18, DiI18, Mc540 and nile red, were dissolved in ethanol at a concentration of 1mg ml^{-1}. These stock solutions in ethanol were then diluted 1:100 with distilled water, or the appropriate culture medium, to give a final concentration of 10µg ml^{-1}.

Fluorescent lipid probes which were supplied from the manufacturer (Sigma) dissolved in chloroform, e.g. NBD-PC and NBD-PE could be incorporated into lipid vesicles (liposomes) for surface-labelling by the following simple method:

Procedure

1. Add 1mg lecithin PC (in 50µl chloroform) to a glass test-tube which had been previously washed with organic solvent to remove any contaminants.
2. Add 0.05mg fluorescent probe (in 200µl chloroform).
3. Mix in the test-tube using a whirlimixer, then dry-down under nitrogen.
4. Add 75µl ethanol to dry lipids and mix gently.
5. Heat 1ml of stirring PBS to 35°C.
6. Inject lipid mixture slowly into the warm, stirring PBS over approx. 2 minutes.
7. Dialyze overnight at 4°C against PBS with several changes.

Fluorescent lipid probes, whether in a very dilute ethanolic mixture, or in liposomes, were then in a suitable state to be added to culture medium containing live nematodes. The labelling period was normally for 10 minutes at room temperature, unless otherwise stated.

2.6.2 Fluorescence labelling of proteins

(a) Fluorescein-5-maleimide (FM) and 6-iodoacetamidofluorescein (IAF) were dissolved in 0.1M sodium bicarbonate (pH 8.2) to give a concentration of 1.0 mg ml^{-1}. These stock solutions were then diluted 1:100 with 0.1M sodium bicarbonate or culture medium to give a final concentration of 10 $\mu g\ ml^{-1}$. Live nematodes were reacted with either of these probes for 30 minutes, after which time they were centrifuged (500 x g for 5 minutes) and the unreacted probe was aspirated. The labelled worms were centrifuged and washed at least a further 5 times before preparation for fluorescence microscopy or SDS-PAGE.

(b) The succinimido-derivatives of biotin were dissolved in either DMSO (NHS-biotin) or distilled water (sulpho-NHS-biotin) to a concentration of 1.0 mg ml^{-1}. This stock solution was diluted 1:10 with 0.1M sodium bicarbonate (pH 8.2) to give a final concentration of 100 $\mu g\ ml^{-1}$. Live worms were incubated in this solution for 30 minutes and then washed 5 times in PBS. Washed worms were then incubated in FITC- streptavidin (100 $\mu g\ ml^{-1}$) for a further 30 minutes. The labelled worms were then centrifuged (500 x g for 5 minutes) and washed at least 5 times before preparation for microscopy.

(c) Dichlorotriazinylaminofluorescein (DTAF) was dissolved in 0.2M sodium borohydride (pH 10.1) to a concentration of 5.0 mg ml^{-1} so that the solution became bright green in colour, and effervescent. Live worms were placed in this solution and incubated for 30 minutes (method adapted from Sheetz et al, 1980). A tris buffer solution (pH 10.1) was used as a control (no sodium borohydride). DTAF-labelled worms were then centrifuged and washed at least 8 times before preparation for fluorescence microscopy or homogenization for SDS-PAGE of labelled proteins.
All the above at room temperature.

2.6.3 Fluorescent lectin-binding

The lectin-binding assay was a modification of that described by Kaushal et al (1984). Known numbers of worms were placed in PBS containing lectin (100 µg/ml) in a total volume of 200 µl. The appropriate competing sugar was added to a corresponding tube at a final concentration of 150mM as a check on specificity of the binding reaction. Incubation was carried out at room temperature for 30 minutes in the dark and was followed by 3 cycles of washing in PBS (4°C) and centrifugation at 300 g for 5 minutes. The sample was then ready for fluorescence microscopy (see section 2.13.1). Single fluorescence quantitation readings were taken on 10 individual nematodes to establish mean values. Specific binding attributable to the presence of specific saccharides was determined by subtracting nonspecific binding (binding with specific saccharide inhibitor present) from total binding (no inhibitor present).

2.6.4 Fluorescent hydrazide-labelling

Nematodes were labelled with lucifer yellow hydrazide (LYH) by a method adapted from the dansyl hydrazine method of Eckhart et al (1976). Oxidation of surface sugars was attempted using sodium periodate or galactose oxidase at room temperature. After thorough washing with medium, this was followed by incubation in LYH, $5mgml^{-1}$ at 4°C.

2.6.5 Fluorescent antibody-labelling

All stages in the life-cycle of wild-type (N2) *C.elegans* (including dauer larvae) were labelled on the surface with rabbit polyclonal anti-surface IgG and then fluorescently-labelled with the second antibody, FITC-conjugated goat anti-rabbit IgG. Mutants of *C. elegans*, srf-2 and srf-3 , were labelled with first antibody which had been pre-adsorbed with wild-type adults. This pre-adsorbed first antibody has a low titre so that 95µl of adsorbed serum is equivalent to 3µl of unadsorbed serum. The mutants were also fluorescently-labelled with FITC-conjugated goat anti-rabbit IgG.

2.6.6 Preparation of samples for Microscopy

Samples which had been fluorescently-labelled with lipid probes, chemical

probes, lectins or antibodies were thoroughly washed and pipetted onto clean microscope slides within a thin border of silicone grease (from Dow Corning). Larger nematodes, e.g. the adult filarial nematodes, were placed inside the square of silicone grease using a cat's whisker. A coverslip was then placed gently over the sample so that the greased edges were sealed, but so that the surface of the nematode was not in contact with the coverslip.

2.7 Radiolabelling studies.

2.7.1 ^{125}I-iodination

Materials

Na ^{125}I (5 mCi, carrier-free) was supplied by the Western Infirmary, Glasgow.

Iodogen (1, 3, 4, 6-tetrachloro-3α, 6α-diphenylglycouril) was supplied by Pierce. Iodogen was dissolved in chloroform and this was used to coat the inside of glass test-tubes under a stream of nitrogen gas. One side of a coverslip could also be coated with Iodogen for radioiodination of the isolated cuticular surface of adult *Ascaris* worms.

Method

Adult worms or larvae were washed in PBS or the appropriate culture medium, and placed in a glass tube containing 50 μCi (1 Ci = 37 GBq) of carrier-free Na^{125} in a total volume of 1 ml of PBS, and 100 μg Iodogen, dried down from a chloroform solution. The reaction was allowed to proceed for 15 minutes and then stopped by adding 100 μl PBS saturated with tyrosine. The adults or larvae were then removed to a conical glass centrifuge tube and washed exhaustively in PBS to remove unbound radiolabel.

Adult *Ascaris* cuticles were iodinated using a slightly different method (adapted from Fetterer, 1988). Clean cuticles were obtained by cutting the whole worm at the extreme anterior and posterior ends, and squeezing out the contents with large forceps. Extreme care must be taken during this procedure as antigens can be thrown up into the air, and un-embryonated eggs can be released. Thin strips of muscle were

removed from the cuticle preparation using fine forceps, leaving behind the clean cuticle (see figure 2.1). The tube-like cuticle was then cut transversely to produce a flat sheet. This was then pinned down onto a small petri-dish containing Silguard, with the external side of the cuticle facing uppermost. The cuticle was iodinated using a droplet (10 µl) of $Na^{125}I$ (20 µCi) and a coverslip coated on only one side with Iodogen. Again this reaction was allowed to proceed for 15 minutes, and the piece of cuticle was washed exhaustively, first in PBS saturated with tyrosine (to stop the reaction) and then with PBS to remove unbound radiolabel.

Radio-iodinated larvae, adults and cuticle pieces could then be prepared for analysis of proteins (section 2.9) or lipids (section 2.10).

2.7.2 Metabolic labelling with ^{35}S-methionine

Materials

L-[^{35}S]-methionine (in 20mM potassium acetate solution, containing 0.1% 2-mercaptoethanol). Batch was 3.75 mCi at the time of the experiment, (1,300 Ci/mmol)

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

Method

Nematode larvae were cultured either as pre- or post-infective larvae, i.e. for pre-infective forms of *N. brasiliensis* culture was in distilled water at room temperature (approx. 18°C), and for post-parasitic forms of these larvae, culture was in MEM w/o methionine at 37°C. In both cases (pre- and post-infective), 200 µM chloramphenicol was included in the incubation medium to try to eliminate bacterial protein synthesis. Bacteria could not be removed by extensive washing in a solution of penicillin and streptomycin and it was thought that they must be adhering strongly to the surface of the worms, hence the requirement for chloramphenicol. In each experiment, 500 worms were radiolabelled with 20 µCi ^{35}S-methionine in a total volume of 1ml. Radiolabelling was allowed to proceed for a period of 4h. After the labelling period, the nematodes were cooled on ice for 10 minutes, then washed 3 or 4 times, by centrifugation at 500g for 1 minute, in 10 ml of ice-cold medium. Complete medium (RPMI) was generally used for washing. After the final wash, all medium was

Figure 2.1 Isolated *Ascaris suum* cuticle

Photomicrograph showing two pieces of cuticle from *Ascaris suum*
The one on the left is clean and suitable for surface-radio-iodination, whereas the one on the right is shown with some muscle still attached for comparison.

removed, and the nematode pellet frozen at -20°C.

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

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

(Protocol based on Pratt, 1984; and Wales,1989).

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

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

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

(ii) Counting Procedure

Samples were counted on a Beckman LS335 scintillation counter. Each sample was counted for 4 minutes.

2.8 Collection of proteins released by nematode larvae during culture

(1) Materials

Dialysis tubing 8/32"	Scientific Instrument Centre Ltd.
or	
PD-10 columns pre-packed with Sephadex G-25M.	Pharmacia
Phenyl methyl sulphonyl fluoride (PMSF), Protease inhibitor made up as 0.1M in acetone.	Sigma
Dichlorodimethylsilane solution, approx. 2% in 1,1,1-trichloroethane ("Repelcote").	BDH Chemicals

(2) Procedure

At the end of a culture period, the nematode larvae were counted, transferred to sterile 13.5ml plastic centrifuge tubes, and cooled on ice for 10 minutes. The larvae were centrifuged at 3,000 r.p.m. for 5 minutes, and the supernatants, containing proteins released by the parasites during culture, were transferred to fresh centrifuge tubes, and the process was repeated. 5µl of PMSF was added per ml of medium. No parasite bodies were still present at the end of the centrifugations.

(i) Dialysis

The culture supernatants, containing PMSF, were transferred to small dialysis bags, and dialysed against 1 litre of distilled water at 4°C. The dialysis water was changed several times over a 48h period. The dialysed solutions were then decanted into 1.5ml polytubes, pre-treated with Repelcote. After freezing in a bath of dry ice and methanol, the supernatants were freeze-dried overnight. The dry proteins were stored at -20°C.

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

A PD-10 column packed with Sephadex G-25M was pre-blocked with 100µl of 10% (w/v) BSA in distilled water, then equilibrated with 25ml distilled water. The culture supernatant was diluted to 2.0ml with distilled water, and added to the column. When the sample had run into the column, 3.0ml distilled water were used to elute the proteins into a glass centrifuge tube treated with Repelcote. The de-salted solution was then freeze-dried.

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

(1) Gel materials

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

(2) Stock solutions

Solution A:	30% (w/v) Acrylamide 0.8% (w/v) N, N'-methylene bisacrylamide

Solution B:	1.5M Tris-HCl buffer, pH 8.8
Solution C:	0.5M Tris-HCl buffer, pH 6.8
Solution D:	10% (w/v) SDS
Solution E:	1.5% (w/v) Ammonium persulphate (made up fresh for each gel).

(3) **Gel composition**

10% (w/v) acrylamide resolving gels were used, and a 3% (w/v) acrylamide stacking gel was used with the following proportions of solutions:

	Resolving gel (10%)	Stacking gel (3%)
A	13.3	2.5
B	5.0	-
C	-	5.0
D	0.4	0.2
E	2.0	1.0
Dist. H_2O	19.3	11.3

Mixed gently, de-gassed if necessary; then added

TEMED	50µl	15µl

(4) **Sample Preparation**

Protein estimation

Protein estimation was carried out by the method of Lowry et al (1951) using the following reagents:

Folin and Ciocalteu's phenol reagent (BDH)

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

4% (w/v) Sodium potassium tartrate

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

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

PBS

Standard protein solutions made from BSA stock solution of 10.0 mg/ml.

Sample buffer:

2% (w/v) SDS

10% (w/v) Sucrose (Koch-Light Labs.)

5% (v/v) 2-mercaptoethanol (Koch-Light Labs.)

0.001% bromophenol blue (BDH Chemicals) in 0.1M Tris-HCl, pH 6.8

(5) Preparing the gels

Materials

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

Spacer set of 3, 1.5mm width.

20-well comb, 1.5mm width.

These materials were all obtained from Bethesda Research Labs.

The glass plates were cleaned with laboratory detergent, rinsed thoroughly with distilled water and then with ethanol.

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

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

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

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

Electrophoresis buffer:

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

Electrophoresis buffer:

0.025M Tris

0.192M glycine (BDH Chemicals)

0.1% (w/v) SDS

(pH 8.3)

(6) **Fixing and Staining gels.**

Staining for 2h at 37°C in 500ml of 0.1% (w/v) kenacid blue (coomassie blue) stain in 50% methanol, 5% acetic acid.

Destain: 25% methanol, 10% acetic acid. Stain detects 0.2-0.5mg of protein in a single band (Hames, 1981).

2. 10 Analysis of Nematode Lipids

2.10.1 Extraction of Lipids

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

Materials

All solvents were Analar grade (see below for suppliers).

Extraction solvents mixture:

chloroform : methanol

2 : 1

"Upper-phase solvent" mixture:

chloroform	: methanol	: water
3	: 48	: 47

Procedure

The nematode pellet, or piece of isolated adult *Ascaris* cuticle, was re-suspended in 100 µl PBS in a graduated glass centrifuge tube. 2 ml of the extraction solvents mixture were added. The extract was vortexed or mixed vigorously by hand, whichever was necessary. This extract was left for 1h at room temperature under nitrogen. In the case of the *Ascaris* cuticle, the '1h at room temp. extract' was aspirated and retained, however the cuticle was then subjected to further extraction in hot (65^{o}C) extraction solvents which were added to the initial extract.

The extract should have appeared as one phase. Solvent was aspirated, leaving behind the larval bodies (there was usually no need for centrifugation), and this was then the crude extract. To this, 800 µl of distilled water was added. The solvent and water were mixed together, then allowed to separate into two phases. The upper aqueous phase was removed. To this, 300 µl of "upper-phase solvents" was added, then removed. This was done 3 times to wash the interface and then a little chloroform : methanol was added to return to one phase. The final washed extract was then blown dry with a slow jet of nitrogen gas, and if necessary, stored under nitrogen at -20^{o}C.

2.10.2 Lipid Standards

All of the following lipid standards were obtained from Sigma, except waxes which were from Koch-Light (Bucks.).

Neutral lipids

Oleic acid (18:1); palmitoleic acid (16:1); stearic acid; monoolein; diolein; triolein; monostearin; distearin; tristearin; cholesterol; cholesteryl oleate (18:1); C_{22} and C_{36} waxes.

Phospholipids

L- α-phosphatidylethanolamine (PE), (type VII from bovine liver);

L- α-phosphatidylcholine (PC), (type III from bovine liver);

L- α -phosphatidic acid (PA), (from egg yolk lecithin, phospholipase D hydrolysis);

L- α -phosphatidyl-DL-glycerol (PG), (from egg yolk lecithin);

L-α-phosphatidylinositol (PI), (from bovine liver);

L-α-lysophosphatidylcholine (lysoPC), (from bovine liver);

L-α-lysophosphatidylethanolamine (lysoPE), (from bovine brain);

Diphosphatidylglycerol (cardiolipin), (from bovine heart).

2.10.3 Thin-layer chromatography of lipids

(i) **Materials**

Silica G plates:

pre-coated plastic sheets (20cm x 20cm)	Camlab.
Polygram Sil G Layer: 0.25mm gel.	

Chromatography tanks:

20cm x 12cm x 20cm.

Chromatography paper 3MM.	Whatman
Pre-calibrated pipettes (10 µl)	L.I.P.

Organic solvents:

Chloroform (Analar)	BDH
Hexane (Spectrosol^{R})	
Diethyl ether (Analar)	
Methanol (Analytical Reagent)	Fisons
Acetic acid glacial (Analytical Reagent)	

Conc. HCl (Analytical Reagent)

acetone (Analar) May & Baker

Detection and identification:

Iodine (vapour produced in tank) BDH

Conc. H_2SO_4 BDH

Orcinol ferric chloride spray reagent (Bial's reagent),
0.9% ferric chloride and 0.55% orcinol in acidified ethanol. Sigma
Detects sugars, glycosides and sulpholipids.

(ii) **Procedure for one-dimensional t.l.c.**

(adapted from Storry & Tuckley, 1967)

Neutral lipids:

Hexane : Diethyl ether : Acetic acid

80 : 20 : 2

Phospholipids lipids:

Chloroform : Methanol : Acetic acid : Water

25 : 15 : 4 : 2

Procedure

T.l.c. plates were heated overnight at 60°C and lightly marked with pencil with

the appropriate markings. Samples were spotted at the origin which was 1 cm up from the edge of the plate, using a pre-calibrated pipette (10 µl) with intermittent drying from a hot air hair-dryer. The t.l.c. tank (for simple or complex lipids) was set up lined with 3MM filter paper at each end, acting as a wick . The appropriate solvent mixture was added to the tank, and the lid was greased well with vacuum grease. The tank was left to equilibrate for 2h. The chromatogram was then placed gently in the tank so that the origin was just above the solvent, and it was allowed to run for approx. 25 minutes or until the solvent front had reached 1 cm from the edge. The chromatogram was then blown dry with hot air. Chromatograms could then be exposed to iodine vapour to develop the spots, or if radioactively labelled, exposed to X-ray film.

2.11 Chemical Analysis of cuticular sugars

Phenol-sulphuric assay for neutral sugars

The procedure described here is a scaled-down version of the method of Dubois et al (1956).

Reagents

Concentrated H_2SO_4 (sp. gr. 1.84).	BDH
Phenol: a 5% w/v aqueous solution of colourless crystals	Formachem Ltd.
Standard D-mannose solution (40 µg/ml) stored at -18°C	Sigma

Procedure

The reaction was carried out in Pyrex test-tubes. To a sample containing about 10 µg neutral sugar dissolved in water (0.5 ml), phenol reagent (0.3 ml) was added and mixed. Concentrated H_2SO_4 (2ml) was then added rapidly from a fast-flowing pipette and mixed immediately (care). The heat generated by mixing acid and water is essential for colour development. After standing for 30 minutes the absorbance was read at 484 nm.

A water blank and standards of 5-40 µg mannose were included in each assay.

2.12 General Equipment

Bench centrifuge for 1.5ml polytubes (MSE Microcentaur) from Beckman Instruments Ltd.

LKB (Wallac) 1209 Rackbeta, Liquid scintillation counter, from Pharmacia-LKB.

Freeze-dryer: LSL Secfroid (Lyolab A).

2.13 Fluorescence-based Biophysical Techniques

2.13.1 Fluorescence microscopy.

Materials & Equipment

Orthoplan U.V. microscope	
U.V. power house 100	
Bright-field power house	Leitz, Wetzlar,
Control panel	Germany.
Camera control unit	
Objective lenses (x10, x 25,	
NPL Fluotar x40 & x100)	
Fluorescence quantitation - MVP Compact.	
400-1600 ASA professional film	Ektachrome
(colour and black & white).	

The computer used for collection of fluorescence quantitation data was a Hewlett Packard 85.

Microscopy and photography

Fluorescence observations and photomicrographs were obtained with a U.V. microscope, a standard camera attachment, and Ektachrome professional film. Exposures were between 1 and 5 minutes for fluorescence photomicrographs. Approriate filter combinations were used to discriminate between the various fluorescent probes used.

Fluorescence quantitation

The Leitz MPV compact consists of a photomultiplier tube and control panel which allows quantitation of fluorescence from a defined area, which was fixed at 400 μm^2. An average background reading, from a relatively non-fluorescent region of the slide, was subtracted for each group of 20 readings. The voltage across the photomultiplier was fixed at 700V and the mains current held constant by a Leitz steady current power pack.

Fluorescence Quenching

The depth of insertion of fluorescent probes in the membrane was examined by measuring the extent to which a non-permeant molecule, trypan blue (0.25, w/v), could quench fluorescence. Trypan blue quenches the fluorescence of fluorescein due to the phenomenon of resonance energy transfer. Spectral overlap between the emission spectra of fluorescein and the absorption spectra of trypan blue is such that if the two molecules are sufficiently close (R_0 value < 3.8nm), energy will be transferred from one to the other and fluorescence will decrease substantially (Stryer, 1978; Foley et al, 1986).

2.13.2 Fluorescence recovery after photobleaching (FRAP)

The biophysical technique of fluorescence recovery after photobleaching (FRAP) is used extensively in the study of lateral diffusion of plasma membrane components (for reviews see Peters, 1981; Wolf, 1988). The following description of FRAP is based on that by Wolf (1989): In FRAP, the molecule whose diffusion is to be measured must be fluorescently labelled directly or indirectly in a noncross-linking manner. A laser beam is focussed using a modified fluorescence microscope to a small

spot on the sample. The light from this spot is monitored and found to be essentially constant with time. The incident light level is then momentarily raised approximately 10,000-fold so as to irreversibly photobleach a significant fraction of the fluorescence within the spot. Thus, when one returns to the monitoring intensity, the fluorescence intensity is found to be significantly reduced. If there is no freedom of motion of molecules in and out of the spot, the fluorescence intensity will remain at this level ad infinitum. This is the condition of no diffusibility. If, however, there is complete freedom to diffuse in and out of the spot, the fluorescence intensity will return to the the prebleach level (as is indicated in figure 1.8). The diffusion coefficient is obtained by fitting the recovery data to diffusion theory (Axelrod et al, 1976). An intermediate condition can also occur where only a fraction of the molecules is free to diffuse. In that case only partial recovery is observed. FRAP thus provides two measures of diffusibility; the fraction or percent of the molecules free to diffuse, %R, and the diffusion coefficient of that fraction D.

Instrumentation used in FRAP

This section gives a brief description of the important features of the FRAP apparatus, a more detailed approach is given by Wolf (1989).

The laser

A 1.0W LEXEL MODEL 85 Argon ion laser was used (Lexel Corp, Palo Alto, Calif.), with a tap-water cooling system, and adjusted for maximal first order diffraction at 488 or 514.5 nm. Argon ion lasers produce the highest visible power levels and have up to ten lasing wavelenghths in the blue and green portion of the spectrum. In addition to being monochromatic, laser light has a very defined mode structure. The fundamental mode, referred to as the TEM_{00} mode, is Gaussian in intensity profile. Axelrod et al (1976) assumes a Gaussian TEM_{00} mode. The word "laser" stands for light amplification by stimulated emission of radiation. A detailed description is given elsewhere (Johnson, 1983).

The beam attenuator

The bleaching and measuring beam intensities are controlled by a method

which uses a digital acousto-optic modulator (Model 304D, Coherent Associates, Danbury, Conneticut) to attenuate the average intensity of the laser beam by variation of the on-off duty cycle. For example, to acheive an attenuation of 10^4 the measuring beam is delivered as 10-μs pulses at 10 Hz, whereas bleaching is achieved by an uninterrupted pulse of 0.1-10s (see Garland, 1981).

Spatial filter

The spatial filter was obtained from Photon Control, Cambridge, U.K. Spatial filtering is simply an opto-mechanical method of separating the wanted beam from the undesirable noise, and then mechanically blocking the unwanted noise.

Fluorescence microscope and Temperature stage.

The Leitz microscope (Ortholux), contains two secondary image planes. The first of these being where the objective focusses the specimen to a real image. This is usually about 170 mm above the objective in an upright microscope. A second secondary image plane is located also about 170 mm beyond the objective, only along the imaginary path of a ray reflected back by the dichroic mirror. If the laser beam is focussed onto this image plane, it will also be in focus on the object plane.

The temperature stage (Leitz) could be heated between 20°C and 37°C, or alternatively, cooled down to 6°C by pumping ice-cold water through it. Temperatures of less than 6°C were not used since condensation would appear on the glass coverslip, hindering optical measurements.

Optical components

The complete FRAP apparatus is arranged on an optical table (Photon Control, Cambridge). Optical components are all mounted on optical rails (Oriel standard rails). Adjustable mirror mounts (Oriel model 1450) are used to align and direct the laser beam.

Control electronics

The FRAP laser controller GUED 729 was designed and constructed by the Department of Electronic Engineering, Glasgow University. The photomultiplier tube

(P.M.T.) model 9924b was obtained from Thorn E.M.I. , U.K, and powered by a Brandenburg P.M.T. power supply. The signal from the P.M.T. was collected by a Coherent P.M.T. signal collector model 304. A Gould digital storage oscilloscope type 4035 was used to 'capture' the signal which produces the FRAP curve. A Hewlett Packard Integral personal computer model 82927A, and plotter model 747OA (supplied by Electroplan) were used to retreive the relevant information about the FRAP curve, using a program which was kindly supplied by
Dr. John Birmingham.

Measuring the beam radius (spot size)

Before data can be expressed as an absolute diffusion coefficient, it is necessary to measure the radius of the beam. A variety of methods exist for performing these measurements (reviewed by Johnson, 1983) and I have used the method of Thompson et al (1980) where essentially one carries out a simple photobleaching experiment on a thin film of FITC-labelled BSA suspended in 95% glycerol at room temperature. A typical FRAP curve for FITC-BSA in 90% glycerol is shown in figure 2.3. The theoretical value for the diffusion of albumin in solution is $6 \times 10^{-7} cm^2 s^{-1}$, which had been determined experimentally by quasi-elastic light scattering (Raj and Flygare, 1974). By taking this theoretical value and the viscosity of glycerol at 37°C, the predicted diffusion coefficient was estimated. This value was calculated to be $7.5 \times 10^{-9} cm^2 s^{-1}$. If we now insert this value into the familiar diffusion equation;

$$D_L = (w^2 / 4t_{0.5})\gamma$$

we have all variables except w. Both γ and $t_{0.5}$ can be obtained from the recovery curve.

2.14 Electron microscopy

Materials

Sodium cacodylate	
glutaraldehyde	BDH
Osmium tetroxide	Sigma

Procedure

Specimens were exposed to 3 consecutive fixatives, according to the system described by Hockley and McLaren (1973):

(i) 2% v/v glutaraldehyde in the cacodylate buffer system of Lewis and Shute (1966), consisting of 0.05M sodium cacodylate/0.002M calcium acetate, pH 7.4. Gluteraldehyde fixation proceeds for 4h at 4°C followed by 3 washes in ice-cold Lewis-Shute buffer.

(ii) 1% (w/v) osmium tetroxide in Lewis-Shute buffer for 2h at 4°C, followed by 10 washes in ice-cold distilled, de-ionized water.

(iii) A 0.5% aqueous solution of uranyl acetate, pH 5.0, containing 45 mg/ml sucrose, for 1.5h at 4°C in total darkness (system developed by de Harven, 1967).

After 3 washes in cold, distilled water, the specimens were dehydrated by a series of incubations in increasing concentrations of ethanol as follows:

(1) 25% ethanol for 10 minutes.

(2) 50% ethanol for 10 minutes.

(3) 75% ethanol for 10 minutes.

The specimens were then resuspended in 100% ethanol, and taken to Dr. T. Downie at the Electron Microscopy Unit in the Western Infirmary, Glasgow, where the specimens were embedded in araldite, thin sections were cut with a diamond knife, and stained with uranyl acetate and lead citrate.

Histochemical staining of nematode sugars was examined, in collaboration with Dr. Walter Robertson, using the periodate-thiosemicarbazide-silver proteinate (PA-TSC-SP) method of Spiegel et al (1983).

2.15 Differential scanning calorimetry

Temperarture-scanning calorimeters are usually constructed as differential instruments (DSC = differential scanning calorimeter), where the experimental temperature changes continuously during the measurement. These instruments, which are heat capacity calorimeters, are of great importance in biothermodynamics, particularly for the characterization of thermally induced transitions of biopolymers and aggregates such as membrane materials (Sturtevant, 1987). The DSC measurements for this study were kindly performed by Dr. Alan Cooper of the Department of Chemistry, Glasgow University.

Equipment

MicroCal - OMEGA reaction cell

MicroCal scanning calorimeter, model MC-2

Samtron computer

Principle and procedure

The MC-2 is a differential instrument, utilizing twin 1.0 ml total-fill cells. The sample cell is normally filled with a dilute solution containing the biological substance. In this case, the sample cell was filled with one of the following suspensions:

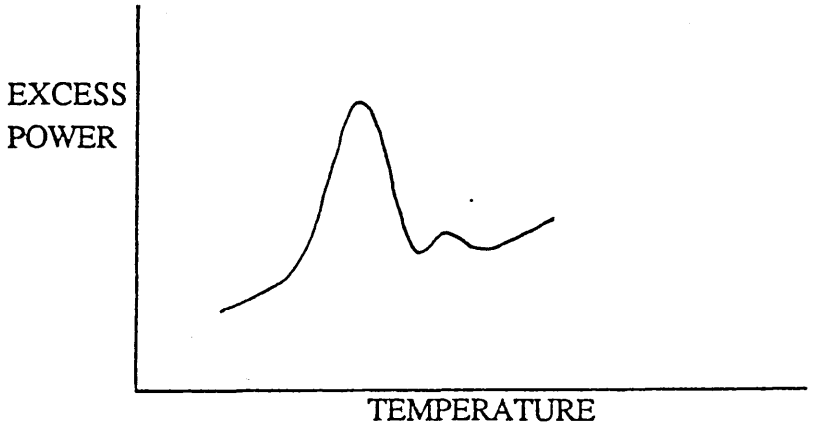
(a) Pre-infective MSL of *T. spiralis* in PBS which had been maintained at room temperature (18^{o}C).

(b) Post-infective MSL of *T. spiralis* in RPMI which had been maintained at 37^{o}C.

(c) Adults of *T. spiralis* in RPMI which had been maintained at 37^{o}C.

The other cell is called the reference cell and is filled with an identical amount of solvent. Each cell has two sets of heaters on its outer surface, i.e. the main and the auxilliary heaters. An experiment is started by connecting the power supply to the main

heaters so that the temperature of the cells begins to rise. The main heaters are pre-trimmed so that the sample cell receives slightly less power than the reference cell, i.e. its temperature rises slightly less rapidly when current passes through the main heaters. There is a thermopile between the sample and reference cells, which senses the off-balance temperature, ΔT and produces a corresponding voltage (0-5 μV). This voltage is then amplified and used to drive (0.5 mA) the auxilliary heater on the sample cell, which then acts to keep the off-balance signal close to zero. A typical display would be;



where peaks are generated if the solute undergoes a structural change.

2.16 Studies on the transition in surface biophysical properties between the pre- and post-parasitic states.

2.16.1 Measurement of the time taken for AF18 insertion

Pre-infective L3's were washed free of medium by 2 consecutive centrifugations and washes, and resuspended in RPMI at 37°C. An identical type of experiment was performed with the model for infective-stage parasites, i.e. the ex-sheathed dauer larva (L3) form of *C. elegans*, except that the stimulus used for

transformation was a fresh bacterial food source.

The time taken for insertion of the AF18 lipid probe into the surface of each of the different worms was recorded from the initial time of exposure to the new external environment. In the case of MSL of *T. spiralis* timings of AF18 insertion were also compared for different combinations and concentrations of RPMI, trypsin and bile. For exsheathed *O. ostertagia* the time was recorded from the initial point of exsheathment.

2.16.2 Measurement of the extent of AF18 insertion using different culture conditions.

The extent of AF18 insertion in different species of L3 was measured using fluorescence quantitation microscopy. Fluorescence was measured in arbitrary fluorescence units and these values could be compared for a particular species using particular incubation conditions (e.g. Grace's insect medium at 27^{o}C *versus* RPMI 1640 at 37^{o}C.

2.16.3 Monitoring the release of FITC-cationised ferritin from the surface

The time taken for the release of FITC-cationised ferritin from the surface of L3's of *Strongyloides ratti* was measured by fluorescence quantitation in worms which had been incubated in (i) distilled water at 18^{o}C; and (ii) RPMI at 37^{o}C. Fluorescence measurements (in arbitrary fluorescence units) were taken from both groups of worms at t=0, t=20min. and t=60min, with washing in between measurements.

2.16.4 Serum effects on AF18 insertion

Normal, hyperlipidaemic and de-lipidated sera (see section 2.2.3), were used to evaluate the effects of serum, in particular the lipids, on AF18 insertion in pre- and post-infective L3's of *B. pahangi* and *A. viteae* . Before addition of the fluorescent lipid probe, as much of the serum as possible was removed by at least 5 consecutive centrifugations and washes in the appropriate medium.

2.17 Physical and chemical stimuli used to promote transformation in a normally non-permissive environment

Various physical and chemical stimuli were used to investigate the mechanism of the transformation event. AF18 insertion was used as a marker for transformation. The so-called pre-infective L3's were maintained in the appropriate conditions which would normally keep them in the pre-infective state (e.g. Grace's insect medium at 27°C for *B. pahangi*), and then subjected to various chemicals which were candidates for the transformation stimuli. These included:

2.17.1 Increasing Na^+ion concentration

For this experiment, L3's which had been maintained in water or Grace's insect medium were exposed to 140mM NaCl. To assess whether any effect of Na^+ ions could be replaced by potassium ions (K^+) or choline ($N^+(CH_3)_3$), 150mM KCl, and 150mM choline-chloride were used as alternatives.

10μM monensin, a Na^+ionophore, was used in the presence or absence of 10mM NaCl.

2.17.2 Increasing HCO_3^- ion concentration

L3's which had been maintained in water or Grace's insect medium were exposed to 40mM $NaHCO_3$ for 1h.

2.17.3 Increasing (more alkaline) pH

The fact that there is an alkaline pH shift in going from Grace's insect medium (pH 6.0) to RPMI (pH 7.4) (see table 2.1), points towards a possible involvement of the H^+ or HCO_3^- ion concentration. In these experiments, only the effect of H^+ ion concentration was investigated. 0.05M Tris-HCl (pH 7.6) was used to adjust the pH of Grace's from 6.0 to 7.4, and 1M HCl was used to adjust the pH of RPMI from 7.4 to 6.0.

2.17.4 Chemicals affecting the activity of Adenylate Cyclase

The stimulation to transform to an AF18 lipophilic surface, or inhibition of this

process, was investigated using the following chemicals.

cAMP (5 mM) and 8-bromo-cAMP (5 mM) were used alone or in combination with forskolin (100 μM) and serotonin (1mM), which are potent activators of adenylate cyclase (Nishizuka, 1984, 1986). Imidazole (20mM) and NH_4Cl (20mM), are cAMP/cGMP phosphodiesterase activators and could reduce levels of these second messengers in the nematode, thus possibly affecting the activity of adenylate cyclase (Kawamoto et al, 1989). IBMX, which was used at 100 μM, is a cAMP/cGMP-phosphodiesterase inhibitor.

2.17.5 Chemicals affecting the activity of Guanylate Cyclase

8-bromo-cGMP (10 mM) was used alone or in combination with sodium nitroprusside (10 μM) and IBMX (100 μM), which were used as they are known as potent activator and inhibitor of cAMP/cGMP-phosphodiesterase, respectively.

2.17.6 Chemicals affecting the activity of Protein Kinase C (PKC)

12-O-tetradecanoylphorbol-13-acetate (TPA) is a tumour promoting phorbol ester which activates PKC (Nishizuka, 1984a & b). TPA (1 mM) obtained from Sigma, was dissolved in DMSO which following dilution did not exceed 1% in each experiment.

2.17.7 Chemicals affecting Ca^{++} levels or action

Nicardipine (Ca^{++} channel inhibitor) was used at 50 μM concentration (stock 10 mM in DMSO). The following drugs were dissolved in distilled water to a stock concentration of 10 mM. TMB-8 (an inhibitor of Ca^{++} release from internal resources) was used at 200 μM concentration. W-7 (calmodulin inhibitor) and W-5 (an inactive analogue of W-7) were used at 100 μM concentration. EGTA (a chelator of Ca^{++}) was used at 10 mM concentration.

The potent Ca^{++} ionophore A23187 was made up in DMSO as a 10 mM stock solution. This stock solution was then used at 5 μM concentration.

Chapter Three

Fluorescence-labelling studies.

3. 1. Introduction

Microscopic studies designed to reveal the presence and topological distribution of surface components on living cells rely heavily on fluorescent probes of known specificity. Extensive use has been made of fluorophore-labelled macromolecules, such as antibodies, lectins and ligands of specific receptors, to locate and chart the behaviour of specific membrane proteins. However, only recently have smaller hydrophobic probes, either naturally fluorescent or conjugated to a fluorophore, begun to yield similar information on the structure and organization of the lipid moiety of the membrane.

In order to gain information on the structure and organization of the intact nematode epicuticle, it was necessary to select fluorescent probes which would insert into, or react chemically with the epicuticle without damaging or penetrating it.

A variety of lipid probes of varying hydrophobicity with different fluorophores (see figure 1.7) were used to probe epicuticle organization. The aminofluorescein (AF) lipid probes and the carbocyanine lipid probes are slightly more polar than phospholipids and tend to bind to membranes with their anionic and cationic fluorophores, respectively, at the aqueous interface and the fatty tail(s) embedded in the hydrophobic layer (Radda, 1975; Axelrod, 1979). Lack of charge in the nitrobenzoxazdiazole (NBD) fluorophore permits 'sensing' of the hydrophobic interior of a membrane and can be conjugated with phospholipids, fatty acids and sterols.

The depth of insertion of these different fluorescent probes can be estimated by measuring the extent by which a non-permeant molecule included in the medium (Trypan blue) can quench the fluorescence of the probe by Forster resonance energy transfer (Foley et al, 1986). A detailed description of the labelling procedures for these lipid probes is given in Chapter 2, section 2.6.1.

Chemically reactive fluorescent probes were used to label surface proteins, which included sulphydryl- and amino group-reactive probes. A detailed description of the labelling procedures for these probes is given in Chapter 2, section 2.6.2.

3.2. Results

3.2.1. Insertion of fluorescent lipid probes in the nematode surface.

The nematode surface is selective for the insertion of fluorescent lipid probes, and this selectivity is exhibited for a wide range of nematode species and stages (tables 3.1-3.7). There are also particularly interesting differences within the infective-stage larvae in that all of the lipid probes are excluded until there is some appropriate stimulation for developmental change (see chapter 6 for more detailed studies on this phenomenon).

The results of fluorescence-labelling of the nematode surface with lipid probes can be summarized as follows:

(i) *Toxocara canis*(table 3.1):

Of the aminofluoresceins, only AF18 inserted into the surface, i.e. the shorter chain-length probes AF12 and AF16 were excluded. AF18 was found to be surface-restricted by trypan-blue quenching, as were NBD-chol and nile red (NR).

(ii) *Trichinella spiralis* (table 3.2):

AF18, NBD-chol and NR were surface-restricted in adults, but were excluded from muscle-stage larvae (MSL) which had been maintained in PBS at 18°C.

AF18, NBD-chol, NR and Mc540 were surface-restricted in new-born larvae (NBL).

The insertion of the cationic lipid probes RH18 and DiI18 in adults was permitted only after the worms had been treated for 10 minutes in 0.01% CTAB detergent, and then treated for 1 hour in $10mgml^{-1}$ protease. This treatment also caused a 42.2% increase in AF18 insertion in adults, when compared to controls.

(iii) *Acanthocheilonema viteae* (table 3.4):

AF18, NBD-PC and NBD-cholesterol (NBD-chol) remained surface-restricted (measured by trypan blue quenching) within a period of approximately 2 hours culture in RPMI 1640 at 37°C. After this time, internalization of the NBD-chol probe began (fig. 3.1). In this same parasitic stage, AF18 and NBD-PC remained surface-restricted for up to 48 hours in RPMI 1640 at 37°C, suggesting that this type of probe is not internalized to any great extent. In contrast, NBD-PE was internalized within minutes (figs. 3.1 & 3.2(b)). The mechanism for

Table 3.1 : **Insertion of fluorescent lipid probes in the surface of *Toxocara canis* infective-stage larvae.**

Probe	Fluorescence Intensity	% Quenching
AF12	-	
AF16	-	
AF18	+++	85.2±4.6 (4)
RH18	-	
DiI18	-	
NBD-chol	++	79.6±8.1 (6)
NR	+++	90.2±5.5 (3)
Mc540	-	

The fluorecently-labelled worms were washed in RPMI 1640 and immobilised using levamisole ($1mgml^{-1}$) or 0.1% (v/v) 2-phenoxy-propanol. The intensity of fluorescence is represented by an arbitrary scale of - to +++.

The experiment was performed on three separate occasions. On each occasion, AF18, NBD-chol and nile red (NR) were found to be confined to the outermost surface, as determined by trypan blue (0.25%) quenching (number of worms tested is shown in brackets).

Table 3.2 : **Insertion of fluorescent lipid probes in the surface of *Trichinella spiralis* adults, infective muscle-stage larvae (MSL), and new-born larvae (NBL).**

Stage in the life-cycle	Probe								
	AF12	**AF18**	**RH18**	**DiI18**	**NBD-chol**	**NR**	**Mc540**	**NBD-PE**	**NBD-PC**
Adult	-	+++	-	-	++	++	internal	internal	+
0.01% CTAB + protease	+++	++++	+++	++	++	++	internal	internal	++
MSL	-	-*	-	-	-	-	-	-	-
NBL	internal	+++	-	-	+	+	++	not done	not done

*Surface lipophilicity for AF18 changes with environmental conditions which mimic the infection process (see Chapter 6).

Worms were incubated for 10 minutes with the above-mentioned probes in RPMI 1640 mammalian cell culture medium for adult worms and NBL, and phosphate-buffered saline for MSL.

The experiment was performed on five separate occasions. The intensity of fluorescence is represented by an arbitrary scale of - to ++++.

AF18 insertion in adults was increased by 42.2% after 0.01% CTAB and 10mgml^{-1}protease.0

Table 3.3 : **Determination of the extent of epicuticular permeability in adults of *Trichinella spiralis* to the membrane-impermeant dye Trypan blue.**

Nematodes (n = 6)	Fluorescence measurement of AF18-labelling (arbitrary units- backround)	% Quenching with 0.25% trypan blue.
1st set of readings	36.9 ± 4.5 (8)	89.2 ± 7.8 (5)
2nd set of readings*	38.4 ± 4.9 (8)	87.7 ± 7.0 (5)

* Trypan blue removed from same worm, fluorescence readings repeated, then Trypan blue quenching readings repeated.

There were 8 separate fluorescence readings for each of the 6 worms; and 5 separate readings for % quenching in each of the 6 worms.

The percentage quenching is equal to:

$$100 \times (F_- - F_+ / F_-)$$

where F_- and F_+ are fluoresence intensities in the absence and presence of Trypan blue, respectively.

The readings were not significantly different between the first and second set of fluorescence measurements ($P < 0.5$) and the first and second set of quenching measurements ($P < 0.6$).

Table 3.4 : **Insertion of fluorescent lipid probes in the surface of *Acanthocheilonema viteae* adults, infective third-stage larvae (L3's), and microfilariae (mf) which are unsheathed.**

Stage in the life-cycle	Probe								
	AF12	**AF18**	**RH18**	**DiI18**	**NBD-chol**	**NR**	**Mc540**	**NBD-PE**	**NBD-PC**
Adults	+++	+++	-	-	+++	++	-	internal	++
L3's	-	-*	-	-	-	-	-	-	-
mf	internal	+++	-	-	+++	internal	+++	not done	not done

* The lipophilicity of the surface of *A. viteae* L3's for AF18 changes with environmental conditions mimicking the infection process (see results section in Chapter 6).

The intensity of fluorescence was measured on an arbitrary scale of - to +++, and internal labelling was determined using trypan blue (0.25%) quenching.

The experiment was performed on four separate occassions for adults and mf, but on ten separate occassions for the L3's.

Figure 3.1 **Time course of the internalization of the fluorescent lipid probes AF18, NBD-PC, NBD-PE and NBD-chol from the surface of adult *Acanthocheilonema viteae***

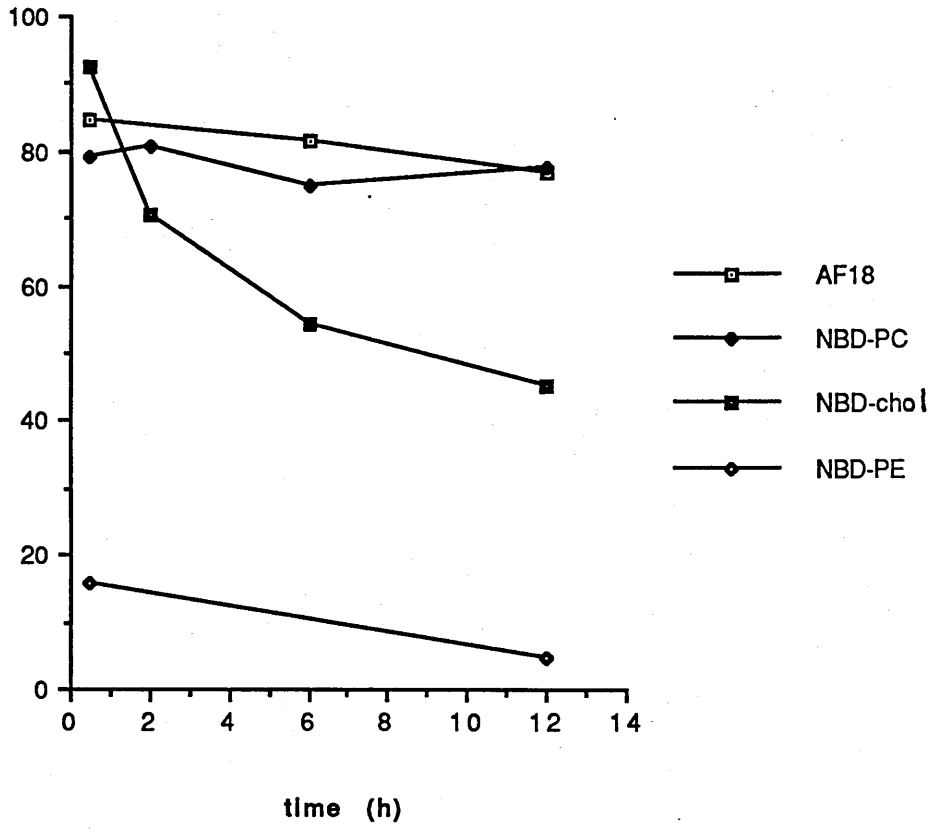


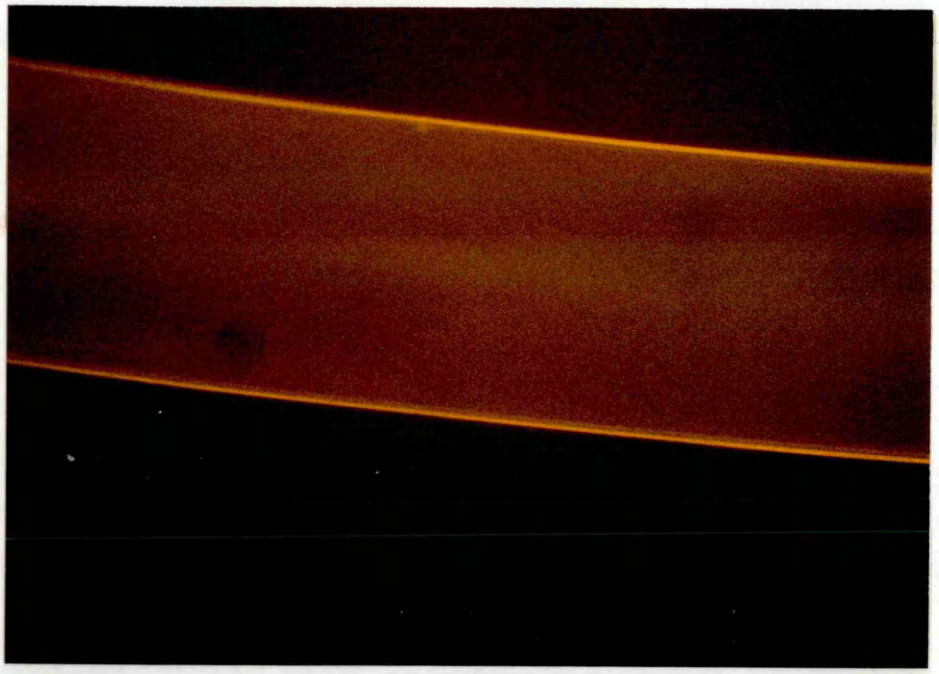
Figure 3.2 **Fluorescent photomicrographs of the labelling of adult *Acanthocheilonema viteae* with:**

(a) NBD-chol

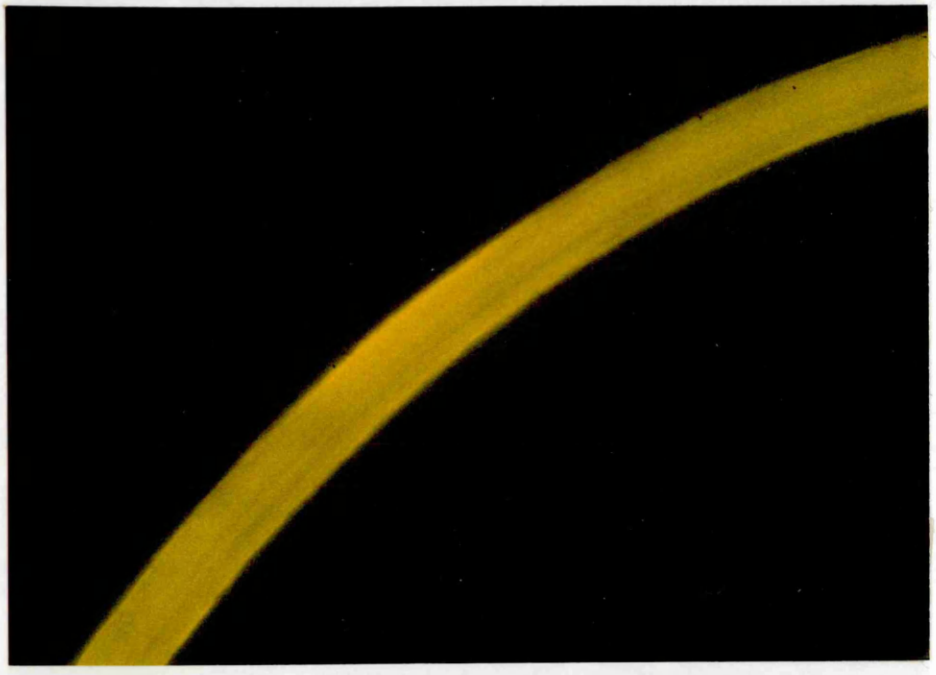
(b) NBD-PE

(c) NBD-PC

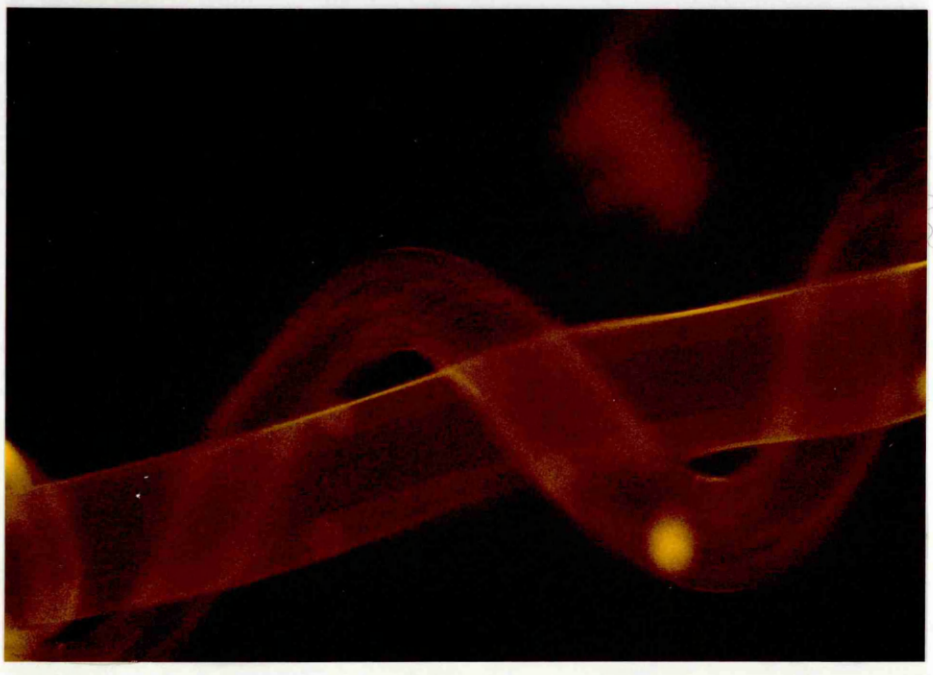
a



b



c



this differential uptake of fluorescent phospholipids was thought not to be due to any active transport of NBD-PE since a combination of low temperature (4^{o}C) and the antimetabolite sodium azide did not prevent uptake (data not shown).

(iv) *Brugia pahangi* and *L. carinii*(table 3.5):

AF12, AF16, AF18 and NBD-chol were surface-restricted in adults of both parasites, although AF18-labelling in *L. carinii* was significantly brighter (fig. 3.3). Sheathed microfilariae (mf) of *L. carinii*, but not those of *B. pahangi*, allowed the insertion of DiI18.

(v) *Ostertagia ostertagi* (table 3.6):

AF18, NBD-chol and NR were surface-restricted in adults, but excluded from the surface of exsheathed L3's (the sheath allowed the insertion of all probes tested). L4's differed from adults in that they excluded the NBD-chol probe.

(vi) *Caenorhabditis elegans* (table 3.7):

AF16, AF18, NBD-chol, NR and Mc540 were surface-restricted in adults, but completely excluded in exsheathed dauer larvae (the sheath allowed the insertion of all probes tested).

The insertion of the cationic lipid probes RH18 and DiI18 was only permitted after the worms had been treated for 10 minutes in 0.01% CTAB detergent followed by treatment for 1 hour in 10mgml^{-1} protease.

Mc540 labelled the surface of L1's.

Assessment of epicuticular permeability to Trypan blue

One potential problem in accurately measuring the degree of surface-restriction of a probe was that although Trypan blue is known to be membrane-impermeant (Wolf, 1988) there was no information, at the outset of these experiments, as to how permeable the nematode epicuticle might be to this dye. This was tested first by labelling the nematode with the appropriate fluorescent probe, quantitating the fluorescence, and then measuring the percentage quenching by Trypan blue. The Trypan blue was then removed from the slide without washing and the fluorescence was quantitated again. A comparison between initial fluorescence values and post-Trypan blue values for adults of *Trichinella spiralis* (table 3.3) proved to be almost identical

Table 3.5 : **Insertion of fluorescent lipid probes in the surface of *Brugia pahangi* and *Litomosoides carinii* adults and microfilariae (mf) which are sheathed.**

Stage in the life-cycle	Probe AF12	AF16	AF18	RH18	DiI18	NBD-chol
B.pahangi						
Adults	++	++	++	-	-	++
mf	internal	++	++	-	-	+
L.carinii						
Adults	+	++	++++	-	-	++
mf	internal	internal	++	-	+++	+

Worms were incubated for 10 minutes at 37°C in RPMI 1640 containing each probe at a final concentration of $10\mu gml^{-1}$.

The experiment was performed on two separate occasions. The intensity of fluorescence is represented by an arbitrary scale of - to ++++, and internal labelling was determined by trypan blue (0.25%) quenching.

Figure 3.3 Fluorescent photomicrograph of the labelling of adult *Litomosoides carinii* with AF18

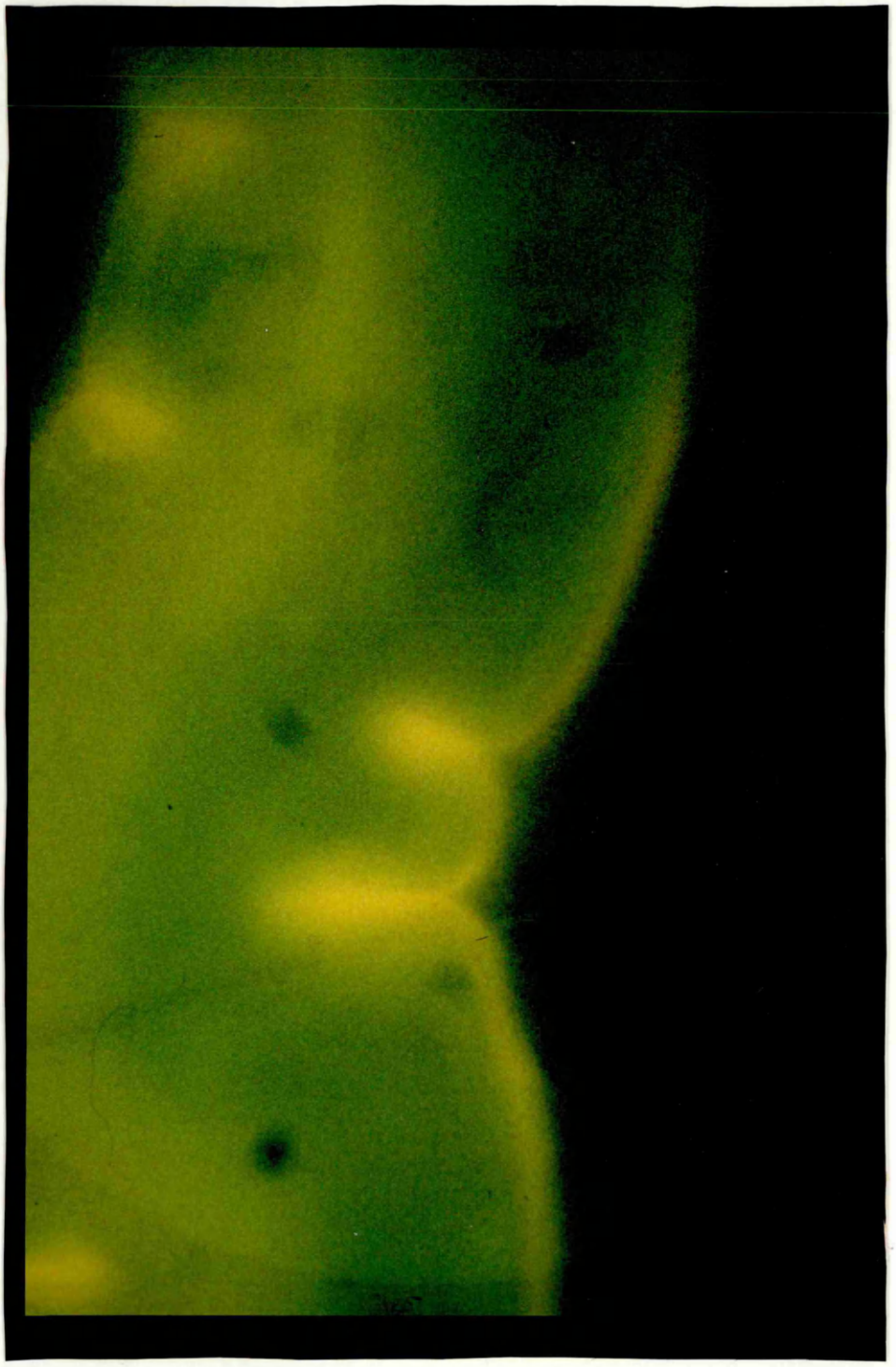


Table 3.6 : **Insertion of fluorescent lipid probes in the surface of *Ostertagia ostertagi* adults, fourth-stage larvae (L4's) and infective third-stage larvae (L3's).**

Stage in the life-cycle.	Probe AF12	AF18	RH18	DiI18	NBD-chol	NR	Mc540
Adult	-	+++	-	-	+++	++	-
L4's	-	+++	-	-	-	+	-
L3's							
sheathed	+++	+++	+++	+++	+	+	++
exsheathed	-	-*	-	-	-	-	-

* Surface lipophilicity for AF18 changes with environmental conditions which mimic the infection process (see Chapter 6).

Exsheathed larvae were recovered immediately after they had started to exsheath spontaneously in RPMI 1640 + excess gaseous CO_2.

The intensity of fluorescence is represented by an arbitrary scale from - to +++.

Table 3.7 : **Insertion of fluorescent lipid probes in the surface of all the stages in the life-cycle of *Caenorhabditis elegans*, including the dauer larvae.**

Stage in the life-cycle.	Probe AF12	AF13	AF16	AF18	RH18	DiI18	NBD-chol	NR	Mc540
Adults*	internal	internal	++	+++	-	-	++	++ (patchy)	+ (+++ in L1's)
0.01% CTAB + protease	internal	internal	+++	+++	+++	+++	++	+++	+ (patchy)
Dauer larvae (sheathed)	+++	+++	+++	+++	+++	+++	+	+++	+++
(exsheathed)	(-	-	-	-)**	-	-	-	-	-

* L4's, L3's, L2's and L1's exhibited labelling patterns which were essentially the same as for adults.

** Surface lipophilicity for the amino lipids becomes positive after exposure of the dauer larvae to a fresh food supply (i.e. an agar plate seeded with *Escherichia coli*, see Chapter 6).

Insertion of cationic lipid probes in adults was permitted after treatment with 0.01% CTAB and subsequent treatment with 10 $mgml^{-1}$ protease.

The intensity of fluorescence is represented by an arbitrary scale of - to +++, and internal labelling was determined by trypan blue (0.25%) quenching.

and this was seen as an assurance that there had been no residual dye within the cuticle. Furthermore, only worms which had been treated with 0.25% CTAB detergent for more than 2h took up Trypan blue in a parallel experiment (data not shown).

3.2.2. Reactivity of fluorescent protein-binding probes with the nematode surface.

Many different species and developmental stages could be labelled with chemically-reactive fluorescent probes on their intact surface (tables 3.8-3.13). The results are summarized as follows:

(i) *Toxocara canis* (table 3.8):

Only DTAF (assisted by sodium borohydride reduction) was successful in labelling the surface of infective-stage larvae of *T. canis*(see figure 3.4). This probe was also found to be surface-restricted, as measured by Trypan blue quenching. The worms remained viable throughout the strong reducing conditions of labelling with DTAF/ borohydride, and could continue to survive in culture in RPMI at 37^{o}C for several days after this treatment.

(ii) *Trichinella spiralis* (table 3.9):

DTAF/borohydride labelled the surface of adults and muscle-stage larvae (MSL), however, new-born larvae (NBL) were killed by this treatment. MSL could also be labelled with NHS-biotin and FITC-streptavidin (Biot/strep).

Adults of *T. spiralis* did not appear to have -SH groups exposed on the surface as there was no binding by fluorescein maleimide (FM) or 6-iodoacetamidoaminofluorescein (IAF). In contrast, MSL could be labelled with FM and IAF.

(iii) *Acanthocheilonema viteae* (table 3.10)

Adults and infective L3's (pre-parasitic in Grace's insect medium) were labelled with FM, IAF, Biot/strep and DTAF/borohydride. Microfilariae (mf), which are unsheathed, were also labelled with DTAF/borohydride and Biot/strep, but were killed in FM and IAF reagents used at the normal final concentration of 10 μgml^{-1}.

Table 3.8 : **Reactivity of sulphydryl group- and amino group-binding probes with the surface of *Toxocara canis* infective-stage larvae.**

Probe	Fluorescence Intensity	% Quenching Trypan blue
Sulphydryl group		
Fluorescein-5-maleimide (FM)	-	
6-Iodoacetamidofluorescein (IAF)	-	
Amino group		
Dichlorotriazinylaminofluorescein (DTAF) with 0.2M sodium borohydride.	++++	85.5 ± 5.3 (4)
Fluorescein isothiocyanate (FITC)	-	
Biotin/FITC-streptavidin (Biot/strep)	-	

After the labelling period, worms were washed thoroughly and immobilised with levamisole (1mgml-1) or 0.1% (v/v) 2-phenoxy-propanol.

The fluorescence intensity is represented by an arbitrary scale of - to ++++.

The experiment was performed on two separate occasions.

DTAF was surface-restricted, as determined by trypan blue (0.25%) quenching (4 separate readings).

Figure 3.4 Fluorescent photomicrograph of the labelling of infective-stage larvae of *Toxocara canis* with DTAF

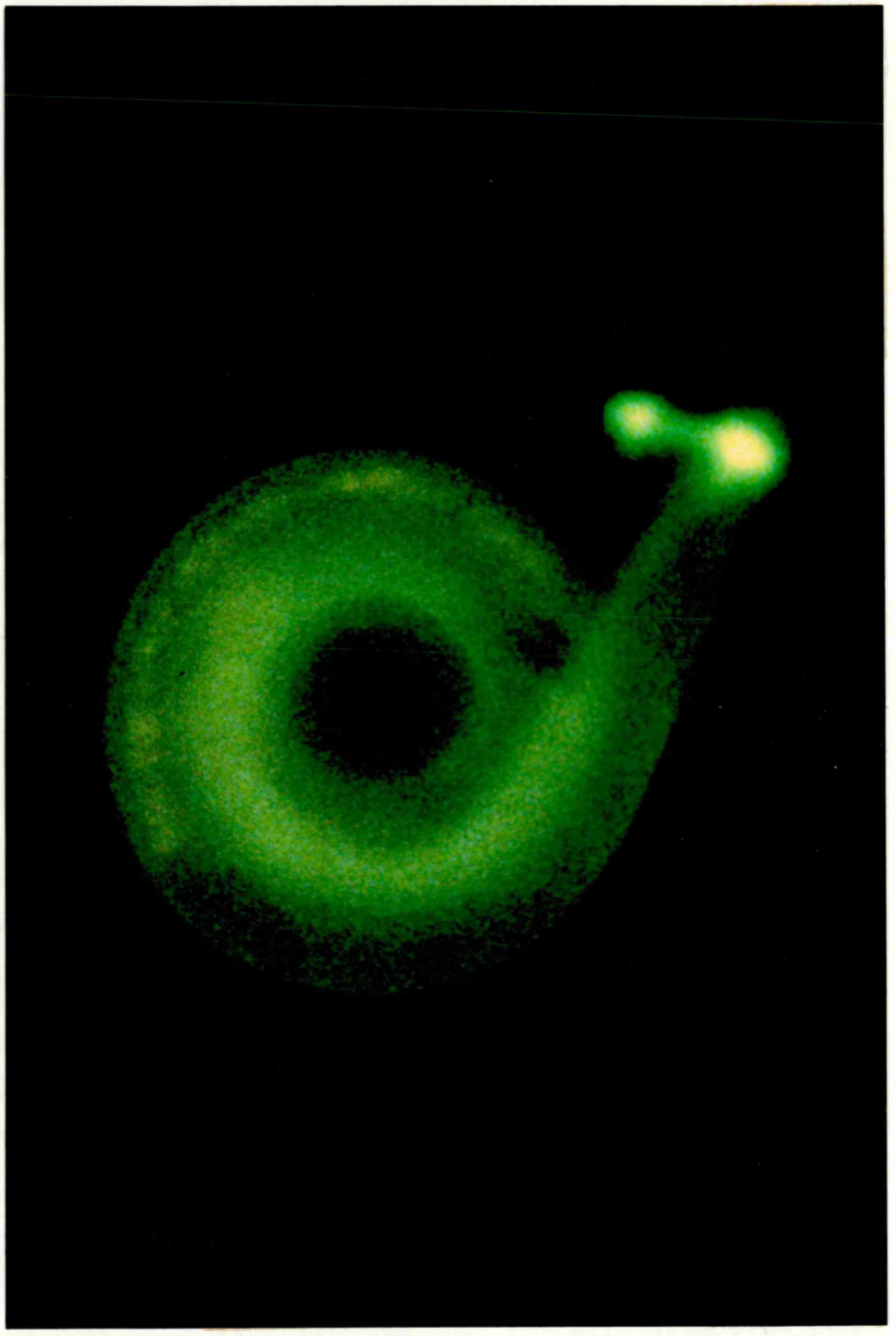


Table 3.9 : **Reactivity of sulphydryl group- and amino group-binding reagents with the surface of *Trichinella spiralis* adults, infective muscle-stage larvae (MSL) and new-born larvae.**

Stage in the life-cycle	Reactive Probe			
	FM	**IAF**	**DTAF (borohydride)**	**Biot/strep**
Adult	-	-	++++	-
MSL	++++	+++	++++	++
NBL	++	++	killed	-

Worms were incubated for 30 minutes with the above-mentioned probes in RPMI 1640 for adults and NBL, and in phosphate-buffered saline for MSL.

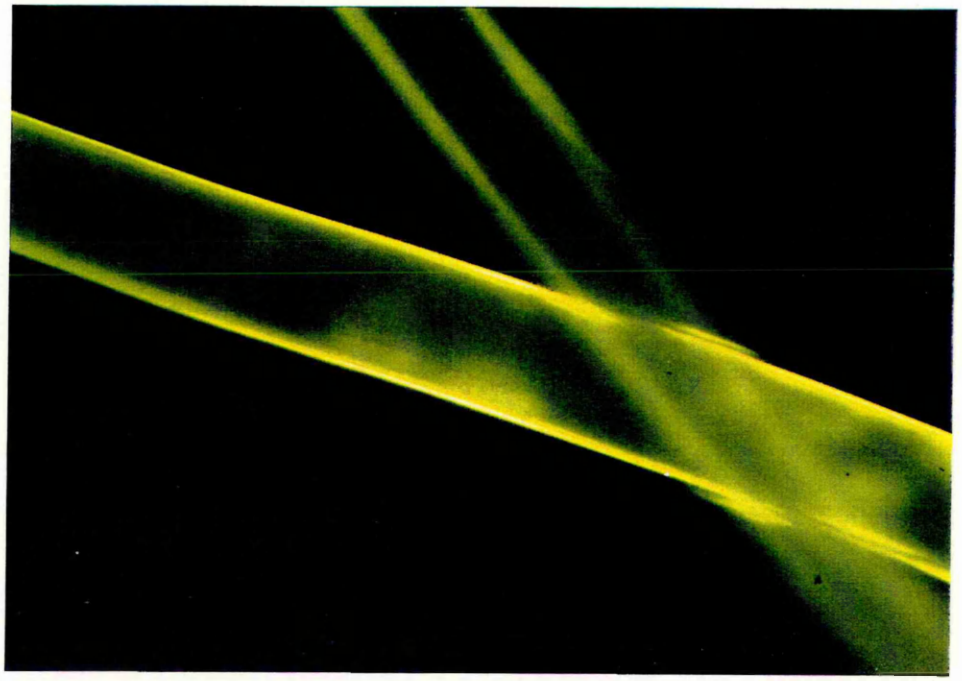
The intensity of fluorescence is represented by an arbitrary scale of - to ++++.

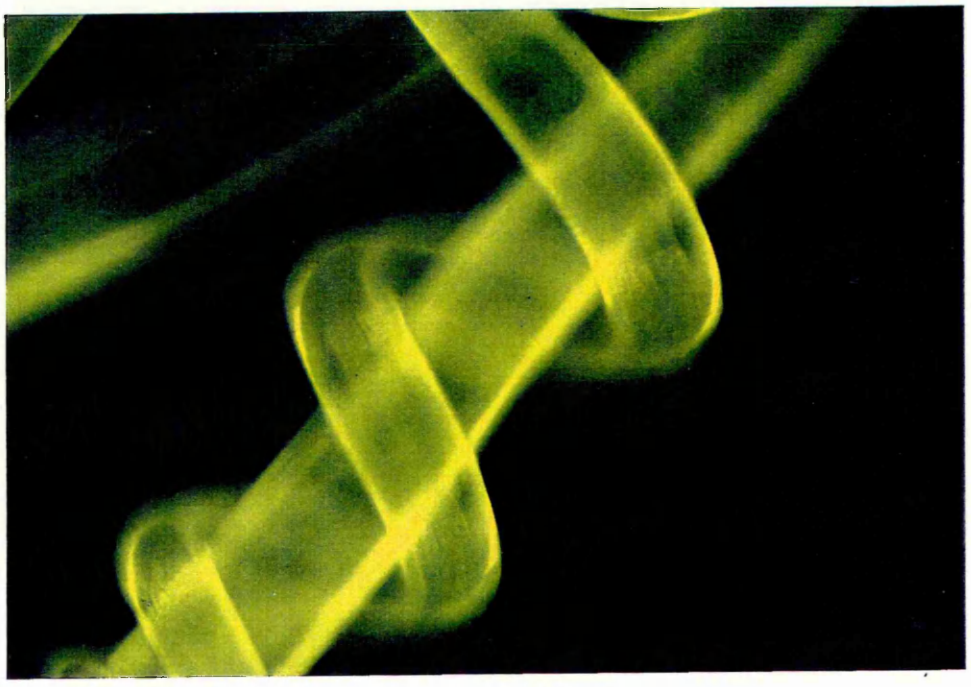
Table 3.10 : **Reactivity of sulphydryl group- and amino group-binding reagents with the surface of *Acanthocheilonema viteae* adults, infective third-stage larvae (L3's), and microfilariae (mf) which are unsheathed.**

Stage in the life-cycle	FM	Reactive Probe IAF	DTAF (borohydride)		Biot/strep
Adults		+++	+++	++++	+
L3's		+++	++	++++	+
mf		killed	killed	++++	+

The intensity of fluorescence was measured on an arbitrary scale from - to ++++.

Figure 3.5 **Fluorescent photomicrographs of the labelling of adult *Acanthocheilonema viteae* with DTAF**





(iv) *Brugia pahangi* and *Litomosoides carinii* (table 3.11)

Adults and microfilariae, which are sheathed, were labelled with FM, IAF (using 10 μgml^{-1}) and Biot/strep.

(v) *Ostertagia ostertagi* (table 3.12)

Adults, L4's and sheathed L3's were labelled with FM, IAF and DTAF/borohydride. Adults were also labelled with Biot/strep. L3's which had exsheathed naturally in RPMI1640 + excess gaseous CO_2, could only be labelled faintly with FM, whereas L3's which had exsheathed by artificial means in 2% sodium hypochlorite could be labelled with FM, IAF and DTAF/borohydride.

(vi) *Caenorhabditis elegans* (table 3.13)

Adults were labelled with FM, IAF and DTAF/ borohydride. Sheathed dauer larvae could also be labelled by these reagents. However, dauer larvae which had exsheathed on a fresh lawn of *E. coli*, could only be labelled with DTAF/ borohydride.

The mutants of *C. elegans*, srf-2 and srf-3 were labelled with FM and IAF, but became damaged (cuticular 'blebbing') in the DTAF/borohydride solution within 5 minutes. This was in contrast to wild-type (N2) *C. elegans* which could survive in identical conditions for up to 20 minutes.

3.2.3 **Fluorescent lectin-binding**

Adult parasites did not bind any of the lectins tested (table 3.14), however, it appeared that in *O. ostertagi* there was a small area around the copulatory bursa which would bind all three lectins. In contrast to adult parasites, the adult free-living nematode, *C. elegans*, bound FITC-Con A, FITC-PNA and FITC-WGA.

The sheaths of L3's of *O.ostertagia* and dauer larvae of *C. elegans* bound lectins, whereas their exsheathed surface did not. Sheathed microfilariae of *B. pahangi* and *L. carinii* also bound lectins, but notably, the unsheathed microfilariae of *A. viteae* did not.

Table 3.11 : **Reactivity of sulphydryl group- and amino group-binding reagents with the surfaces of *Brugia pahangi* and *Litomosoides carinii* adults and microfilariae (mf) which are sheathed.**

	Reactive Probe			
Stage in the life-cycle	**FM**	**IAF**	**DTAF (borohydride)**	**Biot/strep**
Adults	+++	+	not done	++
mf	+	+	not done	+

Fluorescence intensity is represented by an arbitrary scale from - to +++.

Table 3.12 : **Reactivity of sulphydryl group- and amino group-binding probes with the surface of *Ostertagia ostertagi* adults, fourth-stage larvae (L4's) and infective third-stage larvae (L3's).**

Stage in the life-cycle	Reactive Probe FM	IAF	DTAF (borohydride)	Biot/strep
Adults	+++	+	++++	++
L4's	+++	+	++++	not done
L3's				
sheathed	+++	+++	++++	not done
exsheathed	+	-	-	not done
$HClO_3^-$ exsh*	++	+	+++	not done

* exsheathment by incubation for less than 10 minutes in a 12% solution of sodium hypochlorite.
Natural exsheathment was acheived by brief exposure to RPMI 1640 with excess gaseous CO_2.
The intensity of fluorescence is represented by an arbitrary scale from - to ++++.

Table 3.13 : **Reactivity of sulphydryl group- and amino group-binding probes with the surface of *Caenorhabditis elegans* adults and dauer larvae.**

Stage in the life-cycle	Reactive Probe FM	IAF	DTAF(borohydride)	Biot/strep
Adults	+++	+++	++++**	-
Dauer larvae				
(sheathed)	++	++	++++	+
(exsheathed)	-	-	+++	-
Mutants:				
srf-2 +++	+++	damaged*		not done
srf-3 +++	+++	damaged	not done	

*Mutants became damaged in DTAF/sodium borohydride solution within 5 minutes.

**Wild-type (N2) adults survived in DTAF/ sodium borohydride solution for up to 20 minutes.

The intensity of fluorescence is represented by an arbitrary scale of - to ++++.

Table 3.14 : **Fluorescent lectin-binding on the nematode surface.**

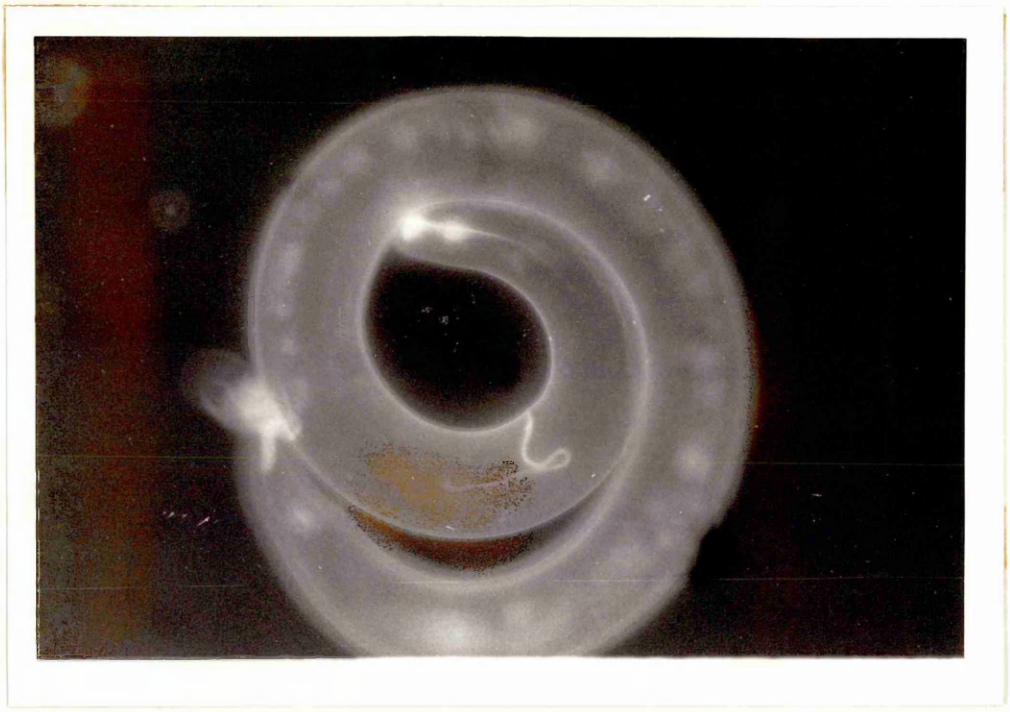
Species (& Stage)	FITC-ConA	FITC-PNA	FITC-WGA
Toxocara canis			
L2	no binding	no binding	non-specific binding
Trichinella spiralis			
Adults, MSL & NBL	no binding	no binding	no binding
Acanthocheilonema viteae			
Adults	non-specific binding	no binding	non-specific binding
L3	++	++	no binding
mf(unsheathed)	no binding	no binding	no binding
Brugia pahangi			
Adults	no binding	no binding	non-specific binding
mf (sheathed)	+	+	++++
Litomosoides carinii			
Adults	no binding	no binding	non-specific binding
mf (sheathed)	+	+	++
Ostertagia ostertagi			
Adults	no binding*	no binding*	no binding*
L3 (sheathed)	+++	+++	+++
L3 (exsheathed)**	no binding	no binding	no binding
Caenorhabditis elegans			
Adults	+++	+++	+++
Dauers (sheathed)	+++	++	++
Dauers (exsheathed)	no binding	no binding	no binding

*Bright fluorescence around the area of the copulatory bursa.

**Exsheathed in RPMI 1640 + excess gaseous CO_2 at 37°C.

Non-specific binding was determined using the appropriate competing sugar at 150mM (see Chapter 2, sections 2.5.3 and 2.6.3).

Figure 3.6 **Fluorescent photomicrograph of the labelling of infective-stage larvae of *Toxocara canis* with lucifer yellow hydrazide (LYH)**



3.2.4 Fluorescence-labelling of sugars with the fluorescent hydrazide Lucifer Yellow.

Infective-stage larvae of *T. canis* did not bind lectins, but their surface could be reacted with periodate to allow binding of Lucifer Yellow Hydrazide (LYH), (see figure 3.6). There was no fluorescence with the control, using only LYH without periodate treatment, and no fluorescence with larvae incubated with galactose oxidase (see Chapter 2, section 2. 6.4 for labelling procedures).

3.2.5 Fluorescent antibody-labelling of *Caenorhabditis elegans*

Wild-type (N2) nematodes of all stages in the life-cycle of *C. elegans* could be labelled on their surface with the polyclonal anti-surface IgG raised in rabbits. Fluorescence-labelling of the surface was then achieved by labelling with the second antibody, FITC-goat anti-rabbit IgG. The dauer larval sheath could also be labelled with these antibodies.

The mutants, srf-2 and srf-3 were labelled with the first antiserum (unadsorbed) or with the same serum which had been pre-adsorbed with N2 adults (not shown).

3.3. Discussion

Lipids

The observed selectivity of insertion of fluorescent lipid probes in adult nematode surfaces may be due primarily to the physico-chemical properties of each probe. Anionic lipid probes such as 5-(N-octadecanoyl)aminofluorescein (AF18) can normally be inserted into the adult nematode surface, whereas cationic lipid probes such as octadecyl rhodamine B (RH18) and dioctadecyltetramethylindocarbocyanine (DiI18) cannot. Since AF18 and RH18 have equal hydrophobicities (i.e. in terms of their acyl chains), selectivity must be largely head-group-dependent. Molecules that contain both charged and hydrophobic residues, such as AF18 and RH18, would normally occupy the polar-nonpolar interface region of a typical plasma membrane (Radda, 1975), so that different charged probes might have slightly different vertical

partitioning in the nematode epicuticle, especially in cases when there are the properties of a charged surface coat to consider.

The exclusion of cationic fluorescent lipid probes in the nematode epicuticle is a property which is shared by the unusual plasma membrane of fertilized *Xenopus* eggs (Dictus et al, 1984). In both cases, exclusion could be due to interfacial interactions with a surface coat. Experiments with adults of *C.elegans* and *T. spiralis* showed that initial treatment with a weak cationic detergent solution (0.01% CTAB), followed by protease treatment, could change the surface properties of these nematodes so that cationic lipid probes could then insert (tables 3.2 and 3.7). In 1985, Pritchard et al demonstrated that 0.25% CTAB detergent allowed effective stripping of antigens from the surface of *T. spiralis* infective larvae. This finding might support the idea that CTAB detergent had been able to remove at least some of the surface coat material which might normally have prevented access to proteases, and that this treatment had removed part of the barrier to cationic lipid probes.

Since protease treatment alone had no effect on the exclusion of cationic lipid probes from the intact surface, it could be that the surface is covered by a protease-resistant, but detergent-susceptible layer. Indeed, some researchers believe that it is this type of surface covering which masks the potential antigenicity in parasitic nematodes (Pritchard et al, 1988; Scott et al, 1988). A protease-resistant layer could be provided by glycolipid, as suggested by Scott et al (1988) who identified a 10- to 6 kDa glycolipid on the surface of the adult filarial nematode *Dirofilaria immitis*. This glycolipid was thought to form a molecular barrier between the cuticular surface and the external environment. It is also conceivable that a 'dense mesh' of glycoconjugates, lying across the surface of the worm might be able to prevent entry of cationic lipid probes, degradative enzymes, and perhaps also immune components.

Many of the results on the interaction of lipid probes with the epicuticle appear to be determined by the charge of the fluorophore. However, too much emphasis should not be placed on this, since some lipid probes with a nonpolar fluorophore behave very differently. For example, NBD-PE is rapidly internalized by the adult filarial nematode *Acanthocheilonema viteae*, whereas NBD-PC is surface-restricted in the same parasite, the only difference in structure being the phospholipid head-groups. This differential uptake was thought to be due to the physical nature of the epicuticle and not active transport processes, since low temperature and various antimetabolites had no effect. However, changes in the overall charge of one

phospholipid but not the other (PC and PE are zwitterionic) could have occurred during chemical derivatization with the fluorophore, causing their overall physico-chemical properties to be different. These results could have implications for drug design in which a slight modification to charge and /or hydrophobicity could greatly enhance drug penetration.

Internalization of fluorescent lipid probes was noted for only two of the probes tested, i.e. rapid internalization of NBD-PE, and a slow, incomplete internalization of NBD-chol. Internalization of NBD-chol did not commence until 2h after surface-labelling, suggesting that a slow transport process might have been involved. It was found recently, that a cuticular protein of the filarial nematode *Brugia malayi* has an amino acid sequence bearing strong homology to the human apoprotein B (Selkirk, M. personal communication). Apoprotein B binds lipids, cholesterol in particular, and transports them in the blood in the form of lipoproteins. Since nematodes cannot synthesize sterols *de novo* (Dutky et al, 1967), the parasitic species must obtain their sterols from their hosts in order to fulfill their requirements for these molecules. Antibodies against the filarial apoprotein-like molecule are able to bind throughout the cuticle (Selkirk, M. personal communication), suggesting that they could be used as a transport mechanism for aquisition of host lipids.

In considering the structure of the epicuticle, one could argue that the insertion of fluorescent lipid probes does not demonstrate the presence of lipid, but merely the presence of lipophilic proteins, for example. However, nile red (NR) is a dye which binds to lipid droplets and was also able to bind to the surface. Likewise NBD-labelled probes, which only fluoresce when inserted in lipid (Haughland, 1987), bound to the nematode surface. In conclusion, it is presumed that surface-restricted probes, such as AF18, NBD-chol and NR, were inserted in the lipid epicuticle.

Reactivity of protein-binding reagents with the epicuticle is discussed in chapter 4.

Sugars

The lack of exposed sugars for lectin-binding on the intact surface of many nematode species and stages is another indication that what we are dealing with is a very unusual biological surface. The lack of exposed sugars has already been noted for adults of *A. viteae* (Kieffer et al, 1989), and for six different species of microfilariae (Paulson et al, 1988). In the

latter case, it was only the microfilarial sheaths which bound lectins, supporting the present findings.

The presence of galactose, glucose, mannose and N-acetylglucosamine on the exposed surface of *C. elegans* was indicated by the experiments of Zuckerman et al (1979) who found the specific binding of three iodinated plant lectins. This is in support of the present results for fluorescent lectin-binding on the surface of *C. elegans*, although it appears that the exsheathed surface of dauer larvae will not bind any of the lectins tested. Similar results were obtained with the exsheathed surface of *O. ostertagi* infective larvae, suggesting that sugars are not exposed for lectin-binding on the true surface.

It was surprising that lectin-binding did not occur on the surface of infective-stage larvae of *T. canis* since there are monoclonal antibodies which can bind to exposed carbohydrate epitopes in *T. canis* (Maizels et al, 1987) and *T. cati* (Kennedy et al, 1987b). Also, surface proteins of *T. canis* are known to be heavily glycosylated with galactose and N-acetyl-galactosamine (Maizels and Selkirk, 1988). Steric hindrence may be the problem if sugars are arranged on the surface in an unusual configuration. Successful labelling of surface sugars of *T. canis* was achieved using periodate oxidation, followed by reaction with the fluorescent hydrazide Lucifer Yellow (LYH). Periodate oxidation could not be replaced by galactose oxidase oxidation in this experiment, suggesting that the mucin-like surface coat may be resistant to the action of extrinsic enzymes.

Antibody-labelling

The polyclonal antiserum raised in rabbits against the surface of a mixed population of *C. elegans*, was used to label different stages in the life-cycle and two mutants. The mutants, srf-2 and srf-3 expose normally hidden antigens on their surface (Politz et al, 1987) so that the antiserum pre-adsorbed with wild-type (N2) nematodes should have had some degree of specificity for the antigens exposed on the mutants. Successful differential labelling of wild-type and mutant strains was achieved, which meant that the lateral diffusion of these different antigens could be measured by fluorescence recovery after photobleaching (results shown in Chapter 5) and subsequently compared.

Chapter Four

Biochemical composition of the epicuticle

4.1. Introduction

Biochemical investigations of the nature of the epicuticle have been hindered by the fact that this structure is extremely resistant to solubilization by methods conventionally employed for membranes and that the isolation of the intact epicuticle from the rest of the cuticle, without the use of strong chemicals, has proved to be almost impossible (Bird, 1957; Cox et al, 1981; Murrell et al, 1983; Betschart and Jenkins, 1987).

In spite of this difficulty, the aims of the work described in this chapter were to extract and characterize epicuticle lipids and proteins, to investigate the nature of the association of fluorescent protein- and carbohydrate-binding reagents with the epicuticle, and to use fluorescence microscopy and transmission electron microscopy to gain some insight into how these molecules are organized in the epicuticle.

4.2. Results

4.2.1. Epicuticle lipids

The osmiophilic nature of the epicuticle (Bird, 1980), and the ease of penetration of hydrophobic anthelmintics (Marks et al, 1968), suggests that it contains lipids. This prompted the investigation into the classes of lipid present in the epicuticle.

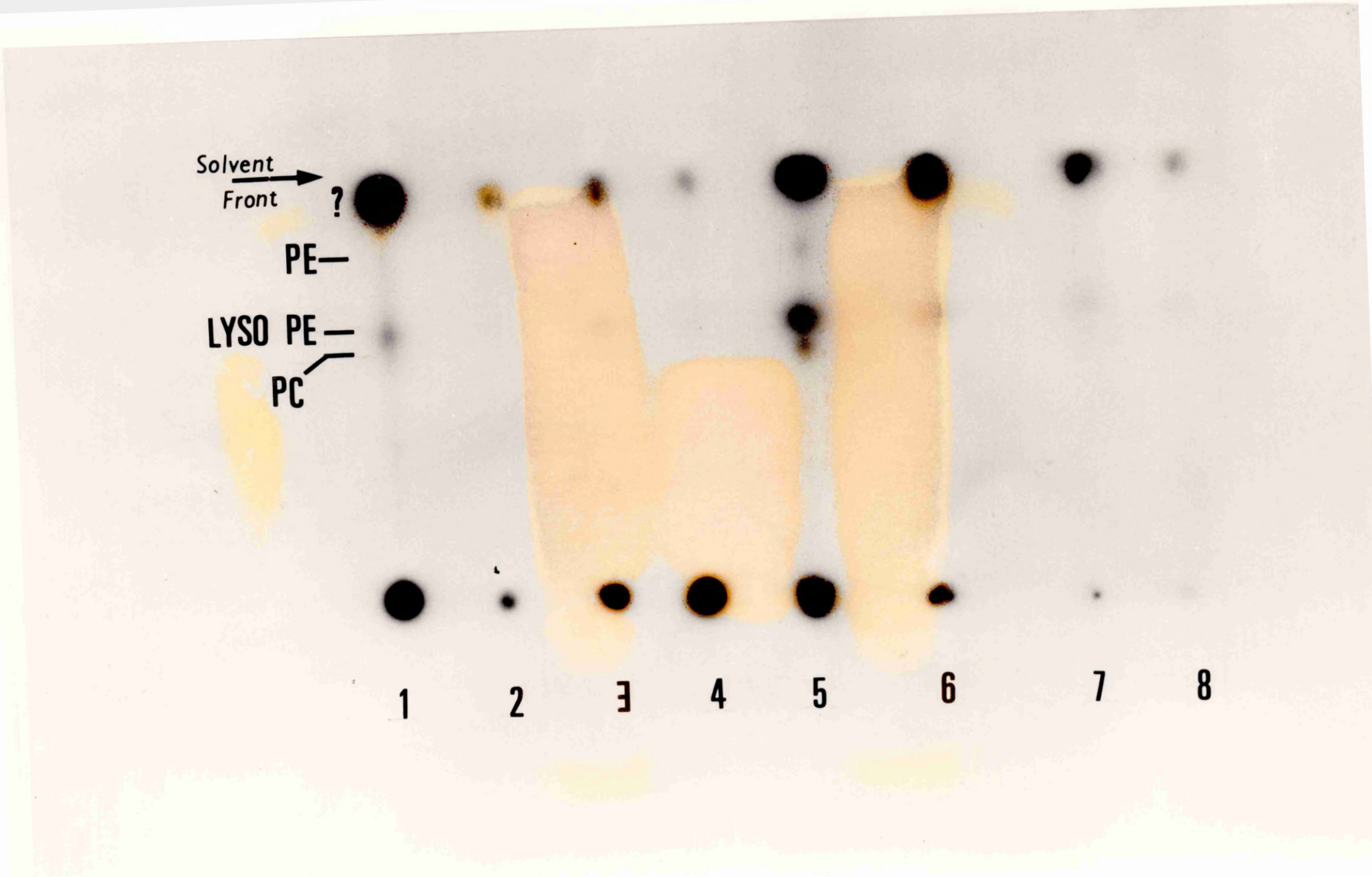
Nematode surface lipids were labelled extrinsically with ^{125}iodine using the surface-restricted reagent Iodogen (as described in section 2.7.1) and extracted using the method of Folch et al (1957). Lipid extracts were measured by one-dimensional thin-layer chromatography (TLC) using the solvent system of Storry and Tuckley (1967) for neutral lipids, and the solvent system of Skipski et al (1964) for phospholipids (see section 2.10.3). The results in figure 4.1 show that the release of radio-iodinated material from the surface of the free-living nematode *Caenorhabditis elegans* could be acheived using SDS detergent alone or in combination with vortexing with small glass beads. Subsequent lipid extraction of whole worms, SDS extracts or SDS/bead extracts revealed that the predominant phospholipid on the surface is probably L-α - lysophosphatidylethanolamine

Figure 4.1 Radio-iodinated phospholipids from the surface of *Caenorhabditis elegans*

1 & 5 - whole worms extracted with chloroform : methanol (2 : 1)

2, 3 & 4 - 2% SDS extracts

6, 7 & 8 - 2% SDS/ vortexing with beads extracts.


Solvent
Front
?
PE
LYSO PE
PC
1
2
3
4
5
6
7
8

(lyso PE) with minor components of L-α-phosphatidylethanolamine (PE) and L-α-phosphatidylcholine (PC). There is also a large amount of unidentified, relatively nonpolar material which runs close to the solvent front.

Similarly, lyso PE was a predominant phospholipid on the surface of the adult parasitic nematode *Litomosoides carinii*, with comparably high levels of L-α-phosphatidic acid (PA) and PC (figure 4.2). Also, it appears that there was slightly less L-α-phosphatidylserine (PS) and PE at the surface, but again, a large amount of heavily labelled nonpolar material was found running close to the solvent front. This material was re-run in a solvent system for neutral lipid classes (figure 4.3) which resolved it as a spot running behind wax esters and cholesterol oleate but just slightly ahead of triolein, so that it was possibly a triacylglycerol.

Ascaris suum cuticle, which had been radiolabelled on the surface with ^{125}iodine using Iodogen (as desribed in section 2.7.1) was extracted in hot (65°C) chloroform : methanol (2 : 1) and the extracted lipids analyzed by one-dimensional TLC for complex lipids (figure 4.4). Three of the heavily radio-iodinated lipids correspond to phospholipid standards of PE, PA and PI, but again there is a heavily labelled spot running close to the solvent front which remains unidentified.

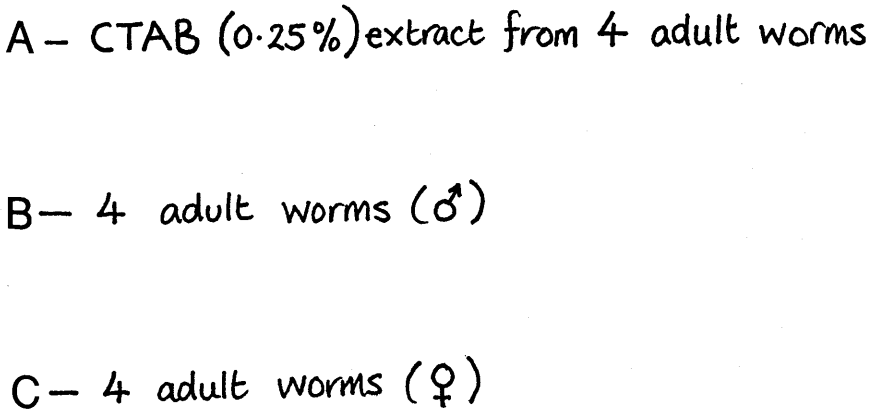
Attempts to degrade or release phospholipids with phospholipase C, or neutral lipids with lipase from the intact surface of adult *L. carinii*, were considered to be unsuccessful since no extractable radio-iodinated lipid material was released into the culture medium by these methods. It should be noted however, that adults of *L. carinii* became extremely hyperactive as soon as the phospholipase C (but not the lipase) had been added to the culture medium.

That none of the extractable lipid from the epicuticle is glycolipid was suggested by a negative reaction for both the α-napthol and orcinol-ferric chloride spray tests (not shown) which give a pink and red colour, respectively, if carbohydrate is present.

4.2.2. Proteins associated with the epicuticle

Earlier, in chapter 3, it was shown that certain fluorescent protein-binding reagents could label the intact surface of many different species and stages of nematode. However,

Figure 4.2 **Radio-iodinated phospholipids from the surface of adult *Litomosoides carinii***



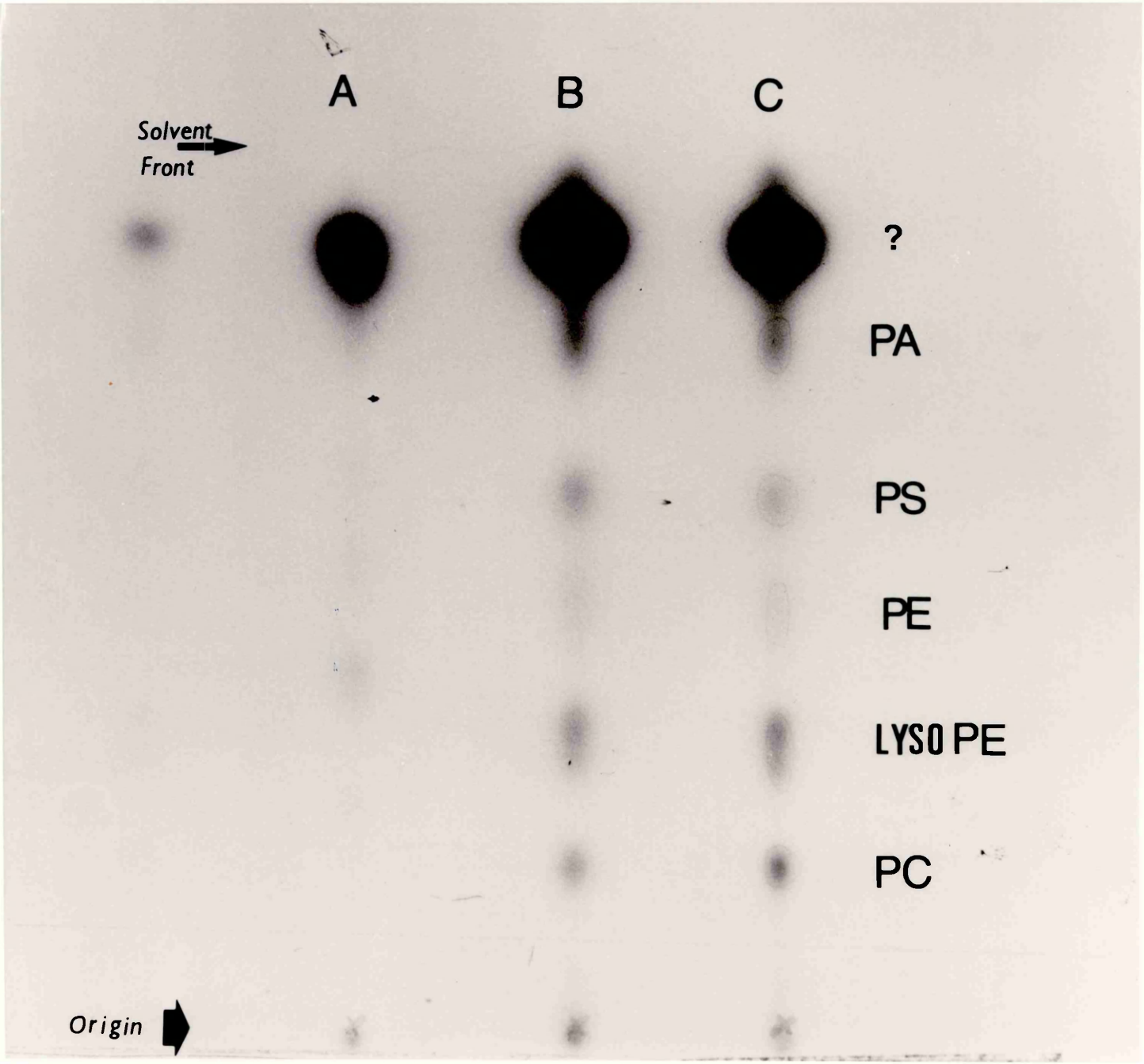

A
B
C
Solvent Front
?
PA
PS
PE
LYSO PE
PC
Origin

Figure 4.3 **Radio-iodinated neutral lipids from the surface of adult *Litomosoides carinii***

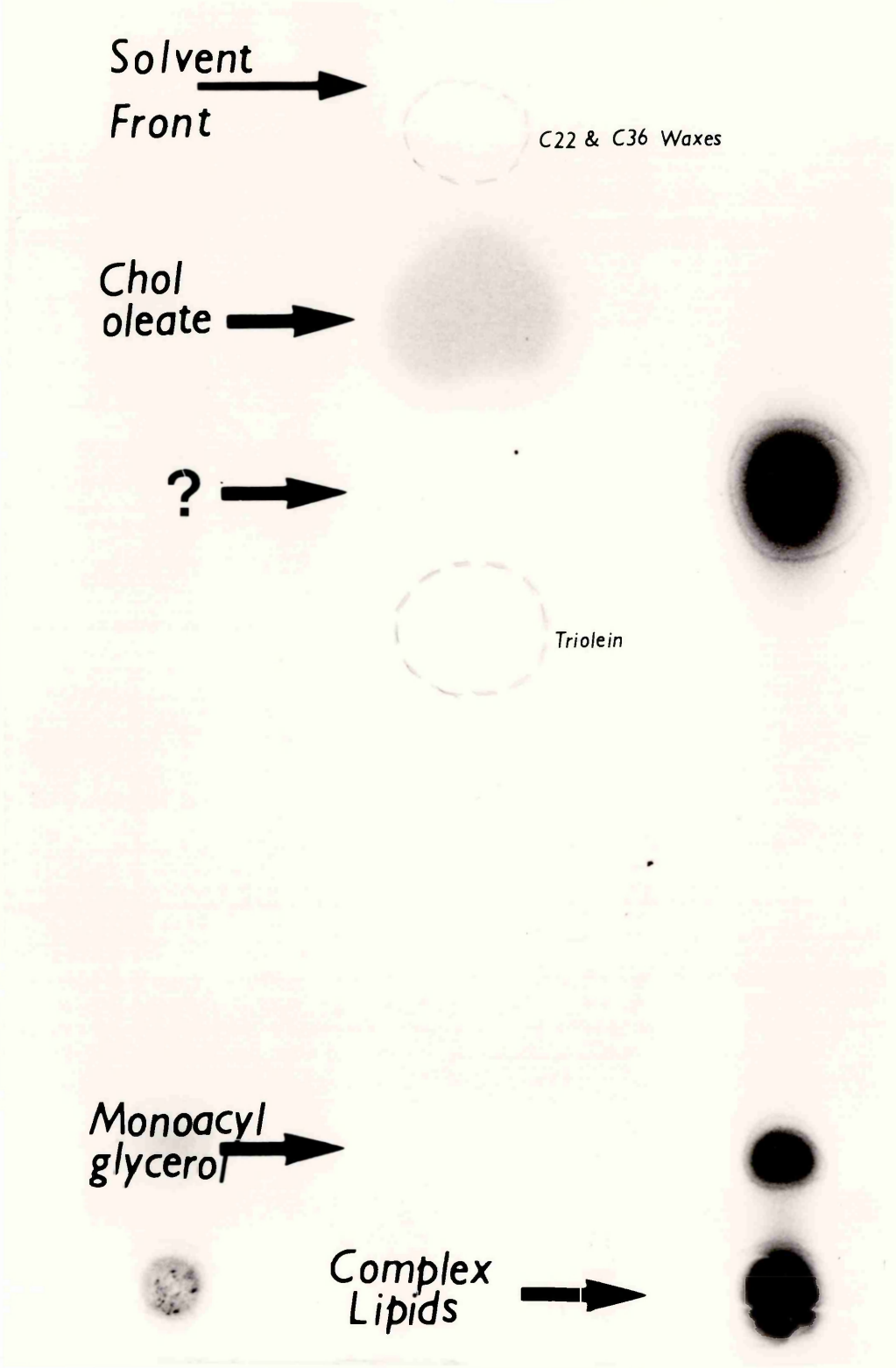
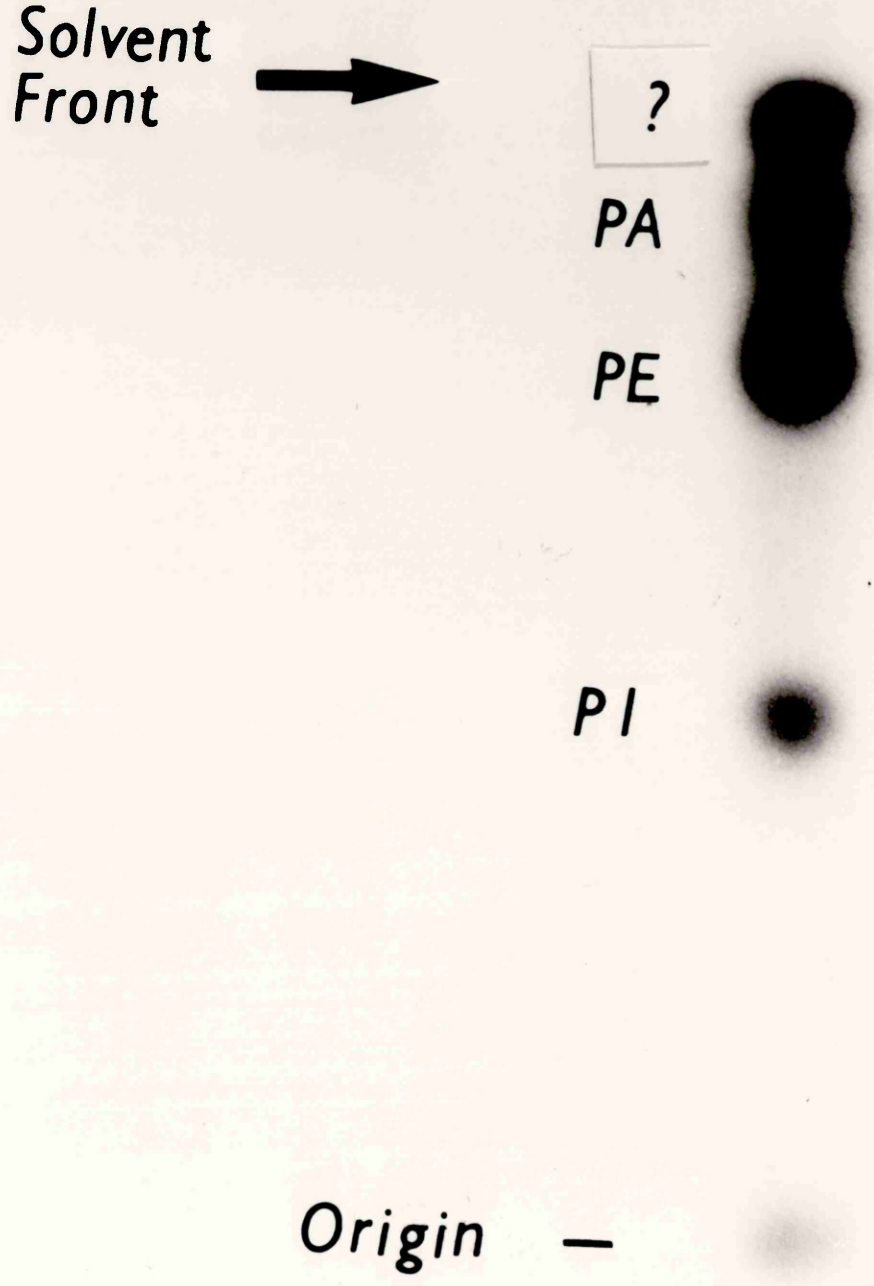

Solvent
Front
C22 & C36 Waxes
Chol
oleate
?
Triolein
Monoacyl
glycerol
Complex
Lipids

Figure 4.4 **Radio-iodinated phospholipids from the isolated cuticle of *Ascaris suum***


Solvent
Front
?
PA
PE
PI
Origin

since fluorescein-5-maleimide (FM) and dichlorotriazinylaminofluorescein (DTAF) react with sulphydryl groups and amino groups, respectively, it was necessary to identify which set(s) of proteins were being labelled. Infective muscle-stage larvae of *Trichinella spiralis*, which label strongly with both FM and DTAF (in the presence of 0.2M sodium borohydride) (see table 3.9), were sonicated in electrophoresis sample buffer (without ß-mercaptoethanol) and then subjected to SDS-PAGE (see section 2.9). The unstained gel (still between the glass plates) was then examined for fluorescent bands, first by eye on exposure to U.V. light on a trans-illuminator, and then by scanning each track with a laser beam. A diagrammatic representation of the observed fluorescent bands is shown in figure 4.5. With FM, a 50- to 55kDa band and a very broad band of 82- to 98kDa were labelled, whereas DTAF-labelling in the presence of borohydride showed a faint band at 50- to 53 kDa and a very broad band of high molecular weight material (> 220 kDa).

To investigate the effects of sodium borohydride reduction (rather than alkaline conditions) on the epicuticle and its associated proteins, MSL of *T. spiralis* were labelled with (i) FM in 0.5M Tris buffer, pH 10; (ii) as in (i) but then exposed to 0.2M sodium borohydride, pH 10; and (iii) DTAF in 0.2M sodium borohydride, pH 10. Fluorescence levels were monitored over a 24h period of culture in RPMI1640 at 37^{o}C, the results of which are shown in figure 4.6. After 2h, the MSL labelled with FM and then exposed to 0.2M sodium borohydride had lost approximately 75% of their original surface fluorescence. In contrast, MSL labelled with FM, which had not been exposed to sodium borohydride, only lost 5% of their original fluorescence in 2h. The results for DTAF-labelling, over the 24h period, were unusual in that instead of decreasing or staying the same, fluorescence levels increased by 60% of the original levels.

In order to investigate further the effects of sodium borohydride reduction on the epicuticle, pieces of isolated *Ascaris* cuticle were subjected to different treatments. First, a piece of cuticle was exposed to 10 mgml^{-1} sodium borohydride for 30 minutes and then examined under transmission electron microscopy (figure 4.7) where there was no evidence of structural damage. Second, a piece of cuticle was exposed to 10 mgml^{-1} sodium borohydride for 24h which lead to its complete solubilization into a viscous gel-like material, with no sign of any epicuticular material left behind (not shown). This effect could not be achieved using ß-mercaptoethanol under similar conditions. Solubilized cuticle was

Figure 4.5 Diagrammatic representation of the observed fluorescent bands after labelling the surface of infective muscle-stage larvae of *Trichinella spiralis* with FM or DTAF

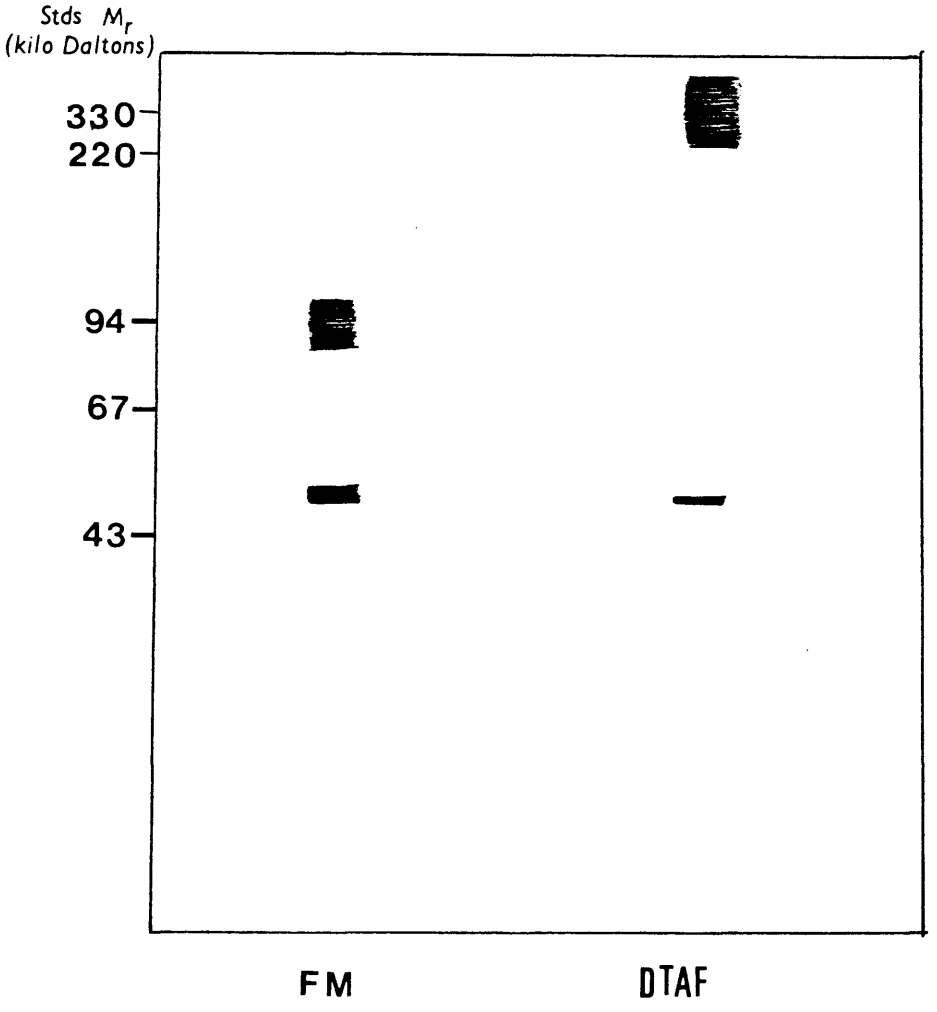


Figure 4.6 **Time course of intensity of surface fluorescence on infective muscle-stage larvae of *Trichinella spiralis* labelled with FM in Tris buffer, FM in $NaBH_4$, or DTAF in $NaBH_4$**

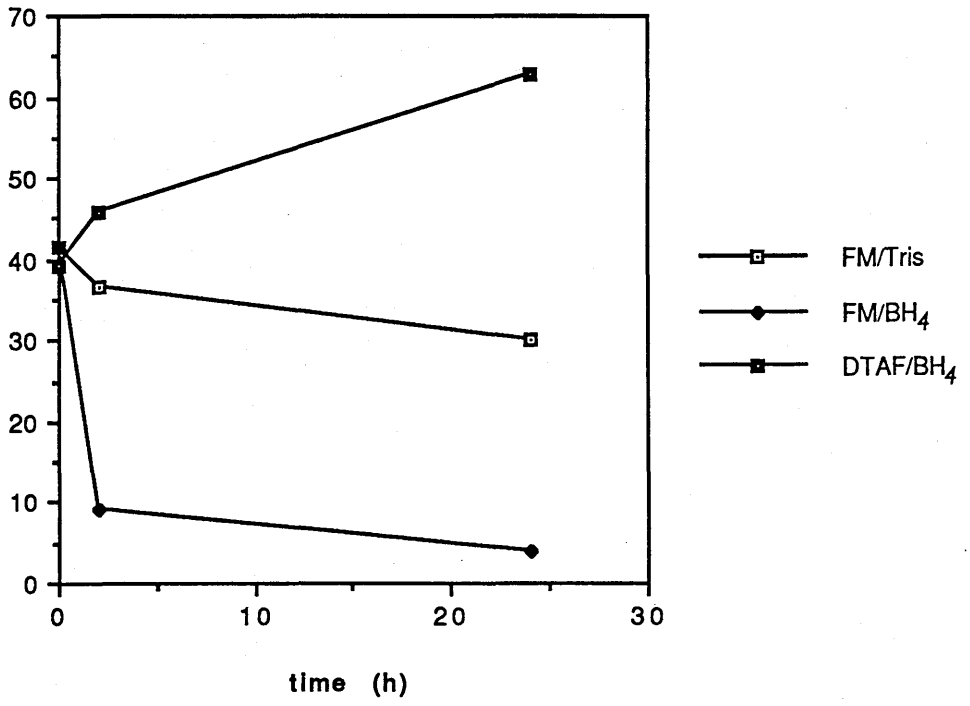
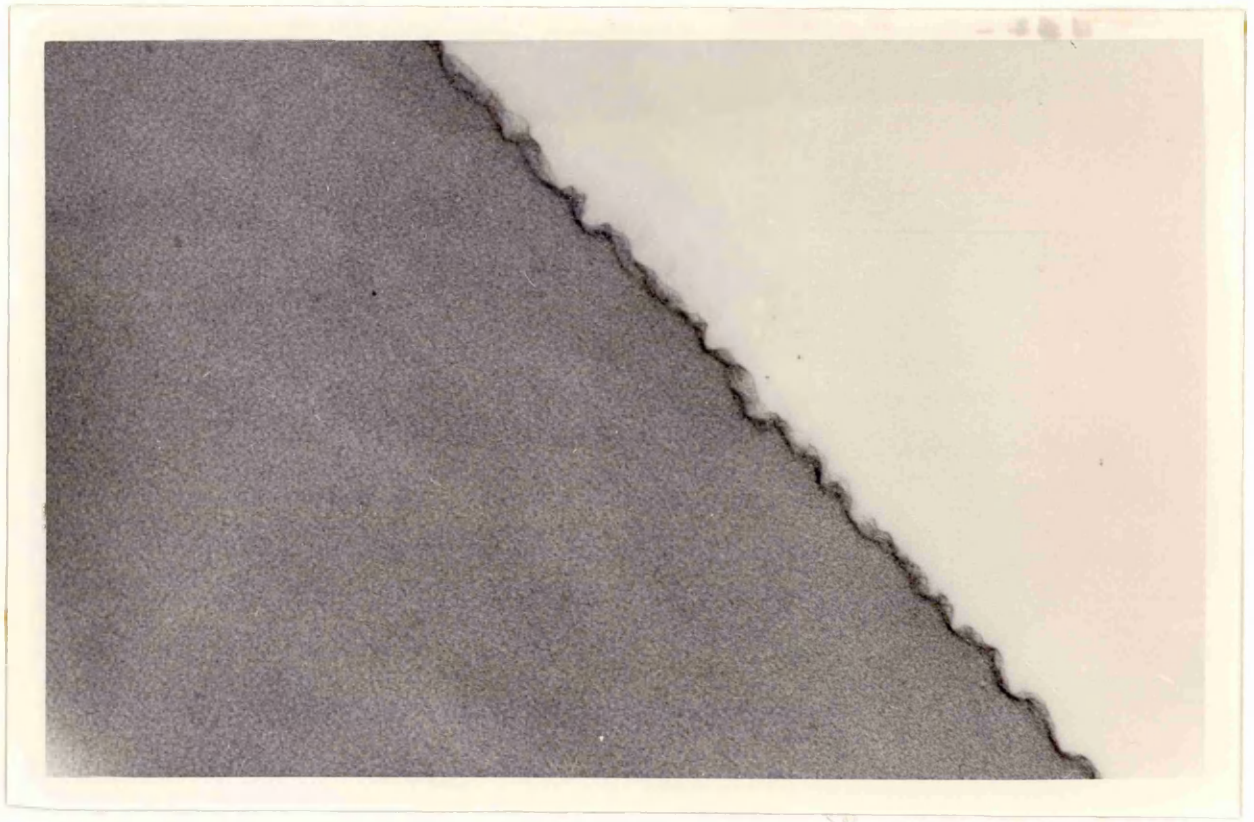
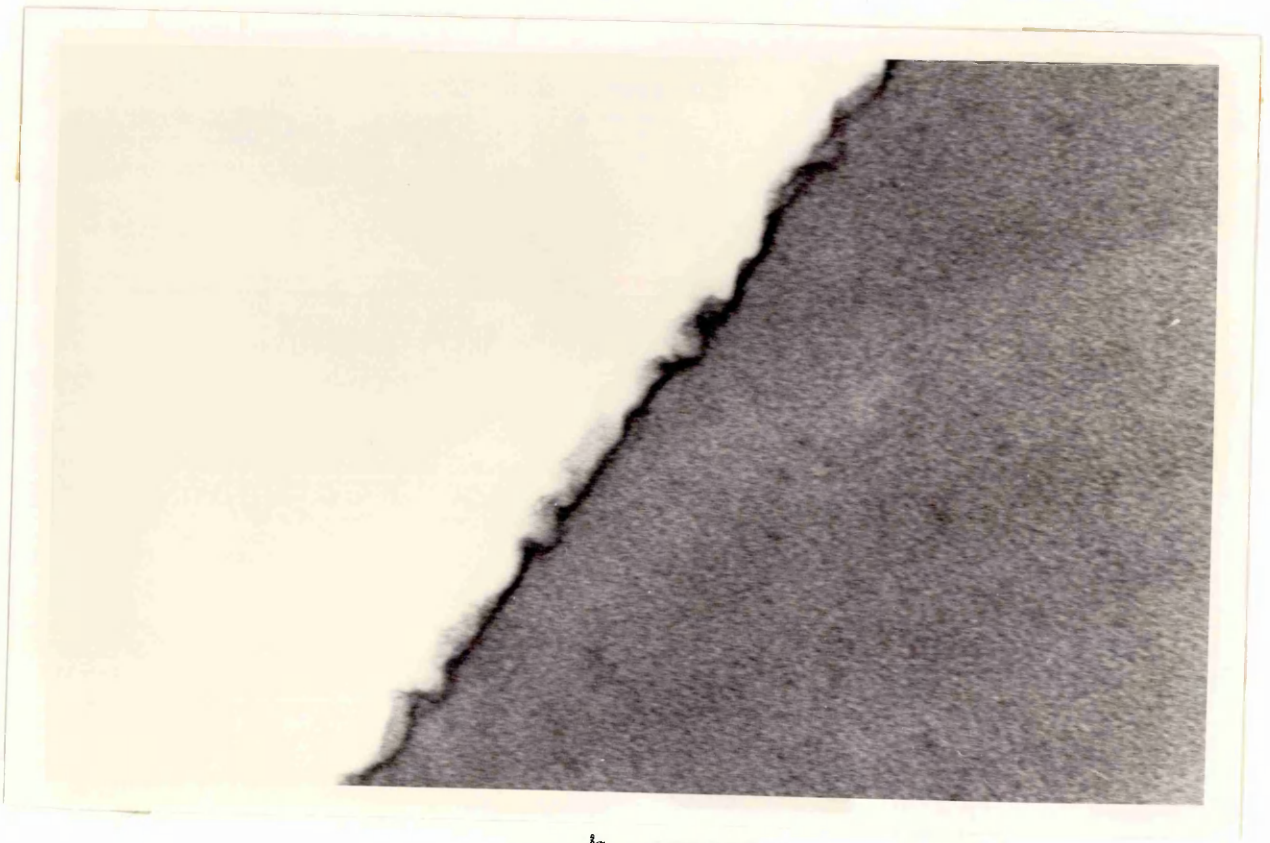


Figure 4.7 **Electron micrographs of isolated *Ascaris suum* cuticle (a) without $NaBH_4$ treatment, and (b) with $NaBH_4$ treatment.**

Section (b) had been pre-treated for 30 minutes in 10mgml^{-1} $NaBH_4$.



a X 57000



b X 78 000

then analyzed for proteins by the method of Lowry et al (1951), and for neutral sugars by the method of Dubois et al (1956) (table 4.1). The third strategy of investigation was to radio-iodinate the intact surface of two pieces of *Ascaris* cuticle (as described in section 2.7.1), one which had been pre-treated on the surface with 5% ß-mercaptoethanol for 30 minutes, and the other which had been pre-treated on the surface with $10mgml^{-1}$ sodium borohydride, also for 30 minutes. Both of the washed, radiolabelled cuticles were then cut into small pieces and homogenized in SDS-PAGE sample buffer with ß-mercaptoethanol. Figure 4.8 illustrates that only the pre-treatment with sodium borohydride had been effective in destroying the epicuticular barrier to radio-iodine and Iodogen in the intact surface to allow labelling of what appeared to be high molecular weight material, possibly collagens, and material of around 10 kDa which was possibly lipid. As the resolution had been very poor on the autoradiograph (figure 4.8 (a)), it was thought necessary to scan the autoradiograph through a densitometer (figure 4.8 (b)). This resolved 5 main peaks from the borohydride-treated cuticle sample but only 3 relatively small peaks from the Tris buffer-treated cuticle sample. Similar results were obtained using fluorescent DTAF-labelling of the intact *Ascaris* cuticle (figure 4.8 (c)), but in this case, three distinct high molecular weight bands (330-, 250- and 195 kDa), and one low molecular weight band (18 kDa) were detected. The highly fluorescent material found running at the gel front was thought to be only free DTAF since no protein staining with coomassie blue was found at this area on subsequent processing of the gel.

4.2.3. **Carbohydrates associated with the epicuticle**

From the results of lectin-binding experiments (see table 3.14), it appears that sugars are not exposed on the intact nematode surface, with the free-living nematode *Caenorhabditis elegans* being the major exception. Therefore, it was thought necessary to look for the presence of epicuticular sugars by methods not employing lectin-binding specificities. Demonstration of sugars associated with the epicuticle in adult *Ostertagia ostertagi*,which does not bind lectins (see table 3.14) was achieved using the periodate-thiosemicarbazide silver-proteinate (PA-TSC-SP) method used by Spiegel et al (1983), shown in figure 4.9. It is interesting to note that, although there is intense staining associated with the epicuticle, there is also intense staining of material in the cuticle extending up into the lateral alae.

Table 4.1 Composition of the epicuticle

	Percentage of total dry weight (%)
Protein (2% SDS-solubilised)	57.0 ± 4.6 (n = 6)
Carbohydrate (solubilised in gel-form cuticle)	5.5 ± 3.2 (n = 5)

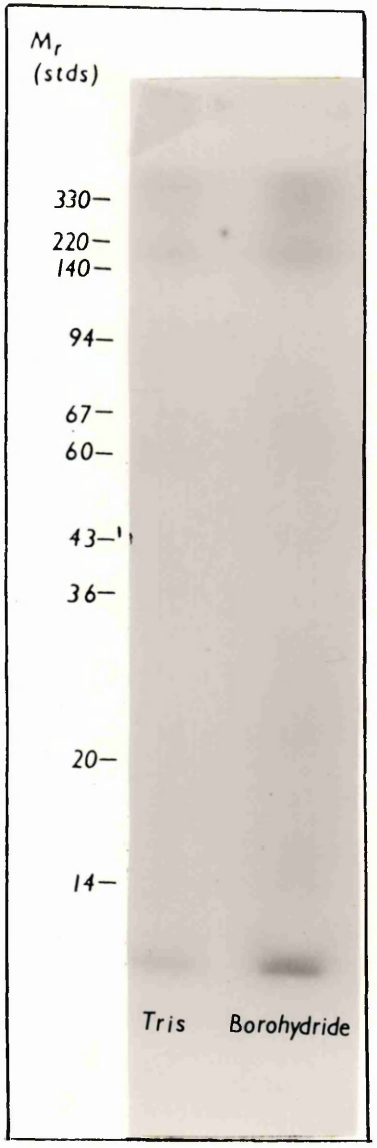
Figure 4.8 Radio-iodination of proteins from isolated *Ascaris* cuticle.

A - Autoradiograph of ^{125}I-iodinated proteins from surface-labelling of isolated cuticle.

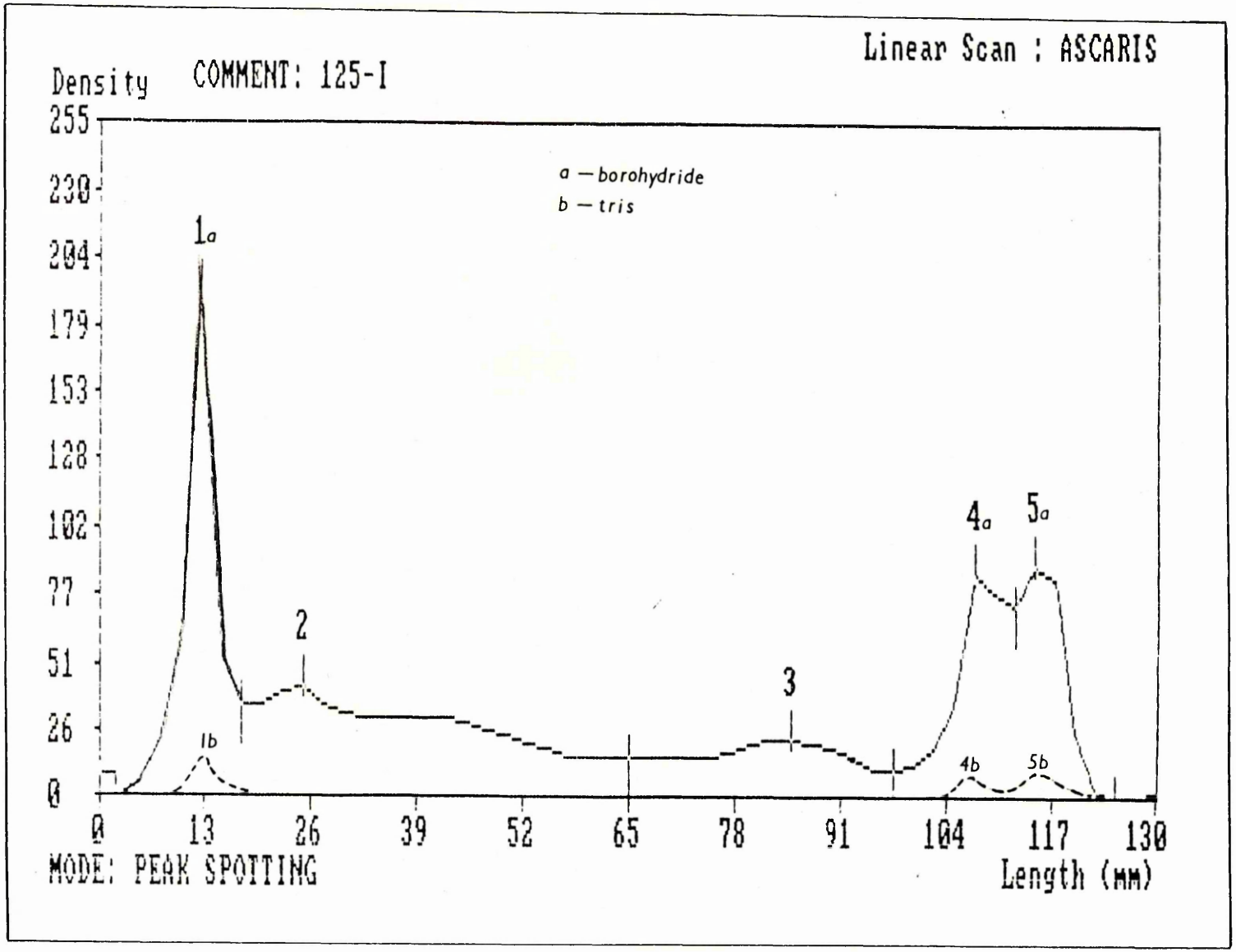
B - Densitometer scan of the autoradiograph from A showing greatly increased labelling at peak positions 1, 4 & 5.

C - Diagrammatic representation of the fluorescent bands observed on an SDS-PAGE gel of DTAF-labelled cuticle.

A


M_r
(stds)
330
220
140
94
67
60
43
36
20
14
Tris
Borohydride

B


Linear Scan : ASCARIS
Density
COMMENT: 125-I
255
230
204
179
153
128
102
77
51
26
0
a — borohydride
b — tris
1a
1b
2
3
4a
4b
5a
5b
0
13
26
39
52
65
78
91
104
117
130
MODE: PEAK SPOTTING
Length (mm)

C

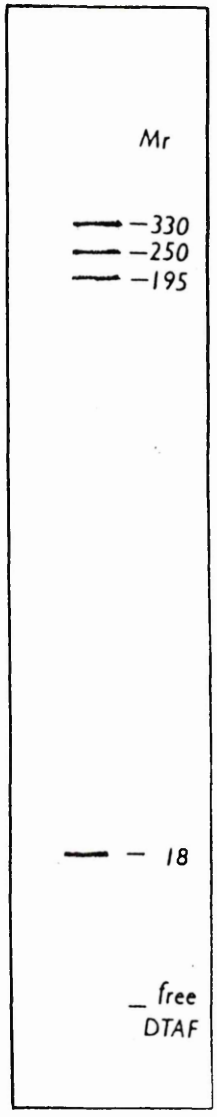
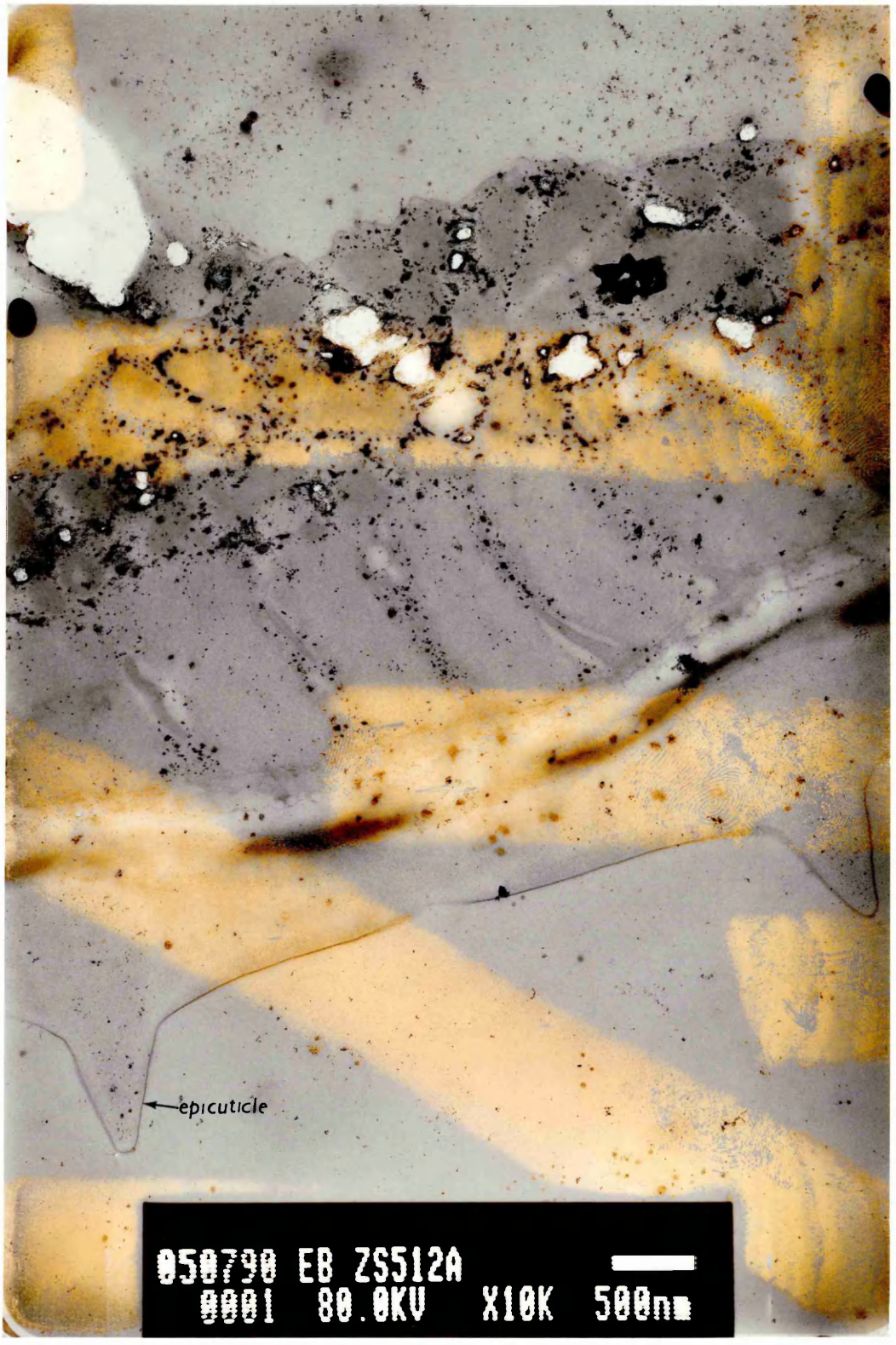

Mr
330
250
195
18
free
DTAF

Figure 4.9 **Electron micrograph of surface-labelling of adult *Ostertagia ostertagi* by the periodate-thiosemicarbazide-silver proteinate (PS-TSC-SP) method for sugars**


epicuticle
050790 EB ZS512A
0001 80.0KV X10K 500nm

4.3. Discussion

4.3.1. Lipids

The results from the lipid analyses show that surface radio-iodination, using Iodogen, was a successful technique in the identification of some of the surface lipids of adult *C. elegans*, *L. carinii* and *A. suum*. Identification of nematode surface lipids by radio-iodination has never been used before, but since it is almost impossible to remove the epicuticle from the rest of the cuticle without changing it chemically, this technique was thought to be the only way to achieve extrinsic labelling of the lipids. In 1979, Hayunga et al reported that mainly lipids were labelled in the tegument of adult *Schistosoma mansoni* after radio-iodination by a number of different techniques. These workers believed that the radioactive iodine had reacted with unsaturated fatty acids in lipids, although the exact nature of this interaction was unknown.

The presence of the phospholipids PE, lyso PE, PC, PS, PI and PA was variable in the three different nematode surfaces examined, as were their relative concentrations. The only invariable lipid was the nonpolar lipid seen near the solvent front. In *L. carinii* it was thought that this nonpolar spot might be a triglyceride, but that it was unlikely to be a wax or sterol ester since these standards migrated much closer to the solvent front.

In contrast to the relative abundance of information on total lipid composition in nematodes, there is a paucity of information on surface lipids. One recent attempt to characterize nematode surface lipids was by Wright and Hong (1988) who tried to remove only surface lipid of MSL of *T. spiralis* by flushing a chloroform/methanol mixture over the worms for less than 5 seconds. The solvent extracts contained mainly PE and some lyso PE, however it would be expected that even rapid rinses with organic solvent could remove some lipids from the internal membranes. Other information comes from extensive studies on *Ascaris* cuticle composition. For example, Fairbairn (1955) obtained a thin, translucent cuticle by scraping away the body wall muscle with a steel spatula. He found that of the total cuticular lipids, phospholipids made up 41%, unsaponifiables made up 16% and triacylglycerols made up 43%, supporting the present view that the radio-iodinated nonpolar

material is a triacylglycerol. Of the phospholipids of the body wall, 45% were of the ether type (which is an unusually high percentage for plasma membrane lipids). Matausic et al (1976) isolated the polar lipids from the cuticle of *A. suum* and found PI, PS and sphingomyelin (SM). A more extensive study of the surface of the related parasite *Ascaridia galli* revealed that the outermost layer contains the polar lipids PE, PC, lyso PC, PA and glycolipids. The nonpolar lipids were acylglycerols, sterol and sterol esters (Parshad and Guraya, 1977).

Regardless of the comparisons with the early literature, the present work using radio-iodination seems to have produced some useful information about the types of lipid which are accessible to Iodogen at the outermost surface. Since it is the nonpolar species which are heavily labelled, it is highly likely that they are present on the outermost surface and may be important in protection mechanisms.

4.3.2. **Proteins**

The different surface proteins of the nematode cuticle appear to fall into one of three categories, (i) the collagenous proteins cross-linked by disulphide bonds, (ii) the detergent/ßME-insoluble proteins linked by covalent interactions, and (iii) the proteins which are soluble under non-reducing conditions, and do not appear to contribute to the structural integrity of the cuticle. These are then the three categories of cuticular protein which might be available for labelling with extrinsic reagents.

From the results on fluorescence labelling of the surface of MSL of *T. spiralis*, it appears that the 50-55 kDa, and the 82- to 98 kDa proteins can be released by the action of sodium borohydride, since proteins of this molecular weight were not present on the SDS-PAGE gel of DTAF/ borohydride-labelled material (figure 4.5). Fluorescence labelling with DTAF/ borohydride was confined to high molecular weight proteins, possibly collagens, although a very small amount of 50- to 53 kDa material was also labelled. The 60% increase in DTAF/borohydride labelling in washed MSL, over 24h culture in RPMI 1640 at 37^{o}C (see figure 4.6), may have been due to the increased exposure of proteins related to developmental changes in the larvae.

Maizels et al (1989) noted that liberation of the 29 kDa glycoprotein from the intact surface of adult *Brugia malayi* parasites requires the addition of a reducing agent. Reducing

agents dissociate the collagenous proteins which are cross-linked by disulphide bonds and may disrupt the loose interaction with certain surface proteins. Philipp et al (1980) were able to iodinate only a few proteins on the surface of MSL of *T. spiralis* which were in a similar molecular weight range to those described here. These iodinated proteins were rapidly shed into the surrounding medium, particularly in response to the presence of serum. Direct comparison between those results and the present results of fluorescently-labelled worms indicates that there may be similarities and that the proteins desribed here are loosely associated with the surface.

Surface proteins of MSL of *T. spiralis* are probably adsorbed onto the epicuticle following synthesis and secretion from the gut and the stichocytes (Silberstein and Despommier, 1984). Adsorption of secreted proteins onto the epicuticle may be common amongst nematodes, although there is also evidence that some surface proteins arrive at the epicuticle by a transcuticular route, as in the 29 kDa glycoprotein of *B. malayi* (Selkirk et al, 1990).

The experiments using the outermost, intact surface of *Ascaris* cuticle indicated that surface proteins were more accessible to radio-iodine and fluorescent protein-binding reagents after treatment of the surface with sodium borohydride (see figure 4.8). There was no apparent damage to the epicuticle in a piece of cuticle treated on the surface with with sodium borohydride (figure 4.7), however, prolonged treatment (24h) of the whole cuticle caused the structure to collapse completely and turn into a gel (not shown), an effect which could not be achieved using ß-mercaptoethanol under the same conditions. This suggests that the sodium borohydride had been able to break other covalent bonds in addition to disulphide bonds.

4.3.3. **Carbohydrates**

The absence of exposed sugars on the nematode parasite surface is intriguing and possibly reflects the need to reduce antigenicity. Nevertheless, most of the surface antigens described so far in nematode parasites are glycosylated (see Maizels and Selkirk, 1988), leaving us with a paradoxical situation. From the results shown in figure 4.9, it is clear that histochemical techniques used with transmission electron microscopy, may provide some of the answers. The labelling procedure involved, first, periodate oxidation of surface sugars,

then reaction with thiosemicarbazide, and lastly overlaying with a solution of silver proteinate. In Chapter 3, section 3.2.4, it was shown that sugars of *T. canis* could be labelled by periodate oxidation and then reaction with a fluorescent hydrazide (LYH), whereas galactose oxidase oxidation and lectin-binding did not work. This suggests that steric hindrance of relatively large molecules, such as enzymes and lectins, was occurring, and that periodate was able to penetrate the surface coat.

For reasons which have already been discussed, it is impractical to isolate the epicuticle from the rest of the cuticle for analysis of its sugars. However, in the future, it may be possible to analyze the sugars and the nature of their interactions by non-extractive techniques, e.g. nuclear magnetic resonance (n.m.r.).

Chapter Five

Lateral diffusion of lipids, proteins & glycoconjugates on the nematode surface as measured by fluorescence recovery after photobleaching (FRAP).

5.1. Introduction

Studies of the lateral mobility of membrane components using fluorescence recovery after photobleaching (FRAP) have, in the past, been able to give some insight into how biological membranes are organized (for reviews see Peters, 1981; Wolf, 1988). For example, we now know that most membrane lipids show rapid lateral diffusion ($D_L = 10^{-6}$ to $10^{-7} cm^2 s^{-1}$; %R = 90 to 100) and slower diffusion of membrane proteins ($D_L = 10^{-9}$ to $10^{-10} cm^2 s^{-1}$), with some proteins completely immobilized by interactions with the cytoskeleton (Schlessinger, 1983). It was, therefore, considered possible that the FRAP technique could be used to probe the lateral organization of the nematode epicuticle. FRAP has several advantages over conventional biochemical techniques in this regard, since the measurements can be performed on live nematodes. Also, this technique may be able to help us to establish whether the epicuticle has a dynamic nature or not.

5.2. Results

5.2.1. Lateral diffusion of fluorescent probes in the epicuticle

The results of lateral diffusion of fluorescent lipid probes and fluorescent protein-binding reagents in the adult epicuticle are given in this section. The results for different species and stages, with a variety of different probes, were as follows:

(i) *Toxocara canis* (table 5.1; figure 5.1)

Infective-stage larvae (L2's):

The majority of the FRAP curves which were obtained for this parasite using the AF18 lipid probe showed immobility. However, with some batches of larvae, there was what could be described as a 'fast component', which is illustrated in fig. 1(a). In contrast, NBD-chol consistently showed mobility, with no 'fast component'.

Sugars labelled on the surface with the fluorescent hydrazide, Lucifer Yellow (LYH), were immobile, as were the molecules (presumed to be proteins) which were labelled with DTAF.

Table 5.1 **Lateral diffusion coefficient and mobile fraction of AF18, NBD-chol, LYH and DTAF in *Toxocara canis* infective-stage larvae.**

Probe	Diffusion coefficient D_L (cm^2s^{-1})	Mobile fraction (% R)	Number of worms D_L (n) ; %R (n)
AF18			
Expt. 1	$5.52 \pm 2.06 \times 10^{-10}$*	36.31 ± 7.53	10 ; 14
Expt. 2	$4.96 \pm 1.79 \times 10^{-11}$*	8.27 ± 4.36	15 ; 17
NBD-chol	$4.37 \pm 1.39 \times 10^{-9}$	61.66 ± 3.40	8 ; 11
LYH	$6.43 \pm 2.09 \times 10^{-10}$	8.45 ± 4.31	7 ; 8
DTAF	$<<10^{-10}$ (immobile)	5.52 ± 1.97	8 ; 10

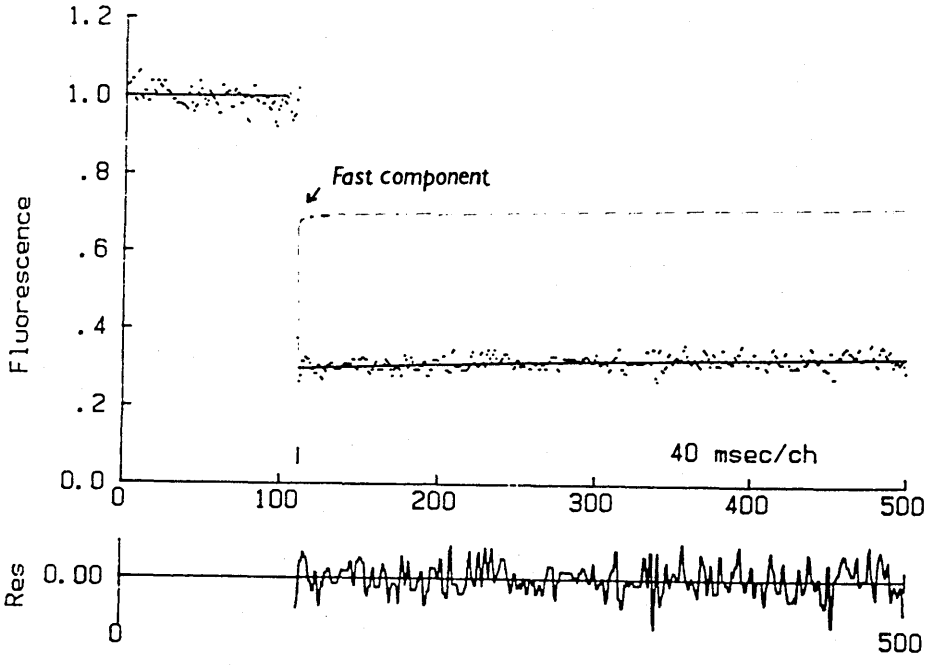
T. canis infective larvae were immobilized with $1 mgml^{-1}$ of the paralyzing drug levamisole

*Occasionally with AF18 diffusion, a 'fast component' appeared giving unusually rapid diffusion, i.e. approx. 10^{-6} cm^2s^{-1}. These unusual curves were not included in the statistical analysis but one of them is represented in figure 5.1.

Figure 5.1 **Representative FRAP curves of (a) AF18 and (b) NBD-chol diffusion in infective-stage larvae of *Toxocara canis***

a

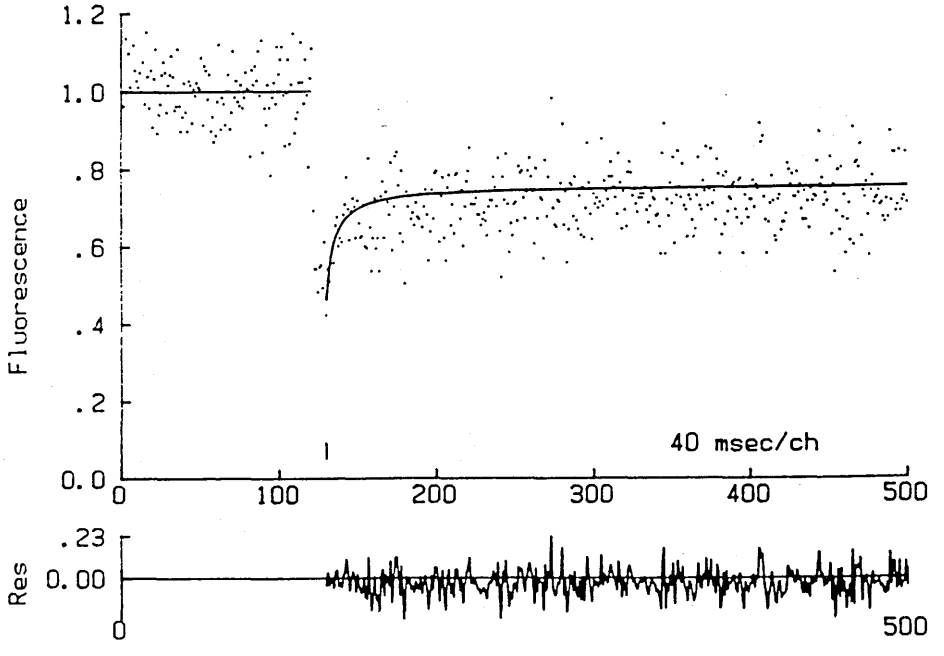
T. canis AF18
%-bleach = 70.3 %-recovery = 10.09 T-half sec = 16.48
Beam um = 0.5 Gamma-2D = 1.30664 D1 = 0.4955 10-10 cm2/sec



Filename : TCNC1

b

T. canis NBD-cholesterol
%-bleach = 61.2 %-recovery = 60.29 T-half sec = 0.16
Beam um = 0.5 Gamma-2D = 1.22421 D1 = 47.820 10-10 cm2/sec



(ii) *Trichinella spiralis* (table 5.2; figs. 5.2-5.3).

Adults: The lipid probe AF18 was immobile, whereas NBD-chol showed mobility.

It was interesting that of 9 anthelminthic drugs tested (see Chapter 2, section 2.4), only the isothiocyanate, CGP 20376 (at 1μM), could 'fluidize' the surface and allow AF18 to become mobile. The diffusion coefficient and mobile fraction in CGP 20376-treated worms were significantly different ($P < 0.0001$) from the control.

Muscle-stage larvae (MSL): Only post-infective larvae could be labelled with AF18 (see Chapter 6),which was immobile on the surface of these larvae. Protein-binding reagents such as FM, IAF and DTAF were also immobile.

New-born larvae (NBL): The lipid probe, AF18 and the protein-binding reagents, FM and IAF were mobile.

(iii) *Acanthocheilonema viteae* (table 5.3; figs. 5.4-5.6).

Adults: The lipid probe AF18 was immobile, whereas NBD-chol and NBD-PC were relatively mobile. Protein-binding reagents FM and DTAF were mobile.

Pre-infective L3's: These larvae had been cultured and labelled in Grace's insect medium at 27°C. Under these conditions there was no AF18-labelling (see Chapter 6).

The protein-binding reagents FM, IAF, Biot/strep and DTAF were mobile, as were the fluoresceinated lectins Con A and PNA.

Post-infective L3's: These larvae had been cultured and labelled in RPMI 1640 at 37°C.

AF18 was relatively mobile compared to the restricted diffusion in the adult surface.

Microfilariae: These microfilariae are unsheathed.

The lipid probes AF18 and NBD-chol were mobile, as were the protein-binding probes Biot/strep and DTAF.

Table 5.2 **Lateral diffusion coefficient and mobile fraction of AF18, NBD-chol, FM, IAF and DTAF in *Trichinella spiralis* adults, infective muscle-stage larvae (MSL) and new-born larvae (NBL).**

Probe		Diffusion coefficient (cm^2s^{-1})	Mobile fraction (% R)	Number of worms D_L(n) ; % R (n)
AF18				
Adults[a]	Expt. 1	$5.45 \pm 2.34 \times 10^{-10}$	10.25 ±5.23	7 ; 7
	Expt. 2	$\ll 10^{-10}$ (immobile)	3.11 ± 1.85	12 ; 12
+ 1μM CGP 20376		$5.69 \pm 2.92 \times 10^{-9}$	47.25 ± 10.66	
MSL	Expt. 1	$4.54 \pm 1.74 \times 10^{-10}$	7.13 ± 3.46	5 ; 10
	Expt. 2	$3.40 \pm 1.43 \times 10^{-10}$	4.44 ± 1.93	7 ; 7
NBL	Expt. 1	$4.89 \pm 2.46 \times 10^{-9}$	59.72 ± 8.38	5 ; 6
	Expt. 2	$6.26 \pm 3.52 \times 10^{-9}$	40.38 ± 9.58	5 ; 7
NBD-chol				
Adults[b]		$3.04 \pm 2.50 \times 10^{-8}$	76.86 ± 13.54	7 ; 7

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Table 5.2 continued.

Probe	Diffusion coefficient (cm^2s^{-1})	Mobile fraction (%R)	Number of worms D_L(n); %R(n)
FM			
MSL	$3.32 \pm 1.56 \times 10^{-10}$	14.63 ± 11.39	4 ; 8
NBL	$1.48 \pm 0.78 \times 10^{-8}$	59.91 ± 5.58	4 ; 8
IAF			
MSL	$\ll 10^{-10}$ (immobile)	6.79 ± 4.43	8 ; 8
NBL	$1.42 \pm 0.19 \times 10^{-8}$	71.89 ± 10.53	5 ; 6
DTAF			
MSL	$1.47 \pm 0.41 \times 10^{-10}$	21.54 ± 7.90	5 ; 7

Figure 5.2 **Representative FRAP curves of AF18 diffusion in (a) adult and (b) infective muscle-stage larvae (MSL) of *Trichinella spiralis*.**

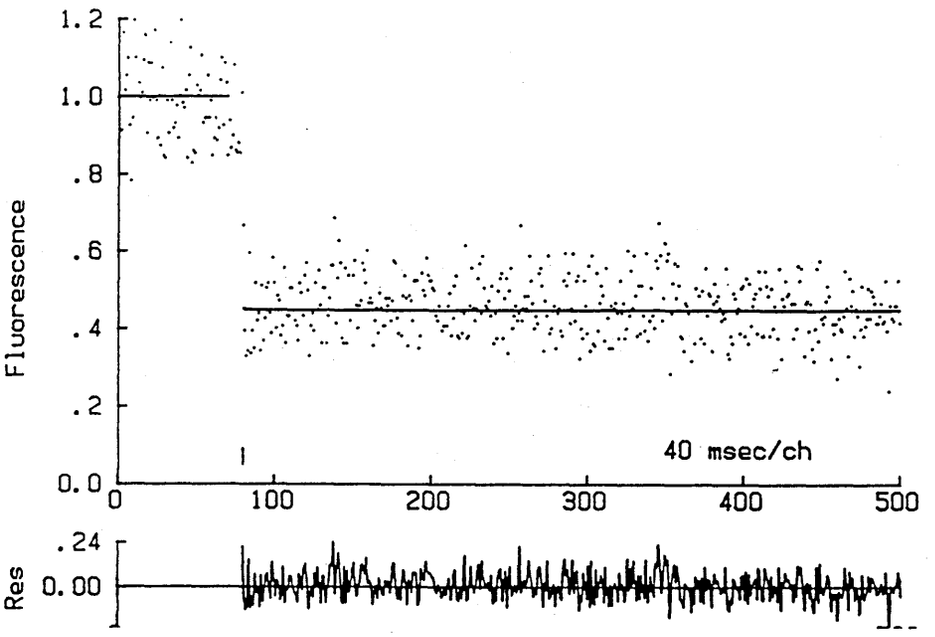
a

Filename : C18A3

Adult T. spiralis; AF18

%-bleach = 54.3 %-recovery = -1.84 T-half sec = 0.04

Beam um = 0.5 Gamma-2D = 1.17848 D1 = 184.13 10-10 cm2/sec



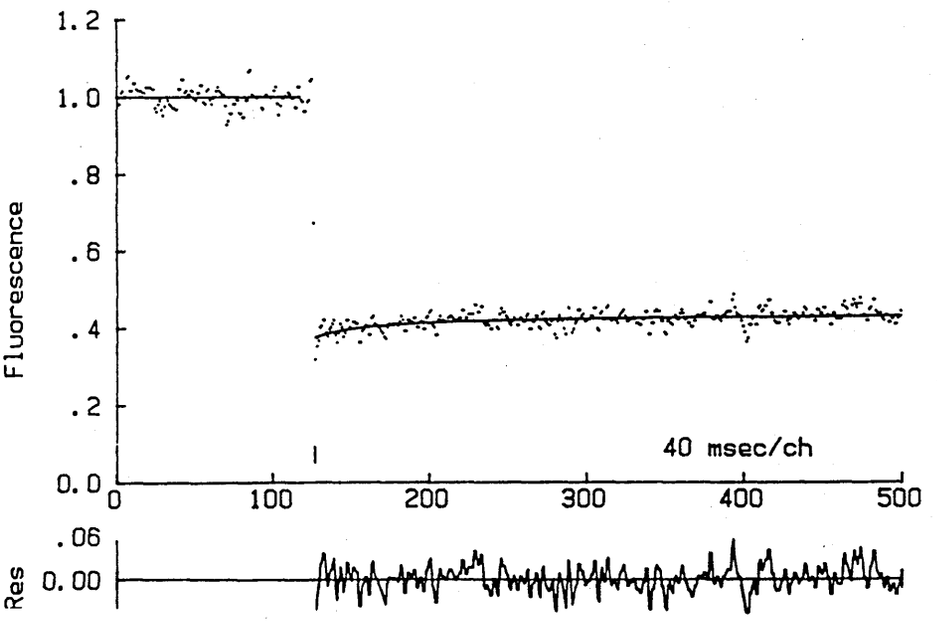
b

Filename : A11

MSL T.spiralis; AF18

%-bleach = 62.6 %-recovery = 9.744 T-half sec = 1.43

Beam um = 0.5 Gamma-2D = 1.23490 D1 = 5.3973 10-10 cm2/sec



Figuree 5.3 Representative FRAP curves of AF18 diffusion in (a) control adults and (b) adults treated with the anthelmintic drug CGP 20376.

CGP 20376 was used at 10^{-8}M concentration.

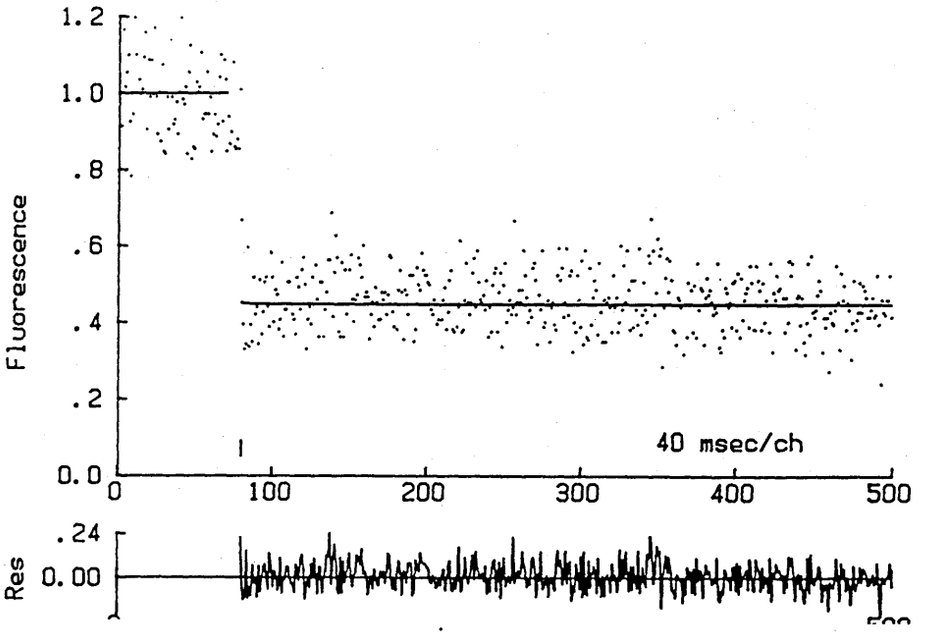
a

Filename : C18A3

Adult T. spiralis; AF18

%-bleach = 54.3 %-recovery = -1.84 T-half sec = 0.04

Beam um = 0.5 Gamma-2D = 1.17848 D1 = 184.13 10-10 cm2/sec



b

Filename : CGPAF4

Adult T.spiralis; CGP 20376 ; AF18

%-bleach = 90.3 %-recovery = 54.04 T-half sec = 0.15

Beam um = 0.5 Gamma-2D = 1.74936 D1 = 72.890 10-10 cm2/sec

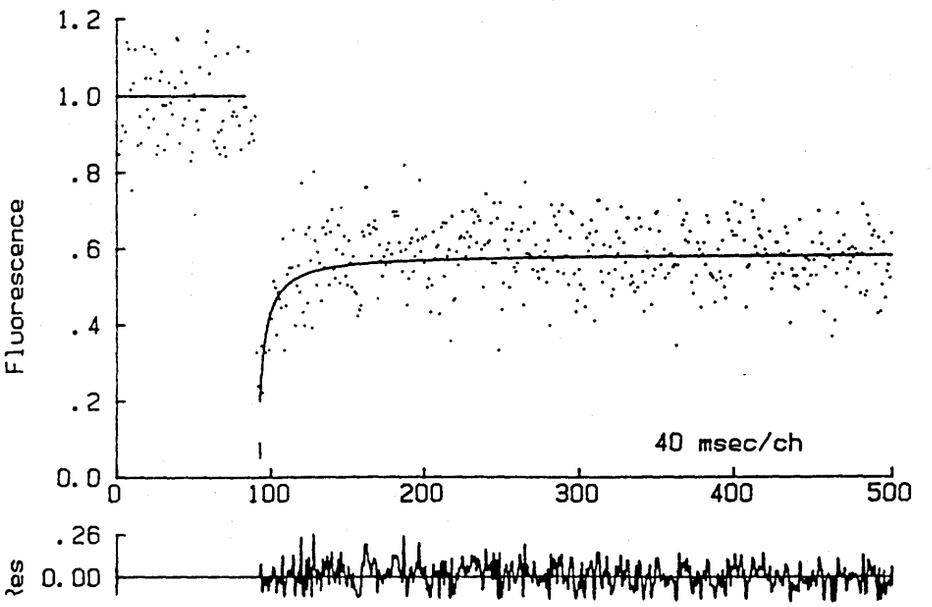


Table 5.3 **: Lateral diffusion coefficient and mobile fraction of AF18, NBD-chol, NBD-PC, FM, IAF, Biot/strep, DTAF and the fluorescent lectins FITC-Con A and PNA in *Acanthocheilonema viteae* adults, 'pre- and post-infective' third-stage larvae (pre- and post-L3) and microfilariae (mf) which are unsheathed.**

		Diffusion coefficient (cm^2s^{-1})	Mobile fraction (% R)	Number of worms $D_L(n)$; % R(n)
AF18				
Adults	Expt. 1	$2.72 \pm 1.11 \times 10^{-8}$	18.26 ± 1.42	7 ; 7
	Expt. 2	$2.23 \pm 0.72 \times 10^{-8}$	12.19 ± 5.53	9 ; 9
post-L3		$7.42 \pm 1.85 \times 10^{-8}$	46.07 ± 3.95	7 ; 7
mf		$1.73 \pm 0.50 \times 10^{-7}$	44.32 ± 6.01	6 ; 12
NBD-chol				
Adults		$1.61 \pm 1.93 \times 10^{-9}$	62.10 ± 6.14	11 ; 11
mf		$8.92 \pm 1.94 \times 10^{-10}$	58.63 ± 8.58	7 ; 7
NBD-PC				
Adults		$1.92 \pm 0.78 \times 10^{-8}$	50.27 ± 12.67	6 ; 7

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Table 5.3 continued.

Probe	Diffusion coefficient (cm^2s^{-1})	Mobile fraction (%R)	Number of worms D_L(n); %R(n)
FM			
Adults	$8.72 \pm 2.24 \times 10^{-8}$	90.59 ± 6.83	6 ; 7
pre-L3	$3.90 \pm 0.71 \times 10^{-8}$	63.83 ± 3.03	5 ; 5
IAF			
pre-L3	$2.11 \pm 0.38 \times 10^{-8}$	74.44 ± 6.38	5 ; 5
Biot/strep			
pre-L3	$1.55 \pm 0.70 \times 10^{-8}$	59.10 ± 4.75	4 ; 4
mf	$3.74 \pm 2.78 \times 10^{-9}$	46.74 ± 6.16	7 ; 7
DTAF			
Adults	$7.07 \pm 4.34 \times 10^{-8}$	80.13 ± 10.61	6 ; 6
pre-L3	$6.55 \pm 2.97 \times 10^{-8}$	72.35 ± 6.72	10 ; 11
mf	$1.39 \pm 0.31 \times 10^{-7}$	72.47 ± 9.95	5 ; 6
FITC-Con A			
pre-L3	$3.71 \pm 0.74 \times 10^{-9}$	52.40 ± 5.33	5 ; 5
FITC-PNA			
pre-L3	$5.24 \pm 2.40 \times 10^{-9}$	60.62 ± 3.51	5 ; 5

Figure 5.4 **Representative FRAP curves of (a) AF18 and (b) NBD-chol diffusion in adults of *Acanthocheilonema viteae*.**

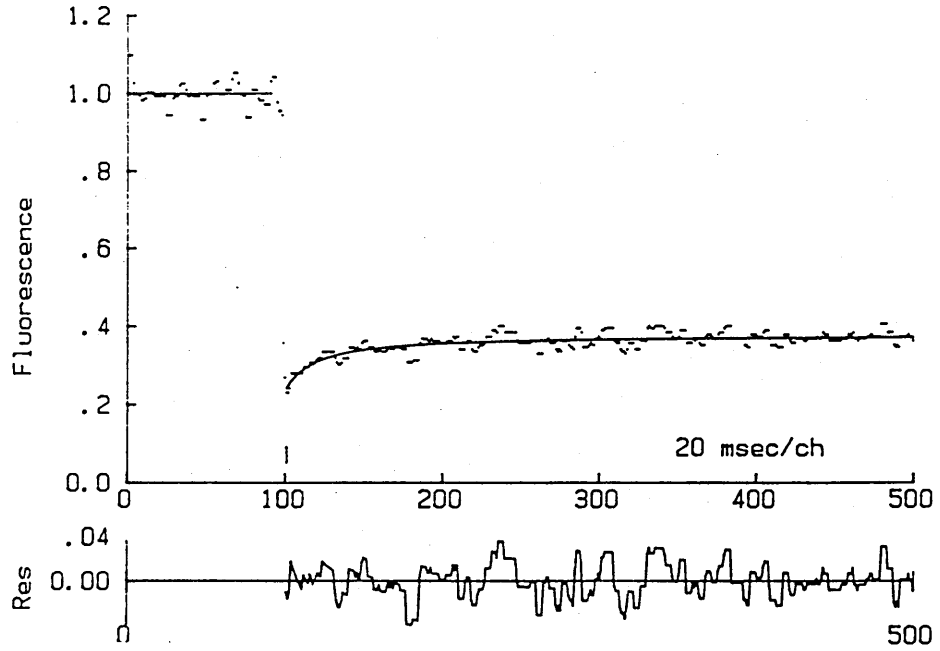
a

Filename : AD1810

Adult A. viteae: AF18

%-bleach = 76.6 %-recovery = 18.79 T-half sec = 0.37

Beam um = 0.5 Gamma-2D = 1.39086 D1 = 23.494 10-10 cm2/sec



b

Filename : ADCH4

Adult A. viteae; NBD-cholesterol

%-bleach = 94.8 %-recovery = 67.93 T-half sec = 0.08

Beam um = 0.5 Gamma-2D = 1.96312 D1 = 153.36 10-10 cm2/sec

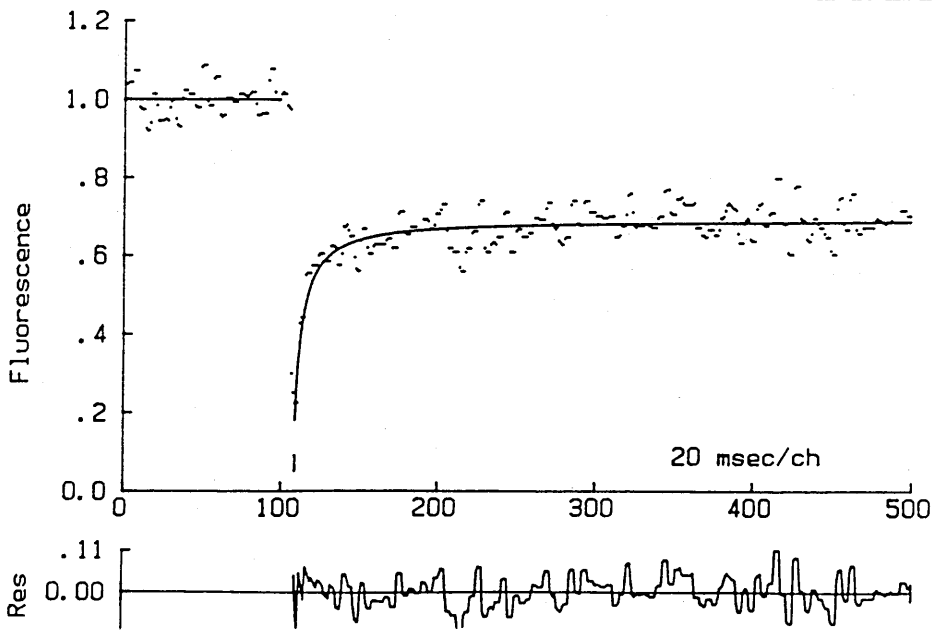


Figure 5.5 Representative FRAP curves of AF18 diffusion in (a) microfilariae and (b) 'post-infective' L3's of *Acanthocheilonema viteae*.

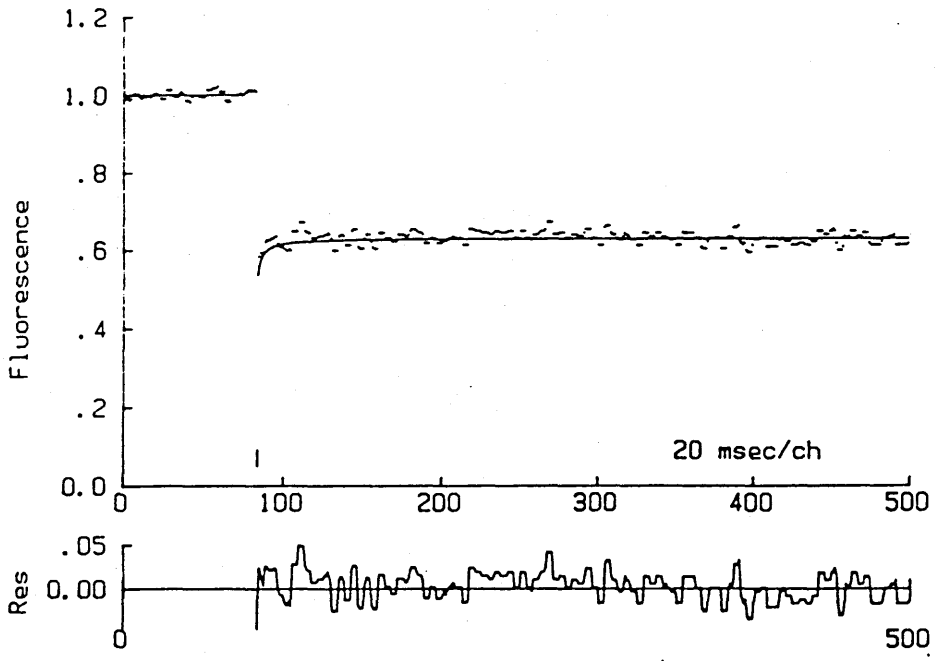
A

Filename : MF181

Microfilaria A.viteae: AF18

%-bleach = 51.2 %-recovery = 27.92 T-half sec = 0.04

Beam um = 0.5 Gamma-2D = 1.16088 D1 = 181.38 10-10 cm2/sec



B

Filename : AA187

"Post-infective" L3 A.viteae: AF18

%-bleach = 66.1 %-recovery = 54.16 T-half sec = 0.04

Beam um = 0.5 Gamma-2D = 1.26442 D1 = 197.56 10-10 cm2/sec

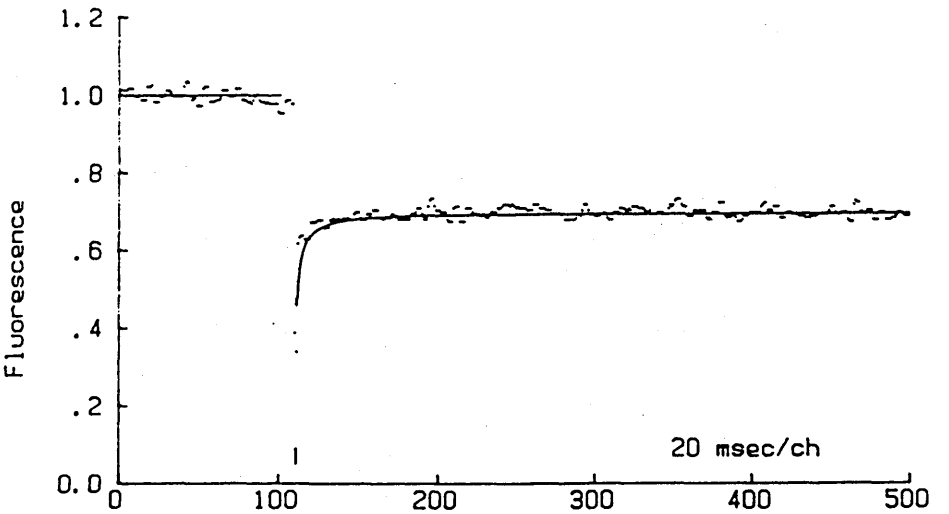


Figure 5.6 Representative FRAP curves of DTAF-labelled protein diffusion in (a) microfilariae and (b) adults of *Acanthocheilonema viteae.*

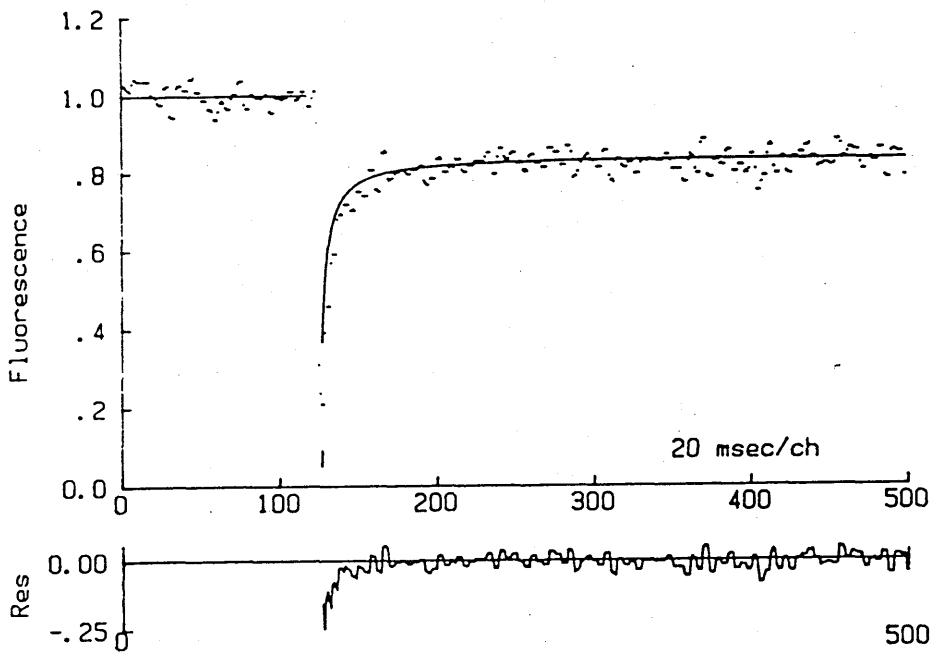
A

Filename : MFDT5

Microfilaria A.viteae: DTAF

%-bleach = 79.1 %-recovery = 79.89 T-half sec = 0.06

Beam um = 0.5 Gamma-2D = 1.43426 D1 = 149.40 10-10 cm2/sec



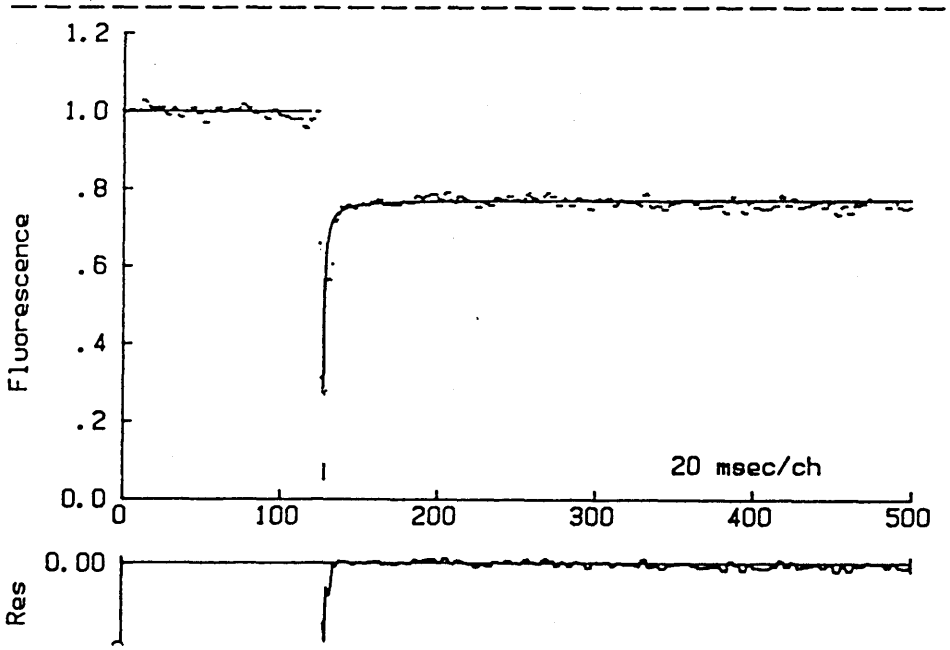
B

Filename : ADDT3

Adult A.viteae: DTAF

%-bleach = 93.1 %-recovery = 75.51 T-half sec = 0.01

Beam um = 0.5 Gamma-2D = 1.87442 D1 = 1171.5 10-10 cm2/sec



(iv) *Brugia pahangi* (table 5.4; figs. 5.7 and 5.8).

Adults: AF18 was immobile, whereas NBD-chol and the protein-binding reagent FM were mobile.

Post-infective L3's: These larvae had been cultured and labelled in RPMI 1640 at 37°C. AF18 was relatively mobile, compared to the restricted diffusion in the adult surface.

Microfilariae: These microfilariae were sheathed, but could be exsheathed using the 'freeze-thawing' method described in section 2.

AF18 was mobile in sheathed microfilariae, but completely immobile in the exsheathed surface (fig. 5.7(a)).

The fluoresceinated lectin, WGA was mobile in the sheath of peritoneal and blood microfilariae, although there is a strong possibility that this probe was diffusing freely in the space between the sheath and the microfilaria (Devaney, 1985).

(v) *Litomosoides carinii* (table 5.5; fig. 5.9)

Adults: In general, AF18 was immobile, however, the same worm could also display patches of rapidly diffusing AF18 from time to time ($D_L \approx 10^{-7}$ cm^2s^{-1}) in no discernible pattern. NBD-chol was consistently mobile with no 'fast component'.

Microfilariae: These microfilariae are sheathed.

Diffusion of AF18 and FM in the sheath was very slow, with approximately half of the probe free to diffuse. The fluoresceinated lectin WGA was confined to the surface of the sheath (as measured by quenching with 0.25% Trypan blue) and was immobile.

(vi) *Ostertagia ostertagi* (table 5.6).

Adults: The lipid probe AF18 was immobile, whereas the protein-binding reagents, FM and DTAF were mobile.

Post-infective L3's: These larvae had been cultured and labelled in RPMI 1640 at 37°C (see Chapter 6). AF18 was relatively mobile compared to the restricted diffusion in adults.

Table 5.4 : **Lateral diffusion coefficient and mobile fraction of AF18, NBD-chol, FM and the fluorescent lectin, FITC-WGA in *Brugia pahangi* adults, post-infective L3's and microfilariae which are sheathed.**

Probe	Diffusion coefficient (cm^2s^{-1})	Mobile fraction (% R)	Number of worms $D_L(n)$; % R(n)
AF18			
Adults	$3.91 \pm 1.83 \times 10^{-10}$	15.50 ± 7.79	10 ; 15
mf (sheath)	$1.64 \pm 0.55 \times 10^{-8}$	47.71 ± 5.34	12 ; 12
mf (exsheathed)	$<<10^{-10}$ (immobile)	immobile	10 ; 10
post-L3	$7.42 \pm 3.40 \times 10^{-8}$	54.22 ± 6.90	6 ; 6
NBD-chol			
Adults	$6.38 \pm 2.04 \times 10^{-8}$	79.58 ± 8.50	5 ; 6
FM			
Adults	$3.27 \pm 1.27 \times 10^{-9}$	74.96 ± 5.95	8 ; 8
FITC-WGA			
peritoneal mf (sheath)	$3.14 \pm 1.20 \times 10^{-8}$	52.35 ± 6.40	7 ; 13
blood mf (sheath)	$6.06 \pm 1.54 \times 10^{-8}$	57.22 ± 6.68	5 ; 5

Figure 5.7 Representative FRAP curves of AF18 diffusion in (a) exsheathed blood microfilariae and (b) adults of *Brugia pahangi*.

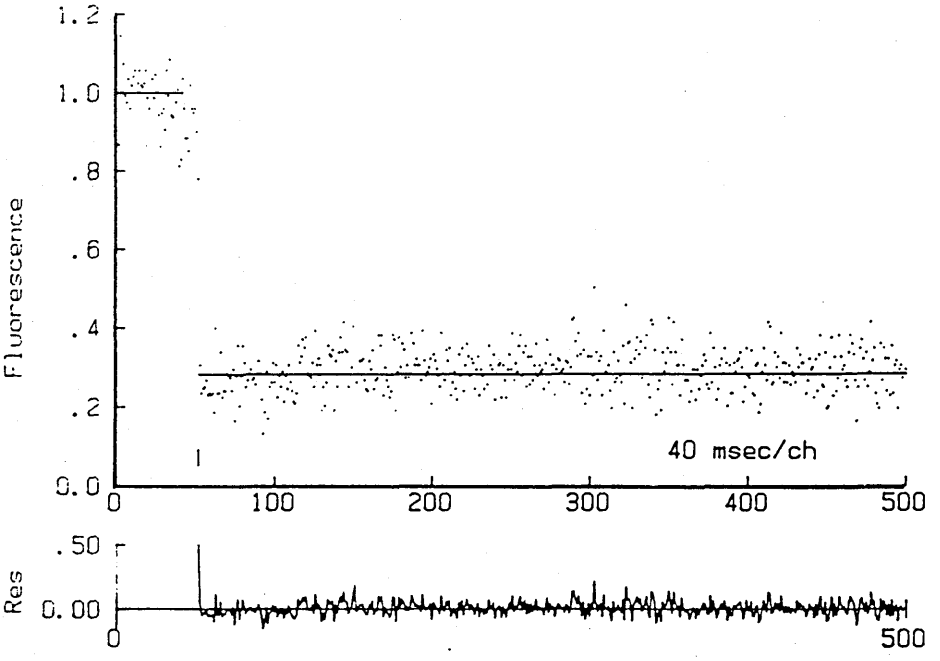
a

Filename : BLUSC4

Exsheathed blood microfilaria of B.pahangi: AF18

%-bleach = 71.9 %-recovery = 0.834 T-half sec = 23.2

Beam um = 0.5 Gamma-2D = 1.32525 D1 = 0.3570 10-10 cm2/sec



b

Filename : AC182

Adult B.pahangi: AF18

%-bleach = 36.9 %-recovery = 0.813 T-half sec = 13.4

Beam um = 0.5 Gamma-2D = 1.09661 D1 = 0.5114 10-10 cm2/sec

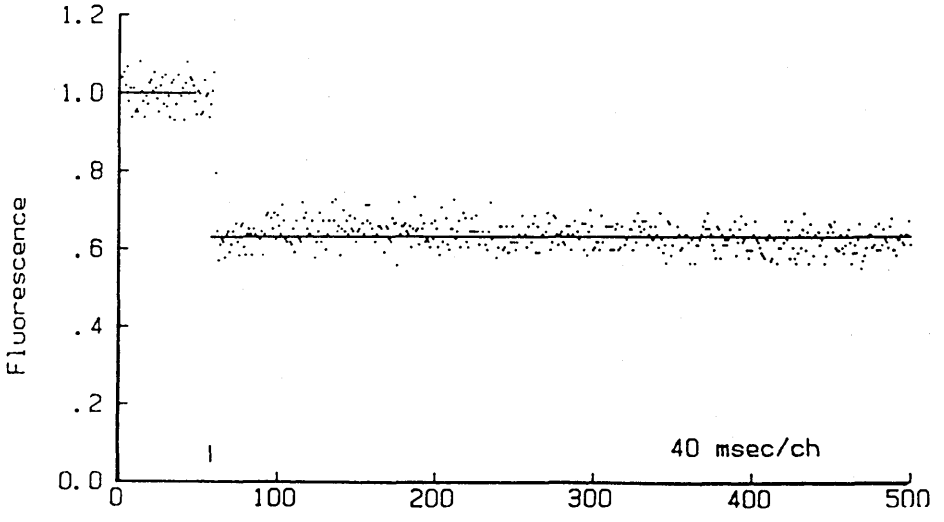


Figure 5.8 **Representative FRAP curves of (a) AF18 and (b) FITC-WGA lectin diffusion in sheathed blood microfilariae of *Brugia pahangi*.**

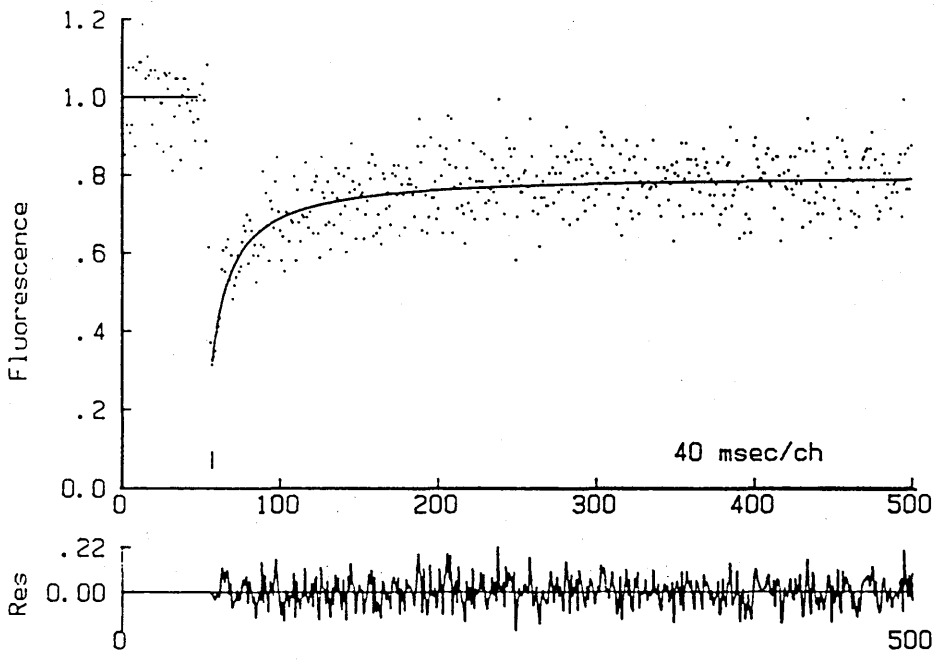
a

Filename : BLC183

Sheathed blood microfilaria: AF18

%-bleach = 71.6 %-recovery = 72.20 T-half sec = 0.49

Beam um = 0.5 Gamma-2D = 1.32164 D1 = 16.857 10-10 cm2/sec



b

Filename : WBGA5

Sheathed microfilaria of B. pahangi: FITC-WGA lectin

%-bleach = 74.6 %-recovery = 82.43 T-half sec = 0.22

Beam um = 0.5 Gamma-2D = 1.36070 D1 = 38.656 10-10 cm2/sec

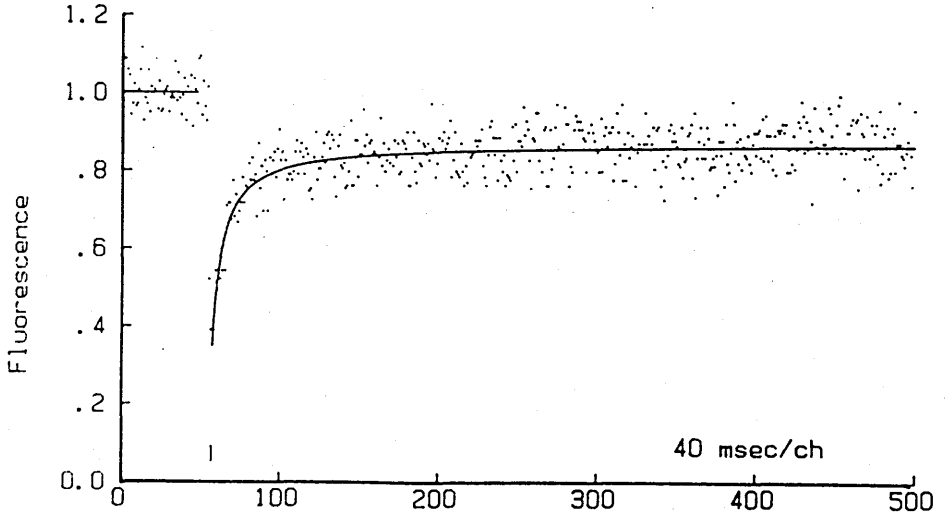


Table 5.5 : **Lateral diffusion coefficient and mobile fraction of AF18, NBD-chol, FM, Biot/strep and the fluorescent lectin, FITC-WGA in *Litomosoides carinii* adults and microfilariae which are sheathed.**

Probe		Diffusion coefficient (cm^2s^{-1})	Mobile fraction (% R)	Number of worms $D_L(n)$; % R(n)
AF18				
Adults	Expt. 1	$2.54 \pm 1.65 \times 10^{-10}$	5.43 ± 5.40	6 ; 8
	Expt. 2	$4.00 \pm 2.90 \times 10^{-8}$	15.72 ± 3.77	5 ; 5
	Expt. 3	$4.95 \pm 4.68 \times 10^{-9}$	21.50 ± 6.72	11 ; 14
	Expt. 4 (fast component)	$1.67 \pm 0.61 \times 10^{-7}$	60.48 ± 17.88	9 ; 13
mf (sheath)		$8.33 \pm 4.86 \times 10^{-10}$	58.62 ± 8.65	18 ; 19
NBD-chol				
Adults	Expt. 1	$1.54 \pm 1.13 \times 10^{-9}$	55.51 ± 6.12	6 ; 6
	Expt. 2	$1.71 \pm 0.53 \times 10^{-9}$	64.65 ± 6.22	6 ; 8
FM				
Adults	Expt. 1	$2.04 \pm 2.47 \times 10^{-8}$	36.54 ± 6.42	10 ; 11
	Expt. 2	$2.79 \pm 2.89 \times 10^{-8}$	36.05 ± 9.43	7 ; 10
mf (sheath)		$5.58 \pm 0.77 \times 10^{-10}$	40.32 ± 7.77	13 ; 13
Biot/strep				
Adults		$1.70 \pm 0.78 \times 10^{-8}$	36.83 ± 4.86	5 ; 6
FITC-WGA				
mf (sheath)		$3.19 \pm 2.42 \times 10^{-9}$	10.51 ± 4.54	4 ; 4

Figure 5.9 Representative FRAP curves of (a) AF18 (immobile component), and (b) AF18 (fast component) in adults of *Litomosoides carinii*.

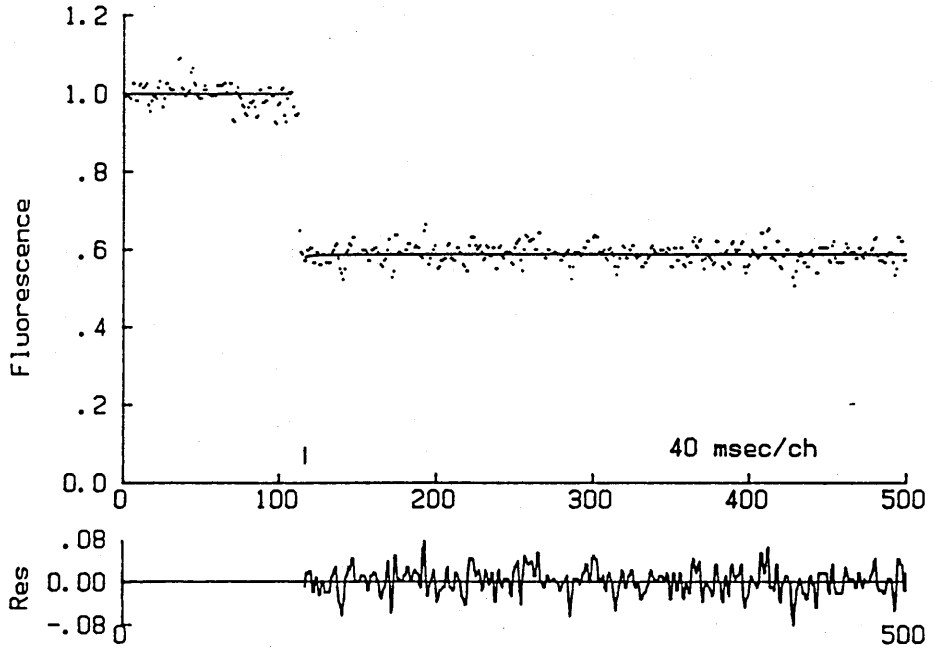
a

Filename : EAF8

Adult L.carinii; AF18 (immobile component)

%-bleach = 42.4 %-recovery = 2.594 T-half sec = 0.08

Beam um = 0.5 Gamma-2D = 1.11843 D1 = 87.377 10-10 cm2/sec



b

Filename : LTAF8

Adult L.carinii; AF18 (fast component)

%-bleach = 71.8 %-recovery = 75.76 T-half sec = 0.08

Beam um = 0.5 Gamma-2D = 1.32404 D1 = 103.44 10-10 cm2/sec

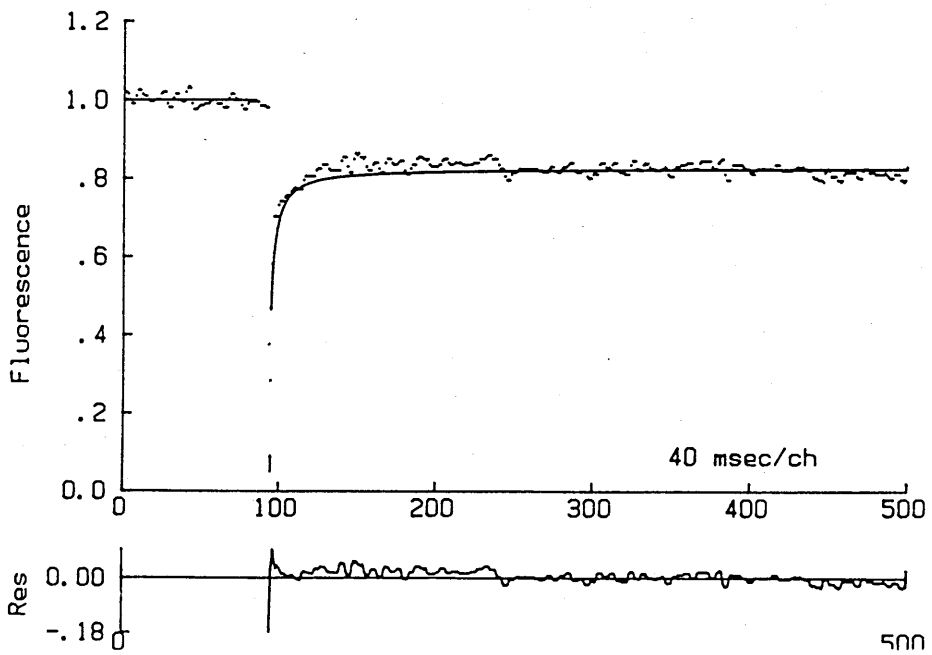


Table 5.6 : **Lateral diffusion coefficient and mobile fractions of AF18, FM and DTAF in *Ostertagia ostertagi* adults and 'post-infective' third-stage larvae (post-L3).**

Probes	Diffusion coefficient (cm^2s^{-1})	Mobile fraction (% R)	Number of worms $D_L(n)$; % R(n)
AF18			
Adults	$2.38 \pm 1.30 \times 10^{-9}$	31.30 ± 12.57	5 ; 7
post-L3	$3.57 \pm 1.25 \times 10^{-8}$	50.84 ± 6.60	7 ; 7
FM			
Adults	$3.51 \pm 1.38 \times 10^{-8}$	76.28 ± 10.24	5 ; 6
DTAF			
Adults	$4.70 \pm 1.86 \times 10^{-8}$	77.06 ± 5.98	7 ; 7

All stages were immobilized with $5mgml^{-1}$ sodium azide.

Table 5.7 : **Lateral diffusion coefficient and mobile fraction of AF18, NBD-chol, DTAF, anti-surface antibody, and the fluorescent lectins FITC-Con A, -PNA and -WGA in *Caenorhabditis elegans* adults and dauer larvae.**

Probes		Diffusion coefficient (cm^2s^{-1})	Mobile fraction (% R)	Number of worms $D_L(n)$; % R(n)
AF18				
Adults	Expt. 1	$4.10 \pm 1.89 \times 10^{-8}$	65.87 ± 6.97	6 ; 6
	Expt. 2	$8.38 \pm 3.66 \times 10^{-8}$	51.84 ± 9.27	8 ; 8
	Expt. 3	$5.45 \pm 1.69 \times 10^{-8}$	64.72 ± 12.93	7 ; 7
Dauers*	Expt. 1	$3.62 \pm 0.96 \times 10^{-9}$	14.48 ± 6.28	9 ; 9
	Expt. 2	$6.37 \pm 1.10 \times 10^{-10}$	17.35 ± 3.25	6 ; 6
	Expt. 3	$1.47 \pm 0.51 \times 10^{-10}$	15.55 ± 3.65	8 ; 8
	Expt. 4	$3.92 \pm 2.39 \times 10^{-10}$	18.06 ± 3.99	6 ; 7
Dauers + 10 µM Suramin**		$1.39 \pm 1.15 \times 10^{-8}$	30.4 ± 7.21	8 ; 8
NBD-chol				
Adults		$4.34 \pm 1.41 \times 10^{-9}$	88.82 ± 4.07	5 ; 6
Dauers*	Expt. 1	$7.06 \pm 0.35 \times 10^{-9}$	75.33 ± 6.52	7 ; 7
	Expt. 2	$1.32 \pm 0.22 \times 10^{-8}$	80.72 ± 2.10	5 ; 5
	Expt. 3	$1.34 \pm 0.47 \times 10^{-8}$	76.04 ± 8.91	6 ; 6

Continued over the page.

Table 5.7 continued.

Probe	Diffusion coefficient (cm^2s^{-1})	Mobile fraction (%R)	Number of worms D_L(n); %R(n)
DTAF			
Adults	$2.93 \pm 1.56 \times 10^{-8}$	81.05 ± 7.99	5 ; 5
Anti-surface antibody			
Adults	$4.66 \pm 2.45 \times 10^{-8}$	72.77 ± 5.42	5 ; 5
srf-3	$6.41 \pm 5.62 \times 10^{-8}$	85.10 ± 11.75	6 ; 7
Dauer*	$<< 10^{-10}$ (immobile)	7.20 ± 2.30	8 ; 8
FITC-Con A			
Adults	$7.12 \pm 1.25 \times 10^{-9}$	66.36 ± 3.70	7 ; 7
FITC-PNA			
Adults	$9.25 \pm 3.26 \times 10^{-8}$	88.36 ± 2.03	4 ; 9
FITC-WGA			
Adults	$6.94 \pm 1.88 \times 10^{-9}$	35.48 ± 11.83	5 ; 5
Dauer	$5.92 \pm 0.49 \times 10^{-9}$	69.98 ± 21.30	9 ; 12

* Dauer larvae retain the cast cuticle of the L2-stage larva, and it is this sheath which becomes labelled, not the surface of the dauer.

Figure 5.10 Representative FRAP curves of (a) AF18, and (b) NBD-chol diffusion in adult, wild-type (N2) *Caenorhabditis elegans*.

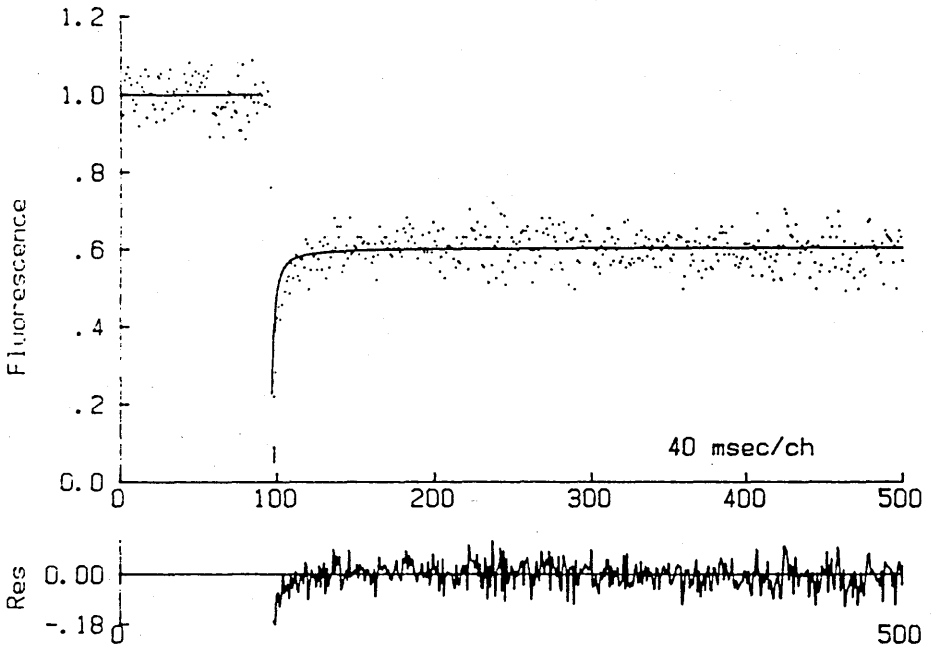
(a)

Filename : CC183

C. elegans (N2) Wild-type: AF18

%-bleach = 83.3 %-recovery = 52.58 T-half sec = 0.04

Beam um = 0.5 Gamma-2D = 1.52528 D1 = 238.32 10-10 cm2/sec



(b)

Filename : CHOL3

Adult C. elegans NBD-cholesterol

%-bleach = 92.8 %-recovery = 89.33 T-half sec = 0.25

Beam um = 0.5 Gamma-2D = 1.85983 D1 = 46.495 10-10 cm2/sec

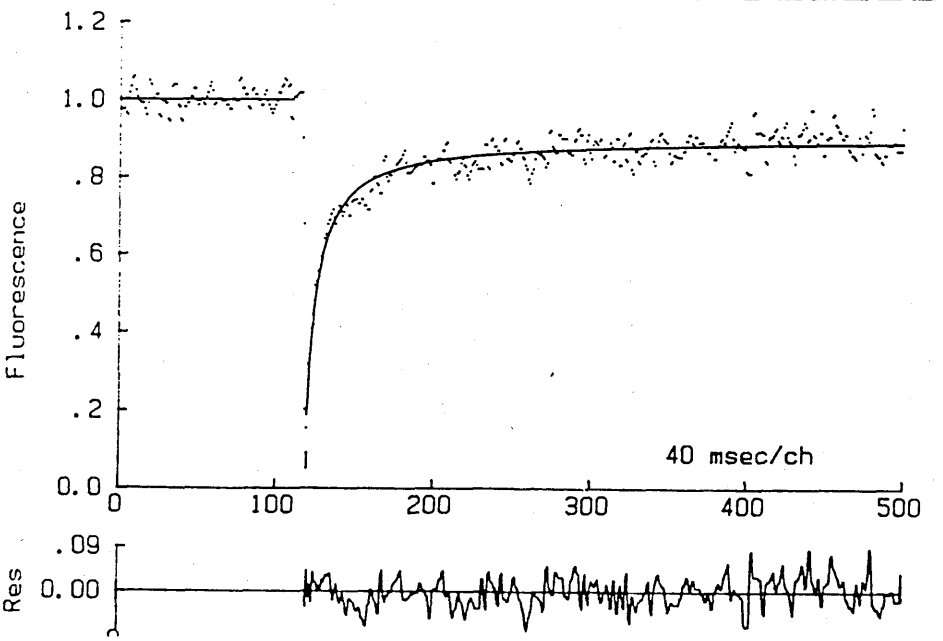
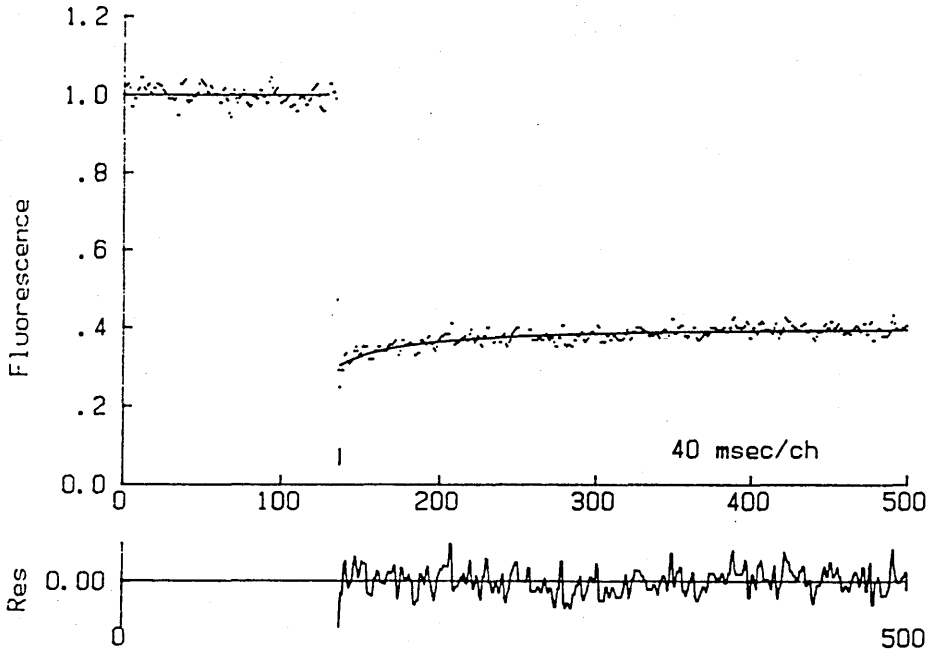


Figure 5.11 **Representative FRAP curves of AF18 diffusion in (a) control, and (b) suramin-treated dauer larvae of *Caenorhabditis elegans*.**

(a)

Dauer L. elegans AF18 (Without drug)
%-bleach = 69.9 %-recovery = 15.16 T-half sec = 1.71
Beam um = 0.5 Gamma-2D = 1.30223 D1 = 4.7596 10-10 cm2/sec



(b)

Filename : 8D1
SURAMIN-treated dauer larvae of C. elegans; AF18
%-bleach = 81.3 %-recovery = 45.51 T-half sec = 0.15
Beam um = 0.5 Gamma-2D = 1.47874 D1 = 61.614 10-10 cm2/sec

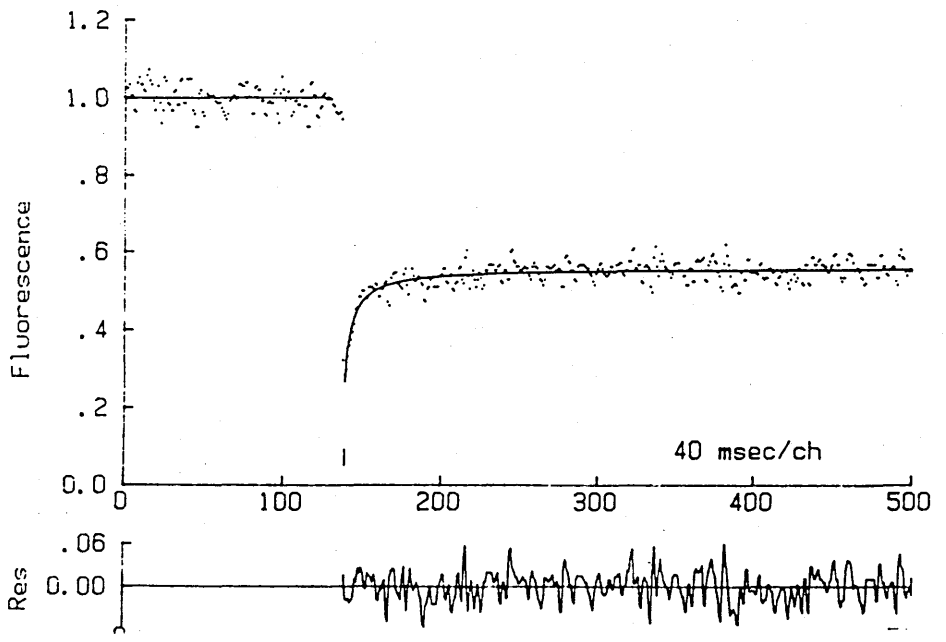


Figure 5.12 **Representative FRAP curves of fluorescent polyclonal antibody diffusion in (a) the dauer sheath, and (b) the true surface of srf-3 mutants of *Caenorhabditis elegans*.**

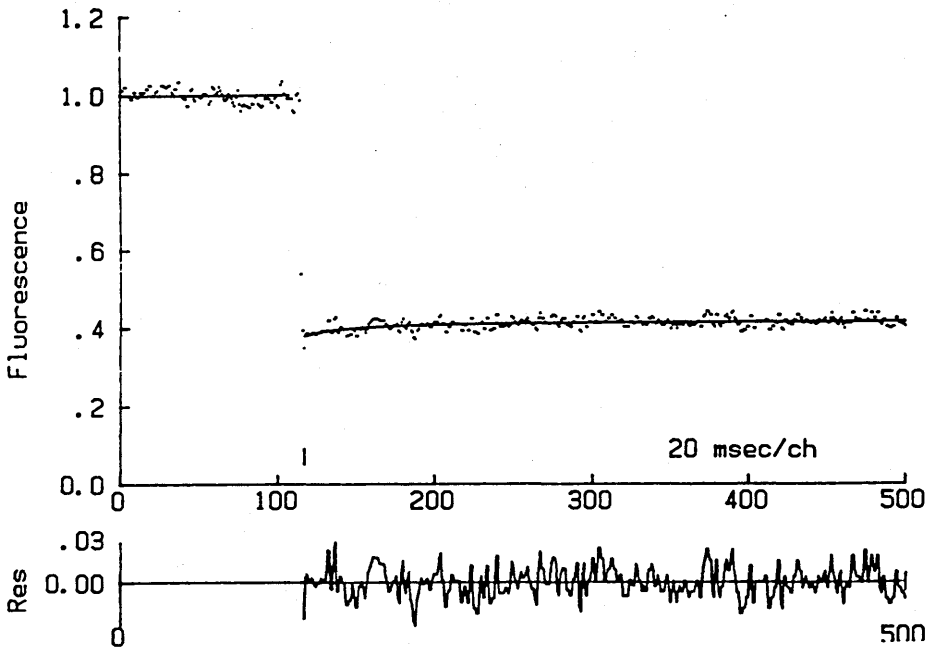
(a)

Filename : SFAB2

Sheath of C. elegans srf-3 Antibody

%-bleach = 62.2 %-recovery = 6.591 T-half sec = 0.55

Beam um = 0.5 Gamma-2D = 1.23179 D1 = 13.997 10-10 cm2/sec



(b)

Filename : SF3UN2

C. elegans Mutant srf-3; Polyclonal Antibody

%-bleach = 72.4 %-recovery = 89.50 T-half sec = 0.03

Beam um = 0.5 Gamma-2D = 1.33141 D1 = 277.37 10-10 cm2/sec

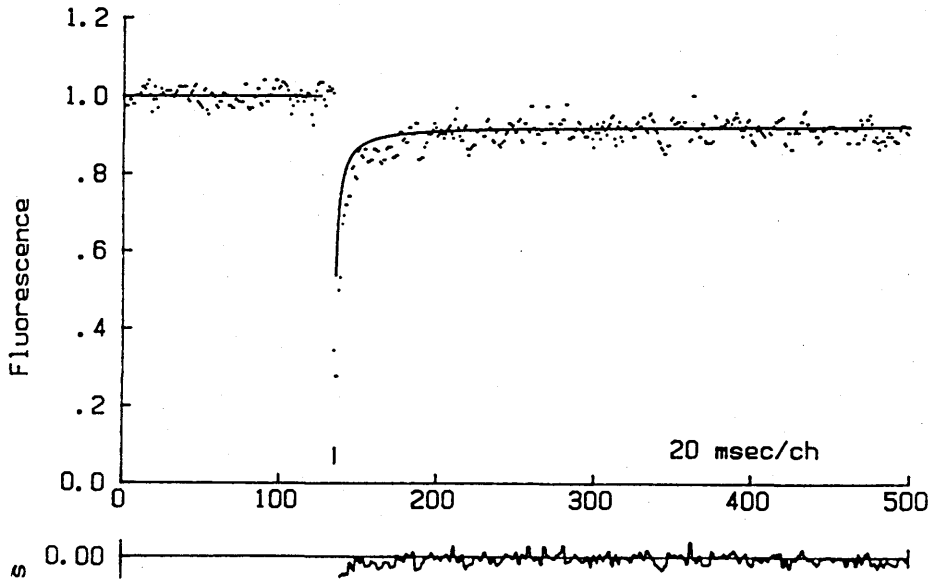
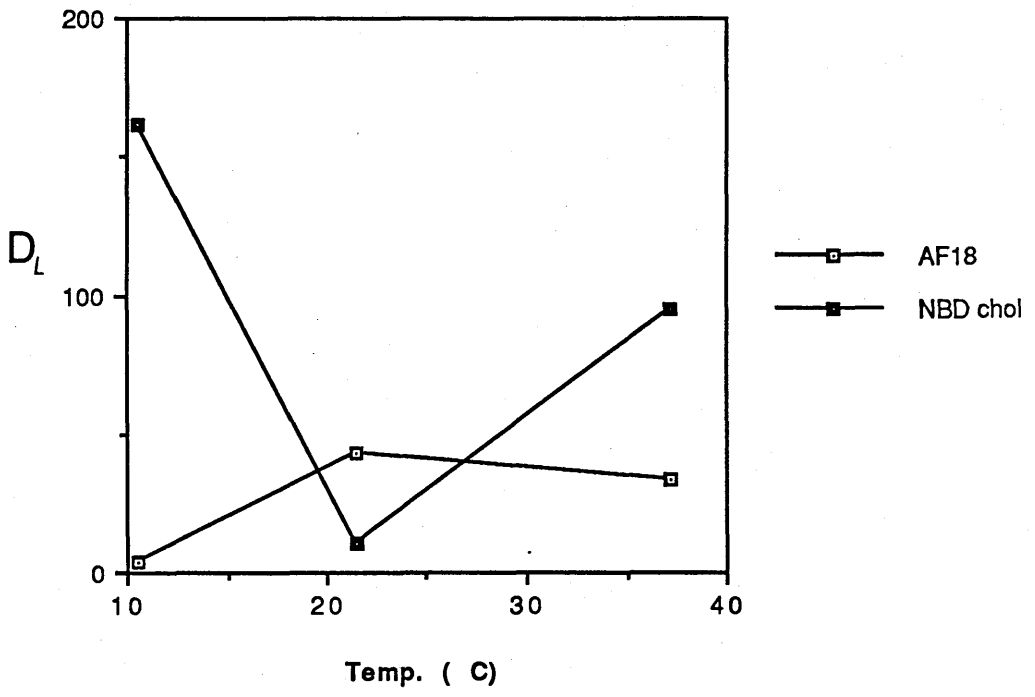
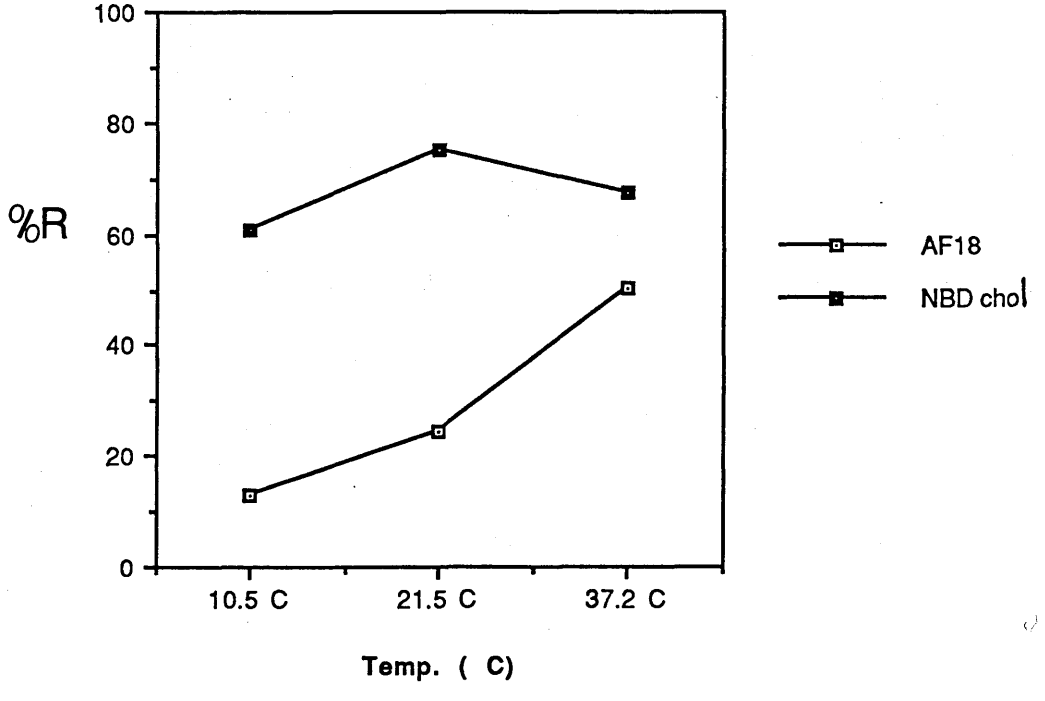


Figure 5.13 Influence of temperature on (a) the diffusion coefficient (D_L), and (b) the percentage recovery (%R) of AF18 and NBD-chol in the dauer sheath of *Caenorhabditis elegans*





(vii) *Caenorhabditis elegans* (table 5.7; figs. 5.10-5.12).

Adults: The lipid probe AF18 was mobile in this free-living nematode, which was in contrast to the restricted diffusion observed in adult parasites. NBD-chol was also mobile.

The protein-binding reagent, DTAF and the anti-surface antibody were completely mobile.

The fluoresceinated lectins Con A, PNA and WGA diffused rapidly on the surface, however their respective mobile fractions were variable, i.e. from 35% to 88%.

srf-3 mutants: These are mutants which expose normally-hidden determinants on their surface (Politz et al, 1987).

Anti-surface antibody was mobile and comparable with that of wild-type (N2) adults, as was AF18 mobility (data not shown).

Dauer larvae: These larvae are sheathed (cast cuticle of L2-stage).

AF18 was immobile in the sheath, but interestingly, the potent anthelmintic suramin was able to 'fluidize' the otherwise immobile sheath. The diffusion coefficient and mobile fraction in suramin-treated dauer larvae were significantly different ($P < 0.0001$) from the control.

NBD-chol and the fluoresceinated lectin, WGA were mobile.

Anti-surface antibody was immobile on the dauer sheath.

Influence of temperature on the mobility of AF18 and NBD-chol in the dauer sheath of *C. elegans*.

When the temperature was varied from 10.5°C to 37.2°C, the diffusion coefficient and mobile fraction of AF18 and NBD-chol varied disproportionately (fig. 5.13). In particular, the diffusion coefficient of NBD-chol varied considerably over this temperature range.

5.3. Discussion

Lipids

FRAP studies on the adult nematode surface immediately showed it to be a highly unusual biological surface with a large non-diffusing fraction of lipid detected in all of the adult parasitic nematodes using the fluorescent lipid probe AF18. However, the same probe was free to diffuse in the infective larvae of *A. viteae*, *B. pahangi* and *O. ostertagi*, and in the adults of the free-living nematode *C. elegans*. Diffusibility of AF18 was less clear in infective-stage larvae of *T. canis* and in adults of *Litomosoides carinii*, where a 'fast component' was occasionally observed in addition to immobility of the probe. This 'fast component' was most pronounced in infective-stage larvae of *T. canis* and could possibly be explained by the interference of a dense surface coat which is present in these larvae (Badley et al, 1987). The 'fast component' of *L. carinii* was exhibited as mobile patches which were distributed in no discernible pattern. These mobile patches have been noted previously in *L. carinii* (Kennedy et al, 1987c) and may be due to the highly-folded cuticle in this parasite (see figure 3.3) which may cause rapid exchange of the AF18 lipid probe within the folds. The reasoning behind this idea comes from experiments on the AF18-labelled surface of the cystacanth envelope of the acanthocephalan parasite, *Moniliformis moniliformis* (data not shown). When two of these labelled envelopes were in juxta-position, a 'fast component, i.e. 10^{-6} to 10^{-7} cm^2s^{-1}, was observed which was unusual since there had been immobility in other areas of the envelope.

It was interesting that the isothiocyanate anthelmintic, CGP 20376 was able to 'fluidize' the epicuticular environment of the AF18 probe in adults of *T. spiralis*, since this probe was normally immobile. It is unlikely that this effect had been due to direct effects of the drug on AF18, since the worms were washed free of the medium containing the drug before fluorescence labelling. It is therefore an attractive hypothesis that this isothiocyanate anthelmintic could exert its effects through alteration of surface properties.

The general immobility of AF18 in the surface of adult mammalian-parasitic forms was in stark contrast to that found in adults of the free-living nematode *Caenorhabditis elegans*. Lateral diffusion of AF18 was relatively unrestricted in *C. elegans*, although the proportion of the probe that was free to diffuse was still much less than the expected value

for a typical plasma membrane. The difference in lipid diffusibility prompts the suggestion that immobility in mammalian-parasitic adults is an adaptation to parasitism.

Although limited recovery of AF18 upon photobleaching implies a totally rigid lipid surface layer, results from the diffusion of other types of probe, such as NBD-chol and NBD-PC imply that there are co-existent regions with rapid diffusion. These observations suggest that there may be differential horizontal partitioning of the lipid probes. From figure 5.13, it is clear that when temperature was varied in the dauer sheath *C. elegans*, the diffusion coefficients of AF18 and NBD-chol varied disproportionately. The critical point is that within the context of a homogeneous fluid membrane, the qualitative behaviour of all probes should be the same at the same temperature (Treistman et al, 1987). Percentage recovery appears to rise steadily for AF18 with temperature, whereas NBD-chol is able to maintain a fairly constant diffusible fraction. One pool of lipid appears to melt out with increasing temperature so as to maintain a constant state of diffusibility, and for another to decrease diffusibility. Differential mobility of fluorescent lipid probes usually indicates the co-existence of solid and fluid microdomains (Karnovsky et al, 1982; Dictus et al, 1984; Treistman et al, 1987). The concept of domains in the nematode epicuticle will be discussed in Chapter 8.

Proteins and Sugars

The immobile fraction of epicuticular lipid presents a paradox when one considers the dynamic functions of the cuticle which include the process of antigen-shedding. However, it would be relatively easy to envisage proteins diffusing rapidly in the fluid canals of the above domain model (see figure 7.2), with proteins being jettisoned from the surface. Alternatively, proteins may not be inserted in the lipid layer at all, but held only by loose ionic interactions.

The results from lateral diffusion of fluorescent protein-binding reagents indicated that a large proportion of the proteins associated with the adult surface are able to diffuse and that they are able to diffuse very rapidly. The exception to this again, is *Litomosoides carinii* with much less than half of its surface proteins free to diffuse. There were, however, some stages in the life-cycle of different species which exhibited immobility of labelled surface

proteins. These included infective-stage larvae of *T. canis*, infective muscle-stage larvae of *Trichinella spiralis*, and dauer larvae of *C. elegans* which were ensheathed. The dynamic nature of the surface of infective-stage larvae of *T. canis* is not disputed, since we already know that glycoproteins can be rapidly released from the surface (Maizels et al, 1984), and that adherent leucocytes can be jettisoned by the surface coat (Badley et al, 1987). However, from these results, it appears that the glycoprotein surface coat does not move in the plane of the epicuticle. Rather, glycoproteins are released vertically from the surface by some unknown mechanism.

The so-called 'accessory layer' of *T. spiralis* has been observed overlying the epicuticle by electron microscopy (Lee et al, 1986) , and it is thought that it may be an inert, protective covering related to passage through the pepsin-HCl environment of the stomach (Stewart et al, 1987). It seems likely that this is the layer which was labelled by fluorescent protein-binding reagents since these probes were found to be immobile by FRAP. Perhaps the accessory layer only has the appearance of a dynamic layer (e.g. in antigen-shedding), when the epicuticle exerts physical changes upon exposure to the mammalian tissue environment (see Chapter 6).

Dauer sheaths, like the sheaths of some infective larvae (e.g. *O. ostertagi*), are essentially inert and afford additional physical protection to the enclosed larva during exposure to the physical and chemical onslaughts of the free-living environment. It is perhaps not surprising then, that proteins were immobile in sheathed dauer larvae, and also in sheathed L3's of *O. ostertagi* (data not shown). An isolated piece of cuticle from *Ascaris suum* , which had been labelled on the surface with either FM or DTAF/borohydride, also exhibited immobility of the probes (data not shown), which was not surprising since the isolated cuticle would be expected to be inert.

It was notable that anti-surface polyclonal antibodies which were mobile on the surface of adult *C. elegans* were immobile on the dauer sheath. Presumably some of the antibodies would recognise the same epitopes on the 'true' epicuticle of the adult and on the inert epicuticle of the dauer sheath, reaffirming that the 'true' epicuticle is dynamic.

Labelling of surface sugars, which could be labelled by fluoresceinated lectins, or a fluorescent hydrazide in *T. canis*, was limited to only a few nematodes (see table 3.14). As expected, the fluorescent hydrazide (LYH) was immobile on the surface of *T. canis*,

confirming the hypothesis that the surface coat is immobile in the plane of the epicuticle in this parasite. FITC-Con A, -PNA and -WGA were immobile on the sheath of dauer larvae of *C. elegans* , but mobile on the adult surface, suggesting that the sheath is inert but that the adult surface is dynamic. Only sheathed microfilariae bound lectins, although it appears that the microfilariae of *B. pahangi* allowed the penetration of FITC-WGA into the sheath space, giving anomalous fast diffusion results. In contrast, FITC-WGA was confined to the outermost surface of the sheath of *L. carinii*, where this lectin was immobile.

Chapter Six

Modification of the biophysical properties of the third-stage epicuticle upon exposure to the environment of the mammalian host.

6.1. Introduction

A critical stage for both the physiological processes of the parasite and the defence mechanisms of the host, is the process of infection. Possession of a surface which is capable of responding rapidly to environmental shifts may be essential when a poikilotherm-infecting parasite is transmitted to a homeotherm, or when a free-living larva penetrates the skin of a mammal. Since any modifications which might be undergone by the parasite during the process of infection might provide targets for chemo- or immunotherapy, it is important that the basic biology and biochemistry of the transition from the pre- to the post-parasitic state should be understood.

In this series of experiments, larvae which enter the mammalian host by different routes and from different environments were examined. Firstly, those which are taken directly into the gastro-intestinal tract, e.g. *Ostertagia ostertagi* and *Trichinella spiralis*. Secondly, those that enter via an arthropod vector, e.g. L3's of *Acanthocheilonema viteae* from the argasid tick, and L3's of *Brugia pahangi* from the mosquito. Thirdly, those which have a free-living infective larva which actively penetrates tissue, e.g. *Nippostrongylus brasiliensis* and *Strongyloides ratti*. The surface biophysical properties of these larvae were examined in the so-called pre-parasitic environment, and then upon exposure to various different infection stimuli, including increased temperature to 37^{o}C and changes in pH and ionic composition.

Virtually nothing is known about the type of signalling, whether biochemical or neuroendocrine, which must occur in nematode parasites in the initial stages of infection. Therefore, it was thought that any surface biophysical marker of change found could be used to study the isolated effects of temperature, pH, ions and chemicals which alter biochemical signalling mechanisms on the transition from one developmental state to another.

In order to investigate the nature of any gross structural modification occurring in response to increased temperature, it was necessary to employ the biophysical technique of differential scanning calorimetry (D.S.C.). This technique used widely to detect overall structural changes in biological macromolecular structures (Sturtevant, 1987). Since infective muscle-stage larvae of *Trichinella spiralis* can be obtained in fairly large quantities, these parasites were used in studies of thermotropic transitions using D.S.C.

6.2. Results

6.2.1. Modification of the infective-stage epicuticle in *Trichinella spiralis*.

AF18 insertion

The so-called pre-infective larva, which has been extracted from muscle using pepsin and HCl, has no affinity for the fluorescent lipid probe AF18. However, after exposure to various different infection stimuli, the surface of the larva becomes lipophilic for AF18. The time taken for the insertion of AF18 in muscle-stage larvae of *T. spiralis* was measured in different incubation conditions, including the cationic detergent cetyltrimethyl-ammonium bromide (CTAB), trypsin, bile, RPMI 1640 and M199 nutrient media (table 6.1). Unlike the rapid response (< 10 min.) to infection stimuli, which will be described for many other nematode species, MSL of *T. spiralis* have a much longer response time (approx. 5h) in *in vitro* conditions. Table 6.1 shows that at room temperature, the nutrient media RPMI 1640 and M199 take approximately 5h to achieve surface changes in the nematode such that the AF18 probe will insert. This time is reduced to approximately 2h if trypsin or bile is included in the nutrient medium, and reduced even further to approx. 1h if trypsin and bile are both present. Note that trypsin and bile together do not promote AF18 insertion if nutrient medium is absent. Treatment of the worms with 0.25% CTAB detergent causes an almost immediate change in behavioural characteristics from coiling/uncoiling movement to sinusoidal movement, and AF18 insertion occurs within 30 min. This might suggest that stripping of material from the surface, or alteration of its properties, by CTAB causes an artificial stimulation of developmental processes related to infection.

The results shown in figure 6.1 indicate that maximal insertion of AF18 could be achieved in RPMI at 37^{o}C. Bile and trypsin were not included in the medium in these experiments, so that only the effects of medium and temperature would be taken into consideration. Nutrient medium alone, or temperature alone were not absolute requirements for surface change in this case, since distilled water at 37^{o}C or RPMI at 18^{o}C promoted significant levels of AF18 insertion.

Table 6.1 The effect of CTAB, bile & trypsin on the time taken for the insertion of AF18 in *Trichinella spiralis* MSL, at room temperature.

Treatment	Time taken in hours	Arbitrary Fluorescence units
0.25% CTAB in PBS	0.5*	36.76 ± 2.70 (7)
0.12% CTAB in PBS	1.0	35.63 ± 1.95 (7)
M199 + bile + trypsin	1.0	25.28 ± 1.45 (4)
RPMI + bile + trypsin	1.5	19.04 ± 3.10 (7)
M199 + trypsin	2.0	19.22 ± 0.51 (5)
M199 + bile	2.0	17.56 ± 0.39 (5)
RPMI + bile	2.0	22.99±2.37 (7)
RPMI + trypsin	2.5	22.06±2.49 (7)
RPMI alone	5.0	14.86±2.80 (10)
M199 alone	5.5	15.77±1.40 (5)

* Treatment with 0.25% CTAB resulted in killing of the worms within 4 hours. AF18 insertion was checked after killing, and was found to be internal (mean trypan blue quenching = 34.8% for a sample of 8 dead worms).

Bile was at a concentration of 5% w/v, and trypsin was at concentration of 0.25% w/v. Bile concentrations above 5% were found to be damaging to the worms so were not used. Before labelling with AF18, bile was removed by at least 5 consecutive centrifugations and washes in the appropriate medium.

The number of worms in each sample is indicated in brackets.

Figure 6.1 The effect of temperature and medium composition on AF18 insertion in MSL of *Trichinella spiralis*.

Infective muscle-stage larvae (MSL) of *T. spiralis* were removed from infected muscle by pepsin-HCl digestion, and incubated in different environmental conditions for a period of 5 hours (see table 6.1). Following incubation, the larvae were examined for their ability to allow surface-insertion of the lipid probe AF18. Insertion of this lipid probe was determined quantitatively (in relative fluorescence units with the background subtracted) using fluorescence quantitation.

The following incubation conditions were used:

4 DW, 18 DW & 37 DW: Distilled water at 4°C, 18°C and 37°C.

4 R, 18 R & 37 R: RPMI 1640 mammalian cell culture medium at 4°C, 18°C and 37°C.

37 PBS: Phosphate-buffered saline (pH 7.4) at 37°C.

Statistical Analysis

4 DW v 4 R - $P < 0.001$
37 DW v 37 R - $P < 0.001$
37 DW v 37 PBS - $P < 0.005$

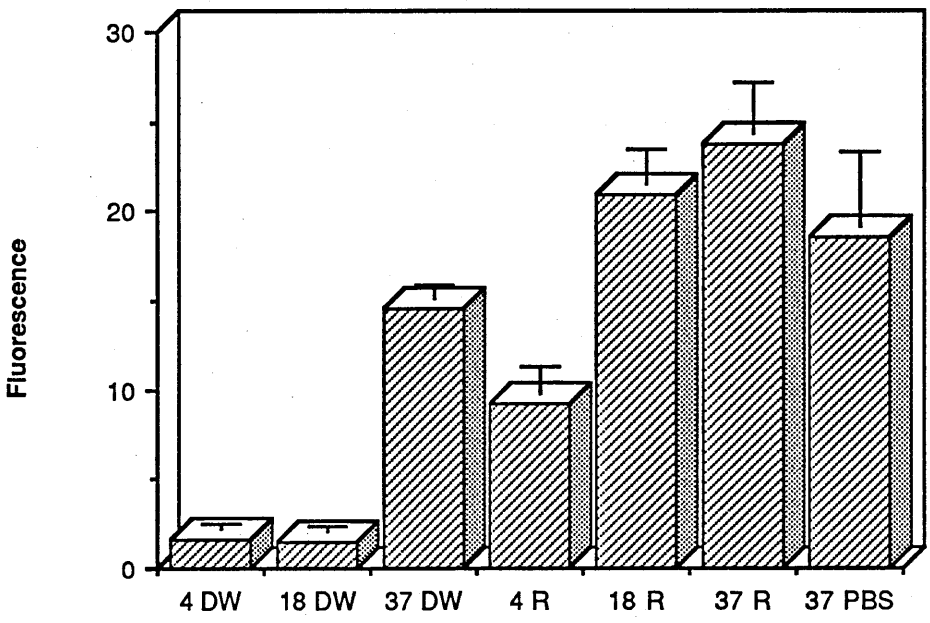

Fluorescence
30
20
10
0
4 DW
18 DW
37 DW
4 R
18 R
37 R
37 PBS

Figure 6.2 Differential scanning calorimetry of infective muscle-stage larvae (MSL), and adults of *Trichinella spiralis*.

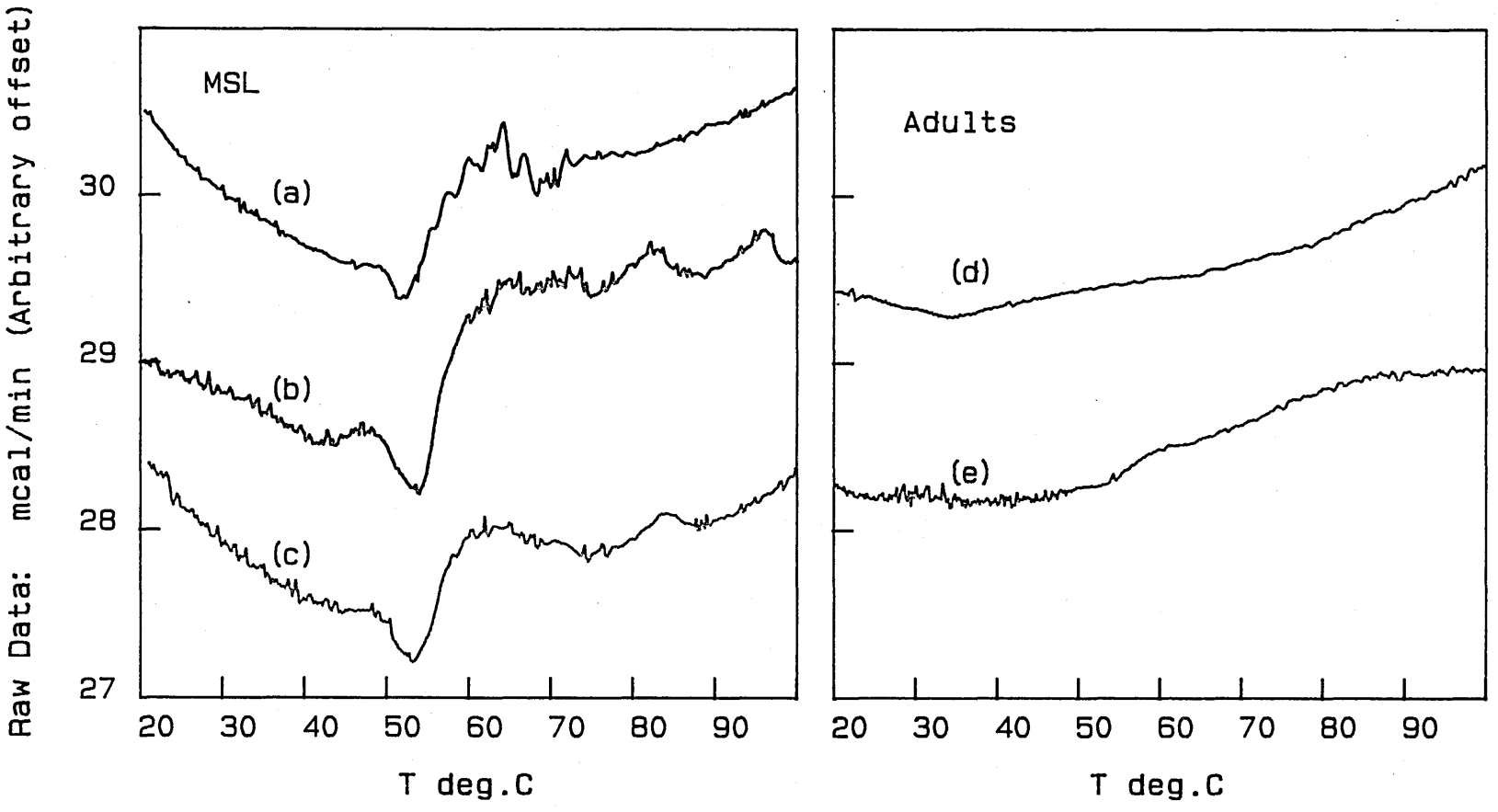
(a) MSL (~800 worms) in PBS;

(b) MSL (~800 worms) in distilled water;

(c) MSL (~800 worms) in RPMI 1640

(d) Adults (~50 worms) in RPMI (experiment 1)

(e) Adults (~50 worms) in RPMI (experiment 2).


Raw Data: mcal/min (Arbitrary offset)
MSL
Adults
(a)
(b)
(c)
(d)
(e)
30
29
28
27
20
30
40
50
60
70
80
90
T deg.C
T deg.C

D.S.C.

Differential scanning calorimetry (D.S.C.) showed that there was a very reproducible thermotropic transition at around 52°C in MSL (figure 6.2 a, b and c) which was not to be found in 12 day old adult worms under identical conditions (figure 6.2 d and e). Since adults are assumed to possess essentially the same types of cell membrane in the hypodermis and body as in MSL, it was thought that the transition could be attributed to a specialized structure related to infection, which was not present in the adult, i.e. the accessory layer of MSL.

6.2.2. Modification of the infective-stage epicuticle in exsheathed larvae of *Ostertagia ostertagi*.

In L3's of *O. ostertagi* it appears that artificial removal of the sheath with sodium hypochlorite had been sufficient to cause changes of the worm surface to allow AF18 insertion. However, in view of the fact that the small number of worms that had exsheathed naturally in PBS were non-lipophilic for AF18, it would appear likely that the hypochlorite had damaged or altered the epicuticle structure and had not stimulated biophysical changes to occur. In addition, hypochlorite altered the surface within 15 minutes, whereas spontaneous exsheathment in RPMI with CO_2 took approx. 2h to allow AF18 insertion.

6.2.3. Modification of the infective-stage epicuticle in *Acanthocheilonema viteae*.

When the vector-stage L3 of *A. viteae* is transferred from insect culture medium (Grace's) at 27°C to mammalian cell culture medium (RPMI 1640) at 37°C, its surface begins to show affinity for AF18 within 10 minutes (figure 6.4). This effect appears to be due to synergism between increased temperature and exposure to the components of the new medium, since temperature elevation to 37°C or medium change alone have only slight effects on lipophilicity. It would also appear that lipophilicity is not confined to AF18 in post-infective larvae, but includes serum lipids (figure 6.5). Hyperlipidaemic sera from the definitive host, *Meriones spp.* included in the medium and then washed out thoroughly before labelling with AF18, caused a dramatic reduction in the amount of fluorescent lipid probe which could be inserted. The inference here being that if host lipid is present in

Figure 6.3 The effect of temperature and medium on AF18 insertion in exsheathed infective larvae of *Ostertagia ostertagi*.

Infective-stage larvae of *O. ostertagi* were obtained from bovine faecal cultures. The epicuticlular surface of these L3's could only be examined after exsheathment by natural or artificial methods. AF18 insertion in the L3 epicuticle was measured by fluorescence quantitation (in relative fluorescence units with the background subtracted).

The following exsheathment and incubation conditions were used:

18 PBS: Natural exsheathment after incubation for 1h in PBS at 18°C.

18 $HClO_3^-$: Artificial exsheathment in 12% sodium hypochlorite (Milton's fluid) in PBS (pH 7.4) for no longer than 15 minutes. Exsheathment rate = 72%. Insertion of AF18 was measured after incubation for 1h in PBS at 18°C.

37 RPMI: Spontaneous exsheathment in RPMI 1640 + excess gaseous CO_2 after 2h incubation at 37°C. Insertion of AF18 was measured after incubation for 1h in RPMI at 37°C.

Incubation of L3's in PBS at 37°C, or in RPMI(with or without CO_2) at 18°C did not result in exsheathment, therefore, AF18 insertion in the L3 epicuticle under these conditions could not be assessed.

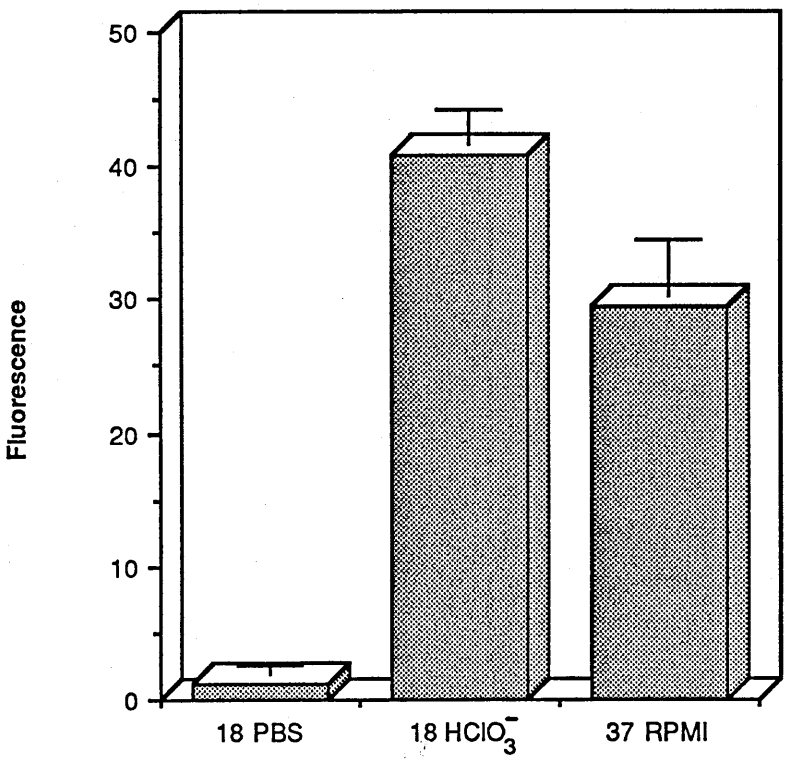

Fluorescence
50
40
30
20
10
0
18 PBS
18 $HClO_3^-$
37 RPMI

Figure 6.4 The effect of temperature and medium composition on insertion of AF18 in infective-stage larvae of *Acanthocheilonema viteae*.

A. viteae L3's were recovered directly from the argasid tick and exposed to a variety of different environmental conditions, including those which mimic the mammalian host environment. AF18 insertion was measured by fluorescence quantitation (relative fluorescence units with the background subtracted).

The following incubation conditions were used:

4 Gr & 27 Gr: Grace's insect medium for 1h at 4°C and 27°C.

4 R & 27 R: RPMI 1640 mammalian cell culture medium for 1h at 4°C and 27°C.

37 Gr(3) & 37 Gr(6): Grace's insect medium at 37°C for 3h and 6h.

37 R: RPMI 1640 mammalian cell culture medium for 10 minutes at 37°C.

Statistical Analysis:

4 G v 4 R - $P < 0.0001$
27 R v 37 G - $P < 0.0001$

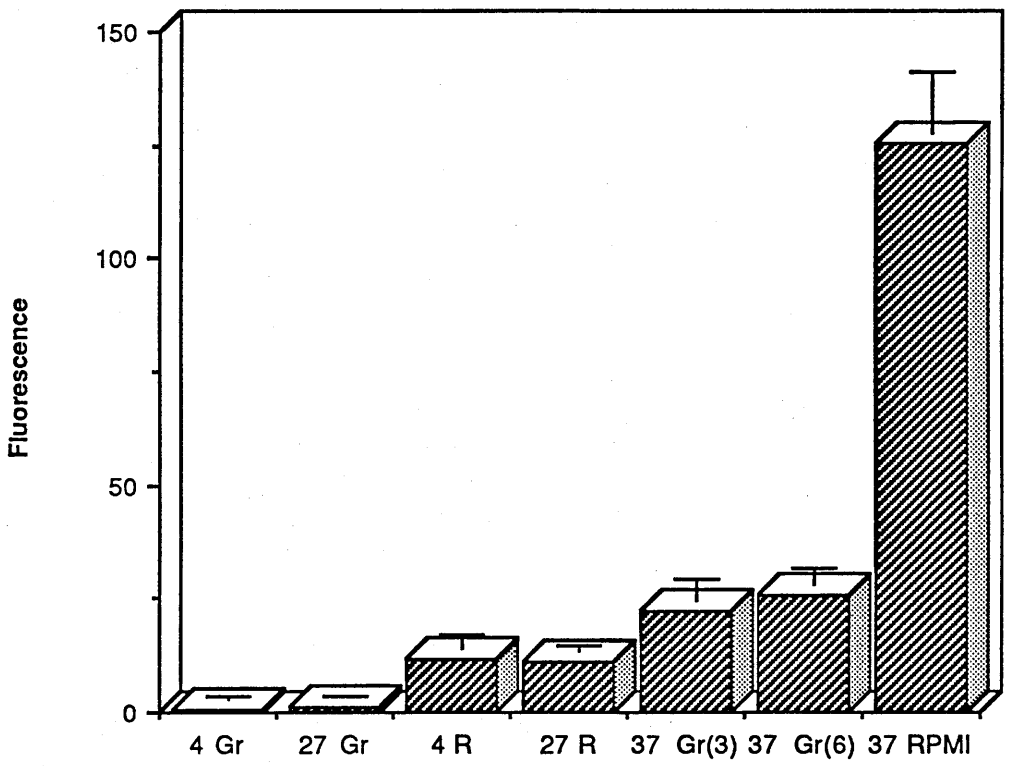

150
100
50
0
Fluorescence
4 Gr
27 Gr
4 R
27 R
37 Gr(3)
37 Gr(6)
37 RPMI

Figure 6.5 Influence of serum on AF18 insertion in infective larvae of *Acanthocheilonema viteae* .

A. viteae L3's were recovered from the argasid tick. These infective L3's were exposed to various different environmental conditions, in the presence or absence of 30% (v/v) serum. Serum was removed by sequential washing and centrifugation with an appropriate medium before labelling with the fluorescent lipid. AF18 insertion was measured by fluorescence quantitation (in relative fluorescence units with the background subtracted).

The following sera were used:

FCS - Foetal calf serum (heat-inactivated).

JSA - jird serum from *Meriones unguiculatus.*
JSB - jird serum from *Meriones libycus.*
JSC - jird serum from *Meriones crasrus.*

The following incubation conditions were used:

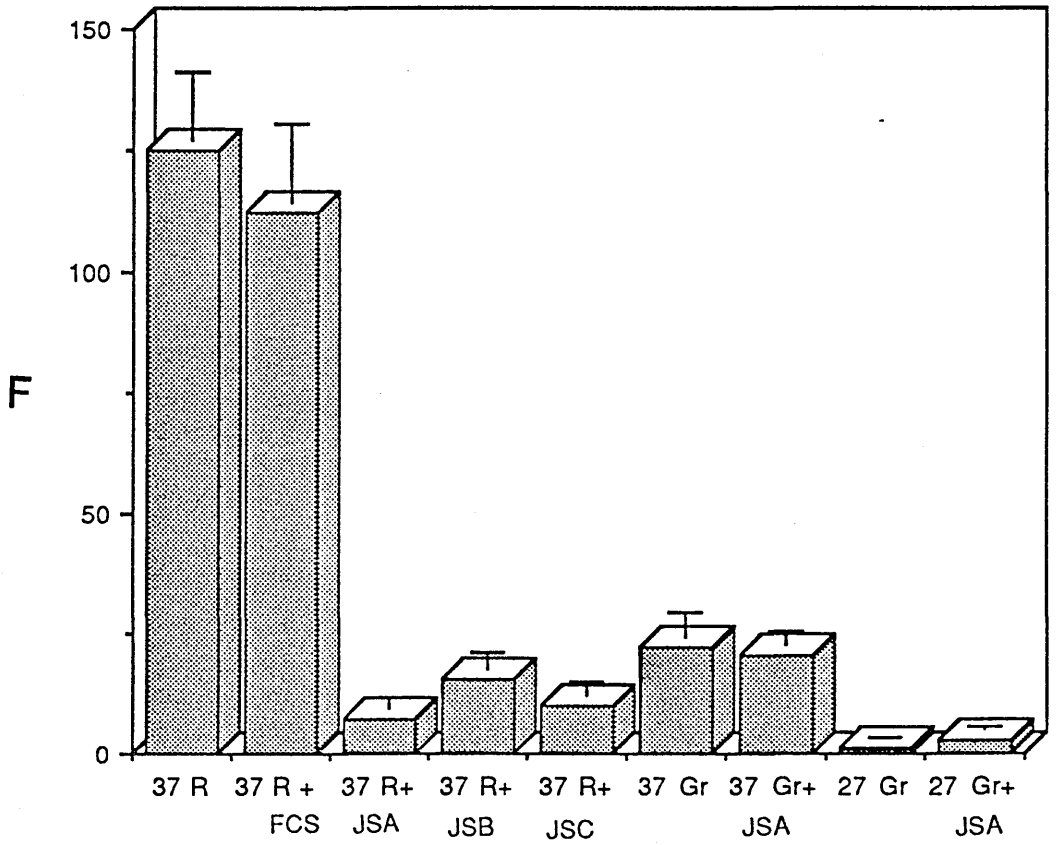
27 G & 27 G + JSA: Grace's insect medium for 1h at 27°C, alone, or with jird serum A.

37 G & 37 G + JSA: Grace's insect medium for 1h at 37°C, alone, or with jird serum A.

37 R & 37 R + JSA, JSB, or JSC: RPMI 1640 for 10 minutes at 37°C, alone, or with jird serum A, B or C.

Statistical Analysis

37 R v 37 R + FCS - $P < 0.1$
37 R v 37 R + JSA - $P < 0.0001$


150
100
50
0
F
37 R
37 R + FCS
37 R+ JSA
37 R+ JSB
37 R+ JSC
37 Gr
37 Gr+ JSA
27 Gr
27 Gr+ JSA

significant quantities, it will saturate the surface to the exclusion of the fluorescent lipid probe. Foetal calf serum does not produce such effects, which may be related to the relatively low lipid levels in this serum (se table 6.2).

6.2.4. Modification of the infective-stage epicuticle in *Brugia pahangi*.

Transfer of mosquito-derived L3's of *B. pahangi* from insect culture medium (Grace's) at 27°C to mammalian cell culture medium (RPMI 1640) at 37°C causes the surface of the larva to change its affinity for AF18 within 10 minutes. In this case, RPMI alone did not have significantly different effects from the controls in Grace's, however elevation of the temperature to 37°C in Grace's did cause significant uptake of the AF18 probe which was maximal at 3h incubation. Pre-incubation in serum from the definitive host *Meriones unguiculatus*(which was not hyperlipidaemic) did not cause any significant reduction in AF18 insertion from the levels in the control (figure 6.7).

6.2.5. Modification of the infective-stage epicuticle in *Nippostrongylus brasiliensis*.

L3's of *N. brasiliensis* , which were recovered from rat faecal cultures, showed no affinity for AF18 at room temperature in distilled water (figure 6.8). However, after exposure to RPMI at room temperature at 37°C for 1h (maximal fluorescence), the surface of the worms became lipophilic for AF18. Interestingly, elevated temperature to 37°C alone (i.e. in distilled water) over 24h gave a gradual increase in lipophilicity.

In experiments looking at the effects of different sera and lipids on AF18 insertion in *N. brasiliensis* (figure 6.9), it was again found that only the hyperlipidaemic serum caused a reduction in AF18 insertion. Also, the fact that de-lipidated human serum and an artificial mixture containing high levels of lipid (Intralipid) did not have any effect on AF18 insertion, suggests that there is a very specific type of interaction, perhaps with serum lipoproteins.

6.2.6. Modification of the infective-stage epicuticle in *Strongyloides ratti*.

L3's of *S. ratti* which had been recovered from rat faecal cultures showed very little affinity for AF18 in distilled water at 18°C (figure 6.10). However, after exposure to RPMI at 18°C or 37°C, the surface of the worms became lipophilic for AF18. Elevation

Figure 6.6 The effect of temperature and medium composition on AF18 insertion in infective larvae of *Brugia pahangi*.

B. pahangi L3's were recovered from mosquitoes and were exposed to various different environmental conditions, including those which mimic the mammalian host environment. AF18 insertion was measured using fluorescence quantitation (in relative fluorescence units with the background subtracted).

The following incubation conditions were used:

4 Gr & 27 Gr: Grace's insect medium for 1h at 4°C and 27°C.

4 R & 27 R: RPMI 1640 mammalian cell culture medium for 1h at 4°C and 27°C.

37 Gr(3) & 37 Gr(6): Grace's insect medium at 37°C for 3h and 6h.

37 RPMI: RPMI 1640 mammalian cell culture medium at 37°C for 10 minutes.

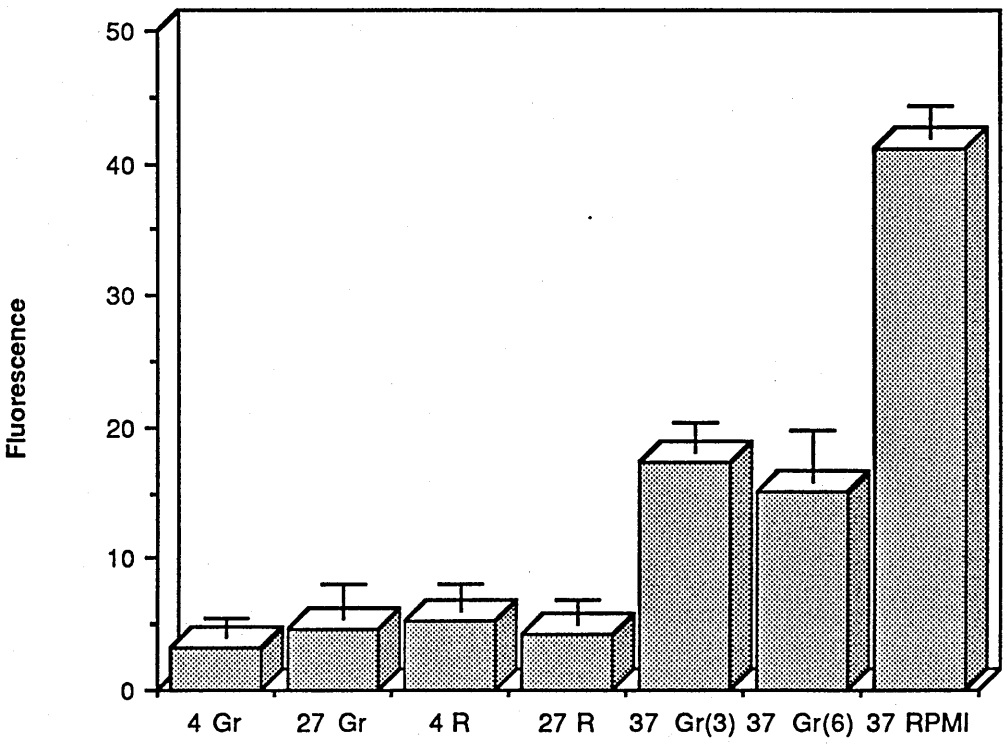

Fluorescence
50
40
30
20
10
0
4 Gr
27 Gr
4 R
27 R
37 Gr(3)
37 Gr(6)
37 RPMI

Figure 6.7 Influence of serum on AF18 insertion in infective larvae of *Brugia pahangi*.

B. pahangi L3's were recovered from mosquitoes and exposed to various different environmental conditions in the presence or absence of 30% (v/v) serum. Exstensive washing was required to remove the serum before labelling with the fluorescent lipid. AF18 insertion was measured by fluorescence quantitation (in relative fluorescence units). Jird serum was from one species only, *Meriones unguiculatus*, and was not considered to be hyperlipidaemic.

The following incubation conditions were used with or without serum:

27 G & 27 R: Grace's insect medium or RPMI 1640 mammalian cell culture medium at 27°C for 30 minutes.

37 G & 37 G + JS: Grace's insect medium for 30 minutes at 37°C, alone, or with jird serum.

37 R & 37 R + JS: RPMI 1640 mammalian cell culture medium for 10 minutes at 37°C, alone, or with jird serum.

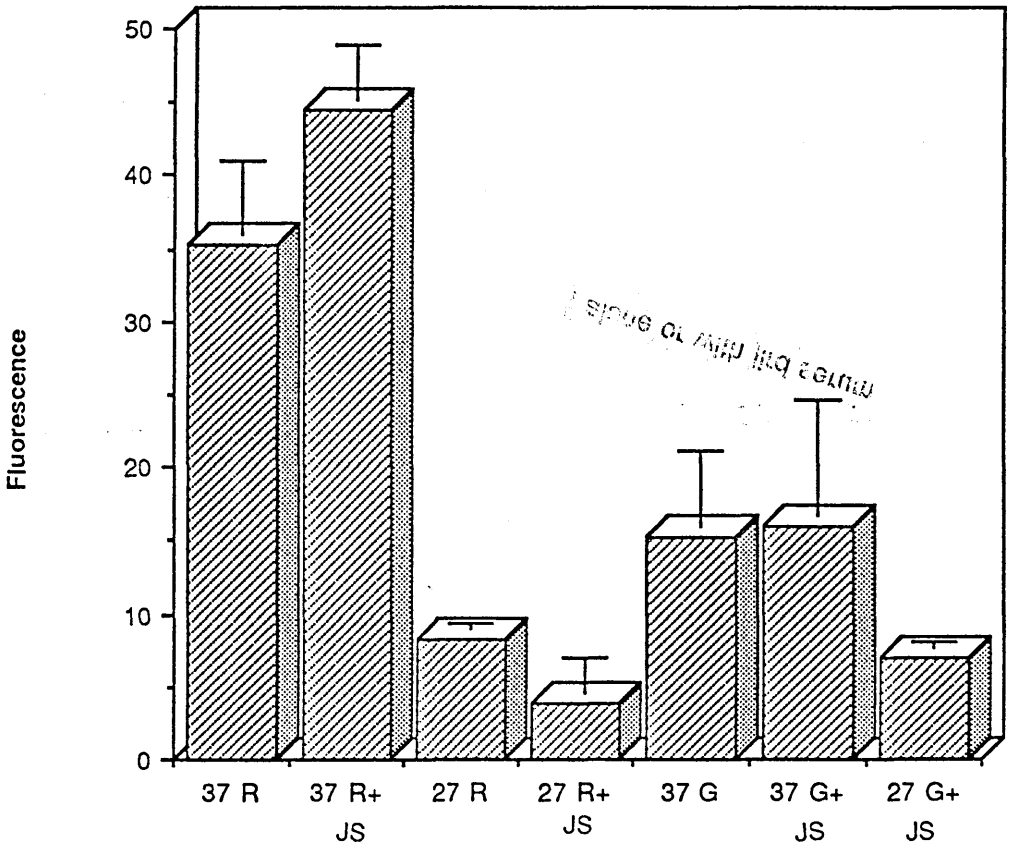

Fluorescence
50
40
30
20
10
0
37 R
37 R+ JS
27 R
27 R+ JS
37 G
37 G+ JS
27 G+ JS

Figure 6.8 The effect of temperature and medium composition on AF18 insertion in infective larvae of *Nippostrongylus brasiliensis* .

N. brasiliensis L3's were recovered from rat faecal cultures on filter paper, and exposed to various different environmental conditions, including those which mimic the mammalian host environment. AF18 insertion was measured by fluorescence quantitation (in relative fluorescence units with the background subtracted).

The following incubation conditions were used:

18 DW & 18 R: Distilled water and RPMI 1640 for 1h at 18^{o}C .

37 DW(3), 37 DW(6) & 37 DW(24): Distilled water for 3h, 6h and 24h at 37^{o}C.

37 R: RPMI 1640 for 1h at 37^{o}C.

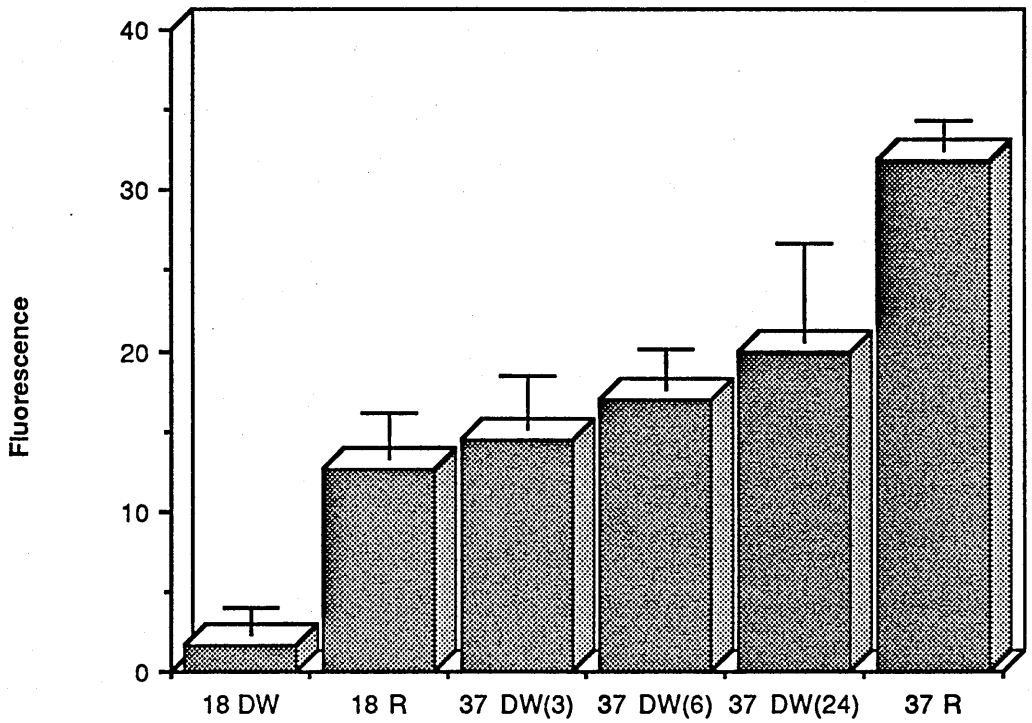

Fluorescence
40
30
20
10
0
18 DW
18 R
37 DW(3)
37 DW(6)
37 DW(24)
37 R

Table 6.2 Cholesterol and TG levels for normal, hyperlipidaemic, and artificial sera used in AF18 labelling experiments.

Serum	Cholesterol (mmol/l)	Triglyceride (mmol/l)
Pooled normal human serum (NHS)	4.5	3.9
Pooled hyperlipidaemic human sera (HHS)	9.5	5.3
Foetal calf serum (FCS)	1.1	1.3
De-lipidated human serum from Sigma, (De-lipHS)	0.0	0.1
Intralipid, artificial mixture of serum lipids (IL)	0.0	47.4
Normal rat serum (NRS)	2.2	3.7
Normal jird serum (NJS)	0.7	1.2
Hyperlipidaemic jird serum A (*M. unguiculatus*)	8.1	3.7
B (*M. libycus*)	5.5	2.7
C (*M. crasrus*)	7.9	4.0

All cholesterol and triglyceride levels were determined using enzymatic/colourimetric kits from Boehringer-Mannheim, on the Roche Cobas-bio centrifugal analyser, courtesy of the Cardiovascular Research Unit, Edinburgh.

Figure 6.9 Influence of serum on AF18 insertion in infective larvae of *Nippostrongylus brasiliensis.*

N. brasiliensis L3's were recovered from rat faecal cultures on filter paper and exposed to various different environmental conditions, in the presence or absence of different types of serum (30%). Extensive washing was required to remove serum before labelling with the fluorescent lipid. AF18 insertion was measured by fluorescence quantitation (in relative fluorescence units).

The following incubation conditions were used:

18 DW & 18 DW + HHS: Distilled water for 1h at 18°C, alone, or in the presence of hyperlipidaemic human serum.

37 R; 37 R + NHS; +HHS; + FCS; +De-lipHS; + IL; + NRS : RPMI 1640 medium for 30 minutes at 37°C, alone, or in the presence of one of the following:

(1) **NHS** - Pooled normal human serum.
(2) **HHS** - Pooled hyperlipidaemic (FFA, TG and chol levels elevated) human sera.
(3) **FCS** - Foetal calf serum.
(4) **De-lipHS** - De-lipidated human serum from Sigma.
(5) **IL** - Intralipid (artificial mixture of serum lipids).
(6) **NRS** - Normal rat serum.

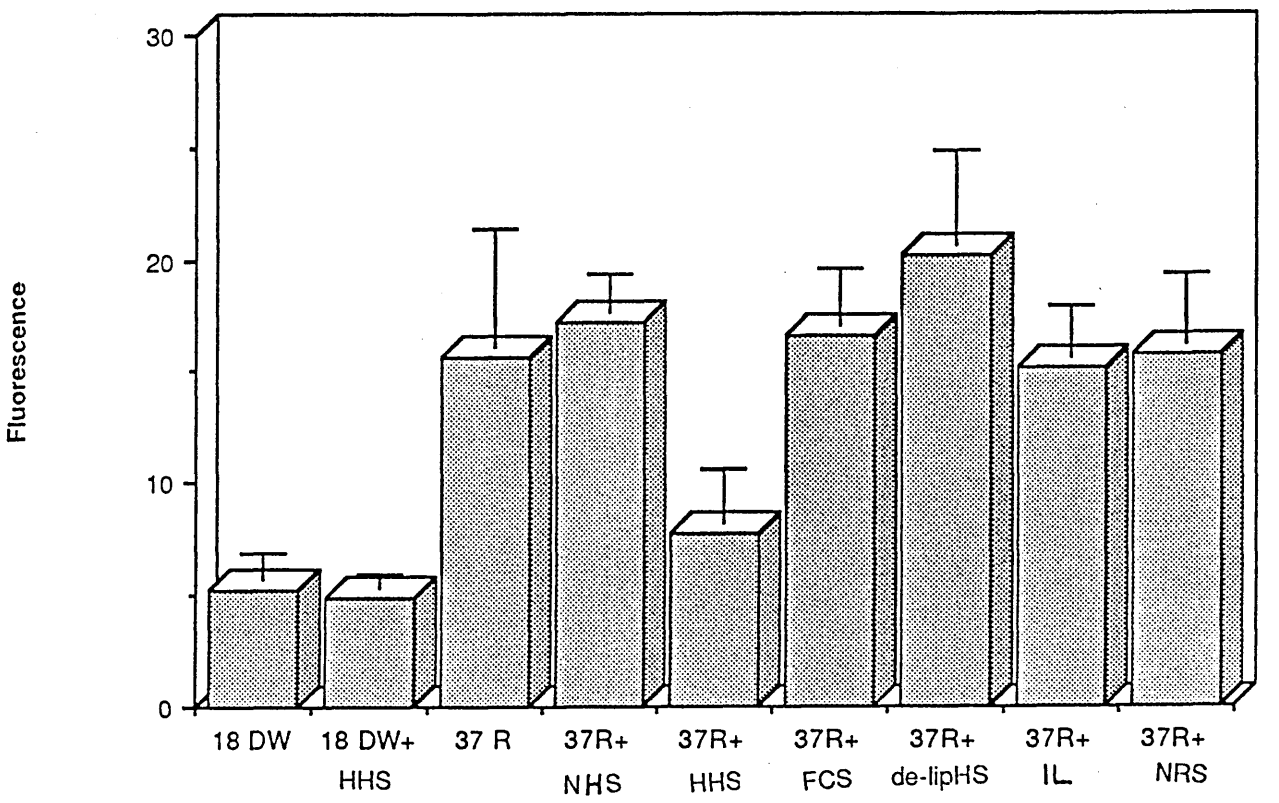
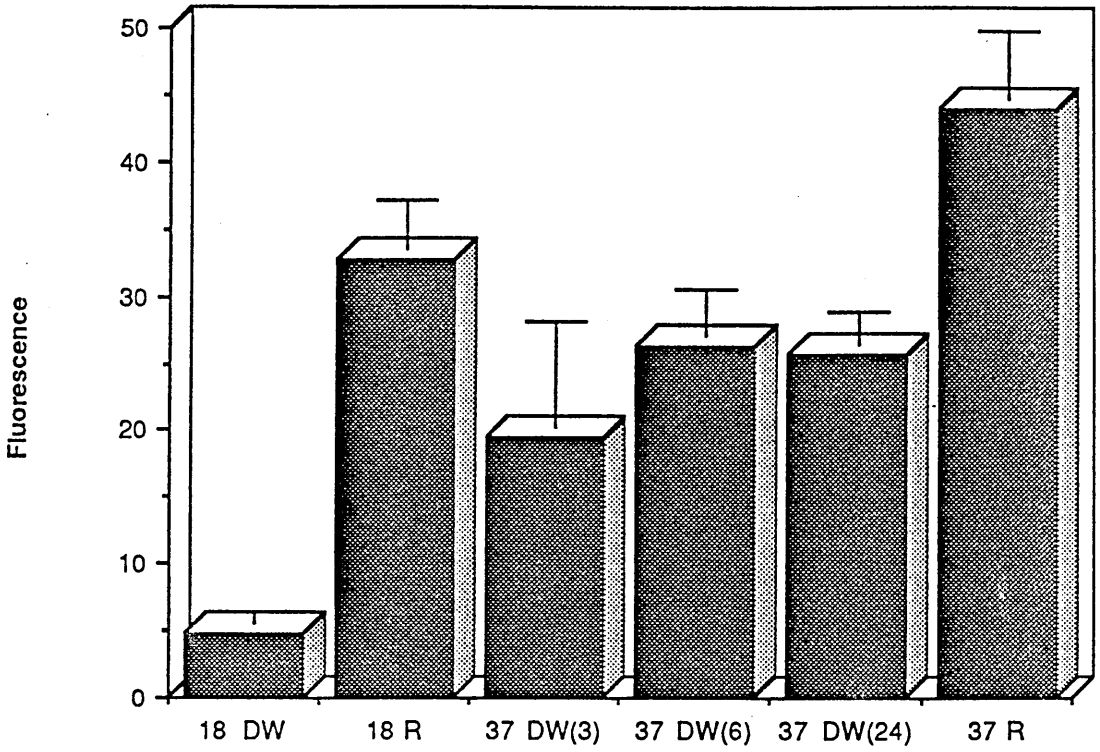

Fluorescence
30
20
10
0
18 DW
18 DW+ HHS
37 R
37R+ NHS
37R+ HHS
37R+ FCS
37R+ de-lipHS
37R+ IL
37R+ NRS

Figure 6.10 **The effect of temperature and medium composition on AF18 insertion in heterogonic infective larvae of *Strongyloides ratti*.**



S. ratti L3's were recovered from rat faecal cultures on filter paper, and exposed to various different environmental conditions, including those which mimic the mammalian host environment. Surface-insertion of AF18 was measured by fluorescence quantitation (in relative fluorescence units with the background subtracted).

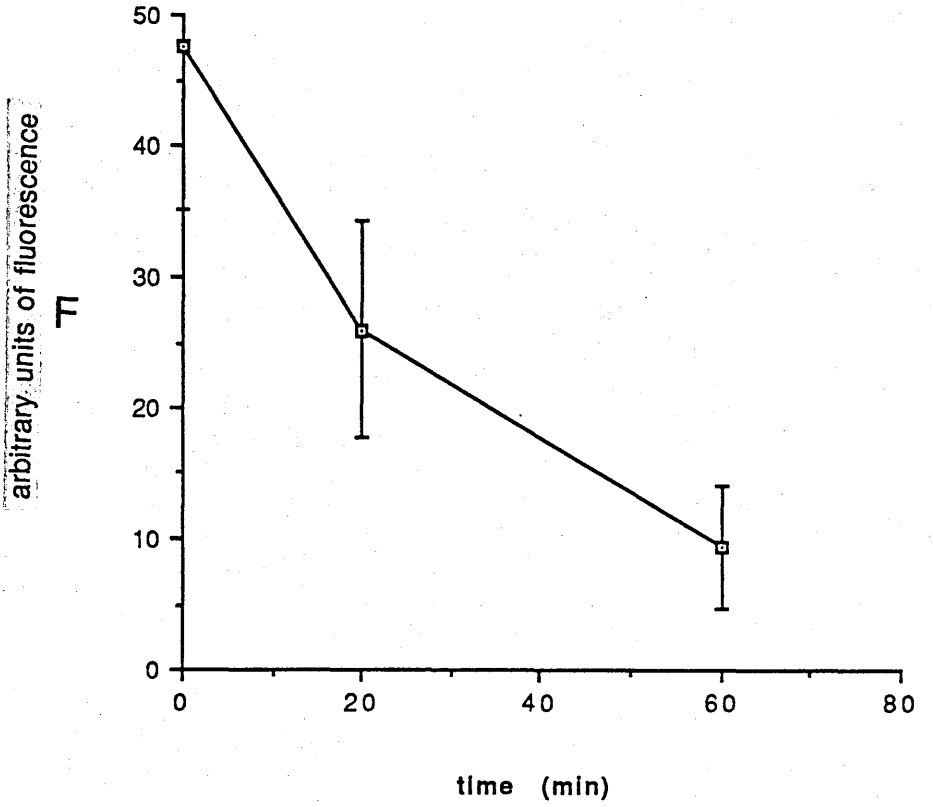
The following incubation conditions were used:

18 DW & 18 R: Distilled water and RPMI 1640 medium for 30 minutes at 18°C.
37 DW(3), 37 DW(6) & 37 DW(24): Distilled water at 37°C for 3h, 6h and 24h.
37 R: RPMI 1640 mammalian cell culture medium for 30 minutes at 37°C.

Statistical Analysis

37 DW(3) v 37 DW(6)	- $P < 0.001$
37 DW(3) v 37 DW(24)	- $P < 0.0001$

Figure 6.11 **Time course of FITC-cationised ferritin release from the surface of *Strongyloides ratti* infective larvae.**



Nematodes were labelled with FITC-cationised ferritin (2mg/ml) in distilled water at room temperature (~18°C) and then transferred to RPMI 1640 at 37°C at t=0.

Figure 6.12 **Fluorescence photomicrographs of the loss of FITC-cationised ferritin from the surface of infective-larvae of *Strongyloides ratti*.**

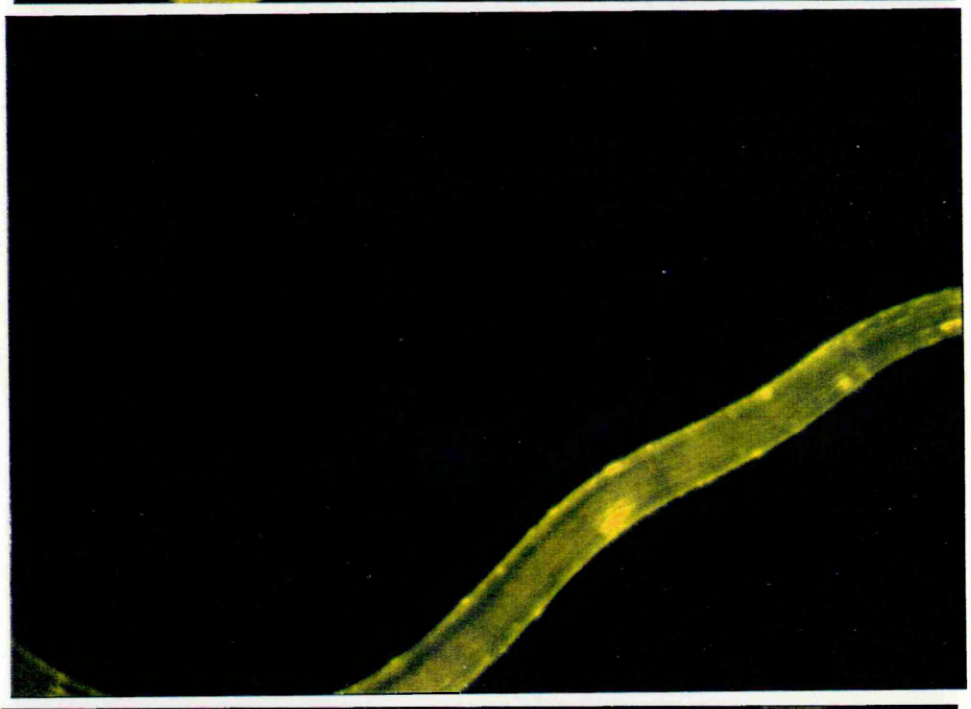
(a) t = 0 (i.e. before exposure to RPMI at 37^{o}C);

(b) t = 20 minutes after exposure to RPMI at 37^{o}C;

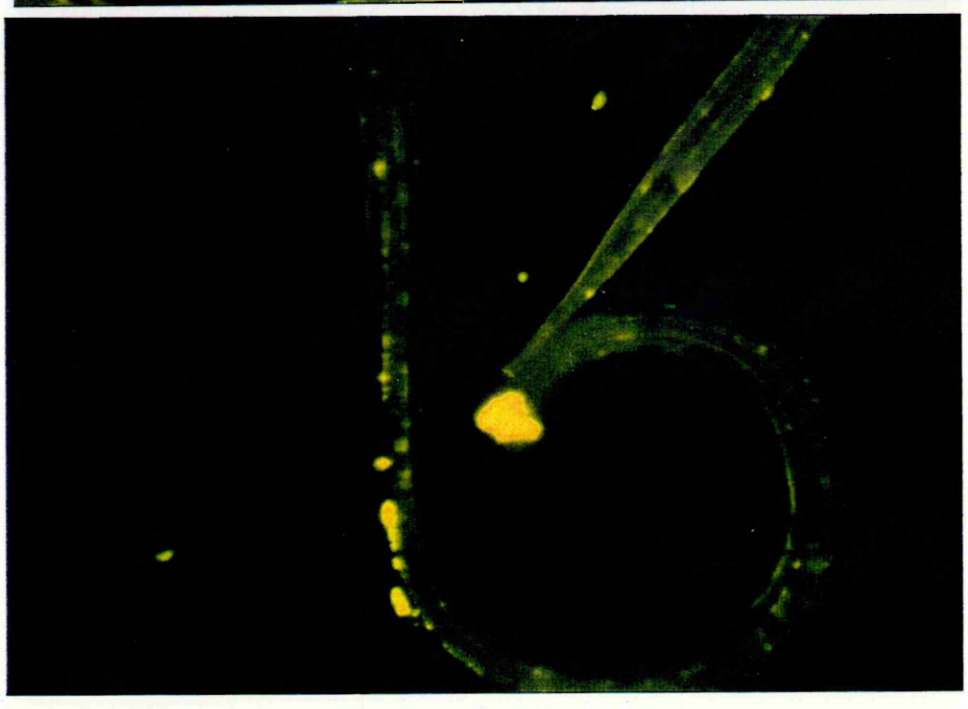
(c) t = 60 minutes after exposure to RPMI at 37^{o}C.

a

b



c



of the temperature to 37°C alone (i.e. in distilled water) caused maximal insertion of AF18 at around 6h and did not increase any further over a 24h period.

The results in figure 6.11 and the photomicrographs in figure 6.12 show that anionic material on the surface of *S. ratti* is rapidly released after exposure to RPMI at 37°C. This indicates that this release may contribute to the increase in lipophilicity which occurs in these parasites under the same conditions. Experiments of this kind (labelling of the surface with FITC-cationized ferritin) were not possible with other parasitic nematodes since labelling in those cases was too faint or did not occur at all.

6.2.7. Role of increasing Na^+ ion concentration in signalling the modification of the infective-stage epicuticle in *Acanthocheilonema viteae*.

Pre-parasitic infective larvae of *A. viteae* do not allow the insertion of the fluorescent lipid probe AF18, whereas larvae which have been exposed to chemical infection stimuli in RPMI 1640 cell culture medium do (see figure 6.4). The concentration of Na^+ ions increases greatly going from Grace's insect medium to RPMI 1640 (see table 2.1), therefore the isolated effects of Na^+ ions or chemicals which influence Na^+ ions on AF18 insertion was studied. All experiments were carried out at 27°C to eliminate the factor of increased temperature which would, cause insertion of the probe by itself.

Phosphate-buffered saline (300mOsm) had a significant effect ($P < 0.0001$) on AF18 insertion above the control. The signal for modification of the epicuticle appeared to be increasing salinity rather than increasing osmolarity because nematodes did not change their surface significantly in 300mOsm mannitol. Monensin, which is a sodium ionophore, had no effect on surface properties suggesting that Na^+ channels were inaccessible to, or unaffected by this drug. The Na^+ effect could not be replaced by K^+ or choline$^+$, the inference being that the effect of Na^+ ions is specific, and not due to non-specific interactions with positively-charged ions.

The results in table 6.3 indicate that the potent inhibitor of Na^+/H^+ antiport, amiloride, was able to inhibit AF18 insertion partially at 1mM. It is unlikely that a Na^+/K^+-ATPase is involved in transformation, however, since ouabain, which is a potent inhibitor of this process, had no effect.

Figure 6.13 Effect of sodium ions, or chemicals which influence or mimic sodium ions, on AF18 insertion in *Acanthocheilonema viteae* infective larvae.

Sodium ions were used to try to influence AF18 insertion in conditions which do not normally result in insertion of the lipid probe, i.e. Grace's insect medium at 27°C. To check the effects of increasing salinity alone, and not increasing osmolarity, a solution of mannitol was included as a control. Choline, and potassium ions were used as controls for the effects of positively-charged molecules in place of sodium ions. Monensin is a sodium ionophore.

The following incubation conditions were used:

Controls 27 G & 27 R: Grace's insect medium and RPMI 1640 mammalian cell culture medium for 1h.

Na^+: 300mOsm (140mM sodium chloride and 10mM sodium phosphate), the mammalian osmolarity (see Samuelson and Stein, 1989) for 1h.

Mannitol: 300mOsm (280mM mannitol and 10mM sodium phosphate), the mammalian osmolarity, for 1h.

K^+: 150mM potassium chloride, for 1h.

Choline$^+$: 150mM choline chloride, for 1h.

Monensin: 10μM monensin, for 1h.

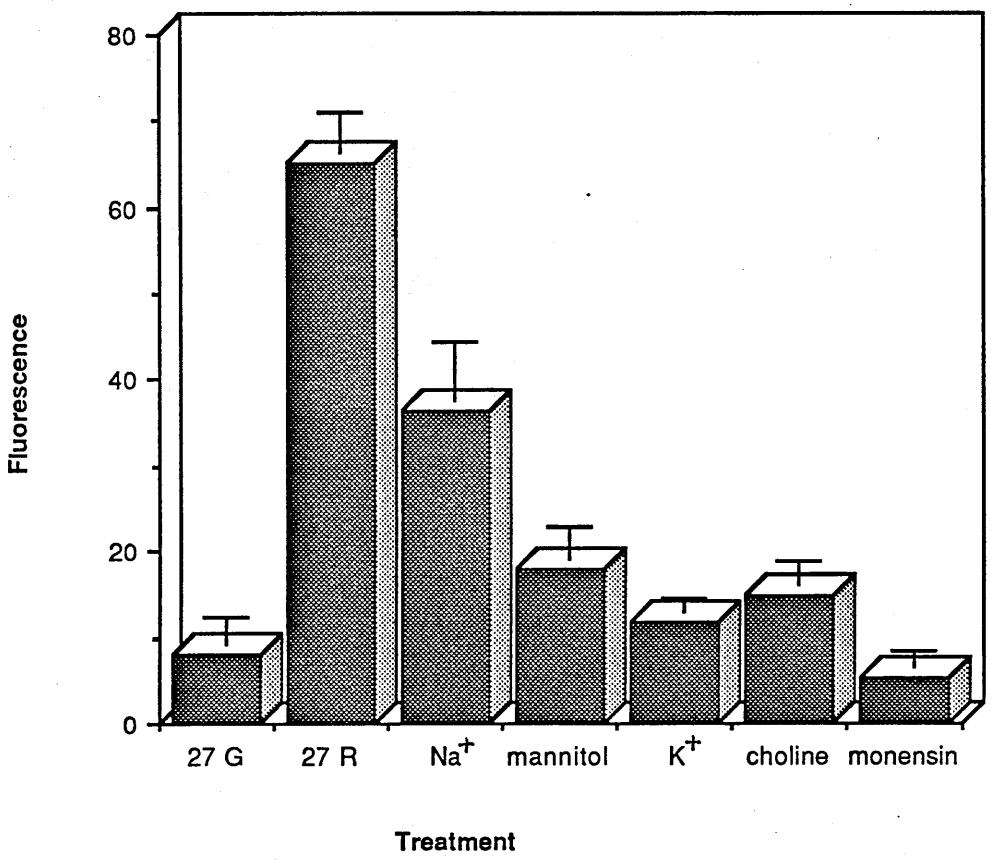

80
60
40
20
0
Fluorescence
27 G
27 R
Na+
mannitol
K+
choline
monensin
Treatment

Figure 6.14 The effect of chemicals which influence pH on the insertion of AF18 in *Acanthocheilonema viteae* infective larvae.

Adjustment of the environmental pH with acidic or basic compounds was employed to assess the effects of pH on insertion of the fluorescent lipid probe AF18 in the surface of *A. viteae* L3's. Amiloride, which is a potent blocker of Na^+/H^+ antiport, was used in conjunction with alkaline environmental conditions to assess the ability of this drug to enhance, or decrease the normal effect which would occur in its absence. The calcium ionophore A23187 was used in conjunction with bicarbonate to assess its effects. AF18 insertion was measured by fluorescence quantitation (in relative fluorescence units with the background subtracted).

The following incubation conditions were used:

Controls:

27 Gr & 27 R : Grace's insect medium and RPMI 1640 mammalian cell culture medium at 27°C.

Gr 7.4 : Grace's insect medium adjusted from pH 6.0 to pH 7.4 by adding 0.05M Tris-HCl dropwise.

Gr 7.4 + amiloride: Grace's insect medium adjusted from pH 6.0 to pH 7.4 by adding 0.05M Tris-HCl dropwise, in the presence of 200µM amiloride.

R 6.0 : RPMI 1640 mammalian cell culture medium adjusted from pH 7.4 to pH 6.0 by adding 1M HCl dropwise.

Gr HCO_3^-: Grace's insect medium and 20mM $NaHCO_3$.

Gr HCO_3^- + A23187: Grace's insect medium and 20mM $NaHCO_3$ in the presence of 50µM A23187.

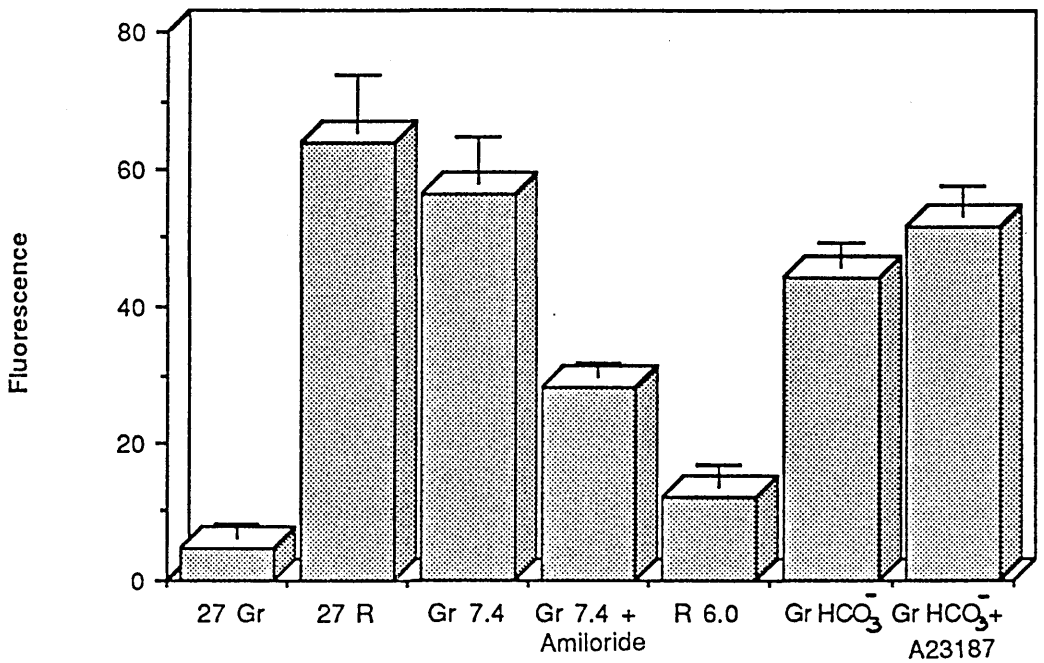

Fluorescence
80
60
40
20
0
27 Gr
27 R
Gr 7.4
Gr 7.4 + Amiloride
R 6.0
Gr HCO_3^-
Gr HCO_3^- + A23187

Figure 6.15 The effect of chemicals which influence pH on the insertion of AF18 in *Trichinella spiralis* infective larvae.

Adjustment of the environmental pH with acidic or basic compounds was employed to assess their effects on insertion of the fluorescent lipid probe AF18 in the surface of *T. spiralis* infective larvae. The calcium ionophore A23187 was used in conjunction with bicarbonate to assess its ability to enhance or decrease an effect. AF18 insertion was measured by fluorescence quantitation (in relative fluorescence units).

The following incubation conditions were used:

<u>Controls</u>:
18 DW & 18 R: Distilled water and RPMI 1640 at 18^{o}C.

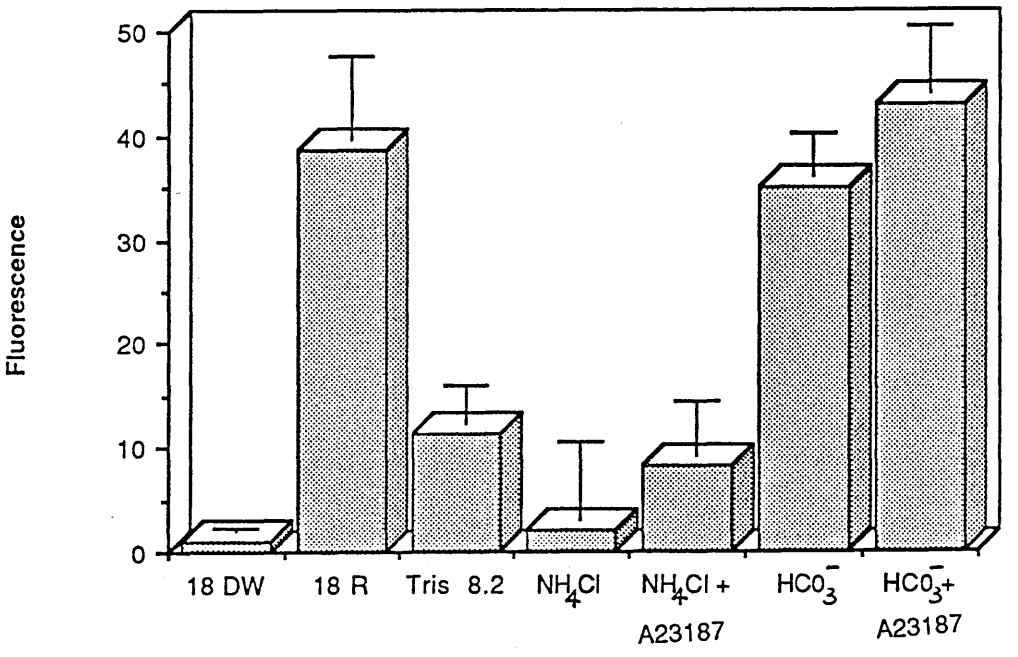
Tris 8.2 : 0.05M Tris-HCl at pH 8.2

NH_4Cl : 20mM ammonium chloride.

NH_4Cl + A23187: 20mM ammonium chloride, in the presence of 50µM A23187.

HCO_3^- : 20mM $NaHCO_3$.

HCO_3^- + A23187: 20mM $NaHCO_3$, in the presence of 50µM A23187.


Fluorescence
50
40
30
20
10
0
18 DW
18 R
Tris 8.2
NH_4Cl
NH_4Cl + A23187
HCO_3^-
HCO_3^-+ A23187

6.2.8. Role of increased pH in signalling the modification of the infective-stage epicuticle in *Acanthocheilonema viteae* and *Trichinella spiralis*.

It is clear from the results in figure 6.14 for *A. viteae*, and in figure 6.15 for *T. spiralis* that pH is a very important factor in the signalling of the modification of the epicuticle. For L3's of *A. viteae*, when the pH of Grace's insect medium was increased from 6.0 to 7.4 there was a dramatic increase in AF18 insertion above the level of the control. Note that the fluorescence intensity of the AF18 probe alone did not change significantly in going from pH 6.0 to pH 7.4. When the pH of RPMI was reduced from 7.4 to 6.0 there was a dramatic decrease in AF18 insertion compared to the control. This suggests that an alkaline pH shift is very important for modification of the biophysical properties of the epicuticle. In addition, when the potent blocker of Na^+/H^+ antiport, amiloride was added to Grace's at pH 7.4, AF18 insertion was inhibited significantly ($P < 0.0001$). Amiloride prevents influx of Na^+ ions and efflux of H^+ ions and would therefore prevent intracellular alkalinization.

In *T. spiralis* the alkaline environment of 0.05M Tris-HCl at pH 8.2 had a small but significant effect on AF18 insertion, however 20mM NH_4Cl had no such effect. Both *A. viteae* and *T. spiralis* responded to HCO_3^- ions with levels of fluorescence for AF18 insertion similar to those achieved with RPMI medium. The HCO_3^- effect was increased slightly for both parasites in the presence of the calcium ionophore A23187 (10μM).

6.2.9. Role of cAMP-dependent pathways in signalling.

The effects of chemicals affecting cAMP-dependent signalling pathways on the modification of surface biophysical properties and behavioural changes were examined in infective L3's of *N. brasiliensis*, *A. viteae* and *B. pahangi* (table 6.4 and table 6.5). In *N. brasiliensis* only ammonium chloride (20mM) and imidazole (20mM) had a positive effect on AF18 insertion. Both of these chemicals are thought to be activators of cAMP/cGMP phosphodiesterases (Kawamoto et al, 1989) and would be expected to reduce intracellular levels of cAMP and cGMP. TPA (1mM) and 8-bromo-cAMP (5mM), which are activators of protein kinase C, caused increased motility in the worms, as did forskolin (100μM) which is an activator of adenylate cyclase (see table 1.2).

In *A. viteae* there was a slight increase in AF18 insertion in the presence of 0.01% DMSO and in TPA (1mM) which is dissolved in a final concentration of 0.01% DMSO, suggesting that in both cases the effect is due to the DMSO. Again, TPA caused the behaviour of the worms to change such that they were hyperactive, forming a 'Medusa head' aggregate, in which they clump together with their heads in the centre.

In *B. pahangi* ammonium chloride (20mM) and imidazole (20mM) caused a significant increase in AF18 insertion, and TPA caused hyperactivity even at a concentration of 100nM. Serotonin, forskolin, and 8-bromo-cAMP are all chemicals which would be expected to increase intracellular cAMP levels, but none of them were able to inhibit AF18 insertion in RPMI 1640 at 27^{o}C.

6.2.10. **Role of cGMP-dependent pathways in signalling**

The effects of chemicals affecting intracellular cGMP concentrations on the modification of the biophysical properties of the epicuticle and behavioural changes were examined in infective L3's of *N. brasiliensis* (table 6.6). Nitroprusside (10μM), which is a potent activator of guanylate cyclase, did not have any significant effect on AF18 insertion or on motility. However, there was a significant effect on AF18 insertion if this chemical was used in conjunction with IBMX (100μM) which is a cAMP/cGMP-phosphodiesterase inhibitor and would therefore have the effect of keeping nitroprusside-elevated cGMP levels high. The membrane-permeant 8-bromo-cGMP was sufficient to stimulate surface change on its own.

6.2.11. **Role of calcium in signalling**

Calcium antagonists varied considerably in their effects on the inhibition of AF18 insertion in infective-stage larvae of *N. brasiliensis* which were incubated in RPMI at 27^{o}C (table 6.7). Treatment of larvae with the Ca^{++} channel blocker nicardipine (50μM) and the calmodulin inhibitor W-7 (100μM), had a significant effect in inhibiting AF18 insertion. In contrast, W-5 the 'inactive' analogue of W-7, TMB-8 an inhibitor of Ca^{++} release from internal sources, and EGTA a Ca^{++} chelator had no effect on AF18 insertion. These results indicate that increased Ca^{++} mobilization plays an important role in transformation of the biophysical properties of the surface.

Treatment with the calcium ionophore A23187 (5μM) had no effect on the

Table 6.3 Effects of chemicals affecting cation transport on AF18 insertion and behaviour in infective-stage larvae of *Acanthocheilonema viteae*.

Treatment	AF18 insertion	Motility
Control (Grace's at 27°C)	0. 91 ± 0.32	+
Control (RPMI at 37°C)	39.56 ± 7.94	+++
Control (RPMI at 27°C)	26.27 ± 5.53	++
+ Ouabain (Na^+, K^+-ATPase inhibitor)		
500µM	23.67 ± 4.61	++
+ Amiloride (Na^+/H^+ exchange inhibitor)		
100µM	28.33 ± 6.35	++
500µM	20.11 ± 3.87	++
1mM	16.74 ± 3.17*	+
10mM	17.21 ± 4.05*	+

AF18 insertion was measured by fluorescence quantitation (in relative fluorescence units-background).

The degree of motility is represented by an arbitrary scale from - to +++.

* significantly different from the control at $P< 0.001$.

Table 6.4 Effects of the chemicals affecting cAMP-dependent pathways on AF18 insertion and worm motility in conditions which would normally prevent transformation in *Nippostrongylus brasiliensis* and *Acanthocheilonema viteae*.

Parasite	Treatment		AF18 insertion	Motility
Nippostrongylus brasiliensis				
	Control (dist. water at 18°C)		-	+
	Serotonin	1mM	-	+
	Forskolin	50μM	-	+
		100μM	-	++
		1mM	-	++
	cAMP	5mM	-	+
8-bromo-cAMP		5mM	-	++
	TPA	1mM	-	++++
	NH_4Cl	20mM	++	+
	Imidazole	20mM	+++	+
Acanthocheilonema viteae				
	Control(Grace's at 27°C)		-	+
	serotonin	1mM	-	+
	forskolin	1mM	-	+
	cAMP	5mM	-	+
8-bromo-cAMP		5mM	-	+
	DMSO	0.01%	+	+
	TPA	1mM	+*	++++**

Fluorescence intensity is represented by an arbitrary scale from - to +++.

The degree of motility is represented by an arbitrary scale from- (totally immobile) to ++++ (extremely rapid movement).

* TPA solution includes DMSO at 0.01% .

**Extremely motile with formation of a 'Medusa head', i.e. heads stick together with tails facing outwards.

Table 6.5 Effects of the chemicals affecting cAMP-dependent pathways on AF18 insertion and worm motility in conditions which would normally (i) prevent or (ii) promote transformation in *Brugia pahangi*.

Parasite	Treatment		AF18 insertion	Motility
Brugia pahangi				
(i)	Control(Grace's at 27°C)		-	+
	serotonin	1mM	-	+
	forskolin	1mM	-	+
	cAMP	10mM	-	+
	8-bromo-cAMP	10mM	-	++
	DMSO	0.01%	+	+
	ß-phorbol	1mM	-	+
	TPA	100nM	-	++++
		100µM	-	++++
		1mM	+*	++++
	NH_4Cl	20mM	+++	+
	Imidazole	20mM	+++	++
(ii)	Control (RPMI at 37°C)		++	+++
	serotonin	1mM		+++
	forskolin	1mM	no inhibition.	+++
	8-bromo-cAMP	10mM		+++

Fluorescence intensity is represented by an arbitrary scale from - to +++.
The degree of motility is represented by an arbitrary scale from - to ++++.
* DMSO effect.

Table 6.6 Effects of the chemicals influencing intracellular cGMP concentration on AF18 insertion and the motility of infective-stage larvae of *Nippostrongylus brasiliensis*.

Treatment	AF18 insertion*	Motility
Control (RPMI at 18°C)	9.22 ± 1.90	++
Control (RPMI at 37°C)	17.37± 3.80	+++
Control (dist. water at 18°C)	0.95± 0.53	+
plus:		
8-bromo-cGMP 10mM	9.24 ± 2.99**	++
IBMX 100µM	1.51 ± 0.93	+
Niroprusside 10µM	4.56 ± 2.34	++
+ IBMX 100µM	8.41 ± 1.55**	++
8-bromo-cGMP 10mM, +Nitroprusside 10µM + IBMX 100µM	8.08± 2.71**	++

The degree of motility is represented by an arbitrary scale from - to +++.

*AF18 insertion was measured in arbitrary fluorescence units with the background subtracted. 20 readings were taken for each different treatment.

** significantly different from the control at $P< 0.0001$.

The experiment was performed on two separate occasions.

Table 6.7 Effects of calcium antagonists and the calcium ionophore A23187 on the insertion of AF18 and motility in infective-stage larvae of *Nippostrongylus brasiliensis*.

Treatment	AF18 insertion	Motility
Control (dist. water at 18°C)	0.28 ± 0.09	+
Control (RPMI at 27°C)	12.66 ± 3.25	+++
+ EGTA 10mM	10.95 ± 1.98	+++
+ Nicardipine 50µM	2.83 ± 2.64*	-
+ W-7 100µM	3.74 ± 1.51*	-
+ W-5 100µM	11.29 ± 4.30	++
+ TMB-8 200µM	8.66 ± 2.26	-
+ A23187 5µM	15.85 ± 1.62	+++

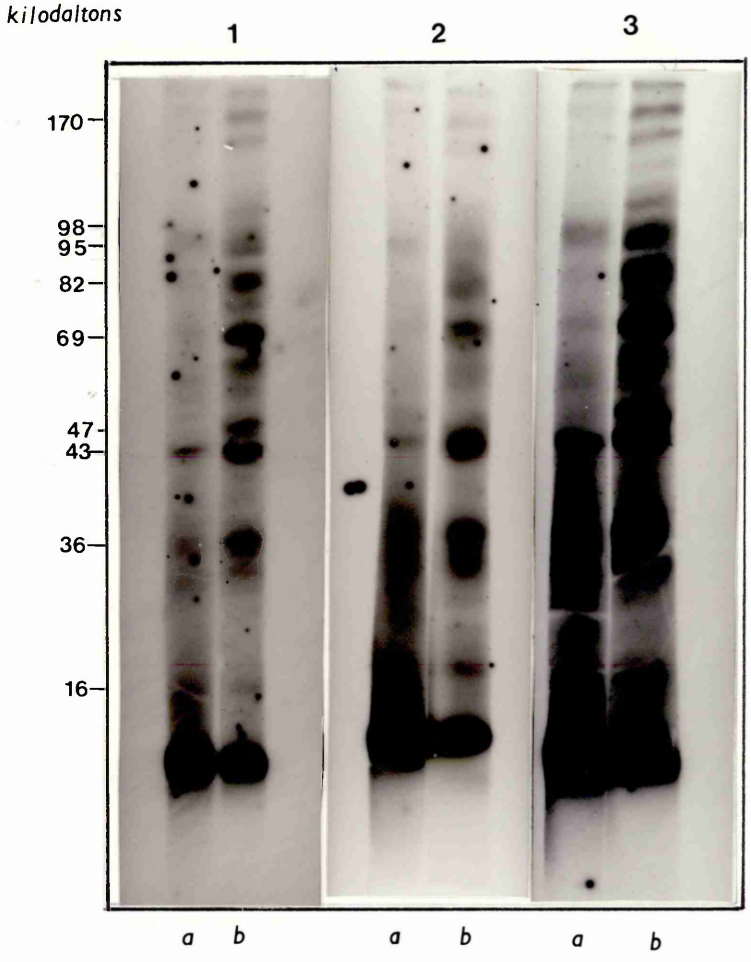
AF18 insertion was measured by fluorescence quantitation (in relative fluorescence units-background).
The degree of motility is represented by an arbitrary scale from - to +++.
*significantly different from the control at $P < 0.0001$.

Figure 6.16 **Fluorograph of ^{35}S-methionine-labelled proteins from pre- and post-infective larvae of *Nippostrongylus brasiliensis* separated by SDS-PAGE.**

1, 2 and 3 represent different batches of larvae (~10,000 in each) which had been incubated for 1h, 2h, and 3h, respectively in either:

(a) distilled water at 18^{o}C, or

(b) RPMI 1640 at 37^{o}C.


Mr
kilodaltons
1
2
3
170
98
95
82
69
47
43
36
16
a
b
a
b
a
b

transformation.

6.2.12. The rate of protein synthesis in *Nippostrongylus brasiliensis* infective larvae after incubation in environmental conditions which (i) mimic the free-living, pre-infective environment, and (ii) mimic the mammalian, post-infective environment.

Metabolic incorporation of ^{35}S-methionine into pre- and post-infective larvae was determined in three different batches of larvae which had been thoroughly washed in a penicillin-streptomycin solution (as described in section 2.7.2) and each batch divided into two groups, one for incubation in distilled water at room temperature and the other for incubation in RPMI 1640 at 37°C. During incubation with the radiolabel, 200μM chloramphenicol was included in the medium to try to eliminate protein synthesis by adhering bacteria which would interfere with the results from larval protein synthesis. From figure 6.16, it is clear that incorporation of ^{35}S-methionine into post-infective larvae occurs more readily than in pre-infective larvae. In addition, in post-infective larvae only, there were 3 new protein bands with molecular weights of 47 kDa, 69 kDa and 82 kDa. Also, some proteins, which were present in the pre-infective larvae, appeared to be synthesized in greater amounts in the post-infective larvae, particularly those with molecular weights of 36 kDa, 43 kDa, 95 kDa and 98 kDa.

6.3. Discussion

6.3.1. Modification of the biophysical properties of the infective-stage nematode epicuticle.

A dramatic change in surface biophysical properties was exhibited in six different species of parasitic nematode upon exposure of the pre-parasitic larva to conditions mimicking the mammalian host environment. The significance of this surface biophysical change during natural infection cannot be underestimated in terms of the implications for development in the nematode, and in terms of what type of surface is presented to the host.

The main surface effects of exposure to infection stimuli in the infective-stage larvae were: (i) a major increase in lipophilicity in all parasitic species tested, but also in the free-

living dauer larva of *C. elegans* (data not shown); and (ii) the release of anionic surface components in *S. ratti*. The work reported in this thesis is the first observation that the infective-stage larva becomes lipophilic upon exposure to infection stimuli. It has been noted before, however, that a surface coat is lost from *S. ratti* during skin-penetration (Grove et al, 1987; McHugh et al, 1989).

Various physical and chemical factors appear to be important in transformation of the surface. It is already well established that trigger cues are implicit in the activation of semi-dormant infective stage parasites when they enter the host environment (Lackie, 1975). For nematodes, these trigger cues would vary according to the mode of infection whether it be through the oral route or the cutaneous route. Muscle-stage larvae (MSL) of *T. spiralis*, which infect by the oral route, may be signalled to reveal a lipophilic surface after passage out of the pepsin-HCl of the stomach and into the mildly alkaline environment of the small intestine, where this process might be aided by the degradative action of trypsin, and the detergent action of bile. This view is supported by the fact that bile, trypsin and RPMI nutrient medium used in combination gave the most rapid transformation of the surface (see table 6.1) , perhaps by stripping off a surface coat. However, in experiments using bile and trypsin without nutrient medium, there was no surface transformation, suggesting that *in vivo* there are a constellation of events occurring from different stimuli which lead to developmental changes.

In larvae which infect by the cutaneous route, either by skin-penetration or direct transfer into a wound, the environmental trigger cues found in host tissues would be very similar to those found in RPMI 1640 mammalian cell culture medium (see table 2.). The results in figures (6.13 to 6.15) indicate that increased Na^+ ion concentration and increased pH are important stimuli for transformation.

In mammalian cells, external 'positive' signals such as hormones and growth factors which induce cellular functions such as protein synthesis and proliferation are mediated by second messengers (Nishizuka, 1984a,b). Recently, it has been shown clearly that intracellular pH (pH_i) is increased as a secondary cellular response to these physiological stimuli (Moolenaar et al, 1983; Thomas, 1984). Therefore, the possibility that alkaline conditions might also mimic stimuli in mammalian tissues was examined. Alkaline conditions created by Tris-HCl and HCO_3^- were able to stimulate transformation to AF18 lipophilicity in infective larvae of *A. viteae* and *T. spiralis*. These results suggest that the alkaline media had been able to mimic the effect of Na^+/H^+ exchange which has been

shown to control pH_i in mammalian cells (Moolenaar, 1986). The involvement of Na^+/H^+ exchange is also suggested by the partial inhibition of AF18 insertion in *A. viteae* by a potent inhibitor of Na^+/H^+ antiport, amiloride. However, complete inhibition could not be achieved with amiloride, indicating that other antiport processes could also be involved. In mammalian cells, several antiporter systems such as Na^+/H^+, $Na^+/ HCO_3^-/H^+/Cl^-$, K^+/H^+ and Cl^-/HCO_3^- have been shown to control pH_i (Thomas, 1984; Moolenaar, 1986). However, the fact that neither KCl nor choline-Cl was able to successfully replace the effects of saline, suggests that the antiporter systems in infective larvae of *A. viteae* do not include those using K^+ or Cl^-, although this data is by no means definitive.

HCO_3^- in the medium had a strong positive effect on surface transformation in *A. viteae* and *T. spiralis*. Since it is highly unlikely that the cuticle is permeable to HCO_3^- ions (Marks et al, 1968), it is more likely that the stimulus comes from the undissociated form, i.e. H_2CO_3 which could pass through the cuticle quite easily (Petronijevic and Rogers, 1987). Alternatively, chemosensors on the surface of the worm might be able to 'sense' HCO_3^- concentration.

Two major pathways have been proposed for signal transduction across the plasma membrane (Nishizuka, 1984a,b): one acts through cAMP and the other is the Ca^{++}-dependent pathway involving inositol lipid turnover. The stimulation of the latter pathway may provoke an increase in cGMP through activation of a particular guanylate cyclase (Garbers, 1989). The results in tables 6.4 and 6.5 indicated that cAMP, or chemicals which elevate cAMP had no effect on AF18 insertion but perhaps acted as a stimulus leading to increased movement. Experiments using chemicals which might be able to reduce levels of cAMP, i.e. the cAMP/cGMP-phosphodiesterase activators NH_4Cl and imidazole (Heyworth et al, 1984; Kawamoto et al, 1989), caused a very significant increase in the insertion of AF18 but very little stimulation of motility. This might explain why chemicals which elevate cAMP levels had had no effect on development.

The protein kinase C activator TPA was always able to produce dramatic effects on nematode behaviour, making the larvae extremely hyperactive. However, this action did not produce any surface effects. This is perhaps a very important observation, since one might have expected nervous stimulation to be involved in changing the surface properties, i.e. the two events are not necessarily connected.

The possible involvement of cGMP in development of the infective-stage larva of *N.*

brasiliensis was indicated in experiments which used the membrane-permeant form, 8-bromo-cGMP, alone or in combination with nitroprusside and IBMX which are a potent activator of guanylate cyclase and an inhibitor of cAMP/cGMP-phosphodiesterase, respectively. The effects of these chemicals are known to be very non-specific and there are many factors which must be taken into account when dealing with cGMP levels that are not usually a problem when dealing with cAMP levels. For example, there are many different types of guanylate cyclase, some of which are activated in a Ca^{++}-dependent manner, and others which are relatively ill-defined (Garbers, 1989).

It is appears likely that Ca^{++} is able to act as a second messenger in infective larvae of *N. brasiliensis*, since AF18 insertion was affected by the Ca^{++} channel blocker, nicardipine and the calmodulin inhibitor W-7. Petronijevic and Rogers (1986,1987) found that mobilization of Ca^{++} within the infective stage of *Haemonchus contortus* was an early and essential feature of the development of the parasitic stage. They also suggested that the induction of development in infective stages may be similar, in some respects, to that seen in the parthenogenic activation of eggs of marine invertebrates. That is, the activation of marine invertebrate eggs by bases is similar, in terms of its Ca^{++} relationships, to the action of basic stimuli on infective stages.

As yet there is no indication of how the initial events in the stimulation of development of the parasitic stage induced by Na^{+}, alkaline media, HCO_3^{-} and increased temperature, finally activates DNA transcription. The results from metabolic incorporation of ^{35}S-methionine into pre- and post- infective larvae of *N. brasiliensis* show that, in post-infective larvae, there is rapid synthesis (<4h) of new proteins and increased synthesis of some of the proteins which were already present in the pre-infective larvae (figure 6.16). This increase in protein synthesis appears to be too rapid for DNA transcription to have taken place, suggesting that the initiation of protein synthesis in infective larvae may be under translational, rather than transcriptional control. There is therefore an urgent need to investigate the connection between surface biophysical changes and developmental changes, in order to take full advantage of the possibilities for novel agents of parasite control.

Chapter Seven

General Discussion

7. General Discussion

7.1. Introduction

The overall aim of this project was to use non-invasive biophysical techniques to try to understand the nature of the nematode epicuticle in different stages of the life-cycle, and to gain insight into the mechanisms underlying dynamic interaction with an environment which is potentially hostile to the parasite. Therefore, the central emphasis of this discussion will be to consider the definition of the epicuticle and its functions, concentrating on the adult and infective stages. I conclude that, the surface biophysical properties of the infective-stage epicuticle are very different from those in adult parasites, requiring a re-definition of the outermost layer of the cuticle.

7.2. Biological significance of the interaction of fluorescent lipophilic probes with the nematode epicuticle.

Adults

Labelling of the intact surface of adult parasites with fluorescent lipophilic probes was found to be highly selective, which was attributed mainly to the charge of the fluorophore, and partly to the acyl chain-length, i.e. hydrophobicity of the probe. A preference for aminofluorescein (AF) lipid probes with an acyl chain-length of 18 carbon atoms was notable. Previously, workers have found that the predominant fatty acid in the nematode *C. elegans* is *cis*-vaccenic acid (Hutzell & Krusberg, 1982) which also has 18 carbon atoms, which might also be the predominant chain-length of fatty acid in lipids at the surface. Also, recently, it has been found that fatty acids with a chain length of 16 or18 carbon atoms are more effective than other chain lengths, or other lipids, in determinining the developmental course of L1's of *S. ratti* in nature (Minematsu et al, 1989). This may reflect the preferential insertion of these fatty acids in the surface of the worms.

Interaction of the fluorescent phospholipids NBD-PC and NBD-PE with the adult surface of the filarial nematode *A. viteae* was very different (see figure 3.1 and figure 3.2). NBD-PC remained at the surface for a long period of time whereas NBD-PE was rapidly internalized. This marked difference was puzzling since the NBD group has no electrical charge, and likewise, PC and PE are zwitterionic and therefore have no overall charge. Also, the rapid

internalization of NBD-PE was independent of low temperature and antimetabolites, suggesting that permeability of the epicuticle is highly selective for differences in phospholipid head-group structure. Very little is known at present, but the prevalence of phosphorylcholine-bearing molecules in nematodes (see Maizels and Selkirk, 1988) may affect binding of the choline phospholipid to the surface. The differential uptake of these phospholipids suggests that the difference in fatty acid composition of NBD-PC and NBD-PE (see section 2.5.1) may be responsible for the selectivity. Differential uptake of NBD-PC and NBD-PE has also been noted in erythrocytes infected with *Plasmodium falciparum* where this membrane behaves differently from that in uninfected erythrocytes (Haldar et al,1989). However, it is also possible that there is a higher metabolic, or biosynthetic requirement for PE rather than PC and this may be reflected in their differential uptake. Results of surface lipid analysis in chapter 4 showed that large amounts of PE and lyso PE were found relative to PC .

Nematodes must have a particularly large requirement for sterols since they cannot synthesize them *de novo* (Dutky et al, 1967) and need them for incorporation into membranes, reproductive tissues, and for the synthesis of hormones, e.g. ecdysones (Howells, 1990, in press). It was, therefore, extremely surprising to find that the fluorescent cholesterol probe NBD-chol was taken up very slowly and a significant proportion of the probe remained on the surface. Perhaps more specific mechanisms are required for cholesterol transport so that lipid-binding proteins would be required throughout the cuticle. Recently, supporting evidence for this hypothesis has come to light, that an apoprotein-like molecule (lipid-binding protein) has been found in large quantities in the cuticle of the filarial nematode *Brugia malayi* (Selkirk, M., unpublished results). However, it is thought that only filarial nematodes and a few others are able to feed transcuticularly (Howells and Chen, 1984) so that this hypothesis might only apply to them and not to gut-feeding nematodes.

All adult nematodes tested excluded the cationic lipid probes DiI18 and RH18 from their surface, which is in stark contrast to adults of the trematode *Schistosoma mansoni* which allow insertion of both these probes (Kusel and Gordon, 1989). This serves to underline a major difference in surface biophysical properties between nematodes and other helminths. Exclusion of cationic lipid probes is a property which is shared by the unusual surface membrane of *Xenopus* eggs (Dictus et al, 1984). These authors attributed this exclusion to the

presence of a thick surface coat, a hypothesis which cannot be applied directly to adult nematodes since they appear to lack a surface coat (Lee et al, 1986; Kieffer et al, 1989).

The basis for the selectivity of insertion and internalization of fluorescent lipid probes in adult parasites is uncertain since there is no obvious candidate for a biophysical barrier, and there can be little speculation on the effects of a surface coat when its presence, in adult *T. spiralis*(Lee et al, 1986) and adult *A. viteae* (Kieffer et al, 1989), is in doubt. However, it is agreed widely that a protease-resistant layer is present on the outermost surface (Pritchard et al 1988; Scott et al, 1988; McKean and Pritchard, 1989) which is thought to be able to mask underlying proteins from the effects of proteolytic digestion. This would have distinct advantages for nematode stages which reside for some time in the gut, or for others which might come under attack from mast cell proteases in the tissues. The experiments in Chapter 3 with adult *T. spiralis* and adult *C. elegans* showed that protease treatment of the intact worms had no effect on the exclusion of cationic fluorescent lipid probes. However, brief exposure to 0.25% CTAB detergent, followed by the protease treatment again, resulted in the intact surface of the worms becoming accessible to DiI18 and RH18 cationic probes. This suggests that the detergent had been able to remove a protease-resistant layer or component which had been masking a protease susceptible layer. In conclusion, it is highly likely that both of these layers had been responsible for the exclusion of cationic molecules from the epicuticle.

Infective-stage larvae

Infective-stage larvae maintained in the pre-parasitic state (i.e. pre-infection environmental conditions) were remarkable in that of all the stages in the life-cycle, they were the only ones which had no affinity for any of the fluorescent lipids tested. That is 'pre-parasitic' or 'pre-infective' larvae appeared to be totally non-lipophilic. This was also true for dauer larvae of *C. elegans* (data not shown) which have similarities with their parasitic counterparts. Manipulation of temperature, ionic composition and pH of the environment, in parasitic forms altered the developmental status of the pre-parasitic larvae such that their surface became lipophilic for the fluorescent lipid probe AF18, and possibly also serum lipids. This will be discussed further in section 7.4.

Possession of a surface which has the capacity to exclude certain lipophilic molecules may be a fundamental feature of parasitic nematodes which protects them against the chemical

and physical onslaughts of tissue environments. Surface-damaging molecules such as phospholipases, fatty acids, and complement components may be unable to insert properly for internalization to take place, so that they might never reach the comparatively fragile membranes of the hypodermal cells and syncytium. If the adult nematode surface is refractory to cationic molecules in general, then this may be extremely advantageous in escaping the effects of, e.g. the released cationic proteins from degranulating eosinophils. Too little is known at present, but the composition of the surface lipid might also have profound effects on interaction with host defence mechanisms, including complement since Mold (1989) has found that primary and secondary amino groups on lipids can activate the alternative pathway. Chemotherapy might then be greatly improved by drugs which are designed to circumvent the restricted entry of certain molecules, perhaps by means of a modification to the charge and/or hydrophobicity of the drug, or means found by which selectivity could be abrogated in order to permit effective immune attack.

Lateral diffusion of fluorescent lipid probes in the adult- and infective-stage epicuticle

FRAP experiments proved to be very useful in trying to envisage the dynamic nature of lipids associated with the epicuticle:

Adults: In nearly all cases, it was found that the AF18 lipid probe was essentially immobile ($D_L << 10^{-10}cm^2s^{-1}$, and %R < 20) in the surface, whereas the NBD-chol lipid probe was very mobile (D_L approx. $10^{-8}cm^2s^{-1}$, and %R approx. 70). As discussed in Chapter 5, it was considered that the AF18 probe was inserted in epicuticular lipid which was somehow stabilized by lipid-lipid interactions or lipid-cuticlin interactions, but that NBD-chol was inserted in fluid areas containing serum-derived sterols.

Post-L3's: In chapter 6, it was described how pre-parasitic L3's became lipophilic for AF18 (and NBD-chol, data not shown) upon exposure to environmental conditions mimicking the mammalian host environment. FRAP measurements of AF18 mobility in these post-parasitic L3's (Chapter 5) revealed that this surface was more mobile (D_L approx. $10^{-8}cm^2s^{-1}$; %R approx. 50), suggesting that the epicuticle of post-parasitic infective-stage larvae is dynamic in comparison with the adult epicuticle. The major exception to this was the MSL of *T. spiralis* which showed immobility of AF18. This may have been due to insertion of the

probe in the lipid of the accessory layer and not the epicuticle.

Differential mobility between AF18 and NBD-chol in adult parasites, and to some extent in infective-stage larvae, prompted the suggestion that the epicuticle is heterogeneously organized into domains. This domain model could also be applied to the more dynamic infective-stage epicuticle, but where restricted recovery upon photobleaching would be attributed to lipid-lipid interactions (see Wolf et al, 1988) instead of covalent lipid-protein interactions which was suggested for adults. Therefore, I have proposed a model for the epicuticle which has the kind of adaptability to cope with stage differences, and perhaps also species differences relating to a particular parasitic environment.

Limited recovery upon photobleaching of a fluorescent lipid probe in a membrane usually indicates the existence of gel-phase microdomains (Karnovsky et al, 1982; Dictus et al, 1984). The immobile lipid fraction of adult parasite surfaces may be stabilized as a consequence of:

(a) covalent interactions with structural protein (e.g. the insoluble cuticlin matrix);

(b) possession of a lipid component which causes the epicuticle to be partially solidified at physiological temperatures;

(c) an unusual lipid composition which favours non-bilayer phases leading to microscopic barriers to diffusion.

With respect to hypothesis (a), a cuticlin matrix similar to that described in Ascaris by Fujimoto and Kanaya (1973) could be the basement protein for attachment of nematode-derived 'structural lipids'. When Fujimoto and Kanaya treated Ascaris cuticle with 1% ß-mercaptoethanol, most of the material was solubilized, however, a small amount of slightly dark, fibrous or membranous material called cuticlin remained. The isolated cuticlin apparently resembles the outermost layers of the cuticle since the latter have grooves at about 20µm and the area between the grooves shows many striations which run parallel to the grooves. A proportion of epicuticular lipid could be covalently attached to cuticlin, forming a lipoprotein layer. In this hypothesis, mobile lipid would be accounted for by host-derived lipid, such as blood cholesterol.

If lipid factors are the cause of co-existent diffusing and non-diffusing lipid fractions, and not structural proteins, as suggested in hypotheses (b) and (c), then the next question is: what distinguishes nematode surface lipids from those of host plasma membranes?

Nematodes are peculiar in their lipid composition in that some have been found to contain

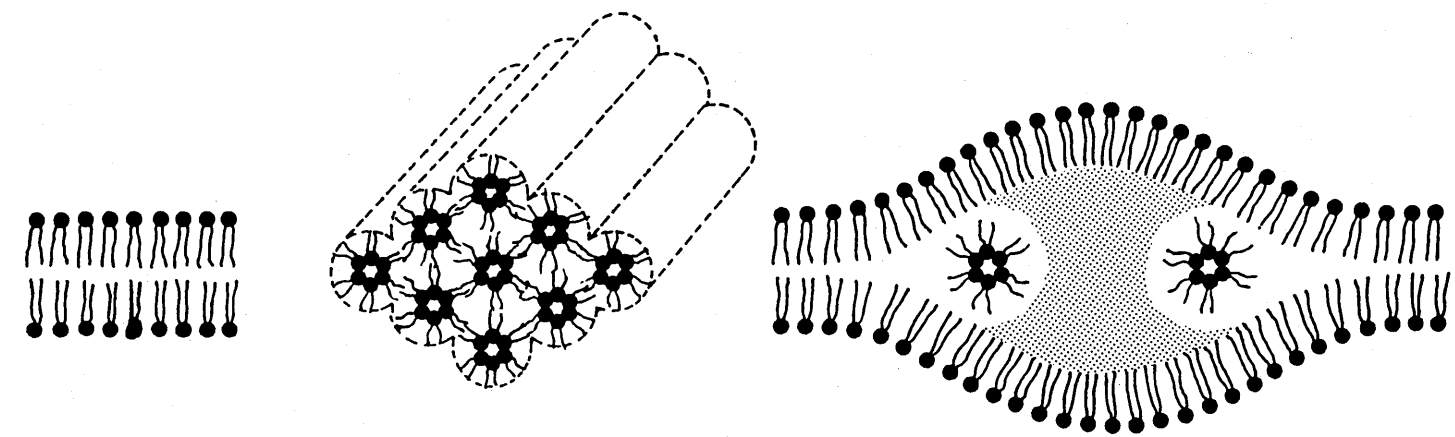
large amounts of plasmalogens and other ether-linked phospholipids. For instance, adults of Litomosoides carinii have 30% of their phospholipids as the ether-linked form (Subrahmanyam, 1967), and of the phopholipids of the body wall of Ascaris cuticle, 45% are of the ether type (Fairbairn, 1955). Plasmalogens appear to play a role in membrane fluidity (Russell, 1984) and in the enhancement of transitions to non-bilayer structures (Goldfine and Langworthy, 1988) (also see figure 7.1). Plasma membranes of both *Leishmania* (Wassef, 1985) and spermatozoa (Evans et al, 1980) possess unusually high levels of ether lipids which, apart from being used in protein anchors, may cause the surface to be highly resistant to lipolytic degradation, a surface property which would be advantageous to a tissue-dwelling nematode. It is also noteworthy that Archaebacteria have membranes which are adapted to cope with environmental stress and contain very high levels of ether lipids to make them more resistant (Goldfine and Langworthy, 1988).

Another possibility, suggested in hypothesis (b), is that there is a lipid component of the epicuticle which causes it to be partially solidified at physiological temperatures. One such lipid is to be found in *Ascaris* reproductive tissues (Fairbairn and Passey, 1955) but possibly also in the cuticle (von Brand and Winkeljohn, 1945), i.e. ascaroside lipid. Free ascarosides have melting points in the range 70-80^{o}C and so produce a solid membrane, whereas the melting point of the ascaroside esters is about 40^{o}C and they are fluid at the body temperature of the host (Barrett, 1981). If ascarosides, or similar lipids were present in the epicuticle, fluidity could be controlled by the release of enzymes which were able to esterify or de-esterify certain lipids.

Mixtures of waxes, ascaroside-like lipids, fatty acids and their esters, ether phospholipids (some of which are thought to be present in the epicuticle, see Chapter 4) or lipoprotein could form a heterogeneous lipid layer with co-existent solid and fluid microdomains. This heterogeneous organization could allow structural flexibility while retaining resistance to chemical and physical agents.

The concept of horizontal partitioning of lipid and lipophilic molecules between domains may have profound implications for the host-parasite interface. A variety of lipophilic molecules and proteins may interact with the epicuticle in such a way that there is the involvement of a unique pattern of partitioning into domains, which is illustrated in figure 7.2. Recently, a similar model was proposed by Mountford and Wright (1988) whereby neutral

Figure 7.1 Lipid polymorphism in membrane structures.



a) Bilayer (L_α) b) Hexagonal (H_{II}) c) Bilayer (L_α) and hexagonal (H_{II})

The type of non-bilayer phase lipid illustrated in (b) and (c) can affect the permeability barrier and increase ion permeability, trans-bilayer (flip-flop) movement of the lipid molecules, and fusion events (De Gier, 1988).

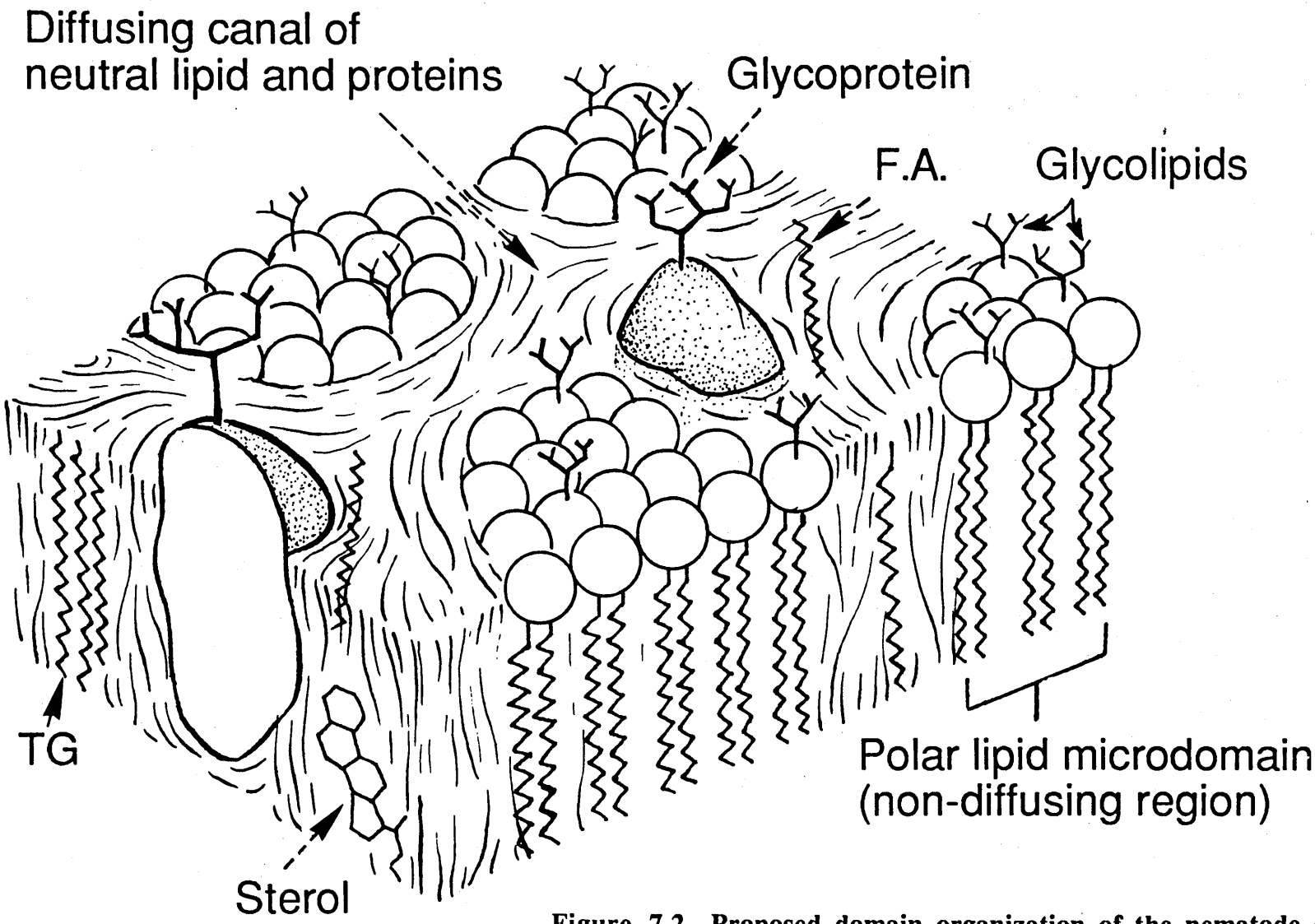


Figure 7.2 Proposed domain organization of the nematode epicuticle

lipid domains were intercalated with the bilayer lipid in the plasma membrane of malignant cells using non-bilayer phase lipid. These authors were particularly interested in the possibility of particles being expelled or 'jettisoned' from the intact surface by mechanisms using the domain organization, which could be readily applied to the nematode epicuticle with regard to insertion of lipophilic molecules and release of antigens.

7.3. Biological significance of the interaction of chemically-reactive fluorescent probes with the nematode epicuticle.

Much of the previous work on the reactivity of the nematode epicuticle with extrinsic reagents has focussd on the identification of surface antigens using radioiodination techniques (see Philipp and Rumjanek, 1984; and Maizels and Selkirk, 1988 for reviews). The majority of these antigens can be easily removed from the surface during homogenization without the aid of detergents (Devaney, 1988; Scott et al, 1988), or removed from the intact surface using reducing agents (Maizels et al, 1988). This suggests that certain antigens present on the surface are associated by weak, ionic interactions and do not contribute to the structural integrity of the epicuticle.

7.3.1 Proteins

The results from Chapters 3, 4 and 5 also suggested that there are loosely attached proteins on the surface. Some of them can be labelled by fluorescein-5-maleimide (FM) which binds to free sulphydryl groups, and some can be labelled by dichlorotriazinylaminofluorescein (DTAF) in the presence of sodium borohydride which binds to amino groups. It appears that (in MSL of *T. spiralis* at least) FM-labelled proteins can be cleaved from the surface by the action of the reducing agent, sodium borohydride (see figure 4.6. When adult worms were examined for lateral diffusion of surface proteins by FRAP, the FM-labelled proteins were free to diffuse quite rapidly over the surface (D_L approx. $10^{-8}cm^2s^{-1}$, and %R approx. 85) which is considerably more mobile than most typical membrane proteins (D_L approx. $10^{-10}cm^2s^{-1}$), (see Peters, 1981). Similarly, surface proteins which were labelled with DTAF were able to diffuse very rapidly (D_L approx. $10^{-8}cm^2s^{-1}$, and %R approx. 80). However, this was surprising , since it had been assumed that the borohydride reduction during labelling with DTAF would remove all of the loosely-associated proteins and label only structural proteins

which would be expected to be immobile. This suggests that not all of the loosely-associated proteins which are free to move in the plane of the epicuticle can be removed by reducing agents acting on the intact surface.

FRAP experiments, measuring surface protein mobility, were very useful in the investigation of the intact epicuticle as a living biological structure. Proteins which were free to move on the 'true' surface (i.e. not cast cuticular sheath) were immobile on the cast cuticle surface. Cast L2 cuticles which remain on the surface of some infective larvae, such as in *O. ostertagi*, and on the surface of dauer larvae of *C. elegans*, had surface proteins which when labelled with FM, DTAF or polyclonal antibody (for dauers only) were completely immobile. This is perhaps not surprising when we consider that the L2 sheath is completely detached from the rest of the worm and therefore, probably inert. However, DTAF was completely immobile on the true surface of isolated cuticle from *A. suum*, suggesting that mobility of surface proteins is energy-dependent and related to close association with the hypodermis. Support for the idea that surface protein mobility was energy-dependent came from results which showed that metabolic inhibitors, e.g. NaN_3 could reduce the diffusion coefficient and percentage recovery in some cases (data not shown).

Evidence for labelling of high molecular weight material with DTAF came from studies of MSL of *T. spiralis* and the isolated cuticle of *A. suum* (see figures 4.5 and 4.8), which were used for this purpose because large quantities of material could be obtained. However, the suitability of these parasites for the study of surface protein dynamics was later in doubt, since the isolated *Ascaris* cuticle is effectively dead once removed from the rest of the worm, and the MSL of *T. spiralis* have an accessory layer overlying the epicuticle which may be unique to this parasite (Lee et al, 1986). In FRAP experiments both of these nematodes showed total immobility of FM- and DTAF-labelled proteins. In the case of MSL of *T. spiralis*, immobility was probably due to labelling the accessory layer; and in *Ascaris* immobility was probably due to the labelling of a dead cuticle.

Survival of intact worms in $10mgml^{-1}$ sodium borohydride during DTAF labelling was something which was completely unexpected and quite remarkable, and from the fluorescence photomicrographs in figures 3.4 and 3.5, it was clear that the cuticular barrier had remained intact throughout the harsh labelling conditions. The fact that the worms survived for days in

RPMI 1640 culture medium after this reducing treatment does not give grounds for optimism about the potential effectiveness of defence mechanisms in the host directed against the surface. Resistance to detergents, proteases and strong reducing agents in nematodes may be partly attributable to the protein referred to as cuticlin which is thought to be associated with the epicuticle (Fujimoto and Kanaya, 1973; Betschart and Jenkins, 1987). This protein is very different from the bulk of the cuticular proteins which are collagenous in nature and are cross-linked by disulphide bonds (Cox et al, 1981a,b; Betschart and Jenkins, 1987; Titanji et al, 1988; Selkirk et al, 1989). Cuticlin may be composed of proteins which are covalently-linked by phenolic tyrosine bridges (Fujimoto et al, 1981), making the epicuticle a formidable barrier to physical and chemical agents.

Results from experiments using the free-living nematode *Caenorhabditis elegans* to examine the effects of borohydride reduction on wild-type nematodes (N2) and mutants (srf-2 and srf-3), showed that the mutants were highly susceptible to damage and that the wild-type nematodes were only moderately susceptible to damage. That is, the mutants, which expose normally hidden protein determinants on their surface (Politz et al, 1987), were not resistant to borohydride reduction at all. The major difference between the free-living nematode and parasitic nematodes in their susceptibility to borohydride reduction suggests that resistance to these reducing conditions is an adaptation to parasitism, where the parasite might encounter harsh chemical attack from, e.g. oxygen radicals in the host. Perhaps the cuticlin layer is defective in the mutants, and if so, it would be important that the genetic basis for the variation be understood.

Special membrane anchors?

Most membrane proteins are unable to diffuse as rapidly as reported here for adult nematode surface proteins, although exceptionally rapid diffusion ($D_L > 10^{-9}cm^2s^{-1}$) has been observed in several cases (Poo and Cone, 1974; Sheetz et al, 1980; Myles et al, 1984). This has been attributed to the absence of the more typical constraints on mobility imposed by interactions of cytoplasmic portions of the proteins with underlying cytoskeletal structures. However, recently a new class of membrane protein has begun to emerge which is anchored by a lipid moiety and is able to diffuse rapidly ($D_L > 10^{-9}cm^2s^{-1}$). These lipid-linked proteins include alkaline phosphatase (Noda et al, 1987), 5'-nucleosidase (Taguchi et al, 1985), Thy-1

(Low and Kincade, 1985), acetylcholinesterase (Low and Finean, 1977) and the variant surface glycoprotein (VSG) of trypanosomes (Ferguson et al, 1985). Several lipid links have now been well characterized and consist of a basic glycosylphosphatidylinositol (GPI) structure (Ferguson et al, 1988b). Scott et al (1988) have already suggested that the glycolipid found on the surface of adults of the filarial nematode *Dirofilaria immitis* might play a role in anchoring or stabilizing peptides to the cuticular surface. This is also an attractive explanation for the ease with which some nematode surface proteins could be released from the surface without the aid of detergents, as nematode phospholipase C could be released during homogenization of the cuticle, cleaving the GPI-linked proteins. Resistance of the 29-30 kDa glycoprotein of *Brugia pahangi* to release with a number of types of phospholipase C indicates that there is no evidence, to date, of a lipid anchor for this protein (Devaney, 1988; Maizels et al, 1989), although more definitive experiments would be required to prove this. Metabolic incorporation of ^{14}C-myristate (which is a common component of GPI-links) has been used to identify and characterize the GPI-linked adult worm immunogens of the trematode *Schistosoma mansoni* (Sauma and Strand, 1988), an experiment which could be repeated for nematode surface proteins.

7.3.2 Sugars

A particularly notable adaptation to parasitism appears to be the reduction of surface antigenicity by concealing sugars. In the results of lectin-binding experiments (see table 3.14), none of the adult parasites bound any of the fluorescent lectins tested on their surface, and only the surface of the free-living nematode *C.elegans* was able to bind lectins. It is also notable that there was no lectin-binding of the intact surface of infective stage larvae of *S. ratti*, *T. canis*, *O. ostertagi* (unsheathed), or *A. viteae*. A glycocalyx-like surface coat is prominent in free-living nematodes (Zuckerman et al, 1979) and in the infective-stage larvae of most parasitic species (Grove et al, 1984; Lee et al, 1986; Badley et al, 1987; Abraham et al, 1988) studied so far. However, Lee et al (1986) noted that adult *T. spiralis* lacked a surface coat, and the same has been noted by Kieffer et al (1989) for adult *A. viteae* using gold-labelled lectins, which adds support to the idea that sugar residues are not exposed on the intact adult parasite surface.

A remarkable feature of some surface glycoproteins in parasitic nematodes is that their

sugar residues are apparently not exposed on the intact surface for lectin-binding. For instance, the 47kDa antigen of *T. spiralis* (Parkhouse et al, 1981; Ortega-Pierres et al, 1984), the 30kDa antigen of *B. pahangi* (Devaney et al, 1988) and the 49kDa antigen of *Dirofilaria immitis* (Scott, A., personal communication) have carbohydrate determinants which are not available for lectin-binding while present on the surface. However, when the 49kDa antigen of *D. immitis* for example, is homogenized out of the epicuticle, it can be recovered on beads coated with concanavalin A (Scott, A., personal communication). Several possibilities exist to explain this phenomenon: firstly, glycoproteins are near to, but not exposed on, the surface yet are iodinatable. Secondly, they are positioned on the outermost surface, but such that lectin-binding is sterically hindered. Lastly, that parasite glycoproteins are inserted in the epicuticle in an unusual configuration with their sugar chains in the hydrophilic part of inverted lipid micelles, as suggested by Wright and Hong (1988).

Sugars are apparently exposed on the intact surface of infective-stage larvae of *T. canis*, to which anti-carbohydrate monoclonals can bind (Maizels et al, 1987). However, I was unable to find fluorescent lectin-binding under any circumstances in this parasite (see table 3.14) and only periodate oxidation with subsequent labelling with a fluorescent hydrazide (figure 3.6) produced surface fluorescence. An attempt to oxidize galactose-rich glycoconjugates (Maizels et al, 1988) of *T. canis* with galactose oxidase was unsuccessful, suggesting that steric hindrance on the surface of the worm had prevented access to this enzyme. There was also an indication that sugars might be present on the surface of adult *O. ostertagi* using the periodate-fluorescent hydrazide technique (not shown), since although labelling was very faint there was no labelling with the control fluorescent hydrazide alone. Future work should investigate the nature of the sugars which are at, or near, the surface, perhaps by combining the techniques of histochemistry and electron microscopy.

7.4. Changes in the surface properties of the infective-stage larva after exposure to the environment of the mammalian host.

It was widely believed, until now, that the basic organization and surface properties of the infective-stage epicuticle remained largely unchanged until the L4 cuticle was revealed at moulting. Indeed most biological assays to assess the suitability of culture conditions for

development to the L4 stage rely on the cuticular moult (Franke and Weinstein, 1984), or in sheathed forms, rely on exsheathment (Petronijevic and Rogers,1987). However, the results from Chapter 6 show very clearly that it is the L3 epicuticle itself which is rapidly altered during adaptation to the new host environment, presenting a surface which is radically different from that in the pre-parasitic larva.

In recent papers there have been references to the surface properties of the 'infective-stage larva', when in fact, some have been dealing with the 'pre-parasitic larva' and others with the 'post-parasitic larva', depending on the culture conditions . This was surely the case when antigen-shedding was observed in 'L3's' of *Dirofilaria immitis* in mammalian cell culture medium (Ibrahim et al, 1989), and similarly, when antibody complexes were shed by 'L3's' of *S. ratti* in saline (Murrell and Graham, 1983). However, many workers are already aware of the surface changes which occur, in going from the pre- to the post-parasitic state, since many of them have remarked on the futility of attempting to radiolabel the surface of the pre-parasitic larva unless it had been cultured in RPMI (Devaney, E., Selkirk, M. and Maizels, R. personal communications).

The results shown in Chapter 6 show that there is real urgency to investigate the mechanisms underlying the surface change in infective-stage larvae since successful establishment in the host is probably dependent on it. Many different aspects of this surface change could be discussed, but the most important considerations are: how does it influence interactions with the host environment; and how is it involved in further development of the parasite? In dealing with the former question, one must consider what components of the larval surface might be released during host invasion, and then, what host components might be inserted in the surface due to its increased lipophilicity. The latter question regarding further development of the parasite may bring in aspects of biochemical signalling and the role of the neuro-endocrine system.

Possible explanations for the surface biophysical change

The increase in lipophilicity for AF18 and possibly serum lipids, and the loss of anionic material in *S.ratti* suggests that there is a general change in the biophysical properties of the surface. The following possible explanations are suggested:

1) There may a rapid re-organization of the surface which changes its affinity for lipids in

general, perhaps by exposing lipid-binding proteins. This would be accompanied by the release of a surface coat which had previously prevented the insertion of lipids.

2) The fact that increasing the temperature to 37°C alone has rapid surface effects to increase lipophilicity, suggests that there is either a temperature-induced lipid phase change, or that certain enzymes at the surface are activated at this temperature to release particular surface molecules.

3) Changes in ionic composition or pH of the environment could affect the operation of various cellular signalling pathways at the hypodermis, leading to rapid relay of signals to the surface. This' signalling' might activate any of the physical changes described above.

4) The sensory apparatus of the nematode is highly specialized, therefore it is also possible that the epicuticle is signalled to change its organization via nervous stimulation. This explanation seems unlikely however, since attempts to block surface lipophilicity for AF18 with the acetylcholinesterase inhibitors eserine sulphate and carbamyl choline were unsuccessful (data not shown).

There are quite a number of publications which have concentrated on molecules which are released from the post-parasitic larva, including from *T. spiralis* (Philipp et al, 1980), *N. brasiliensis* (Maizels et al, 1983), *T. canis* (Maizels et al, 1984), *Brugia* (Marshall and Howells, 1986; Carlow et al, 1987) and *D. immitis* (Ibrahim et al, 1989). However, only in the skin-penetrating larva, *Strongyloides ratti* was it possible to label the polyanionic surface coat (Murrell et al, 1983a; Grove et al, 1987) which is thought to contain sulphated proteoglycan (Murrell et al, 1983b). This negatively-charged surface coat could be labelled with fluoresceinated cationised ferritin, and over a period of 1 hour in culture with RPMI at 37°C, the rapid release of the fluorescent label was observed (figures 6.11 and 6.12). It was assumed that this was due to the release of anionic material, i.e. the sulphated proteoglycan. The release of anionic material coincided with the increase in lipophilicity for AF18, so that in this case at least, one could speculate that the surface coat had been preventing access to the fluorescent lipid probe.

The MSL of *Trichinella spiralis* have a surface coat which has been referred to as the accessory layer (Lee et al, 1984). This is a heterogeneous layer distinct from the epicuticle, with sensitivity to trypsin and bile at 37°C (Stewart et al, 1987), and it is expected that the

action of bile and trypsin in the small intestine would cause a rapid surface transformation with the release of surface proteins and an increase in lipophilicity. Bile contains amphipathic bile salts such as deoxycholic acid which might be able to insert into the lipophilic surface of a newly-infecting worm. This might explain how bile salts are able to act as determinants of host specificity in certain parasites (Chappell, 1979). The unusual thing about the surface of MSL, however, is that the accessory layer remains on the surface of the worm until the cuticular moult (Capo et al, 1989). Therefore, in this case, the exclusion of the fluorescent lipid probe AF18 cannot be explained by the release of a surface coat. However, recent work by Wright and Hong (1988) showed that the accessory layer is composed of an external layer of antigenic material and an internal layer of lipid filaments. These workers speculated that the lipid filaments were the result of non-bilayer phase lipid (see figure 7.1) formed predominantly by phosphatidylethanolamine (PE) which has a tendency towards polymorphism (De Kruijff et al, 1981; Cullis et al, 1985; Gruner et al, 1985). This type of lipid arrangement would be highly flexible to changes in the environment so that AF18 insertion could have been allowed by a lipid phase change in the accessory layer. I believe that the large transition found in MSL by differential scanning calorimetry (D.S.C.) (see figure 6.2), which was absent in adults, could be due to the re-organization of the accessory layer in response to increased temperature.

An important indicator of the surface change leading to increased lipophilicity in MSL of *T. spiralis* was a simultaneous change in behaviour, switching from coiling/uncoiling movement to sinusoidal movement. This behaviour might represent a response to developmental changes which require that the worm should burrow into the lumen of the gut and commence moulting. Clearly, the change in surface biophysical properties to allow the insertion of lipophilic molecules is a vital step for progression to the parasitic state.

Serum lipids

A very interesting feature which was revealed in the experiments with serum, was that if hyperlipidaemic serum was included in the culture medium and then removed before labelling, there was a dramatic decrease in the affinity of the surface of the nematode for the fluorescent lipid probe AF18. These results were obtained with hyperlipidaemic serum from jirds in *A. viteae*, and human hyperlipidaemic serum in *N. brasiliensis*, with no such effect using de-lipidated human serum in *N. brasiliensis*. The inference is, therefore, that host lipid is taken

up from serum and saturates the surface to the exclusion of the fluorescent lipid probe. Saturation of the surface with normal levels of host lipid would not normally be expected to occur, although my experiments do indicate that there will be a close interaction between the nematode surface and blood lipids upon infection.

It has long been known that lipids on the surface of the skin stimulate trematode cercariae to skin-penetrate (Wagner, 1959; Clegg, 1969; Stirewalt, 1971). The unsaturated fatty acid fraction seems to be important for *Schistosoma mansoni*, whereas the active fraction for *Austrobilharzia terrigalensis* is cholesterol (Clegg, 1969). Haas and Schmitt (1982) found that the penetration response by cercariae of *S. mansoni* into agar was stimulated only by aliphatic hydrocarbon chains with a hydrophilic as well as a lipophilic group. This raises the question as to what role skin lipids have in stimulation of penetration behaviour by nematodes, and how they are 'sensed'.

The increased lipophilicity upon exposure to infection stimuli may be vital to the further development of the parasite. This could be important in terms of acquisition of host lipids for metabolic and biosynthetic purposes, and, from an immunological point of view, the insertion of host lipids might allow masking of nematode antigens. This attractive hypothesis is supported by recent findings that certain surface antigens of *B. malayi* and *A. viteae* (which can be labelled with fluorescent monoclonal antibodies) 'disappear' from the surface when the worms are incubated in serum (Philipp, M. and Kaltmann, B., personal communications). Thus, instead of being released into the culture medium, some surface antigens may remain on the surface but become masked by host lipids. In addition, Philipp et al (1980_ noted that a marked increase in the rate of release of surface proteins could be achieved by co-culturing the MSL of *T. spiralis* with rat neutrophils in the presence of immune rat sera or normal sera, suggesting that the attachment of these components had somehow increased molecular signalling across the cuticle. In the present experiments using normal serum with *B. pahangi* and *N. brasiliensis*(figures 6.7 and 6.9), it was notable that the serum had significant effects on the behaviour of the parasites, causing them to become hyperactive. This indicated that specific host factors are required for normal development. It is interesting that in a recent paper (Hawdon and Schad, 1990), it has been shown that canine serum is necessary for normal development of the infective-stage larva of the canine hookworm *Ancylostoma caninum* so that feeding resumes. A temperature shift, mammalian cell culture medium (RPMI 1640) and

changes in pH triggered exsheathment of this larva, however, there was an absolute requirement for canine serum before feeding could be initiated.

Further development of the infective-stage larva in the host

To return to one of the questions which was asked earlier in this section; how does the surface change influence further development in the parasite? To answer this question fully, one would have to undertake a whole new series of experiments looking at, for example, the rapid activation of protein synthesis. However, at the present time, it may be useful to draw comparisons with other 'activation' systems, e.g. in the eggs of marine invertebrates.

A well-characterized 'developmental switch' occurs at fertilization in sea-urchin eggs, which is particularly appropriate to this discussion since the switch has been correlated with the release of surface proteins (Mazia et al, 1975; Johnson and Epel, 1975). At fertilization, peripheral proteins of the egg plasma membrane are released which causes functional modification of the membrane and leads to derepression of various developmental processes within the egg. It is possible, therefore, that release of surface proteins in infective nematode larvae initiates similar processes. Recent work on fertilization in sea-urchin eggs has begun to elucidate the mechanism of rapid activation of protein synthesis (reviewed by Winkler, 1988). Fertilization results in the activation of a variety of metabolic and biosynthetic pathways, including a 20- to 40-fold increase in the rate of protein synthesis by 2h after fertilization. In the present experiments, it was shown that metabolic incorporation of ^{35}S-methionine into certain proteins of *N. brasiliensis* L3's was greatly increased after only 4h incubation in RPMI at 37^{o}C (see figure 6.16) when compared to pre-parasitic larvae. It is known that the increase in protein synthesis in sea-urchin eggs is regulated at a purely translational level without the need for new transcription, and this is accompanied by changes in the intracellular environment (Winkler, 1988). The most important of these changes, in terms of protein synthesis, is the increase in intracellular pH mediated by the activation of a Na^+/H^+ ion exchange (Johnson et al, 1976). A second regulator of protein synthesis in sea-urchin eggs is the transient release of intracellular calcium. It is interesting to note that probably the most important determinant for increased lipophilicity of the surface in infective-stage nematodes is pH. Experiments with amiloride (see table 6.3 and figure 6.14) suggest that Na^+/H^+ antiport is involved to some extent. Also, in infective-stage nematodes, the increase in surface lipophilicity was inhibited

by calcium antagonists, suggesting that release of intracellular calcium is required for further development. There is therefore scope to continue research on nematode developmental mechanisms using other invertebrate systems as a guide.

Perhaps a more appropriate system to use as a guide for future research would be the infective-stage nematode egg. Also, some of the work on induction of permeability in the egg-shell prior to hatching may provide some clues for the induction of surface changes in larvae (reviewed by Perry, 1989). For example, Na^+, HCO_3^- and Ca^{++} ions have been implicated in the induction of egg-shell permeability (Clarke and Perry, 1988) which is comparable with the present results for infective-stage larvae (see figures 6.13 and 6.14, and table 6.7). Clarke and Perry (1988) also proposed an intriguing hypothesis for the induction of permeability. This was that HCO_3^- ions insert in the lipid layer, and assisted by thermally-induced changes in permeability, cause the formation of pores by mutual repulsion of HCO_3^- ions and lipid. These ideas regarding the physical effects of stimulatory chemicals could be incorporated into my own hypothesis of the flexible microdomain structure, which was illustrated in figure 7.2. In this case, a temperature increase and the insertion of ions could induce lipid-phase separations which would lead to the formation of permeable interdomain boundaries.

Future research in this field should be able to make use of the insertion of the fluorescent lipid probe AF18 as a marker for surface transformation. Since this change appeared to be universal among different species of nematode which had totally different modes of infection, there is optimism for the development of new chemotherapeutic strategies which could be designed to interfere with early signalling in the parasite. This may be far into the future, but it is hoped that the infective-stage epicuticle should now be seen as a structure capable of dynamic change.

7.5. The nature of the nematode epicuticle- a re-assessment.

In the face of evidence for rapid biophysical changes, release of surface proteins and dynamic exchange of low molecular weight substances, it would be misleading to argue that the infective-stage epicuticle is a totally inert layer. In fact, if signal transduction does occur across the surface, then the infective-stage epicuticle must be considered as dynamic plasma membrane rather than an inert envelope. The main difficulty in seeing the epicuticle as a membrane is that it is separated from the hypodermis by a thick, proteinaceous cuticle and there

is no evidence, to date, of transcuticular channels which might assist biochemical signalling to the hypodermis. Also, one might expect that signalling for a surface change to occur would be mediated through the sensory nervous system of the nematode. However, this is not necessarily so, since the present experiments showed that behavioural stimulation (hyperactivity) induced by drugs did not always coincide with surface changes (e.g., the results with TPA shown in tables 6.4 and 6.5) and that acetylcholine inhibitors had no effect (data not shown). The infective-stage epicuticle may, therefore, be able to alter its biophysical properties independently of the nervous system.

In contrast to the situation with the infective-stage, the argument for a membrane-like epicuticle in the adult is very weak. A significant proportion of its surface lipid is immobile in most species, and this may be associated with a formidable protein barrier, the cuticlin layer. However, adult filarial nematodes do feed across their surface in a selective manner (Howells and Chen, 1984) so that the adult epicuticle must be assigned some dynamic properties. This is where the lipid microdomain model may be most useful, as the adult epicuticle would not have to be a membrane, in the typical sense, to allow dynamic exchange and selectivity of low molecular weight substances. For example, there is good evidence that gram-negative bacteria use the microdomain organization in their outer membrane/envelope to form a selective diffusion barrier(Lugtenberg and van Alphen, 1982; Nikaido, 1989). Filarial nematodes could make use of a similar lipid organization in their epicuticle. Nevertheless, there is still a strong argument for an inert adult epicuticle having similarities with the cuticulin membrane of insects (Howells and Blainey, 1983).

Insect cuticulin contains structural lipid, in the form of a lipoprotein layer, which renders the cuticulin membrane highly resistant (Filshie and Waterhouse, 1969; Wigglesworth, 1973; 1985), but a layer which is also selectively permeable, allowing the passage of the products of digestion of the old cuticle for uptake by the hypodermis (Locke, 1982). There is also a waxy coating over the structural lipid which can be continually replenished giving insects their phenomenal resistance to water loss (Lockey, 1988). The lipoprotein layer is trilaminate when first deposited but becomes hardened into a uniform structure by oxidation and polymerization (Dennell and Malek, 1954; Wigglesworth, 1985) and is probably extensively cross-linked by methylene groups (Hackman, 1986). The composition of the insect cuticulin layer has been investigated using n.m.r., infra-red spectroscopy and U.V. absorption (see Lockey, 1988 for

a review). These are biophysical techniques which should now be employed in nematode research for the determination of epicuticle composition.

Locke (1982) stated that "some cells in almost all groups from bacteria to vertebrates have the ability to secrete an envelope directly through or above their plasma membranes, and that envelopes are not plasma membranes". I believe that the adult epicuticle falls into this category and should be referred to as the 'adult envelope'. Like the bacterial outer membrane/ envelope, the adult envelope would form a physical barrier to the environment so that the metabolically-active hypodermis could control the intervening space, i.e. the rest of the cuticle. Increased specificity at the adult envelope may occur due to the presence of nutrient- binding proteins analogous to bacterial porins, and to the presence of lipid-binding proteins. That is, the apparent dynamic properties of the adult envelope may be due to insertion of proteins, which were synthesized in the hypodermis, during and after cuticle formation.

The confusion in the literature as to whether the outermost surface of the nematode cuticle should be considered a membrane or an envelope may be well-founded, as we have limited information on its biochemical structure. Also, we cannot seriously consider the infective-stage surface and the adult surface as having exactly the same type of structure, since they have widely differing biophysical properties. Wright (1987) did attempt to form a consensus of opinion on this controversial subject, however, the problem of stage-differences had not been sufficiently addressed. Furthermore, in the light of new information from this thesis, I propose that the biochemical nature of the outermost surface of the adult- and the infective-stage should be investigated more fully, and that the nomenclature should be re-assessed to avoid further confusion.

Additional References:

Anya, A.O. (1966) The structure and chemical composition of the cuticle. Parasitology, 56, 179-198.

Bird, A.F. (1971) The Structure of Nematodes. Academic Press, New York and London, 318.

Estey, S.J. and Mansour, T.E. (1987) Nature of serotonin-activated adenylate cyclase during development of *Schistosoma mansoni*. Molecular and Biochemical Parasitology, 26, 47-60.

Fetterer, R.H. and Urban, J.F. (1988) Developmental changes in cuticular proteins of *Ascaris suum*. Comparative Biochemistry and Physiology. 90B(2), 321-327.

Garland, P.B. (1981) The Acousto-optic modulator. The Biophysical Journal, 33, 481-482.

Mackenzie, C.D., Preston, P.M. and Ogilvie, B.M. (1978) Immunological properties of the surface of parasitic nematodes. Nature, 276, 826-828.

Madshus, I.H. (1988) Regulation of intracellular pH in eukaryotic cells. Biochemical Journal, 250, 1-8.

Mansour, T.E. (1984) Serotonin receptors in parasitic worms. Advances in Parasitology, 23, 1-36.

Murrell, K.D., Graham, C.E. and McGreevy, M. (1983) *Strongyloides ratti* and *Trichinella spiralis*: Net charge of the epicuticle. Experimental Parasitology, 55, 331-339.

Myles, D.G. et al (1984) A localized cell surface protein of guinea pig sperm exhibits free diffusion in its domain. Journal of Cell Biology, 98, 1905-1909.

Petronijevic, T., Rogers, W.P. and Sommerville, R.I. (1986) Organic and inorganic acids as the stimulus for exsheathment of infective juveniles of nematodes. International Journal of Parasitology, 16, 163-168.

Poo, M.M. and Cone, R.A. (1974) Nature (London), 247, 438-441.

Riding, I.L. (1970) Microvilli on the outside of a nematode. Nature (London) 226, 179-180.

Sheetz, M.P. et al (1980) Nature (London) 285, 510-512.

Thompson, N.L. and Axelrod, D. (1980) Reduced lateral mobility of a fluorescent lipid probe in cholesterol-depleted red blood cell membranes. Biochimica et Biophysica Acta, 597, 155-165.

REFERENCES

Abraham, D., Grieve, R.B., & Mika-Grieve, M. (1988) *Dirofilaria immitis*: suface properties of third- and fourth-stage larvae. Experimental Parasitology, **65**, 157-167.

Aizenbud, B.M. & Gershon, N.D. (1982) Diffusion of molecules on biological membranes of non-planar form. A theoretical study. Biophysical Journal, **38**, 287-293.

Axelrod, D., Koppel, D.E, Schlessinger, J., Elson, E., & Webb, W.W. (1976) Mobility measurement by analysis of fluoresence photobleaching recovery kinetic. Biophysical Journal, **16**, 1055-1069.

Badley, J.E., Grieve, R.B., Rockey, J.H. & Glickman, L.T. (1987) Immune-mediated adherance of eosinophils to *Toxocara canis* infective larvae: the role of excretory-secretory antigens. Parasite Immunology, **9**, 133-143.

Barrett, J. (1981) Nutrition and biosynthesis: Ascaroside synthesis. In Biochemistry of Parasitic Helminths, (Macmillan Ltd., London) 204-205.

Benz, R. (1985) Porin from bacterial and mitochondrial outer membranes. In Critical Reviews in Biochemistry, 19, (CRC Press, Boca-Raton, Florida), pp. 145-190.

Betschart, B. & Jenkins, J.M. (1987) Distribution of iodinated proteins in *Dipetalonema viteae* after surface labelling. Molecular and Biochemical Parasitology, **22**, 1-8.

Bird, A.F. (1957) Chemical composition of the nematode cuticle. Observations on individual layers and extracts from these layers in *Ascaris lumbricoides* cuticle. Experimental Parasitology, **6**, 383-403.

Bird, A.F. (1958) Further observations on the structure of nematode cuticle. Parasitology, 48, 32-37.

Bird, A.F. (1968) Changes associated with parasitism in nematodes. III. Ultrastructure of the egg-shell, larval cuticle, and contents of the subventral esophageal glands in *Meloidogyne javanica*, with some observations on hatching. Journal of Parasitology, **54**, 475-489.

Bird, A.F. (1980) The nematode cuticle and its surface. In Nematodes as Biological Models, edited by B.M. Zuckerman (Academic Press, New York), pp. 213-236.

Bird, A.F. (1984) Nematoda. In Biology of the Integument, edited by J. Bereiter-Hahn, A.G. Matoltsy & K.S. Richards (Springer, Berlin), pp. 212-231.

Bird, A.F. and Rogers, G.E. (1965) Ultrastructure of the cuticle and its formation in *Meloidogyne javanica*. Nematologica, **11**, 224-230.

Bluemink, J.G., Tertoolen, L.G.J., Ververgaert, P.H.J.T., and Verkleij, A.J. (1976) Freeze fracture electron microscopy of preexisting an nascent cell membrane in cleaving

eggs of *Xenopus laevis*. Biochimica et Biophysica Acta, **443**, 143-155.

Boggs, J. M. & Moscarello, M.A. (1978) Structural organisation of the human myelin membrane. Biochimica et Biophysica Acta, **515**, 1-21.

Bonner, T.P. and Weinstein, P.P. (1972) Ultrastructure of cuticle formation in the nematodes *Nippostrongylus brasiliensis* and *Nematospiroides dubius*. Journal of Ultrastructural Research, **40**, 261-271.

Borovjagin, V.L., Sabelnikov, A.G., Tarahovsky, Y.S., Vasilenko, I.A. (1987) Polymorphic behaviour of gram-negative bacteria membranes. Journal of Membrane Biology, **100**, 229-242.

Branton, D. & Deamer, D.W. (1972)Membrane Structure, (Springer-Verlag, Berlin & New York).

Brindley, P.J. & Sher, A. (1987) The chemotherapeutic effect of praziquantel against *Schistosoma mansoni* is dependent on host antibody response. Journal of Immunology, **139**, 215-220.

Burnell, E., van Alphen, L., Verkleij, A., de Kruijff, B. & Lugtenberg, B. (1980) Non-bilayer phase lipid in ^{31}P-n.m.r. spectra of *Escherichia coli* outer membrane. Biochimica et Biophysica Acta, **597**, 518-532.

Campbell, A.K. (1983) Intracellular Calcium-Its Universal Role as Regulator. John Wiley & Sons, Inc., New York.

Capo, V.A., Despommier, D.D. & Silberstein, D.S. (1984) The site of ecdysis of the L1 larva of *Trichinella spiralis*. Journal of Parasitology, **70**, 992-994.

Carlow, C.K.S., Perronne, J., Spielman, A. & Philipp, M. (1987) A developmentally regulated surface epitope expressed by the infective larva of *Brugia malayi* which is rapidly lost after infection. UCLA Symposium on Molecular and Cellular Biology, **60**, 301-310.

Cassada, R.C. & Russell, R.L. (1974) A positive selection for behavioural and develpomental mutants of a nematode (*Caenorhabditis elegans*). Federation Proceedings, **33**, 1476.

Cassada, R.C. & Russell, R.L. (1975) The dauer larva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. Developmental Biology, **46**, 326-342.

Chapell, L.H. (1979) Establishment and growth of parasites. In " Physiology of Parasites", Blackie & Son Ltd., Glasgow, 129-160.

Chitwood, D.J. & Krusberg, L.R. (1981) Diacyl, alkylacyl and alkenylacyl phospholipids of the nematode *Turbatrix aceti* . Comparative Biochemistry and Physiology, **69B**, 115-120.

Clarke, A.J. & Perry R.N. (1988) The induction of permeability in egg-shells of *Ascaris suum* prior to hatching. International Journal for Parasitology, **18**, 987-990.

Clegg, J.A. (1969) Skin penetration by cercariae of the bird schistosome *Austrobilharzia terrigalensis*: the stimulatory effect of cholesterol. Parasitology, **59**, 973-989.

Cox, G.N., Kusch, M. & Edgar, R.S. (1981) Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. Journal of Cell Biology, **90**, 7-17.

Crompton, D.W.T. (1973) The sites occupied by some parasitic helminths in the alimentary tract of vertebrates. Biological reviews, **48**, 27-83.

Cullis, P.R., Hope, M.J., de Kruijff, B., Verkleij, A.J., & Tilcock, C.P.S. (1985) Structural properties and functional roles of phospholipids in biological membranes. In Phospholipids and Cellular Regulation, Volume 1, edited by J.F. Kuo (CRC Press, Boca Raton, Florida), 3-49.

De Gier, J. (1988) The use of liposomes in a search for an understanding of the significance of membrane lipid diversity. Biochemical Society Transactions, **16**, 912-914.

Dennell, R. & Malek, S.R.A. (1954) The cuticle of the cockroach, *Periplaneta americana*-II. The epicuticle. Proceedings of the Royal Society of London (B), **143**, 239-257.

De Laat, S.W., Luchtel, D., & Bluemink, J.G. (1973) The action of cytochalasin B during egg cleavage in *Xenopus laevis*: Dependance on cell membrane permeability. Developmental Biology, **31**, 163-177.

De Laat, S.W., & Bluemink, J.G. (1974)New membrane formation during cytokinesis in normal and cytochalasin B treated eggs of *Xenopus laevis*. 2. Electrophysiological observations. Journal of Cell Biology, **60**, 529-540.

De Laat, S.W., Wouters, W., Marques Da Silva Pimenta Guarda, M.M., & Da Silva Guarda, M.A. (1975) Intracellular ionic compartmentation, electrical membrane properties and cell membrane permeability before and during first cleavage in the *Ambystoma* egg. Experimental Cell Research, **91**, 15-30.

Derzko, Z. & Jacobson, K. (1980) Comparative lateral diffusion of fluorescent lipid analogues in phospholipid multibilayers. Biochemistry, **19**, 6050-6057.

Devaney, E. (1985) Lectin-binding characteristics of *Brugia pahangi* microfilariae. Tropenmedizin und Parasitologie, **36**, 25-28.

Devaney, E. (1988) The biochemical and immunological characterisation of the 30 kilodalton surface antigen of *Brugia pahangi*. Molecular and Biochemical Parasitology, **27**, 83-92.

Dictus, W.J.A.G., van Zoelen, E.J.J., Tetteroo, P.A.T., Tertoolen, L.G.J., de Laat, S.W. and Bluemink, J.G. (1984) Lateral mobility of plasma membrane lipids in *Xenopus* eggs: regional differences related to animal/vegetal polarity become extreme upon fertilization. Developmental Biology, **101**, 201-211.

Dubois, M., Giles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Colourimetric method for determination of sugars and related substances. Analytical Chemistry, **28**, 350-356.

Evans, R.W., Weaver, D.E. & Clegg, E.D. (1980) Diacyl, alkenyl and alkyl ether phospholipids in ejaculated, in utero-, and in vitro- incubated porcine spermatozoa. Journal of Lipid Research, **21**, 223-228.

Fairbairn, D. (1955) The muscle and integument lipids in female *Ascaris lumbricoides*. Canadian Journal of Biochemistry and Physiology, **34**, 39-45.

Fairbairn, D. & Passey, B.I. (1955) The lipid components in the vitelline membrane of *Ascaris lumbricoides* eggs. Canadian Journal of Biochemistry and Physiology, **33**, 130-134.

Ferguson, M.A.J., Low, M.G. & Cross, G.A.M. (1985) Glycosyl-sn-1,2-dimyristylphosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. Journal of Biological Chemistry, **260**, 14547-14555.

Ferguson, M.A.J. & Homans, S.W. (1988a) Parasite glycoconjugates: towards the exploitation of their structure. Parasite Immunology, **10**, 465-479.

Ferguson, M.A.J. & Williams, A.F. (1988b) Cell-surface anchoring of proteins via glycosylphosphatidylinositol structures. Annual Reviews of Biochemistry, **57**, 285-320.

Filshie, B.K. & Waterhouse, D.F. (1969) The structure and development of a surface pattern on the cuticle of the green vegetable bug *Nezara viridula*. Tissue and Cell, **1**, 367-385.

Folch, J., Lees, M. and Stanley, G.H.S. (1957) Journal of Biological Chemistry, **226**, 497.

Foley, M., MacGregor, A.N., Kusel, J.R., Garland, P.B., Downie, T. and Moore, I. (1986) The lateral diffusion of lipid probes in the surface membrane of *Schistosoma mansoni* . Journal of Cell Biology, **103**, 807-818.

Franke, E.D. & Weinstein, P.P. (1984) In vitro cultivation of *Dipetalonema viteae* third-stage larvae: evaluation of culture media, serum, and other supplements. Journal of Parasitology, **70**, 618-628.

Frelin, C., Vigne, P., Ladoux, A. & Lazdunski, M. (1988). The regulation of the intracellular pH in cells from vertebrates. European Journal of Biochemistry, **174**, 3-14.

Fujimoto, D. and Kanaya, S. (1973) Cuticlin: a noncollagen structural protein from

Ascaris cuticle. Archives of Biochemistry and Biophysics, **157**, 1-6.

Fujimoto, D., Horiuchi, K., Hirama, M. (1981) Isotrityrosine, a new cross-linking amino acid isolated from *Ascaris* cuticle collagen. Biochemical and Biophysical Research Communications, **99**. 637-643.

Garbers, D.L. (1989) Cyclic GMP and the second messenger hypothesis. Trends in Neurobiological Sciences, Nov/Dec, 64-67.

Goldfine, H. & Langworthy, T.A. (1988) A growing interest in bacterial ether lipids. Trends in Biochemical Sciences, **13**, 217-221.

Grencis, R.K., Crawford, C., Pritchard, D.I., Behnke, J.M., Wakelin, D. (1986) Immunization of mice with surface antigens from the muscle larvae of *Trichinella spiralis*. Parasite Immunology, **8**, 587-596.

Grove, D.I., Northern, C., Warwick, C. & Lovegrove, F.T. (1984) Loss of surface coat by *Strongyloides ratti* infective larvae during skin penetration: evidence using larvae radiolabelled with 67-Gallium. Journal of Parasitology, **70**, 689-693.

Grove, D.I., Wharton, A., Northern, C. & Papadimitriou, J.M. (1987) Electron microscopical studies of *Strongyloides ratti* infective larvae: loss of the surface coat during skin penetration. Journal of Parasitology, **73**, 1030-1034.

Gruner, S.M., Cullis, P.R., Hope, M.J. & Tilcock, C.P.S. (1985) Lipid polymorphism: the molecular basis of non-bilayer phases. Annual Review of Biophysics and Biophysical Chemistry, **14**, 211-238.

Hackman, R.H. (1986) The chemical nature of the outer epicuticle from *Lucilia cuprina* larvae. Insect Biochemistry, **16**, 911-916.

Haughland, R.P. (1989) Handbook of fluorescent probes and research chemicals. Molecular Probes Inc. Eugene, Oregon.

Hawdon, J.M. & Schad, G.A. (1990) Serum-stimulated feeding *in vitro* by third-stage infective larvae of the canine hookworm *Ancylostoma caninum*.Journal of Parasitology, **76**, 394-398.

Hayunga, E.G., Murrell, K.D., Taylor, D.W., & Vannier, W.E. (1979) Isolation and characterization of surface antigens from *Schistosoma mansoni*. 1. Evaluation of techniques for radioisotope labeling of surface proteins from adult worms. Journal of Parasitology, **65**, 488-496.

Heyworth, C.M., Wallace, A.V., Wilson, S.R. & Houslay, M.D. (1984) An assessment of the ability of insulin-stimulated cyclic AMP phosphodiesterase to decrease hepatocyte intracellular cyclic AMP concentrations. Biochemical Journal, **222**, 183-187.

Himmelhoch, S., & Zuckerman, B.M. (1978) *Caenorhabditis briggsae*: ageing and the

structural turnover of the outer cuticle surface and the intestine. Experimental Parasitology, **45**, 208-214.

Hockley, D.J., & McLaren, D.J. (1973) *Schistosoma mansoni*: changes in the outer membrane of the tegument during development from cercariae to adult worm. International Journal of Parasitology, **3**, 13-25.

Howells, R.E. & Blainey, L.J. (1983) The moulting process and the phenomenon of inter-moult growth in the filarial nematode *Brugia pahangi*. Parasitology, **87**, 493-505.

Howells, R.E., Mendis, A.M. & Bray, P.G. (1983) The mechanisms of amino acid uptake by *Brugia pahangi* in vitro. Zeitschrift fur Parasitenkunde, **69**, 247-253.

Howells, R.E. (1987) Dynamics of the filarial surface. In Filariasis , Ciba Foundation Symposium 127, edited by D. Evered & S. Clark (John Wiley & Sons Ltd, Chichester, U.K.), pp. 94-102.

Hutzell, P.A. & Krusberg, L.R. (1982) Fatty acid compositions of *Caenorhabditis elegans* and *C. briggsae*. Comparative Biochemistry and Physiology, **73B**, 517-520.

Ibrahim, M.S., Tamashiro, W.K., Moraga, D.A. & Scott, A.L. (1989) Antigen shedding from the surface of the infective stage larvae of *Dirofilaria immitis*. Parasitology, **99**, 89-97.

Jain, M.K. (1988) Introduction to Biological Membranes, second edition (Wiley-Interscience).

Johnson, J.D. & Epel, D. (1975) Relationship between release of surface proteins and metabolic activation of sea urchin eggs at fertilization. Proceedings of the National Academy of Sciences (U.S.A.), **72**, 4474-4478.

Karnovsky, M.J., Kleinfeld, A.M., Hoover, R.L. & Klausner, R.D. (1982) The concept of lipid domains in membranes. Journal of Cell Biology, **94**, 1-6.

Kato, K. & Bito, Y. (1978) Relationship between bactericidal action of complement and fluidity of cellular membranes. Infection and Immunity, **19**, 12-17.

Katsuki, S., Arnold, W., Mittal, C. & Murad, F. (1977) Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. Journal of cyclic nucleotide research, **3**, 23-25.

Kaushal, N.A., Simpson, A.J.G., Hussain, K. & Ottesen, E.A. (1984) *Brugia malayi* : stage specific expression of carbohydrates containing N-acetyl-D-glucosamine as the sheathed surfaces of microfilariae. Experimental Parasitology, **58**, 182-187.

Kawamoto, F., Shozawa, A., Kumada, N. & Kojima, K. (1989) Possible roles of cAMP and Ca2+ in the regulation of miracidial transformation in *Schistosoma mansoni*. Parasitology Research, **75**, 368-374.

Kawamoto, F., Alejo-Blanco, R., Fleck, S.L., Kawamoto, Y., & Sinden, R.E. (1990) Possible roles of Ca2+ and cGMP as mediators of the exflagellation of *Plasmodium berghei* and *P. falciparum.* (In press).

Kennedy, M.W., Foley, M., Kuo, Y.-M., Kusel, J.R. and Garland, P.B. (1987a) Biophysical properties of the surface lipid of parasitic nematodes. Molecular and Biochemical Parasitology, **22**, 233-240.

Kennedy, M.W., Maizels, R.M., Meghji, M, Young, L., Qureshi, F. & Smith, H.V. (1987b) Species-specific and common antigens of *Toxocara cati* and *Toxocara canis* infective larvae. Parasite Immunology **9**, 407-420.

Kennedy, M.W., Foley, M., Knox, K., Harnett, W., Worms, M.J., Kusel, J.R., Birmingham, J. & Garland, P.B. (1987c) Are the biophysical properties of the surface lipid of filariae different from other parasitic nematodes? In Molecular Paradigms for Eradicating Helminthic Parasites (ed. A.J. MacInnis), pp 289-300. Alan R. Liss, Inc., New York.

Kieffer, E., Rudin, W. & Hecker, H.(1989) Cytochemical demonstration of lectin binding-sites in the cuticle and tissues of *Acanthocheilonema viteae* (Filarioidea). Acta Tropica, **46**, 3-15.

King, C.A. & Preston, T.M. (1977) Fluoresceinated cationised ferritin as a membrane probe for anionc sites at the cell surface. FEBS Letters, **73**, 59-63.

Klausner, R.D., Kumar, N., Weinstein, J.N., Blumenthal, R. & Flavin, M. (1981). Interaction of tubulin with phospholipid vesicles. Journal of Biological Chemistry, **258**, 5879-5885.

Klausner, R.D. & Wolf, D.E. (1980) Selectivity of fluorescent lipid analogues for lipid domains. Biochemistry, **19**, 6119-6203.

Kusel, J.R. (1972) Protein composition and protein synthesis in the surface membranes of *Schistosoma mansoni.* Parasitology, **65**, 55-62.

Kusel, J.R. & Gordon, J. (1989) Parasite Immunology, **11**, 431-451.

Lackie, A.M. (1975) The activation of infective stages of endoparasites of vertebrates. Biological Reviews, **50**, 285-323.

Lackie, J.M. & Rotheram, S. (1972) Observations on the envelope surrounding *Moniliformis dubius* (Acanthocephala) in the intermediate host, *Periplaneta americana.* Parasitology, **65**, 303- 308.

Lee, D.L., Wright, K.A. & Shivers, R.R. (1984) A freeze-fracture study of the surface of the infective-stage larva of the nematode *Trichinella.* Tissue and Cell, **16**, 819-828.

Lee, D.L., Wright, K.A. & Shivers, R.R. (1986) A freeze-fracture study of the body wall of adult in utero larvae and infective-stage larvae of *Trichinella* (Nematoda). Tissue and Cell, **18**, 219-230.

Lee, D.L. & Bonner, T.P. (1982) Freeze etch studies on nematode body wall. Proceedings of the British Society of Parasitology, 12-16 April 1981, The Netherlands. Parasitology, **84**, x1iv.

Locke, M. (1982) Envelopes at cell surfaces - a confused area of research of general importance. In Parasites - Their World and Ours, edited by D.F. Mettrick & S.S. Desser (Elsevier Biomedical Press, Amsterdam), pp. 73-78.

Lockey, K.H. (1988) Lipids of the insect cuticle: origin, composition and function. Comparative Biochemistry and Physiology, **89B**, 595-645.

Low, M.G. & Finean, J.B. (1977). Non-lytic release of acetylcholinesterase from erythrocytes by a phosphatidylinositol-specific phospholipase C. Federation of the European Biochemical Society Letters, **82**, 143-146.

Low, M.G. & Kincade, P.W. (1985) Phosphatidylinositol is the membrane-anchoring domain of the Thy-1 glycoprotein. Nature , **318**, 62-64.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurment with the Folin Phenol reagent. Journal of Biological Chemistry, **193**, 265-275.

Lugtenberg, B. & van Alphen, L. (1982) Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochimica et Biophysica Acta, **737**, 51-115.

Lumsden, R.D. (1975) Surface ultrastructure and cytochemistry of parasitic helminths. Experimental Parasitology, **37**, 267-339.

Maizels, R.M., Meghji, M., Ogilvie, B.M. (1983) Restricted sets of parasite antigens from the surface of different stages and sexes of the nematode *Nippostrongylus brasiliensis*. Immunology, **48**, 107-121.

Maizels, R.M., de Savigny, D. & Ogilvie, B.M. (1984) Charaterization of surface and excretory-secretory antigens of *Toxocara canis* infective larvae. Parasite Immunology, **6**, 23-27.

Maizels, R.M., Kennedy, M.W., Meghji, M., Robertson, B.D., Smith, H.V. (1987) Shared carbohydrate epitopes on distinct surface and secreted antigens of the parasitic nematode *Toxocara canis*. Journal of Immunology, 139, 207-214.

Maizels, R.M. & Selkirk, M.E. (1988) Immunobiology of nematode antigens. In The Biology of Parasitism, edited by P.T. Englund & A. Sher (Alan Liss, Inc., New York), pp. 285-308.

Maizels, R.M., Gregory, W.F., Kwan-Lim, G.-E. and Selkirk, M.E. (1989) Filarial surface antigens: the major 29 kilodalton glycoprotein and a novel 17-200 kilodalton complex from adult *Brugia malayi* parasites. Molecular and Biochemical Parasitology, **32**, 213-228.

Marks, C.F., Thomason, I.J. and Castro, C.E. (1968) Dynamics of the permeation of nematodes by water nematicides and other substances. Experimental Parasitology, **22**, 321-323.

Marshall, E. & Howells, R.E. (1986) Turnover of surface proteins of third and fourth larval stages of *Brugia pahangi*. Molecular and Biochemical Parasitology, **18**, 17-24.

Martin, S.K., Miller, L.H., Nijhout, M.M. & Carter, R. (1978) *Plasmodium gallinaceum*: Induction of male gametocyte exflagellation by phosphodiestease inhibitors. Experimental Parasitology, **44**, 239-242.

Martinez-Palomo, A. (1978) Ultrastructural characterization of the cuticle of *Onchocerca volvulus* microfilariae. Journal of Parasitology, **64**, 127-136.

Minematsu, T. , Mimori, T., Tanaka, M. & Tada, I. (1989) The effect of fatty acids on the developmental direction of *Strongyloides ratti* first-stage larvae. Journal of Helminthology, **63**, 102-106.

Mok, M., Grieve, R.B., Abraham, D., & Rudin, W. (1988) Solubilization of epicuticular antigen from *Dirofilaria immitis* third-stage larvae. Molecular and Biochemical Parasitology, **31**, 173-182.

Mold, C. (1989) Effect of membrane phospholipids on activation of the alternative pathway of complement. Journal of Immunology, **143**, 1663-1668.

Moolenaar, W.H. (1986) Effects of growth factors on intracellular pH regulation. Annual review of Physiology, **48**, 363-376.

Moolenaar, W.H., Tsien, R.Y., Van der Saag, P.T. & De Laat, S.W. (1983) Na^+/H^+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. Nature, **304**, 645-648.

Mountford, C.E. and Wright, L.C. (1988) Organization of lipids in the plasma membranes of malignant and stimulated cells: a new model. Trends in Biochemical Sciences, **13**, 172-177.

Murrell, K.D. & Graham, C.E. (1983) Shedding of antibody complexes by *Strongyloides ratti* (Nematoda) larvae. Journal of Parasitology, **69**, 70-73.

McBride, O.W. and Harrington, W.F. (1967) Biochemistry, **6**, 1484-1498.

McHugh, T.D., Jenkins, T. & McLaren, D.J. (1989) *Strongyloides ratti*: studies of cutaneous reactions elicited in naive and sensitized rats and of changes in surface

antigenicity of skin-penetrating larvae. Parasitology, **98**, 95-103.

Nikaido, H. (1989) Role of the outer membrane of gram-negative bacteria in antimicrobial resistance. In Microbial Resistance to Drugs. Handbook of Experimental Pharmacology, **91**, edited by L.E. Bryan (Springer-Verlag, Berlin, Heidelberg).

Nikaido, H. & Vaara, M. (1985) Molecular basis of bacterial outer membrane permeability. Microbiological Reviews, **45**, 1-32.

Nishizuka, Y. (1984a) Turnover of inositol phospholipids and signal transduction. Science, **225**, 1365-1370.

Nishizuka, Y. (1984b) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature, **308**, 693-697.

Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. Science, **233**, 305-312.

Noda, M., Yoon, K., Gideon, A.R. & Koppel, D.E. (1987) High lateral mobility of endogenous and transfected alkaline phosphatase: a phosphatidylinositol-anchored membrane protein. Journal of Cell Biology, **105**, 1671-1677.

O' Brien, V. (1988) The role of the envelope of *Moniliformis moniliformis* in immune evasion. Ph. D. Thesis.

Ortega-Pierres, G., Chayen, A., Clark, N.W.T. & Parkhouse, R.M.E. (1984) The occurence of antibodies to hidden and exposed determinants of surface antigens of *Trichinella spiralis*. Parasitology, **88**, 359-369.

Parkhouse, R.M.E., Philipp, M. & Ogilvie, B.M. (1981) Characterization of the surface antigens of *Trichinella spiralis* infective larvae. Parasite Immunology, **3**, 339-352.

Paulson, C.W., Jacobson, R.H. & Cupp, E.W. (1988) Microfilarial surface carbohydrates as a function of developmental stage and ensheathment status in six filariids. Journal of Parasitology, **74**, 743-747.

Perry, R.N. (1989) Dormancy and hatching of nematode eggs. Parasitology Today, **5** (12), 377-383.

Peters, R. (1981) Translational diffusion in the plasma membrane of single cells as studied by fluorescence microphotolysis. Cell Biology International Reports, **5**, 733-760.

Peters, R. & Richter, H.P. (1981) Translational diffusion in the plasma membrane of sea-urchin eggs. Developmental Biology, **86**, 285-293.

Petronijevic, T. & Rogers, W.P. (1987) The physiology of infection with nematodes: The role of intracellular pH in the development of the early parasitic stage. Comparative Biochemistry and Physiology, **88A**, 207-212.

Philipp, M., Parkhouse, R.M.E. & Ogilvie, B.M. (1980) Changing proteins on the surface of a parasitic nematode. Nature, **287**, 538-540.

Philipp, M. & Rumjaneck, F.D. (1984) Antigenic and dynamic properties of helminth surface structures. Molecular and Biochemical Parasitology, **10**, 245-268.

Piessens, W.F. & Beldekas, M. (1979) Diethylcarbamazine enhances antibody-mediated cellular adherence to *Brugia malayi* microfilariae. Nature (London), **282**, 845-847.

Podesta, R.B. (1982) Membrane biology of helminths. In Membrane Physiology of Invertebrates, edited by R.B. Podesta (Dekker, New York), 121.

Politz, S.M., Chin, K.J. and Herman, D.L. (1987) Genetic analysis of adult-specific surface antigenic differences between varieties of the nematode *Caenorhabditis elegans*. Genetics, **117**, 467-476.

Pritchard, D.I., Crawford, C.I. & Behnke, J.M. (1985) Antigen stripping from the nematode epicuticle using the cationic detergent cetyltrimethylammonium bromide (CTAB). Parasite Immunology, **7**, 575-585.

Pritchard, D.I., McKean, P.G. & Rogan, M.T. (1988) Cuticular collagens- a concealed target for immune attack in hookworms. Molecular and Biochemical Parasitology, **28**, 275-284.

Proudfoot, L. , Kusel, J.R., Smith, H.V., Harnett, W., Worms, M.J. & Kennedy, M.W. (1990) The surface lipid of parasitic nematodes: organization, and modifications during transition to the mammalian host environment. Acta Tropica, **47**, 323-330.

Radda, G.K. (1975) Dynamic aspects of membrane structure. In Biological Membranes, edited by D.S. Parsons (Oxford Clarendon Press), 81-95.

Russell, N.J. (1984) Mechanisms of thermal adaptation in bacteria : blueprints for survival. Trends in Biochemical Sciences, **9**, 108-112.

Rutherford, T.A., Webster, J.M. & Barlow, J.S. (1977) Physiology of nutrient uptake by the entomophilic nematode *Mermis nigrescens* (Mermithidae). Canadian Journal of Zoology, **55**, 1773-1781.

Saffman, P.G. & Delbruck, M. (1975) Brownian motion in biological membranes. Proceedings of the National Academy of Sciences (U.S.A.), **72**, 3111-3113.

Samuelson, J.C. & Stein, L.D. (1989) *Schistosoma mansoni*: Increasing saline concentration signals cecariae to transform to schistosomula. Experimental Parasitology, **69**, 23-29.

Sauma, S.Y. & Strand, M. (1990) Identification and characterization of glycosylphosphatidylinositol-linked *Schistosoma mansoni* adult worm immunogens.

Molecular and Biochemical Parasitology, 38, 199-210.

Schimshick, E.J. and McConnell, H.M. (1973) Lateral phase separation in phospholipid membranes. Biochemistry, **12**, 2351-2360.

Schlessinger, J. (1983) Mobilities of cell membrane proteins: how are they modified by the cytoskeleton? Trends in Neurological Sciences, **8** , 360-363.

Schultz, K.D., Schultz, K. and Schultz, G. (1977) Sodium nitroprusside and other smooth muscle-relaxants increase cyclic GMP levels in rats ductus deferens, Nature, **265**, 750-751.

Schwartz, L.M. and Truman, J.W. (1984) Cyclic GMP may serve as a second messenger in peptide-induced muscle degeneration in an insect. Proceedings of the National Academy of Sciences, **81**, 6718-6722.

Scott, A.L., Chamberlain, D., Moraga, D.A., Ibrahim, M.S., Redding, L. & Tamashiro, W.K. (1988) *Dirofilaria immitis* : Biochemical and immunological characterization of the surface antigens from adult parasites. Experimental Parasitology, **67**, 301-307.

Selkirk, M. E., Denham, D. A., Partono, F., Sutanto, I. & Maizels, R.M. (1986) Molecular characterization of antigens of lymphatic filariae. Parasitology, **91**, S15-S38.

Selkirk, M.E., Gregory, W.F., Yazdanbakhsh, M., Jenkins, R.E. and Maizels, R.M. (1990) Cuticular localisation and turnover of the major surface glycoprotein (gp 29) of adult *Brugia malayi*. Molecular and Biochemical Parasitology, **42**, 3-44.

Siffert, W. & Akkerman, J.W.N. (1987) Activation of sodium-proton exchange is a prerequisite for Ca^{++} mobilization in human platelets. Nature, **325**, 456-458.

Silberstein, D.S. and Despommier, D.D. (1984) Antigens from *Trichinella spiralis* that induce a protective response in the mouse. Journal of Immunology, **132**, 898-904.

Singer, S.J., & Nicolson, G.L. (1972) The fluid mosaic model of the structure of cell membranes. Science, **175**, 720-731.

Skipski, V.P., Peterson, R.F. & Barclay, M. (1964) Biochemical Journal, **90**, 374.

Skou, J.C. (1965) Enzymatic basis for active transport of Na^{+} and K^{+} across cell membrane. Physiological reviews, **45**, 596-617.

Smith, H.V., Quinn, R., Kusel, J.R. & Girdwood, R.W.A. (1981) The effect of temperature and antimetabolites on antibody-binding to the outer surface of second-stage *Toxocara canis* larvae. Molecular and Biochemical Parasitology, **4**, 183-193.

Sommerville, R.I. and Rogers, W.P. (1987) The nature and action of host signals. Advances in Parasitology, **26**, 240-284.

Spiegel, Y., Robertson, W.M., Himmelhoch, S. and Zuckerman, B.M. (1983) Electron microscope characterization of carbohydrate residues on the body wall of *Xiphinema index*. Journal of Nematology, **15**, 528-534.

Steim, J.M. (1968) Advances in Chemistry Series, **84**, 259-274.

Steim, J.M. (1972)Federation of the European Biochemical Society (FEBS) Proceedings, **28**, 185-196.

Stewart, G.L., Despommier, D.D., Burnham, J. & Raines, K.M. (1987) *Trichinella spiralis* : Behaviour, structure and biochemistry of larvae following exposure to components of the host enteric environment. Experimental Parasitology, **63**, 195-204.

Stirewalt, M.A. (1971) Penetration stimuli for schistosome cercariae. In "Aspects of the biology of symbiosis" (T.C. Cheng, ed.). University Park Press, Baltimore, 1-23.

Storey, D.M., Ogbogu, V.C., Kershaw, W.E. (1988) The fine structure of the cuticle of third-stage larvae and adults of *Litomosoides carinii* . Tropical Medicine & Parasitology, **39**, 83.

Storry, J.E. & Tuckley, B. (1967) Lipids, **2**, 501.

Sturtevant, J.M. (1974) Some applications of calorimetry in biochemistry and biology. Annual Review of Biophysics and Bioengineering, **3**, 35-51.

Sturtevant, J.M. (1987) Biochemical applications of differential scanning calorimetry. Annual Review of Physical Chemistry, **38**, 463-488.

Subrahmanyam, D. (1967) Glyceryl ether phospholipids and plasmalogens of the filarial parasite *Litomosoides carinii*. Canadian Journal of Biochemistry, **45**, 1195-1197.

Sukhdeo, M.V.K. and Mettrick, D.F. (1987) Parasite behaviour: understanding platyhelminth responses. Advances in Parasitology, **26**, 73-124.

Taguchi, R., Asahi, Y. & Ikezawa, H. (1985) Ectoenzyme release from rat liver and kidney by phosphatidylinositol-specific phospholipase C. Journal of Biochemistry, **97**, 911-921.

Tanabe, K. (1984) Inhibitory effect of rhodamine 123 on the growth of rodent malaria parasites, *Plasmodium yoelii*. Journal of Protozoology, **31**, 310-313.

Taylor, P.W. (1983) Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. Microbiological Reviews, **47**, 46-83.

Thomas, R.C. (1984) Experimental displacement of intracellular pH and the mechanism of its subsequent recovery. Journal of Physiology, **354**, 3-22.

Titanji, V.P.K., Mbacham, W.F., Mbakop, A. (1988). Isolation and biochemical

composition of the cuticle of *Onchocerca volvulus*. Tropical Medicine and Parasitology, **39**, 100-104.

Treistman, S.N., Moynihan, M. M., and Wolf, D.E. (1987) Influence of alcohols, temperature and region on the mobility of lipids in neuronal membrane. Biochimica et Biophysica Acta, **898**, 109-120.

Turco, S.J. (1988) The lipophosphoglycan of Leishmania . Parasitology Today, **4**, 255-257.

Verkleij, A.J. & Ververgaert, P.H.J.Th. (1978) Freeze-fracture morphology of biological membranes. Biochimica et Biophysica Acta, **515**, 303-327.

Vickerman, K. (1974) Antigenic variation in African trypanosomes. In Parasites in the Immunized Host: Mechanisms of Survival, Ciba Foundation Symposium, no. 25 (Elsevier, Amsterdam), 53-70.

Von Brand, T. & Winkeljohn, M.I. (1945) Sterols in the adult body wall of *Eustrongyloides ignotus*. Proceedings of the Helminthological Society of Washington, D.C., **12**, 62.

Wagner, A. (1959) Stimulation of *Schistosomatium douthitti* cercariae to penetrate their host. Journal of Parasitology, **45**, 16.

Wassef, M.K., Fioretti, T.B. & Dwyer, D.M. (1985) Lipid analyses of isolated surface membranes of *Leishmania donovani* promastigotes. Lipids, **20**, 108-115.

Wharton, D.A., Preston, C.M., Barrett, J. & Perry, R.N. (1988) Changes in cuticular permeability associated with recovery from anhydrobiosis in the plant parasitic nematode *Ditylenchus dipsaci*. Parasitology, **97**, 317-330.

Wigglesworth, V.B. (1973) The role of the epidermal cells in moulding the surface pattern of the cuticle in *Rhodnius* (Hemiptera). Journal of Cell Science, **12**, 683-705.

Wigglesworth, V.B. (1985) The transfer of lipid in insects from the epidermal cells to the cuticle. Tissue and Cell, **17**, 249-265.

Winkler, M. (1988) Translational regulation in sea-urchin eggs: a complex interaction of biochemical and physiological regulatory mechanisms. Bioessays, **8**, 157-161.

Wolf, D.E., Handyside, A.H. & Edidin, M. (1982) Effect of microvilli on lateral diffusion measurements made by the fluoresence photobleaching recovery technique. Biophysical Journal, **38**, 295-297.

Wolf, D.E., Lipscomb, A.C. & Maynard, V.M. (1988) Causes of non-diffusing lipid in the plasma membrane of mammalian spermatozoa. Biochemistry, **27**, 860-865.

Wolf, D.E. (1988) Probing the lateral organization and dynamics of membranes. In Spectroscopic Membrane Probes, CRC Critical Reviews , edited by L. Loew (Boca

Raton, Florida), 194-216.

Wolf, D.E. & Voglmayr, J.K. (1984) Diffusion and regionalization in membranes of maturing ram spermatozoa. Journal of Cell Biology, **98**, 1678-1684.

Wright, K.A. (1987) The nematode's cuticle- its surface and the epidermis: function, homology, analogy. Journal of Parasitology, **73** (6), 1077-1083.

Wright, K.A. & Hong, H. (1988) Characterization of the accessory layer of the cuticle of muscle larvae of *Trichinella spiralis*. Journal of Parasitology, **74**, 440-451.

Zaman, V. (1987) Scanning electron microscopy of *Brugia malayi*. In Filariasis , Ciba Foundation Symposium 127, edited by D. Evered & S. Clark (John Wiley & Sons Ltd, Chichester, U.K.) pp. 77-90.

Zuckerman, B.M., Kahane, I. & Himmelhoch, S. (1979) *Caenorhabditis briggsae* and *C. elegans* : partial characterization of cuticle surface carbohydrates. Experimental Parasitology, **47**, 419-424.

Additional References:

Anya, A.O. (1966) The structure and chemical composition of the cuticle. Parasitology, 56, 179-198.

Bird, A.F. (1971) The Structure of Nematodes. Academic Press, New York and London, 318.

Estey, S.J. and Mansour, T.E. (1987) Nature of serotonin-activated adenylate cyclase during development of *Schistosoma mansoni*. Molecular and Biochemical Parasitology, 26, 47-60.

Fetterer, R.H. and Urban, J.F. (1988) Developmental changes in cuticular proteins of *Ascaris suum*. Comparative Biochemistry and Physiology. 90B(2), 321-327.

Garland, P.B. (1981) The Acousto-optic modulator. The Biophysical Journal, 33, 481-482.

Lewis, P.R. and Shute, C.C.D (1966) Journal of Cell Science, 1, 381-390

Mackenzie, C.D., Preston, P.M. and Ogilvie, B.M. (1978) Immunological properties of the surface of parasitic nematodes. Nature, 276, 826-828.

Madshus, I.H. (1988) Regulation of intracellular pH in eukaryotic cells. Biochemical Journal, 250, 1-8.

Mansour, T.E. (1984) Serotonin receptors in parasitic worms. Advances in Parasitology, 23, 1-36.

Murrell, K.D., Graham, C.E. and McGreevy, M. (1983) *Strongyloides ratti* and *Trichinella spiralis*: Net charge of the epicuticle. Experimental Parasitology, 55, 331-339.

Myles, D.G. et al (1984) A localized cell surface protein of guinea pig sperm exhibits free diffusion in its domain. Journal of Cell Biology, 98, 1905-1909.

Petronijevic, T., Rogers, W.P. and Sommerville, R.I. (1986) Organic and inorganic acids as the stimulus for exsheathment of infective juveniles of nematodes. International Journal of Parasitology, 16, 163-168.

Poo, M.M. and Cone, R.A. (1974) Nature (London), 247, 438-441.

Pratt, J.M. (1984) In 'Transcription and translation : a practical approach chapter 7, p188, Oxford Washington, I.R.L. Press!

Riding, I.L. (1970) Microvilli on the outside of a nematode. Nature (London) 226, 179-180.

Sheetz, M.P. et al (1980) Nature (London) 285, 510-512.

Stryer, L. (1978) Annual Review of Biochemistry, 47, 819-846

Thompson, N.L. and Axelrod, D. (1980) Reduced lateral mobility of a fluorescent lipid probe in cholesterol-depleted red blood cell membranes. Biochimica et Biophysica Acta, 597, 155-165.

Wales, A. (1989) Biochemical effects of irradiation of larvae of *Schistosoma mansoni*.